Progress in Inflammation Research
Series Editors: Michael J. Parnham · Achim Schmidtko

Slobodan Vukicevic
Kuber T. Sampath *Editors*

Bone Morphogenetic Proteins: Systems Biology Regulators

Springer

Progress in Inflammation Research

More information about this series at http://www.springer.com/series/4983

Slobodan Vukicevic • Kuber T. Sampath
Editors

Bone Morphogenetic Proteins: Systems Biology Regulators

Springer

Editors
Slobodan Vukicevic
School of Medicine
University of Zagreb
Zagreb
Croatia

Kuber T. Sampath
perForm Biologics Inc
Holliston
Massachusetts
USA

Series Editors
Michael J. Parnham
Fraunhofer IME & Goethe University
Frankfurt
Germany

Achim Schmidtko
Goethe University
Frankfurt
Germany

Progress in Inflammation Research
ISBN 978-3-319-47505-9 ISBN 978-3-319-47507-3 (eBook)
DOI 10.1007/978-3-319-47507-3

Library of Congress Control Number: 2016963598

Printed on acid-free paper

This Springer imprint is published by Springer Nature
The registered company is Springer International Publishing AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Preface

In mid-1960, Marshall Urist described the phenomenon that demineralized bone matrix (DBM) contained "bone morphogenetic protein, BMP," which has the ability to induce new bone in vivo. As the formation of new bone involves a cascade of cellular events such as cell migration, proliferation, and differentiation into endochondral bone, mimicking embryonic bone development, it was long believed that more than one protein was involved.

In early 1980, the discovery that the proteins responsible for bone induction in DBM could be extracted and reconstituted with collagenous bone matrix and assayed for in vivo bone forming activity at ectopic sites has made possible the identification of BMP by employing protein purification and molecular cloning. A single recombinant BMP is capable of inducing the full cascade of cellular events leading to endochondral bone formation and able to restore the lost bone with function both in preclinical models and in man. This has allowed the approval of BMPs for clinical use in delayed long bone fractures and anterior lumbar interbody fusion. In spite of its clinically proven application, the challenges are the safety concerns and its wider application in orthopedic and dental medicine. The unwanted safety has been attributed to higher doses of BMP employed and animal-sourced collagenous scaffold used as substratum. It is likely that utilizing a BMP that has little or no affinity to BMP antagonist like noggin, autologous substratum, and optimal concentration could provide a safe and robust outcome required in the clinic. Although BMPs are originally identified in bone matrix, they are expressed in many tissues and are highly conserved both structurally and functionally from fly to man. Though *Drosophila* does not have bone, the *Drosophila* BMP orthologue is capable of inducing new bone in mammals, and vice versa human BMP can restore the loss of function of *Drosophila BMP* orthologue mutants.

BMPs are members of the TGF-beta superfamily of proteins, also called osteogenic proteins (OPs) and growth and differentiation factors (GDFs), which are required for the development of many organs during embryogenesis, are responsible for ectoderm-mesoderm inductive events, and recapitulate in part during adult tissue repair and regeneration to restore function. BMPs act not only on bones, and the activity is rendered by specific BMP receptors and downstream signaling and

modulated by BMP-specific antagonists and extracellular matrices. In addition to its morphogenic role in cartilage, bone, and dentin regeneration, BMPs have profound influence on providing protection against inflammation, immune-modulation, and angiogenesis and parenchymal fibrosis. Recent advances suggest BMPs play a metabolic role in glucose, calcium and phosphate, and iron homeostasis. Genetic linkage analysis has revealed that BMP signaling is responsible for certain rare disorders like fibrodysplasia ossificans progressiva (FOP), pulmonary arterial hypertension (PAH), hemochromatosis (HH), and hereditary hemorrhagic telangiectasia (HHT). Furthermore, BMP signaling is capable of impacting tumor growth and progression, both positively and negatively; the effects are dependent on the dose, context, and stage of tumor development.

We have edited three BMP-related books in the past, namely, (1) *BMPs: From Laboratory to Clinical Practice*, (2) *BMPs: Regeneration of Bone and Beyond*, and (3) *BMPs: From Local to Systemic Therapeutics*. This book is attributed to "Systems Biology of BMPs" and contains several chapters that cover advances made on various fronts as described above. We sincerely thank our authors, who have made original discovery in their respective fields, for their contribution and presenting the salient features in the BMP field. This book would not have been possible without the authors of the chapters and their hard work. We also thank Ms. Sowmya Ramalingam, Production Editor, Springer, SPI Global, for timely publishing this book.

Slobodan Vukicevic — Zagreb, Croatia
Kuber T. Sampath — Holliston, USA

Contents

List of Contributors

Andreas Benn Institute for Chemistry and Biochemistry, Freie Universität Berlin, Berlin, Germany

DFG Graduate School 1093 Berlin School of Integrative Oncology, Berlin, Germany

DFG Graduate School 203 Berlin-Brandenburg School for Regenerative Therapies, Berlin, Germany

Johanna Bolander Prometheus, Division of Skeletal Tissue Engineering, KU Leuven, Leuven, Belgium

Skeletal Biology and Engineering Research Center, Department of Development and Regeneration, KU Leuven, Leuven, Belgium

Tatjana Bordukalo-Niksic University of Zagreb School of Medicine, Center for Translational and Clinical Research, Laboratory for Mineralized Tissues, Zagreb, Croatia

Yoke Chin Chai Prometheus, Division of Skeletal Tissue Engineering, KU Leuven, Leuven, Belgium

Skeletal Biology and Engineering Research Center, Department of Development and Regeneration, KU Leuven, Leuven, Belgium

Susan Chubinskaya Department of Pediatrics, Rush University Medical Center, Chicago, IL, USA

Igor Erjavec University of Zagreb School of Medicine, Center for Translational and Clinical Research, Laboratory for Mineralized Tissues, Zagreb, Croatia

Peter ten Dijke Department of Molecular Cell Biology and Cancer Genomics Centre Netherlands, Leiden University Medical Center, Leiden, The Netherlands

Ludwig Institute for Cancer Research, Science for Life Laboratory, Uppsala University, Uppsala, Sweden

Ivo Dumic-Cule University of Zagreb School of Medicine, Center for Translational and Clinical Research, Laboratory for Mineralized Tissues, Zagreb, Croatia

Peter V. Giannoudis, MB, BSc, MD, FACS, FRCS(Eng) Academic Department of Trauma & Orthopaedics, School of Medicine, University of Leeds, Leeds, UK

NIHR Leeds Biomedical Research Unit, Chapel Allerton Hospital, West Yorkshire, Leeds, UK

Lovorka Grgurevic University of Zagreb School of Medicine, Center for Translational and Clinical Research, Laboratory for Mineralized Tissues, Zagreb, Croatia

School of Medicine, University of Zagreb, Zagreb, Croatia

Munish C. Gupta, MD Department of Orthopedics, Washington University, St. Louis, MO, USA

Sachin Gupta, BS, MD Cand George Washington University, 900 23rd ST NW, Washington DC, USA

Julia Haupt Institute for Chemistry and Biochemistry, Freie Universität Berlin, Berlin, Germany

Susanne Hildebrandt Institute for Chemistry and Biochemistry, Freie Universität Berlin, Berlin, Germany

DFG Graduate School 203 Berlin-Brandenburg School for Regenerative Therapies, Berlin, Germany

Wei Ji Prometheus, Division of Skeletal Tissue Engineering KU Leuven, Leuven, Belgium

Skeletal Biology and Engineering Research Center, Department of Development and Regeneration, KU Leuven, Leuven, Belgium

Christian Kaehler Institute for Chemistry and Biochemistry, Freie Universität Berlin, Berlin, Germany

Nobuhiro Kamiya, MD, PhD Department of Sports Medicine, Tenri University, Tenri, Nara, Japan

Nikolaos K. Kanakaris Academic Department of Trauma & Orthopaedics, School of Medicine, University of Leeds, Leeds, UK

Frederick S. Kaplan Departments of Orthopaedic Surgery Medicine, and Genetics, and the Center for Research in FOP and Related Disorders, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA, USA

Hiroki Katagiri Prometheus, Division of Skeletal Tissue Engineering KU Leuven, Leuven, Belgium

Skeletal Biology and Engineering Research Center, Department of Development and Regeneration, KU Leuven, Leuven, Belgium

Takenobu Katagiri Division of Pathophysiology, Research Center for Genomic Medicine, Saitama Medical University, Hidaka-shi, Sa-tama, Japan

Petra Knaus Institute for Chemistry and Biochemistry, Freie Universität Berlin, Berlin, Germany

DFG Graduate School 1093 Berlin School of Integrative Oncology, Berlin, Germany

DFG Graduate School 203 Berlin-Brandenburg School for Regenerative Therapies, Berlin, Germany

Herbert Y. Lin Division of Nephrology, Program in Membrane Biology, and Center for Systems Biology, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA

Frank P. Luyten Prometheus, Division of Skeletal Tissue Engineering KU Leuven, Leuven, Belgium

Skeletal Biology and Engineering Research Center, Department of Development and Regeneration, KU Leuven, Leuven, Belgium

Marina Marechal Prometheus, Division of Skeletal Tissue Engineering KU Leuven, Leuven, Belgium

Skeletal Biology and Engineering Research Center, Department of Development and Regeneration, KU Leuven, Leuven, Belgium

Drazen Maticic University of Zagreb School of Veterinary Medicine, Clinic for Surgery, Orthopedics and Ophthalmology, Zagreb, Croatia

Yuji Mishina, PhD University of Michigan, School of Dentistry, Department of Biologic and Materials Sciences, Ann Arbor, MI, USA

Vivek Mohan, MD, MS Orthopaedic Spine Surgeon, Spine Center at DuPage Medical Group, Naperville, IL 60540, USA

Kristof Nolan Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati, Cincinnati, OH, USA

Marija Lipar University of Zagreb School of Veterinary Medicine, Clinic for Surgery, Orthopedics and Ophthalmology, Zagreb, Croatia

Martina Pauk University of Zagreb School of Medicine, Center for Translational and Clinical Research, Laboratory for Mineralized Tissues, Zagreb, Croatia

Marko Pecin University of Zagreb School of Veterinary Medicine, Clinic for Surgery, Orthopedics and Ophthalmology, Zagreb, Croatia

Mihaela Peric University of Zagreb School of Medicine, Center for Translational and Clinical Research, Department for Intercellular communication, Zagreb, Croatia

A. Hari Reddi Lawrence Ellison Center for Musculoskeletal Regeneration, Department of Orthopedics Surgery, University of California at Davies, Sacramento, CA, USA

Jiang Ren Department of Molecular Cell Biology and Cancer Genomics Centre Netherlands, Leiden University Medical Center, Leiden, The Netherlands

David C. Rueger Rueger Consulting, Southborough, MA, USA

Kuber T. Sampath PerForm biologics Inc., Holliston, MA, USA

Eileen M. Shore, PhD University of Pennsylvania, Department of Orthopaedic Surgery, Philadelphia, PA, USA

Cristiano Susin, DDS, MSD, Dr Odont Laboratory for Applied Periodontal and Craniofacial Regeneration – The Dental College of Georgia, Augusta University, Augusta, GA, USA

Thomas B. Thompson Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati, Cincinnati, OH, USA

Vladimir Trkulja University of Zagreb School of Medicine, Department of Pharmacology, Zagreb, Croatia

Slobodan Vukicevic University of Zagreb School of Medicine, Center for Translational and Clinical Research, Laboratory for Mineralized Tissues, Zagreb, Croatia

Ulf M.E. Wikesjö, DDS, DMD, Dr Odont Laboratory for Applied Periodontal and Craniofacial Regeneration – The Dental College of Georgia, Augusta University, Augusta, GA, USA

Peiran Yang, PhD Brigham and Women's Hospital, Division of Cardiovascular Medicine, Department of Medicine, Boston, MA, USA

Paul B. Yu, MD, PhD, FAHA Brigham and Women's Hospital, Division of Cardiovascular Medicine, Department of Medicine, Boston, MA, USA

Historical Perspective of Bone Morphogenetic Proteins

Kuber T. Sampath and A. Hari Reddi

Abstract The bone morphogenetic proteins (BMPs) are growth and differentiation factors and form a large family of proteins structurally related to TGF-βs and activins. BMP-2 and BMP-7 containing osteogenic devices (InFuse® and OP-1®, respectively) have been used as bone graft substitutes for the repair of long-bone fractures and anterior lumbar interbody and posterior-lateral fusion of vertebrae in humans. The PMA (premarket approval) and HDE (humanitarian device exemption) approval of BMP-2 and BMP-7 for orthopedic use demonstrates that signals responsible for ectopic bone formation can form therapeutic principles for bone repair, regeneration, and restoration. This article describes a historical perceptive on the discovery, structure, and function of bone morphogenetic proteins.

Keywords BMP Discovery • BMP Structure and Function • BMP Orthopedic Medicine, InFuse® - OP-1®

1 Bone Formation: Auto-induction

It has long been known that bone has the capacity to heal and repair itself. Hippocrates believed that bone has "endogenous" substances that exhibit considerable healing potential which could be exploited for clinical use to repair bone. Senn [1] practiced the use of antiseptic decalcified bone for the construction of bone following osteomyelitis and applied it to repair bone deformities. Pierre Lacroix [2] postulated the

K.T. Sampath (✉)
perForm biologics Inc., Holliston, MA, USA
e-mail: kuber.sampath@performbiologics.com; kuber.sampath@gmail.com

A.H. Reddi (✉)
Lawrence Ellison Center for Musculoskeletal Regeneration, Department of Orthopedics Surgery, University of California at Davies, Sacramento, CA, USA
e-mail: ahreddi@ucdavis.edu

S. Vukicevic, K.T. Sampath (eds.), *Bone Morphogenetic Proteins: Systems Biology Regulators*, Progress in Inflammation Research,
DOI 10.1007/978-3-319-47507-3_1

presence of inductive osteogenic substances in the bone that may be responsible for osteogenesis.

Although skin and bone are composed of type I collagen, only collagen in bone undergoes mineralization. To ask the question whether bone-derived collagen could be remineralized, Urist performed an experiment in which he demineralized the rabbit bone chips with 0.6 N HCl and then implanted the water-washed and dried bone chips into the thigh muscle. To his surprise, what he observed was not the remineralization process but the formation of highly vascularized and remodeled functional new bone that contained new bone marrow elements within a shape similar to the original bone chip [3]. He described this phenomenon as "bone formation by auto-induction." His observation was the first proof that indeed nonliving acellular bone matrix has a morphogenic activity capable of inducing new bone at ectopic sites as postulated by Hippocrates long ago. In collaboration with Huggins, Urist further showed that demineralized dentin matrix also induced the formation of new bone *in vivo*, and he named the bone- and dentin-derived substances as "bone morphogenetic proteins" [4].

2 Matrix-Induced Bone Formation: A Biological Cascade

Implantation of demineralized bone matrix (74–420 μm) at subcutaneous sites initiates a cascade of cellular events [5] that involve the recruitment and proliferation of fibroblast-like mesenchyme stem cells within 24–72 h, which then undergo differentiation into chondrocytes in 5–7 days, calcify, and, with an advent vascular ingrowth, form new bone containing osteoblasts that lay down extracellular matrix and mineralization by day 9–11. With a concurrent formation of osteoclasts, the newly formed bone undergoes remodeling by day 14, and an ossicle containing new bone marrow elements appear, red and white blood cells and megakaryocyte, by day 18–21 (Fig. 1). Thus, the implantation of nonliving demineralized extracellular bone matrix resulted in the formation of new cartilage, bone, and bone marrow. It was then believed that the bone matrix contained a set of morphogenic proteins responsible for the cascade of cellular events associated with matrix-induced new bone formation.

3 Discovery of Bone Morphogenetic Proteins

Though Marshall Urist coined the phrase "bone morphogenetic proteins" and demonstrated its presence in the bone matrix, the progress in identifying "BMPs" responsible for new bone formation was slow due to the difficulty in isolating protein from the insoluble bone matrix and the lack of a defined bioassay to qualify the bone-inducing activity *in vivo*. It was demonstrated that proteins responsible for bone formation could be extracted from the demineralized bone matrix

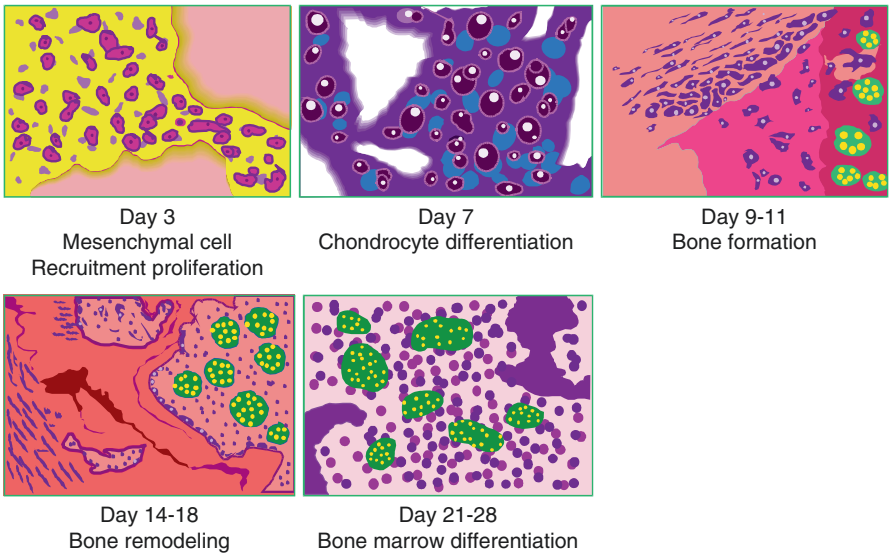


Fig. 1 Demineralized bone matrix induces new bone formation

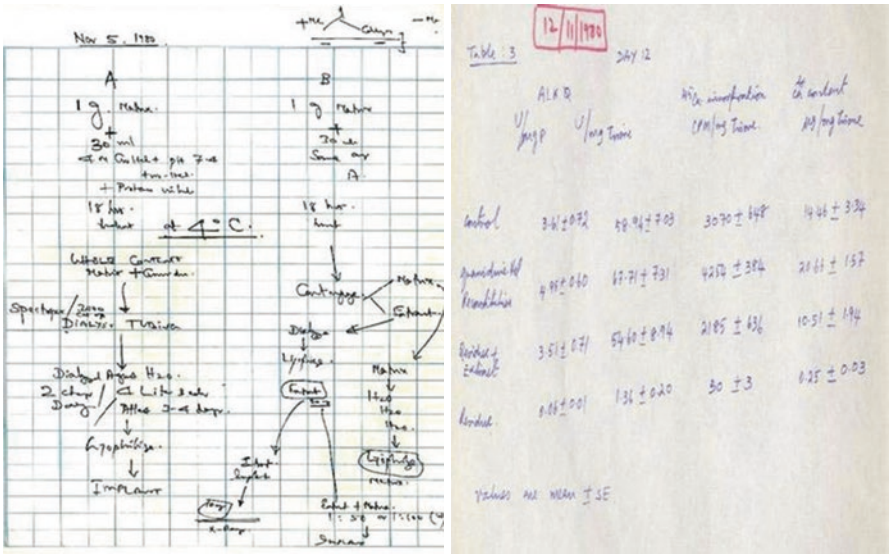


Fig. 2 Notes on first reconstitution assay

by dissociative agents and then reconstituted with a collagenous substratum and be assayed reproducibly in a dose-dependent manner for their bone-forming ability at rat subcutaneous sites [6, 7] (Fig. 2). This advance provided a reproducible

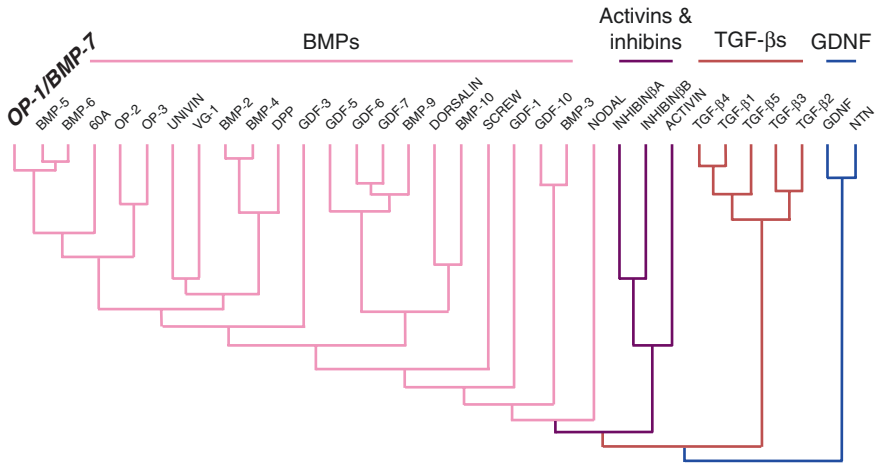


Fig. 3 BMPs represent a large group of the TGF-β superfamily proteins

bioassay and permitted the isolation, characterization, and identification of several bone morphogenetic proteins from bovine bone matrix. It was then identified for the first time the genes encoding "putative" bone morphogenetic proteins (BMP-1, BMP-2, BMP-3, and BMP-4) utilizing the amino acid sequences obtained from enriched bone-inductive protein fractions isolated from bovine bone [8]. Of them, BMP-2, BMP-3, and BMP-4 are members of TGF-beta family of proteins, whereas BMP-1 is a contaminant, a mammalian tolloid proteinase responsible for processing extracellular matrix proteins such as collagens and processing certain member of the TGF-beta family of proteins including TGF-beta and GDFs [9]. The highly purified bovine osteogenic protein is composed of homodimers of osteogenic protein-1 (OP-1, also called BMP-7) and BMP-2 [10]. The OP-1 (BMP-7) gene was identified using a consensus gene construct based on amino acid sequences obtained from highly purified bovine osteogenic protein and related *Drosophila* DPP and *Xenopus* Vg-1 cDNAs [11]. Subsequently, several BMP-related genes have been identified from human cDNA and genomic libraries using oligonucleotide probes whose construction was based on known BMP gene sequences; they are called as "growth and differentiation factors" or GDFs [12, 13] (Fig. 3).

BMPs and GDFs are members of the TGF-β superfamily [14] and are involved in the developmental process of several organs during embryogenesis [15] and play a role in morphogenesis during tissue regeneration and repair in post-fetal life. The induction of new bone by *Drosophila* BMP orthologs (*dpp* and *60A*) when implanted in rats suggests that the formation of new bone is governed by the responding cell types and the microenvironment at the injury site rather than by the morphogenic signals [16]. Thus BMP-induced new bone formation serves as a prototype for tissue engineering and demonstrates the biological principles of regenerative medicine.

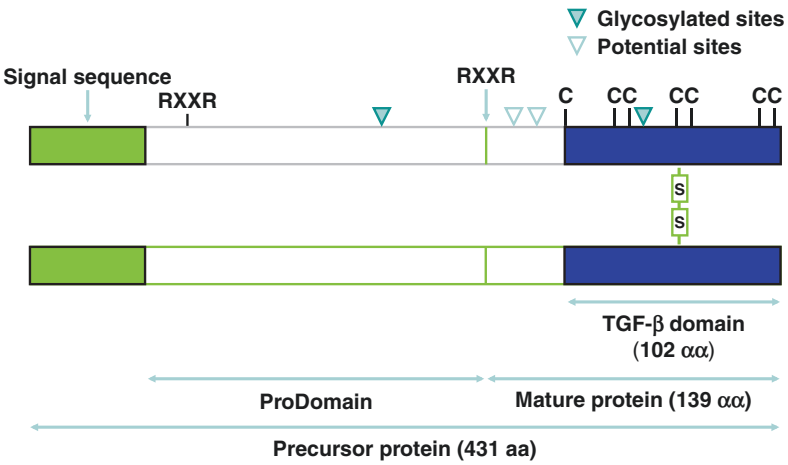


Fig. 4 Structure of OP-1/BMP-7

4 BMP Structure

As a member of TGF-beta superfamily, BMP is synthesized as a large precursor and then processed as a mature disulfide-linked dimer [17]. For example, BMP-7 cDNA (Fig. 4) predicts a protein of 431 amino acids that contains a 29-amino acid signal peptide, a 29–292-amino acid prodomain, and the 293–431 amino acids as processed mature protein containing a 7-cysteine domain, a hallmark of TGF-beta family of proteins. The protein is synthesized in the cell as a monomer and forms disulfide-linked dimer at the C-terminal fourth cysteine and then is cleaved at the RXXR maturation site in an acidic cellular compartment before it is secreted into the medium as disulfide-linked homodimer. Upon secretion, the prodomains remain associated non-covalently with the disulfide-linked mature dimer as a soluble complex under physiological conditions and are biologically active both *in vitro* and *in vivo* [18]. The prodomain alone is not biologically active but may facilitate protein folding, solubility, and transport and participate in tissue targeting by binding to extracellular matrices to guide in establishing receptor specificity. For a BMP to be active, disulfide-linked dimerization is a requirement. The products approved for clinical use employ mature disulfide-linked BMP homodimer applied locally in combination with a collagenous substratum.

5 BMP Receptors

BMP exerts its function by binding to a specific Ser/Thr kinase receptor complex [19] composed of one type I receptor (e.g., ALK-2, ALK-3, and ALK-6) and one type II receptor (e.g., BMPR-II, ActRII-A, and ActRII-B). The ligand-receptor

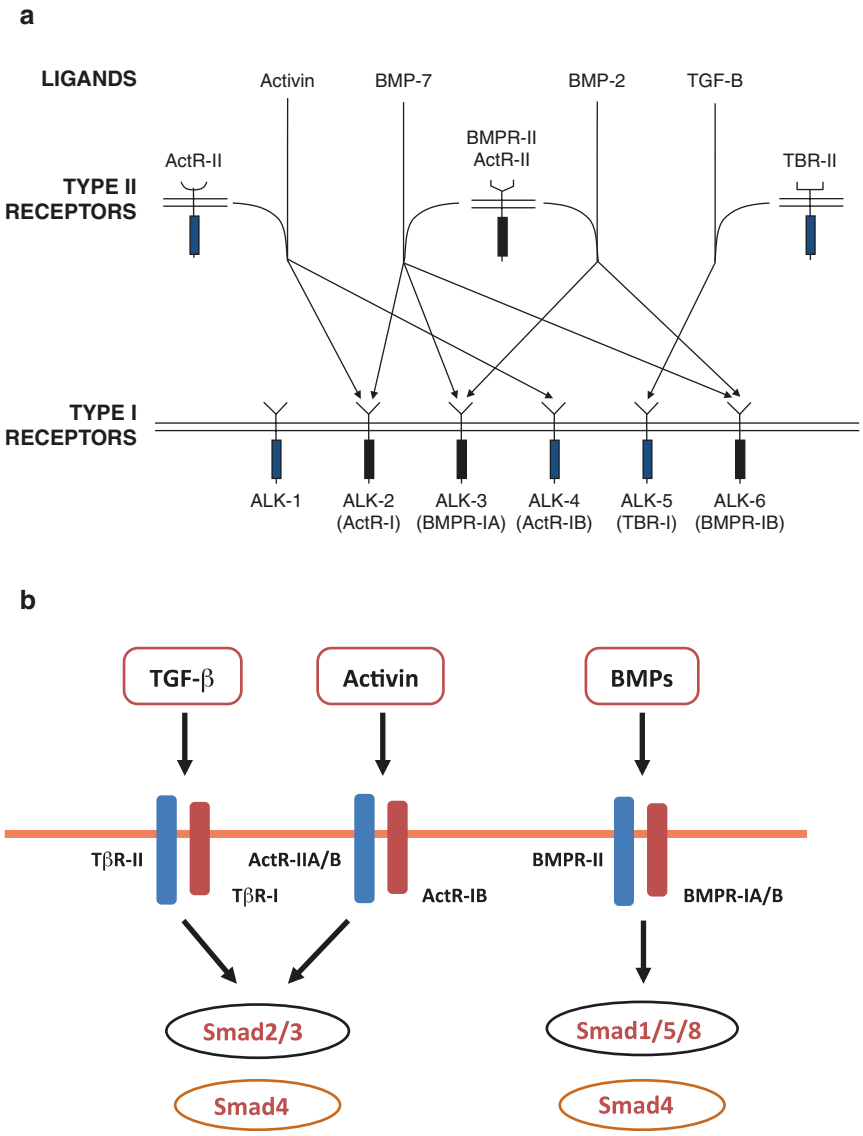


Fig. 5 (**a**, **b**) BMP-7 binding to type I and type II receptors. Signaling pathway of TGF-β superfamily

complex subsequently induces the phosphorylation of intracellular transcription factors signaling SMAD1/5/8 (Fig. 5a, b). The P-SMAD-1/5/8 engages the co-smad-4; the signaling/co-smad-complexes then translocate into the nucleus to switch on/off a set of genes that are involved in tissue protection, repair, and regeneration [20, 21]. The binding of BMP to its receptor complex is tightly controlled

in the extracellular milieu with endogenous antagonists (e.g., noggin, follistatin, sclerostin, twisted gastrulation, gremlin, DAN, and USAG-1/Wise/SOSTdc1) [22]. The intracellular downstream signaling is also regulated via the interaction of P-smad-1/5/8 with anti-smad-6/7 and subsequently by degradation of signaling-, co-, and anti-smad ubiquitination thru smurf1 and E2/E3 ubiquitin ligases.

6 BMP Preclinical Studies

Recombinant BMP when implanted with an appropriate collagenous matrix is capable of inducing new bone at ectopic sites, and this effect is dependent on the dose. BMP-2 [23, 24], BMP-7 [25, 26], and BMP-6 [27] all have been shown to restore large segmental defects when implanted with collagenous matrices in diaphyseal bone defects. The doses employed vary based on the BMP used and selected substratum. The efficacy of BMP-2, BMP-6, and BMP-7 has been shown to exhibit a comparable bone-forming activity at subcutaneous sites and in preclinical models, and this activity is dose dependent. A single BMP is sufficient to elicit this response in diaphyseal segmental defect models of small and large animals.

7 BMP-7 in Articular Cartilage Repair

BMPs are potent chondrogenic morphogens and are capable of inducing differentiation of MSCs into a cell lineage of hyaline cartilage expressing markers associated with a chondrocyte phenotype *in vitro* [28, 29]. Several studies have demonstrated that BMPs when applied alone or in combination with appropriate scaffolds into chondral or osteochondral defects are capable of inducing new articular cartilage formation in vivo [30, 31]. However, the newly formed chondrocytes fail to maintain the cellular morphology and expression of articular cartilage phenotype over time, thus leading to the degeneration of repair tissue in the preclinical studies. It is likely that providing BMPs at periodic intervals (instead of one-time application at the beginning as used to repair bone fractures) may help to maintain the regenerated cartilage and to attain function over time under mechanical loading. The combination of responding cells with an appropriate scaffold and BMP signaling in situ will have added advantage in the enhancement of chondrocyte differentiation and maintenance of phenotypic expression in order to sustain function for a long time.

BMP-7 has been shown to be anabolic to human articular chondrocytes in culture [32]. It stimulates type II collagen synthesis and cartilage-specific proteoglycans and overcomes the IL-1-beta-mediated degradation of cartilage extracellular matrix components [33]. Thus BMP-7 has also been shown to be effective in stimulating type II collagen and proteoglycans in human osteoarthritic explant culture where BMP levels were suppressed as compared to normal, suggesting that BMP-7 may overcome the structural damage that occurs in OA cartilage. This anabolic effect is reproduced in vivo using a preclinical model of chondral defects in sheep by

continuous delivery of BMP-7 locally [34]. BMP-7 is also shown to prevent the progression of existing cartilage degeneration by weekly intra-articular injections in an anterior cruciate ligament transection (ACLT) OA model in rabbits [35]. A phase 1/2 clinical study showed that a single administration of BMP-7 intra-articularly was able to relieve pain in a number of enrolled patients [36]. It remains to be seen whether application of a BMP-7 at intervals will provide sustainable pain relief in the clinic.

8 BMPs Beyond Bone

While BMPs are capable of forming bone at ectopic and bony sites, they are expressed in tissues other than bone [37, 38]. Studies on gain and loss of function indicate that in addition to a morphogenic role in the musculoskeletal system [39], BMPs serve as inductive signals for a number of tissues during organogenesis, suggesting that they may have therapeutic utility in tissues beyond bone [40]. For example, BMP-2 has a developmental role in cardiac tissue [41], BMP-4 (a structurally close member to BMP-2) in lung development [42], and BMP-7 in kidney morphogenesis [43, 44]. The loss of BMP-6 exhibits hemochromatosis [45], and gain of function results in anemia through disturbance in iron-hemojuvelin-hepcidin loop. The loss of GDF-8 results in enhanced skeletal myogenesis with high metabolic activity exhibiting a lean phenotype [46], whereas the GDF-11 (closely related to GDF-8) exerts to have a positive role in aging process [47]. It remains to be seen whether agonizing and antagonizing BMP/GDF signaling has any therapeutic utility against disorders of iron homeostasis (anemia and thalassemia) and cardiac hypertrophy and obesity.

9 BMP Clinical Studies

Several clinical trials have been conducted to assess the safety and efficacy of recombinant BMP-containing devices for the treatment of acute diaphyseal bone fractures and delayed union, tibial nonunion, and spinal fusion. Two BMP products, rhBMP2 [48] (InFUSE®) and rhBMP7 [49, 50] (OP-1® and OP-1 Putty®), were approved under a PMA and HDE, respectively, in the United States.

The InFUSE® is available as a lyophilized powder in vials; after reconstitution (1.5 mg/mL), the solution is applied to a provided type I collagen sponge prepared from bovine Achilles tendon (“carrier”) and used immediately as a wet sponge [51–53]. InFUSE® is also used in combination with osteoconductive bulking agents for an HDE [54, 55] The OP-1® device is composed of approximately 3.5 mg of recombinant BMP-7 dispersed in 1 g of bone type I collagen and lyophilized in a vial. The OP-1 Putty® is provided as two components. Each unit is comprised of one vial of OP-1® Implant containing 1 g of a sterile dry powder consisting of

bovine collagen and 3.5 mg of rhBMP-7 and a 10 mL vial of putty additive containing 230 mg of sterile carboxymethylcellulose.

The OP-1® Implant used in the first human clinical study performed to assess the efficacy of recombinant human rhBMP7 (OP-1®) for the treatment of tibial nonunion in a prospective, randomized, and controlled clinical trial involving 122 patients with tibial nonunion fractures at 17 centers in the United States [56]. This clinical study demonstrated that OP-1® Implant was safe and effective treatment modality for tibial nonunion fractures; the outcome was comparable to the use of autograft but failed to achieve a statistical difference. However, BMP-containing device demonstrated advantages over autograft bone, including a reduction in the amount of operative blood loss, decreased incidence of osteomyelitis at the surgical site, elimination of donor-site complications and pain, as well as a decrease in the use of postoperative pain medication [56]. Figure 6 shows the radiograph analysis (pre, 6 months, 5 years, and 10 years, respectively) of the first tibial nonunion patient who received a recombinant OP-1 Implant®. This patient was a 19-y/o who fractured his tibia through motorcycle accident and underwent several reconstructive procedures including bone graft substitutes. The OP-1 Putty® device was evaluated in a posterolateral fusion (PLF) clinical study, wherein outcomes measured at 12 months of follow-up showed promise but did not meet a statistical difference, and again received an HDE approval for use as an alternative to autograft [57].

Regulatory agencies, clinical and patient communities, and payers are concerned with off-label use of current BMP products. The concern is centered on the high dose of BMPs (e.g., hrBMP-2 applied 12–40 mg for single-level fusion), the use of

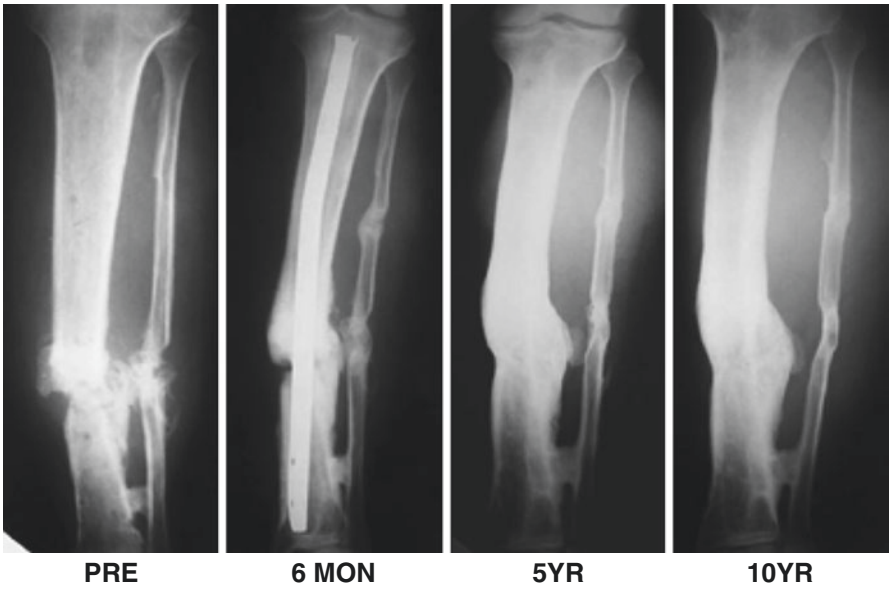


Fig. 6 First patient OP-1® Implant: 10-year clinical follow-up

animal-sourced collagen (bovine type I collagen), and synthetic ceramics (hydroxyapatite and tricalcium phosphate) as substratum to deliver rhBMP-2 at the implant site [58, 59]. Animal-sourced collagens and ceramics as carriers induce inflammatory cytokine release and immune reactions at the local implant sites. Lower doses of BMPs with appropriate biocompatible and bio-friendly scaffold may provide the optimal bone formation without provoking unwanted ectopic bone formation. Future BMP studies should be directed to utilize BMPs such as BMP-6 that have less affinity to endogenous BMP antagonists (e.g., noggin) [60] and delivered with an autologous substratum, which does not provoke inflammatory signals and immune responses.

10 Conclusion

BMPs were originally purified from bone extracts employing a composite signal-scaffold matrix based on subcutaneous implant assay for bone induction. By utilizing the primary amino acid sequences obtained from purified bovine bone-inductive proteins, several BMP genes have been identified from human cDNA and genomic libraries. They are called BMPs and GDFs and constitute a large family of the TGF-beta superfamily of proteins. BMP proteins are highly conserved from fly to mammals, expressed in many organs during embryogenesis, which can be recapitulated during adult tissue repair. BMPs signal through a set of specific Ser-Thr kinase receptors and act under the influence of a concentration gradient, which is governed by extracellular matrix proteins and a family of cysteine-knot proteins, called BMP antagonists. Though recombinant BMP protein-containing osteogenic devices are approved for therapeutic use in orthopedic medicine, there are numerous challenges due to the high doses employed, lack of autologous scaffold for sustained release, and need for adjunct instrumentation for biomechanical stability.

References

1. Senn N (1989) On the healing of aseptic bone cavities by implantation of antiseptic decalcified bone. Am J Med Forensic Sci 98:219–243
2. Lacroix P (1945) Recent investigations on the growth of bone. Nature 156:576–577
3. Urist MR (1965) Bone formation by auto induction. Science 150:893–899
4. Huggins CB, Urist MR (1970) Dentin matrix transformation: rapid induction of alkaline phosphatase and cartilage. Science 6:896–898
5. Reddi AH, Huggins CB (1972) Biochemical sequence in the transformation of fibroblasts into cartilage and bone. Proc Natl Acad Sci U S A 69:1601–1605
6. Sampath TK, Reddi AH (1981) Dissociative extraction and reconstitution of extracellular matrix components involved in local bone differentiation. Proc Natl Acad Sci U S A 78:7599–7602
7. Sampath TK, Reddi AH (1983) Homology of bone inductive proteins from human, monkey, bovine and rat extracellular matrix. Proc Natl Acad Sci U S A 80:6591–6595

8. Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Kriz RW, Hewick RM, Wang EA (1988) Novel regulators of bone formation: molecular clones and activities. Science 242:1528–1534
9. Reddi AH (1996) BMP-1: resurrection as procollagen C-proteinase. Science 27:463
10. Sampath TK, Coughlin JE, Whetstone RM, Banach D, Corbett C, Ridge RJ, Ozkaynak E, Oppermann H, Rueger DC (1990) Bovine osteogenic protein is composed of dimers of OP-1 and BMP-2A, two members of the transforming growth factor-β superfamily. J Biol Chem 265:13198–13205
11. Ozkaynak E, Rueger DC, Drier EA, Corbett C, Ridge RJ, Sampath TK, Oppermann H (1990) OP-1 cDNA clones, an osteogenic protein in the TGF-β family. EMBO J 9:2085–2093
12. Lee SJ (1990) Identification of a novel member (GDF-1) of the transforming growth factor-beta superfamily. Mol Endocrinol 4(7):1034–1040
13. Chang SC, Hoang B, Thomas JT, Vukicevic S, Luyten FP, Ryba NJ, Kozak CA, Reddi AH, Moos M Jr (1994) Cartilage-derived morphogenetic proteins. New members of the transforming growth factor-beta superfamily predominantly expressed in long bones during human embryonic development. J Biol Chem 269:28227–28234
14. Massague J (1990) The transforming growth factor-β family. Annu Rev Cell Biol 6:597–641
15. Hogan BL (1996) Bone morphogenetic proteins in development. Curr Opin Genet Dev 6:432–443
16. Sampath TK, Rashka KE, Doctor JS, Tucker RF, Hoffmann FM (1993) Drosophila transforming growth factor β superfamily proteins induce endochondral bone formation in mammals. Proc Natl Acad Sci U S A 90:6004–6008
17. Sampath TK, Rueger DC (1994) Structure, function and orthopedic application of osteogenic protein-1 (OP-1). Complicat Orthopedics 9:101–107
18. Sampath TK, Maliakal JC, Hauschka PV, Jones WK, Sasak H, Tucker RF, White KH, Coughlin JE, Tucker MM, Pang RH et al (1992) Recombinant human osteogenic protein-1 (hOP-1) induces new bone formation in vivo with a specific activity comparable with natural bovine osteogenic protein and stimulates osteoblast proliferation and differentiation in vitro. J Biol Chem 267:20352–20362
19. ten Dijke P, Yamashita H, Sampath TK, Reddi AH, Estevez M, Riddle DL, Ichijo H, Heldin C-H, Miyazono K (1994) Identification of type I receptors for osteogenic protein-1 and bone morphogenetic protein-4. J Biol Chem 269:16985–16988
20. Massagué J (1998) TGF-β signal transduction. Annu Rev Biochem 67:753–791
21. Heldin C-H, Miyazono K, ten Dijke P (1997) TGF-β signaling from cell membrane to nucleus via Smad proteins. Nature 390:465–471
22. Yanagita M (2008) Bone morphogenetic protein antagonists and kidney. In: Vukicevic S, Sampath TK (eds) Bone morphogenetic proteins: from local to systemic therapeutics. Birkhauser, Basel, pp. 213–232
23. Yasko A, Lane J, Fellinger E, Rosen V, Wozney J, Wang E (1992) The healing of segmental defects induced by recombinant human morphogenetic protein (rhBMP-2). J Bone Joint Surg (USA) 74A:59–671
24. Boden S, Moskovitz P, Morone M, Toribitake Y (1996) Video-assisted lateral intertransverse process arthrodesis: validation of a new minimally invasive lumbar spinal fusion technique in the rabbit and nonhuman primate (rhesus) models. Spine 21:2689–2697
25. Cook SD, Wolfe M, Salkeld S, Rueger D (1995) Recombinant human osteogenic protein-1 (rhOP-1) heals segmental defects in nonhuman primates. J Bone Joint Surg (USA) 77A:734–750
26. Cook SD, Rueger DC (2002) Preclinical models of recombinant BMP induced healing of orthopedic defects. In: Vukicevic S, Sampath TK (eds) Bone morphogenetic proteins: from laboratory to clinical practice. Birkhauser Verlag, Basel
27. Vukicevic S, Oppermann H, Verbanac D, Jankolija M, Popek I, Curak J, Brkljacic J, Pauk M, Erjavec I, Francetic I, Dumic-Cule I, Jelic M, Durdevic D, Vlahovic T, Novak R, Kufner V, Bordukalo Niksic T, Kozlovic M, Banic Tomisic Z, Bubic-Spoljar J, Bastalic I, Vikic-Topic S, Peric M, Pecina M, Grgurevic L (2014) The clinical use of bone morphogenetic proteins revis-

ited: a novel biocompatible carrier device OSTEOGROW for bone healing. Int Orthop 38:635–431
28. Chen P, Vukicevic S, Sampath TK, Luyten FP (1993) Bovine articular chondrocytes do not undergo hypertrophy when cultured in the presence of serum and osteogenic protein-1. Biochem Biophys Res Commun 197:1253–1259
29. Flechtenmacher J, Huch K, Thonar EJ, Mollenhauer JA, Davies SR, Schmid TM, Puhl W, Sampath TK, Adelotte MB, Kuettner KE (1996) Recombinant human osteogenic protein-1 is a potent stimulator of the synthesis of cartilage proteoglycans and collagens by human articular chondrocytes. Arthritis Rheum 39:1896–1904
30. Sellers RS, Peluso D, Morris EA (1997) The effect of recombinant human bone morphogenetic protein-2 (rhBMP-2) on the healing of full-thickness defects of articular cartilage. J Bone J Surg Am 79-A(10):1452–1463
31. Cook SD, Patron LP, Salkeld RDC (2003) Repair of articular cartilage defects with osteogenic protein-1 (BMP-7) in dogs. J Bone J Surg Am 85-A(Suppl 3):116–123
32. Lietman S, Yanagishita M, Sampath TK, Reddi AH (1997) Stimulation of proteoglycan synthesis in explants of porcine articular cartilage by recombinant osteogenic protein-1. J Bone J Surg Am 79-A(10):1132–1137
33. Rueger DC, Chubinskaya S (2004) Bone morphogenetic proteins in articular cartilage repair. In: Vukicevic S, Sampath TK (eds) Bone morphogenetic proteins: regeneration of bone and beyond. Birkhauser Verlag, Basel
34. Jelic M, Pecina M, Haspl M, Kos J, Taylor K, Marticic D, McCartney J, Yin S, Rueger D, Vukicevic S (2001) Regeneration of articular cartilage chondral defects by osteogenic protein-1 (bone morphogenetic protein-7) in sheep. Growth Factors 19:101–113
35. Hayashi M, Muneta T, Takahashi T, Ju YJ, Tsuji K, Sekiya I (2010) Intra-articular injections of bone morphogenetic protein-7 retard progression of existing cartilage degeneration. J Orthop Res 28:1502–1506
36. Hunter DJ, Pike MC, Jonas BL, Kissin E, Krop J, McAlindon T (2010) Phase 1 safety and tolerability study of BMP-7 in symptomatic knee osteoarthritis. BMC Musculoskelet Disord 11:232
37. Ozkaynak E, Schnegelsberg PNJ, Oppermann H (1991) Murine osteogenic protein (OP-1): high levels of mRNA in kidney. Biochem Biophys Res Commun 179:116–123
38. Vukicevic S, Latin V, Chen P, Batorsky R, Reddi AH, Sampath TK. Localization of osteogenic protein-1 (bone morphogenetic prtoien-7) during human embryonic development: high affinity to basement membrane. Biochem Biophys Res Commun. 1994;198:693–700.
39. Reddi AH. Role of morphogenetic proteins in skeletal tissue engineering and regeneration. Nat Biotechnol. 1998;16:247–52
40. Vukicevic S, Sampath TK (eds) (2008) Bone morphogenetic proteins: from local to systemic therapeutics. Birkhauser Verlag, Basel
41. Zhang H, Bradley A (1996) Mice deficient for BMP2 are nonviable and have defects in amnion/chorion and cardiac development. Development 122:2977–2986
42. Bellusci S, Henderson R, Winnier G, Oikawa T, Hogan BL (1996) Evidence from normal expression and targeted misexpression that bone morphogenetic protein (Bmp-4) plays a role in mouse embryonic lung morphogenesis. Development 122:1693–1702
43. Vukicevic S, Kopp JB, Luyten FP, Sampath TK (1996) Induction of nephrogenic mesenchyme by osteogenic protein 1 (bone morphogenetic protein 7). Proc Natl Acad Sci U S A 93:9021–9026
44. Vukicevic S, Basic V, Rogic D, Basic N, Shih MS, Shepard A, Jin D, Dattatreyamurty B, Jones W, Dorai H, Ryan S, Griffiths D, Maliakal J, Jelic M, Pastorcic M, Stavljenic A, Sampath TK (1998) Osteogenic protein-1 (bone morphogenetic protein-7) reduces severity of injury after ischemic acute renal failure in rat. J Clin Invest 102:202–214
45. Andriopoulos B Jr, Corradini E, Xia Y, Faasse SA, Chen S, Grgurevic L, Knutson MD, Pietrangelo A, Vukicevic S, Lin HY, Babitt JL (2009) BMP6 is a key endogenous regulator of hepcidin expression and iron metabolism. Nat Genet 41:482–467

46. McPherron AC, Lawler AM, Lee SJ (1997) Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. Nature 387:83–90
47. Sinha M, Jang YC, Oh J, Khong D, Wu EY, Manohar R, Miller C, Regalado SG, Loffredo FS, Pancoast JR, Hirshman MF, Lebowitz J, Shadrach JL, Cerletti M, Kim MJ, Serwold T, Goodyear LJ, Rosner B, Lee RT, Wagers AJ (2014) Restoring systemic GDF11 levels reverses age-related dysfunction in mouse skeletal muscle. Science 344:649–652.
48. Medtronic Sofamor Danek USA, Inc. INFUSE Bone Graft product information: Oral/Facial. Memphis2006. Available online at www.accessdata.fda.gov/cdrh_docs/pdf5/P050 053c.pdf. Last accessed Feb 2010.
49. Stryker Biotech. OP-1 Implant® product information. Hopkinton; 2009. Available online at www.stryker.com/stellent/groups/public/documents/web_prod/126737.pdf. Last accessed Feb 2010.
50. Stryker Biotech. OP-1 Putty® product information. Hopkinton; 2009. Available online at www.stryker.com/stellent/groups/public/documents/web_prod/127024.pdf. Last accessed Feb 2010.
51. Boden SD, Zdeblick TA, Sandhu HS et al (2000) The use of rhBMP-2 in interbody fusion cages. Definitive evidence of osteoinduction in humans: a preliminary report. Spine 25:376–381
52. Burkus JK, Gornet MF, Dickman CA et al (2002) Anterior lumbar interbody fusion using rhBMP-2 with tapered interbody cages. J Spinal Disord Tech 15:337–349
53. Burkus JK, Dorchak JD, Sanders DL (2003) Radiographic assessment of interbody fusion using recombinant human bone morphogenetic protein type 2. Spine 28:372–377
54. Dawson E, Bae HW, Burkus JK et al (2009) Recombinant human bone morphogenetic protein-2 on an absorbable collagen sponge with an osteoconductive-bulking agent in posterolateral arthrodesis with instrumentation. A prospective randomized trial. J Bone Joint Surg Am 91:1604–1613
55. Dimar JR 2nd, Glassman SD, Burkus JK et al (2009) Clinical and radiographic analysis of an optimized rhBMP-2 formulation as an autograft replacement in posterolateral lumbar spine arthrodesis. J Bone Joint Surg Am 91:1377–1386
56. Friedlaender GE, Perry CR, Cole JD, Cook SD, Clerny G, Muschler GF, Zych GA, Calhoun JH, LaForte AJ, Yin S (2001). Osteogenic protein-1 (bone morphogenetic protein-7) in the treatment of tibial nonunions: a prospective randomized clinical trial comparing rhOP-1 with fresh bone autograft. J Bone Joint Surg Am 83(Suppl 1):S151–8. Vaccaro AR, Patel T, Fischgrund J, et al (2004). A pilot study evaluating the safety and efficacy of OP-1 Putty (rhBMP-7) as a replacement for iliac crest autograft in posterolateral lumbar arthrodesis for degenerative spondylolisthesis. Spine 29:1885–92.
57. Vaccaro AR, Lawrence JP, Patel T et al (2008) The safety and efficacy of OP-1 (rhBMP-7) as a replacement for iliac crest autograft in posterolateral lumbar arthrodesis: a long-term (>4 years) pivotal study. Spine 33:2850–2862
58. Wong DA, Kumar A, Jatana S et al (2008) Neurologic impairment from ectopic bone in the lumbar canal: a potential complication of off-label PLIF/TLIF use of bone morphogenetic protein-2 (BMP-2). Spine J 8:1011–1018
59. Govender PV, Rampersaud YR, Rickards L et al (2002) Use of osteogenic protein-1 in spinal fusion: literature review and preliminary results in a prospective series of high-risk cases. Neurosurg Focus 13:e4
60. Song K, Krause C, Shi S et al (2010) Identification of a key residue mediating bone morphogenetic protein (BMP)-6 resistance to noggin inhibition allows for engineered BMPs with superior agonist activity. J Biol Chem 285:12169–12180

The Systems Biology of Bone Morphogenetic Proteins

Kuber T. Sampath

Abstract BMPs are originally identified based on their ability to induce new bone *in vivo* and represent large members of the TGF-β superfamily of proteins. BMPs serve as inductive signals for cell migration, growth, and subsequently differentiation in many organ developments during embryogenesis and are shown to modulate inflammation, angiogenesis, and immune responses and thus provide biological cues for adult tissue repair, protection, and regeneration. BMP-2- and BMP-7-containing osteogenic devices have been approved for use as bone graft substitutes for spine fusion and long bone fractures. BMP-7 biology has been considered positively against parenchymal tissue fibrosis to improve function. In this chapter, I summarize the biology of BMPs to emphasize its (1) morphogenic role in skeletal tissue repair and regeneration; (2) modulatory role in curtailing inflammation, governing angiogenesis, suppressing apoptosis, and reducing fibrosis following immunological and mechanical insults; (3) metabolic role in glucose, calcium, and phosphate and iron homeostasis; and (4) cytoprotective role to maintain skeletal and vascular integrity. The importance of BMP biology is further corroborated in rare genetic disorders (e.g., pulmonary arterial hypertension, hemochromatosis, fibrodysplasia ossificans progressiva, and osteogenesis imperfecta) and in cancer.

Keywords BMP receptors and signaling • BMP antagonists • BMPs in cartilage, bone and dentin repair • BMP-7 in inflammation, angiogenesis and fibrosis • BMP-7 in caclium and posphate homeostasis • BMPs in diabetes • BMP type II receptor in pulmonary arterial hypertension • BMP-6 in hemochromatosis and anemia • BMP signaling in skeletal rare disorders

1 BMPs During Development

BMPs are potent chemoattractants (motogens) [1, 2], mitogens [3], and morphogens [4, 5] which act across a concentration gradient during embryogenesis [6, 7]. BMPs recruit stem cells and determine the fate of the responding cells to undergo

K.T. Sampath, PhD
perForm biologics Inc., Holliston, MA 01746, USA
e-mail: kuber.sampath@performbiologics.com; kuber.sampath@gmail.com

S. Vukicevic, K.T. Sampath (eds.), *Bone Morphogenetic Proteins: Systems Biology Regulators*, Progress in Inflammation Research,
DOI 10.1007/978-3-319-47507-3_2

condensation (proliferation) and subsequently trigger their differentiation by serving as an inductive signal at specific tissue compartment in order to promote morphogenesis. During embryogenesis, in general, ectoderm expresses BMPs as secretary proteins, which bind to extracellular matrix (e.g., heparin sulfate proteoglycans and type IV collagen) and specific BMP antagonists and subsequently released as needed for mesoderm to respond. The cells that express BMPs also express BMP antagonists in order to establish a concentration gradient for ligand-receptor binding to induce downstream signaling [7, 8]. For example, during embryogenesis, the ureteric bud synthesizes BMP-7 and nephrogenic mesenchyme response to it, which then undergo condensation and differentiation into S- and comma-shaped tubules that become a functional nephron [9, 10]. Likewise, BMP-2 is required for cardiac mesoderm condensation and morphogenesis [11], while BMP-4 is responsible for lung epithelial morphogenesis [12]. Hence, the loss of function of BMP-2 and BMP-4 is embryonically lethal, and they die early at days 11–14 of embryo due to impaired cardiac function, whereas the loss of BMP-7 function results in death during birth due to the lack of functional kidney. The BMP signal-based tissue morphogenesis is so tightly controlled in space and time during embryogenesis, and thus the loss of a given BMP function at given tissue compartment can result in tissue malformation. Furthermore, BMP signaling cross talks with TGF-beta and activin signaling, the other members of TGF-beta superfamily proteins, as well as with Wnt and hedgehog signaling to govern tissue morphogenesis [8, 13].

BMPs are responsible for endochondral bone formation during development, and the cellular events that are responsible for embryonic endochondral ossification can be recapitulated in postnatal life by implanting an osteogenic BMP with appropriate collagenous scaffold at subcutaneous sites to induce mesenchymal cell migration, proliferation, and differentiation to form the cartilage and bone [14–16]. The biological function of BMP is concentration-dependent, the lower amount is motogenic (chemotaxis), medium concentrations are mitogenic (proliferation), and higher concentrations are morphogenic (differentiation). The biological activities of BMPs with respect to chemotaxis, proliferation, and differentiation have been demonstrated in vitro using Boyden chamber, cell proliferation, and differentiation assays in cultures using a BMP and responding mesenchymal stem cells. The role of BMPs and its canonical downstream signaling with cross talk with Wnt signaling during embryonic skeletal and craniofacial development and osteo- and dentinogenesis are described in the chapter on "Embryonic Skeletogenesis and Craniofacial Development".

2 Structure and Function

BMPs are homodimers, and all have the hallmark of "7-cysteine domain" held by an inter-disulfide bridge at the fourth cysteine between two monomers and are highly conserved from fly to humans. BMPs are produced as a large precursor with

signal peptide, pro-domain, and mature "7-cystein TGF-beta domain." They synthesized as monomer with three intra-disulfide bridges and then undergo dimerization in endoplasmic reticulum by forming inter-disulfide bridge at the fourth cysteine and processing at RXXR site before they are secreted into extracellular space [17, 18]. The secreted BMP protein is a dimer at the mature TGF-beta domain, which is biologically active, whereas pro-domain is not active but can interact with mature, processed dimer by non-covalent interactions. The mature protein loses its biological activity if inter-disulfide bridge is broken. The crystal structure reveals that the BMP dimer is aligned antiparallel with Finger 1 and Finger 2 and Heal region [19]. A cysteine knot with intra- and inter-disulfide bridges holds the dimer protein, and because of this, it is very stable, even against proteases like trypsin.

BMPs signal through ser-thr kinase receptors type I and type II. Although both type I and type II bind to the ligand and form a complex, type I receptor renders specificity and recruits intracellular kinases signaling SMAD-1/5/8 and subsequently triggers phosphorylation, which forms a complex with a co-smad-4 and translocates into the nucleus to switch on and off a set of genes responsible for tissue morphogenesis, repair, and regeneration [20]. ALK-2, ALK-3, and ALK-6 are known BMP-specific type I receptors, and BMPRII, ActRII-A, and ActRII-B serve as type II receptors; BMPs employ a specific type I receptor and type II receptor depending on the cell type and type of cellular responses it triggers [21]. There are several BMP co-receptors that have been described to activate or inhibit BMP signaling to trigger specific cellular function and outcome [22]. These include the Dragon family of protein, hemojuvelin, receptor tyrosine kinases (RTKs) TrkC, TGF-β type III receptors, BAMBI, betaglycan, and endoglin. Two downstream inhibitors, smads 6 and 7, are identified to play a functional role as checkpoints by de-plugging the BMP downstream signaling to modulate the biological activity. BMP ligands can also trigger non-canonical downstream signaling directly or indirectly that are SMAD independent, such as MAPK, ERK, NK, p38, PI3K, Akt, RANK and RANKL, as well as substantial cross talk with the Wnt, hedgehog, and VEGF signaling cascades. In addition, known BMP antagonists like noggin, chordin, follistatin, gremlin, sclerostin, and USAG-1 are shown to govern the availability of BMP ligand to its receptor by binding avidly at the extracellular space to render specificity and establish a concentration gradient [23]. For more details, refer to the chapter on "BMP and BMP Regulation: Structure and Function".

3 BMP: In Vitro and In Vivo Model Systems for Endochondral Bone Differentiation

The systems biology of BMP with respect to skeletal tissue morphogenesis has been well documented in vivo [24]. The embryonic cellular events that culminate the formation of the new cartilage and bone can be recapitulated in post-fetal life by

implanting an osteogenic BMP (e.g., BMP-2, BMP-4, BMP-6, and BMP-7) with a carrier in the rat subcutaneous site and in diaphysis fracture, segmental defect, and lumbar spine fusion models. The presence of BMP is a must in the implant in order to attract sufficient amount of mesenchymal stem cells and induce proliferation and differentiation into the bone.

In Vitro Model Systems Several in vitro cell cultures have been used to examine BMP-like activity. Primary cultures generated from the chick [25] and mouse limb bud [26], synovial tissue [27], periosteum [28], primary bovine articular chondrocytes [29], calvarial-derived primary osteoblasts [16], established rat osteosarcoma cell lines [30], C2C12 mouse myoblast cell line [31], and bone marrow-derived W-29 stromal cells [32] have been routinely employed. To examine for chondrogenic and osteogenic responses, the early responsive genes like *id*-1, *id*-2, and *id*-3 [33], differentiation determinants like *sox*-5 and *sox*-9 [34] for chondrocyte and *osterix* and *Runs*-2 for osteoblast [35, 36], markers of chondrocyte phenotype like type II collagen and cartilage-specific proteoglycan [37], and markers of osteoblast phenotype, alkaline phosphatase, and osteocalcin are routinely monitored [16]. Identification of BMP-responding elements in the promotor region of the BMP-SMAD-dependent responding genes has allowed to engineer several established stable cell lines linking with luciferase enzyme to specifically qualify the biological activity of BMP from cell and tissue extracts and body fluids and for release assays for the recombinant BMP production [38]. Furthermore, pluripotent stem cells generated from patients from musculoskeletal disorder are being employed to drive chondrogenesis and osteogenesis in order to understand the loss or gain of function and to establish screens to select small molecules [39]. For more information, refer to the chapter "Novel In Vitro Assay Models to Study Osteogenesis and Chondrogenesis for Human Skeletal Disorders".

In Vivo Model Systems BMP alone when implanted with an appropriate collagenous matrix can induce new bone formation at ectopic or orthotopic sites. This serves as a prototype for tissue engineering [40]. BMP serves as signal and collagen serves as scaffold. The local implant site provides a microenvironment to recruit the responding cells, and they attach onto the collagenous scaffold in order to promote the differentiation into endochondral bone. This BMP-induced new bone formation is dose-dependent [16] up to certain doses based on a given substratum used; however, at a higher dose, BMP can trigger a more number of progenitors' recruitment and proliferation, which results in hematoma and cyst-like condensation and delays the differentiation into the bone. This high-dose cyst phenomenon is observed both in ectopic and orthotopic sites.

The most important component in BMP-based osteogenic device is scaffold. The current BMP-based osteogenic device utilizes bovine-derived collagen alone or in combination with ceramics (hydroxyapatite and tricalcium phosphate), and because of ceramics and an animal-derived collagen, the device triggers initially inflammation and immune responses and promotes the expression of makers associated with

fibroblast phenotype. In order to overcome this unwanted fibrogenic biology, high doses of BMP-2 (12–40 mg) are employed in the current osteogenic device. In addition, because of low affinity to collagen/ceramics, BMPs are diffused out readily from the implant site and induce unwanted ossification at the distant sites. These unwanted safety issues were observed in the clinical studies for posterolateral fusion which has been ascribed to a high dose of BMP and animal-derived collagen.

As the cells are prerequisite for BMP to signal, a situation wherein the site is compromised due to nonunion as seen in tibial diaphysis where the responding cells are not readily available in sufficient quantity, Efforts are being attempted to implant autologous bone marrow with BMP-containing scaffold. Autologous bone marrow-derived mesenchymal stem cells and periosteal-derived mesenchymal stem cells are also being considered for such BMP implants. It is likely that selecting autologous mesenchymal stem cells with specific cell surface markers that have high levels of BMP receptor expression at the cell surface may be beneficial to implant with a BMP and scaffold in certain rare indications like tibial nonunion, pseudo anthrosis and atypical fractures associated with long-term bisphosphonate or steroid use. The preferred components of bone tissue engineering are (1) BMP that lacks affinity for BMP antagonists as a signal, (2) autologous substratum (instead of animal-derived collagen), and (3) autologous responding cells, where they are short supplied. More details on this subject are discussed in the chapter "Towards Advanced Therapy Medicinal Products (ATMPs) Combining Bone Morphogenetic Proteins (BMP) and Cells for Bone Regeneration".

4 Role of BMPs in Cartilage Repair and Regeneration

The therapeutic engineering of tissue formation requires three biological components: signaling molecules, responding cells, scaffold and permissive microenvironment. Carticel® (autologous chondrocyte implantation, ACI), the first FDA-approved cell-based therapy for the articular cartilage repair, employs the autologous cells and the live periosteum as scaffold, two of the biological components required for tissue engineering [41]. Bone morphogenetic proteins (BMPs) are potent chondrogenic morphogens and are capable of inducing differentiation of MSCs into cell lineage of hyaline cartilage and maintenance of the expression of markers associated with chondrocyte phenotype *in vitro* and *in vivo* [42, 43]. Several studies have demonstrated that BMPs when applied alone or in combination with appropriate scaffold onto chondral or osteochondral defects are capable of inducing new articular cartilage formation *in vivo* [44]. However, the newly formed chondrocytes fail to maintain the cellular morphology and expression of articular cartilage phenotype over time, thus leading to the degeneration of the repaired tissue in the preclinical studies. It is likely that providing BMPs continuously or at periodic intervals instead of a one-time application in the beginning as used to repair bone fractures may induce sustainable cartilage differentiation readily and maintain the regenerated cartilage to attain articularization (surface, mid- and deeper zone) and function over

time under mechanical loading [45, 46]. The combination of responding cells with an appropriate scaffold and providing BMP signaling *in situ* will have added advantage in the enhancement of chondrocyte differentiation and maintenance of phenotypic expression in order to sustain function over long time. As BMP-2, BMP-4, BMP-6, and BMP-7 are more osteogenic and CDMP-1/GDF-5/BMP-14 and CDMP-2/GD-6/BMP-13 are more chondrogenic *in vitro* and *in vivo* model systems [42], it remains to be seen which BMP is likely to render an expected outcome in articular cartilage and intervertebral disk repair and regeneration in the human clinical trials.

The first human clinical trial for cartilage repair was conducted to evaluate BMP-7 to treat symptomatic knee OA with emphasis to reduce pain [47]. This was a double-blind, randomized, multicenter, placebo-controlled, single-dose escalation safety study that examined four doses 0 (placebo), 0.03, 0.1, 0.3, and 1.0 mg in 5 % lactose, injected intra-articularly, evaluated at 4, 8, 12, and 24 weeks. Patients receiving the BMP-7 injections at the midrange doses (0.1 and 0.3 mg) reported some symptomatic improvement, while high- and low-dose cohorts do not have the same. For more details, refer to the chapter "BMP Signaling in Articular Cartilage Repair and Regeneration: Potential Therapeutic Opportunity for Osteoarthritis".

5 Role of BMPs in Bone Repair and Regeneration

Several clinical trials have been conducted to assess the safety and efficacy of recombinant human BMP-containing devices for the treatment of acute diaphysis bone fractures and delayed union, tibial nonunion, and anterior lumbar interbody fusion (ALIF) and posterolateral lumbar fusion (PLF). Two BMP products, rhBMP2 (InFUSE®) [48] and rhBMP-7 (OP-1® [49] and OP-1 Putty®) [50], are licensed under PMA and HDE for marketing and clinical application in the USA.

OP-1® Implant: The first human clinical study was performed to assess the efficacy of recombinant human rhBMP-7 (OP-1®) for the treatment of tibial nonunion in a prospective, randomized, and controlled clinical trial [51]. The conclusion of this clinical study demonstrated that OP-1® Implant was a safe and effective treatment modality for tibial nonunion and the outcome was comparable to the use of bone autograft but failed to achieve a statistical significance as the number of patients included in the study is not sufficient, and because of this, it has gotten only HDE approval in the USA.

OP-1 Putty®: It is an OP-1® Implant containing 230 mg of sterile carboxymethyl cellulose to provide putty-like property. The OP-1 Putty® device was evaluated in the PLF clinical study to treat symptomatic single-level degenerative lumbar spondylolisthesis and spinal stenosis without instrumentation [52, 53]. Outcomes measured at 12 months of follow-up showed a promise but did not again meet a statistical difference. Therefore, OP-1 Putty® received again HDE approval for use as an alternative to autograft in compromised patients requiring revision posterolateral (inter-transverse) lumbar spinal fusion.

InFUSE® (rhBMP-2) was approved by FDA via premarketing approval (PMA) process, in conjunction with the LT-Cage Lumbar Tapered Fusion device for spinal fusion procedures via an anterior approach; the specific indication is for spinal fusion procedures in skeletally mature patients with degenerative disk disease (DDD) at one level from L2-S1 [54–56]. However, large clinical studies conducted using a high dose (40 mg/single-level fusion) of InFUSE® with compressive resistant matrices bulking agents (Amplify™) did not result in a positive outcome; autologous ICBG was used as comparator [57, 58].

The FDA issued a public health notification regarding life-threatening complications associated with InFUSE® in cervical spine fusion used as off-label [59]. These complications were associated with swelling of the neck and throat tissue, which resulted in compression of the airway and/or neurological structures in the neck. Some reports described difficulty in swallowing, breathing, or speaking. Though fewer documented adverse events can be attributed to BMP, certain complications and safety issues are of concern. Adverse events that have been reported include but are not limited to inflammation, unwanted ectopic bone formation, infection, immune responses, vertebral osteolysis, and vertebral edema.

Regulatory agencies, clinical and patient communities, and payers are concerned with the off-label use of current BMP products. The concern is centered on whopping dose of BMPs (e.g., hrBMP-2 applied 12–40 mg for single-level fusion) and the use of animal-sourced collagen (bovine type I collagen) and synthetic ceramics (hydroxyapatite and tricalcium phosphate) as substratum to deliver rhBMP-2 at the implant site [60]. Animal-sourced collagens and ceramics as carriers induce inflammatory cytokine release and immune reactions at the local implant sites. Lower doses of BMPs with appropriate biocompatible and bio-friendly autologous scaffold may provide the optimal bone formation without provoking unwanted ectopic bone formation detailed in the chapter "Osteogrow: A Novel Bone Graft Substitute for Orthopedic Reconstruction." Future BMP studies are directed to utilize BMPs that have little or no affinity to endogenous BMP antagonists [61] and delivered with an autologous substratum, which does not provoke inflammatory signals and immune responses. For more details, refer to the chapters "BMPs in Orthopedic Medicine: Promises and Challenges" and "Biology of Spine Fusion and Application of Osteobiologics in Spine Surgery".

6 BMPs in Dentin Repair and Regeneration

Although autograft is a gold standard in dental medicine, because of donor site-associated mobility, BMP-containing bone graft substitutes (BGS) are preferred as it provides robust therapeutic benefit than osteoinductive (e.g., DBM) and osteoconductive (e.g., HA/TCP) biomaterials [62, 63]. The application of BMP-based BGS has its clinical utility in several dentin indications that include alveolar ridge and maxillary sinus augmentation, alveolar cleft and mandibular reconstruction, osteointegration following dentin implants, and periodontium repair. BMP-2- and BMP-7-containing

collagen implants and GDF-5-containing hyaluronic implants have been evaluated in dentin preclinical models and in the clinic for various dental indications [64–66]. Obtaining a robust bone formation to speed up the osteointegration for dental implants and avoiding ankyloses to regenerate periodontium with new cementum, ligaments containing sharpie fibers and regeneration of alveolar bone are unmet needs in dental medicine [67]. Application of a given BMP with an appropriate dose and acceptable autologous scaffold in a permissive microenvironment is lacking. The promises and challenges still remain in order to deliver BMP locally with a bio-scaffold that allows lesser inflammation and immune responses and thus allow dental tissue repair and regeneration in space and time. It is unlikely the same dose and same bio-scaffold will serve as therapeutic benefit for all the dental tissue repair and regeneration. For details refer to the chapter "BMPs in Dental Medicine: Promises and Challenges".

7 BMP-7 in Acute and Chronic Kidney Failure

Although BMP-7 is originally isolated from bone matrix, the predominant site for its synthesis is the kidney [68]. The loss-of-function studies revealed that it is absolutely required for kidney development during embryogenesis [69] and it plays a functional role in the adult kidney and is responsible for vascular and skeletal integrity and modulates calcium and phosphate homeostasis. In preclinical studies, BMP-7 has been shown to provide protection against acute kidney injury (AKI) [70], glomerulosclerosis, diabetic nephropathy, chronic kidney disease (CKD), renal osteodystrophy, lupus nephropathy, and Alport's syndrome [71, 72]. BMP-7 is available in circulation, and its level correlates with renal function. The mechanism of action studies indicates that BMP-7 suppresses inflammation, improves renal blood flow, preserves tubular structure, reduces interstitial fibrosis, and governs calcium and phosphate homeostasis and subsequently vascular calcification by improving disordered bone remodeling. As BMP-7 is a potent bone-inducing morphogenic protein and forms ectopic ossification at the injection sites, it is believed that enhancing its biology through mimetics and secretagogoues may provide a safe and viable therapy than administering BMP-7 protein systemically. More details can be found in the chapter "Bone Morphogenetic Protein-7 and Its Role in Acute Kidney Injury and Chronic Kidney Failure".

8 BMPs in Glucose Homeostasis

By employing a functional genomic approach, BMP-9, expressed in the liver, was first identified as a factor that regulates glucose homeostasis as it was shown to suppress hepatic glucose production to reduce insulin resistance and glycemia in diabetic mice [73]. In concurrence with the observation that the kidney is a major site for BMP-7 expression, it serves as autocrine survival factor for podocytes [74]

and maintains expression of structural proteins of the foot processes such as synaptopodin and podocin. BMP-7 also inhibits the TGF-β1-activated signaling pathway in mesangial cells and podocytes *in vitro*. In preclinical models of diabetic nephropathy, BMP-7 was shown to attenuate tubular pro-inflammatory responses by suppressing oxidative stress and multiple inflammatory signaling pathways in the mesangium and proximal tubular epithelium [75]. It is likely that BMP-7 may be useful in delaying diabetic glomerulosclerosis and reversing early podocyte injury. To support BMP-7 biology role in diabetics, a recent study indicates that removal of USAGA-1/Sostdc1, a BMP-7 antagonist, is able to enhance insulin secretion and glucose homeostasis by improving β-cell function under metabolic stress [76]. A metabolic approach of managing glucose homeostasis is through systemic energy homeostasis. Brown adipose tissue (BAT) is responsible for energy utilization by promoting thermogenesis [77]. Again BMP-7 has been shown to promote BAT differentiation and promote thermogenesis *in vitro* and *in vivo* suggesting a therapeutic role against obesity [78] and thus to improve glucose uptake and reduce insulin sensitivity. For details, refer to the chapter "Role of BMPs in Inflammation."

9 BMP-7 and Calcium and Phosphate Homeostasis

The kidney is the site for the production of active 1,25-dihydroxy vitamin D3 from its precursor 25-dihroxy vitamin D3, and the loss of renal function results in vitamin D deficiency (Rickets) which then leads to secondary parathyroidism. The secondary hyperparathyroidism occurs in CKD, which produces a high turnover osteodystrophy that is associated with peritrabecular fibrosis. In animal models of CKD, BMP-7 treatment was shown to eliminate peritrabecular fibrosis, increased "active" osteoblast number, osteoblast surface, mineralizing surface, and significant decrease in the eroded surface [79, 80]. Loss of renal function is also associated with hyperphosphatemia and elevated calcium x phosphate (Ca x P) product, leading to vascular stiffness, dysfunction, and calcification. Hyperphosphatemia has been a known predictor of cardiovascular death, particularly in hemodialysis patients. Vascular smooth muscle cells (VSMC) are very responsive to changes in elevated serum phosphate and undergo a loss of phenotypic expression and differentiate into cell types of the osteoblast lineage. Although phosphate is managed through binders, it is becoming increasingly important to improve vascular tone and elastic modulus of vessel in ESRD patients. Hyperphosphatemia induces the loss of phenotype in VSMCs and induces dedifferentiation into myofibroblast and subsequently their proliferation in culture. In CKD models of hyperphosphatemia, BMP-7 treatment reduces the loss of VSMC phenotype and vascular calcification [81]. The effect of BMP-7 on osteoblast differentiation also reduces the systemic phosphate level thus indirectly has a positive influence on reducing phosphate levels in circulation. In summary, application of BMP-7 biology agonists may likely reduce hyperphosphatemia, secondary parathyroidism-associated osteodystrophy (osteitis fibrosa), and

the loss of VSMC phenotype, thus reducing vascular stiffness, dysfunction, and calcification, bone pain, and high fracture incidence in patients with loss of kidney function.

10 BMPs in Iron Homeostasis

Currently, erythropoiesis-stimulating agents (ESA) like erythropoietin, EPO, or iron supplements have been used to manage anemia in CKD/ESRD patients. About 1/3 of patients, however, do not respond to EPO. Oral dietary iron serves as an alternative but is not effective, and IV iron supplement provides some relief but does not overcome anemia successfully. High doses of EPO to manage anemia led to cardiovascular events, stroke, progression of cancer, and death, and because of this, the FDA issued black box warning on the EPO label. Patients nonresponsive to IV iron and EPO end up in iron overloading that associates with high levels of hepcidin in the blood.

Hepcidin is the iron regulatory hormone (25 amino acid peptides), and its expression is regulated tightly by circulating iron levels [82, 83]. Hepcidin is a ligand for ferroportin, an iron exporter [84]. Upon binding to ferroportin, hepcidin induces an internalization (endocytosis) and subsequently its degradation (proteolysis in lysosomes) [85]. Hepcidin inhibits the export of iron from enterocytes in the duodenum (obtained through dietary intake), reticular endothelial macrophages (recycled through senescent erythrocytes), and hepatocytes (stored intracellularly through ferritin) into the plasma. High level of hepcidin results in "anemia," and low level of hepcidin results in "hemochromatosis," a rare hematological disorder.

BMP-6 has been shown to regulate the expression of hepcidin through its downstream smad-1/5/8-dependent pathway [86]. Hemojuvelin (HJV), a glycophospholipid inositol (GPI)-anchored membrane protein, functions as a co-receptor for BMP-6 to enhance the effectiveness of BMP signaling-dependent SMAD pathway to stimulate hepcidin expression by acting on its promoter [87].

Inflammatory cytokine IL-6/JAK2/STAT3 pathway can also stimulate hepcidin expression; however, BMP-HJV-SMAD pathway-based functional SMAD binding is necessary for IL-6/JAK2/STAT3 pathway to effectively enhance hepcidin expression. BMP-6 (−/−) knockout mice showed reduced hepcidin levels in circulation and resemble "hemochromatosis" phenotype [88, 89]. A similar phenotype was also observed in HJV (−/−) mice [90]. Recently, three heterozygous missense mutations in BMP-6 were identified in patients with unexplained iron overload; these mutations lead to loss of signaling to SMAD proteins and reduced hepcidin production [91].

Inhibition of BMP-HJV-SMAD pathway is therefore a novel target to reduce the production of hepcidin in the liver. There are several ways one could approach, for example, the use of a drug that can antagonize BMP signaling (dorsomorphin) and the use of BMP antagonist proteins like gremlin, anti-BMP-6-neutralizing monoclonal antibody, Fc-soluble ActRII-A receptor, activin/BMP/GDF ligand trap, anti-

hemojuvelin-neutralizing antibody, and Fc-soluble hemojuvelin, all of which may have some safety concerns, as they are not addressing the specific role of iron-sensing BMP-6 in regulating hepcidin expression with respect to iron homeostasis. For more details on the role of TGF-beta superfamily of proteins in iron homeostasis, refer to the chapter on "The Central Role of BMP Signaling in Regulating Iron Homeostasis".

11 BMPs' Role in Rare Genetic Disorders

Pulmonary Arterial Hypertension (PAH) PAH is a rare disease that occurs neonatal and young children due to poor vascular dilation and abnormal muscularization characterized by a progressive increase in pulmonary vascular resistance [92]. In older children and adults, abnormal vessel and enhanced muscularization occurs in the distal artery [93], all results in progressive intimal and medial thickening leading to occlusive changes and hence elevation in pulmonary arterial pressure [94]. An imbalance between vasodilators and vasoconstrictors has been linked to the onset of PAH [95]. Genetic studies showed a link to mutations in BMPRII among familial PAH (60 %) and idiopathic PAH (10 %–20 %) patients [96–98]. The mutations are spread along the ligand binding domain, kinase domain, and long cytoplasmic tail, all of which can affect negatively BMP-smad downstream signaling. *id*, a BMP-responding gene, is paramount in governing endothelial and smooth muscle cell growth, perturbing *id* expression will have consequences [99]. That said, there are people who have BMPRII mutations who do not develop PAH [100]. This makes sense that BMPs do also engage the other type II receptors, ActRII-A and ActRII-B, for signaling, and likely in the absence of functional BMPRII, these receptors may compensate function in certain PAH patients. BMP-9 and TGF-beta utilize ALK-1, type I receptor, and endoglin, a co-receptor to mediate signaling in endothelial cells (ECs). Mutations in endoglin have also been linked to hereditary hemorrhagic telangiectasia which has been linked in some patients with PAH [101, 102]. Overall, no doubt BMP signaling is paramount in governing normal growth of EC and SMC of the pulmonary artery, and perturbation of BMP-smad signaling may have detrimental effects for the onset of PAH. For more information, refer to the chapter on "BMP Signaling in Pulmonary Arterial Hypertension".

Hereditary Hemochromatosis (HH) HH is a genetic disorder of iron overload characterized by an excess iron entry into the bloodstream surpassing the requirements for erythropoiesis, resulting in tissue iron deposition and organ dysfunction [103]. As there is no regulated mechanism for the removal of excess iron from the body and the excess iron in patients with HH deposits in other tissues, most notably parenchymal cells of the liver, pancreas, heart, and pituitary gland generate reactive oxygen species leading to tissue damage and ultimately resulting in cirrhosis, diabetes, cardiomyopathy, hypogonadism, arthropathy, and increased skin pigmentation that is characteristic of this disease. Mutations in *hfe* gene are identified as a causal for HH [104–106]. *hfe* is atypical major histocompatibility class-I-like

protein [107] that competes with transferrin for binding to transferrin receptor-1 as well as transferrin receptor-2 (TRF1/TRF2). Hence, mutations in TRF1 or TRF2 can also result in HH. It is believed that TFR1 in the liver sequesters HFE and when serum levels increase, iron-saturated transferrin displaces *hfe* from TFF1; thereby, HFE can regulate hepcidin expression possibly by interacting with TRF2 [108, 109]. The precise mechanism by which *hfe* regulates hepcidin expression is still unknown. The loss of function of *hfe* studies in mice showed impaired BMP downstream smad signaling and low level of hepcidin expression. This is further corroborated that BMP-6 (−/−) mice and HJV (−/−) mice both exhibit hemochromatosis phenotype and have low level of hepcidin in circulation. For more details, refer to the chapter on "The Central Role of BMP Signaling in Regulating Iron Homeostasis".

Fibrodysplasia Ossificans Progressiva Fibrodysplasia ossificans progressiva (FOP) is a rare genetic disorder characterized by progressive extra-skeletal (heterotopic) ossification [110]. Patients with FOP develop progressive heterotopic ossification within soft connective tissues by recapitulating a developmental cascade of endochondral ossification in which cartilage forms initially at the lesion site and is subsequently replaced by the bone [111]. The effects of FOP are accelerated by inflammation and trauma, precluding surgical intervention, and there is an urgent need for an effective treatment. Linkage analysis has led to the identification of a recurrent heterozygous mutation (617G A; R206H) in the type I BMP receptor ALK-2 (*ACVR1*) [112, 113]. Additional FOP mutations have since been identified in both the GS and kinase domains of ALK-2 that differentially affect the age of onset of ossification, as well as the extent of skeletal malformation. Analyses of a subset of ALK-2 FOP mutants including L196P, R206H, and G356D suggest that FOP mutations are more weakly activating than constitutively active ALK-2, but show similar potential to induce osteogenic differentiation through reduced FKBP12 binding to ALK-2 and increased Smad1/5/8 phosphorylation [114]. A recent study suggests that nonenzymatic scaffolding function provided by type II receptors is required for mutant ALK-2 to exert its function independent of a BMP ligand [115].

The FOP condition can be recapitulated in cultures using muscle cell lines transfected with mutant ALK-2 and in animal models by transgenic overexpression of caALK-2 [116], a classic constitutively active ALK-2 receptor containing the artificial mutation Q207D and knock-in R206H mutation in mice [117]. Furthermore, pluripotent stem cells generated from FOP patients are also being pursued to screen for small molecules that could inhibit chondrocyte/osteoblast differentiation [118, 119]. By using a dorsalization function assay in zebrafish, researchers in Harvard (MGH/Brigham) have identified a BMP inhibitor called *dorsomorphin* that led to the development of LDN compounds which tend to render a specificity to ALK-2 kinase inhibition and functionally inhibit ALK-2 kinase activity *in vitro* and ectopic endochondral ossification in mutant ALK-2 FOP transgenic mouse model [120]. Based on ALK-2 crystal structure and kinase inhibition assay, researchers at Oxford have identified yet another BMP inhibitor specific to ALK-2 [121]. In addition to SM BMP inhibitors, researchers are looking at the possibility of intervening FOP

mutant ALK-2 activity using siRNA and/or antisense oligonucleotide. Attempts are also being made to inhibit the ectopic differentiation of endochondral ossification using retinoic acid receptor γ agonist [122], a potent stimulator of chondrocyte differentiation.

However, it remains to be established what are the cell types that are cued to manifest heterotopic ossification as a result of FOP-ALK-2 insult. Fascia/skeletal muscle-derived satellite cells/myoblasts, vascular endothelium-derived pericytes/smooth muscle cells, blood-borne inflammatory cells, and endothelial-mesenchymal transition, all of these are contemplated as potential responding cell types. Still it remains elusive how the mechanical/inflammatory signals promote the FOP-ALK-2 insult *in vivo*. A recent study suggests that anti-activin antibody and ActRII-A/ActRII-B trap are shown to provide therapeutic benefit against FOP mice [123]. For more details, refer to the chapter "BMP Signaling in Fibrodysplasia Ossificans Progressiva, a Rare Genetic Disorder of Heterotopic Ossification".

Osteogenesis Imperfecta Osteogenesis imperfecta (OI), also known as "brittle bone disease," is a collagen-related disorder characterized by low bone mass, increased bone fragility, and decreased bone strength. Dominant osteogenesis imperfecta is caused by defects in the quantity or quality (structure) of type I procollagen, which affects the bone at multiple levels, for example, matrix structure and mineralization. Recessive osteogenesis imperfecta is caused by deficiency of proteins that interact with collagen process collagen and/or affect its posttranslational modification or folding, such as CRTAP, P3H1, and PPIB and Serpin H1 and FKBP10 [124]. The common features of dominant and/or recessive osteogenesis imperfecta are delayed collagen folding and increased endoplasmic reticulum stress effects in the bone and are likely to be the key to understanding its pathogenesis. Bisphosphonates are widely administered to individuals with osteogenesis imperfecta, with positive effects on bone mass and vertebral geometry, but cause a decline in bone material quality in time [125]. In its various types, OI occurs in ~1 in 15,000 in the USA (~20,000–50,000) with mostly autosomal dominant inheritance (about 85 %) and lesser with autosomal recessive (15 %).

The clinical overlap in both dominant and recessive phenotypes of OI is comparable. A recent study for the first time demonstrated an excessive TGF-β signaling as evidenced by an increased ratio of pSMAD2/SMAD2 proteins and higher in vivo SMAD2 reporter activity that corresponds with higher expression of TGF-beta target genes. It is suggested that an alteration in collagen posttranslational modifications results in a dysregulation of matrix-cell signaling contributing to phenotype manifestation [126, 127]. Furthermore, anti-TGF-beta antibody (1D11) treatment demonstrated that treatments restored bone volume, trabecular number, trabecular thickness, and reduced trabecular separation in the lumbar and femur of OI mice comparable to WT mice. Biomechanical testing of femurs showed mice treated with the 1D11 showed significant improvements in bone strength as well. Hence, altered TGF-β matrix-cell signaling is a primary mechanism in the pathogenesis of OI.

As BMP downstream signaling counteracts TGF-β activity, it is likely that BMP biology may serve as therapeutic avenue for OI. To support this notion, recent study showed anti-sclerostin, a BMP antagonist, antibody also effectively restored OI phenotype in mice [128]. Genetic linkage studies found mutations in BMP-1 and collagen C-peptidase as a causal for OI in man [129]. BMP-1 is also responsible for processing certain BMP family proteins from pro-form into active and BMP antagonists like chordin [130]. Since BMPs have direct influence on the differentiation of both bone-forming (osteoblast) and bone-resorbing (osteoclast) cells and the bone undergoes a high turnover in OI skeleton, BMP biology-based therapy could be administered intermittently in combination with antiresorptive agents like bisphosphonate.

12 BMP in Oncology

The BMP signaling pathway involves many ligands, receptors, and antagonists extracellularly and downstream signaling smads-1/5/8 and co-smad-4 and inhibitory smads-6/7 intracellularly, all of which are capable of impacting tumor growth and progression, both positively and negatively [131]. The effects of BMP on tumor growth are based on specific BMP, are dose- and context-dependent, and are associated with either increased or decreased survival. For example, in ovarian carcinoma, the MSCs that recruited at the tumor microenvironment exhibit a phenotype that expresses high levels of BMP-2, BMP-4, and BMP-6 [132]. On the contrary, in primary mammary tumor, BMP-7 expression is reduced which is accompanied by enhanced TGF-beta activity and EMT transition that leads to bone metastasis [133]. Aberrant expression of BMP ligands and their respective receptors and subsequently dysregulation of downstream signaling can influence growth inhibitory genes (e.g., *id1-3*) [134] and tumor suppressor genes (e.g., *p53*) [135, 136] and promote epithelial-mesenchymal transition [137], stromal cell proliferation [132], angiogenesis [138], inflammation, and immunosuppression to promote tumor growth and metastasis. Depending on the tumor cell type (carcinoma versus sarcoma) and stage (primary versus metastasis), BMPs can affect cancer growth and its progression and modulate responsiveness to endocrine and metabolic factors [139].

As an example, low expression of BMP-7 can shift a cell phenotype from androgen-dependent to androgen-independent activity in primary prostate tumor cells, and the loss of endogenous BMP-7 may encourage the prostate cancer cells to be more aggressive [133]. However, BMP-7 can be reexpressed once cancer cells metastasized in the bone suggesting when to consider BMP-based therapy for targeting to curtail cancer growth [140, 141]. Likewise, not all BMPs are the same when it comes to angiogenesis; BMP-2, BMP-4, BMP-6, BMP-7, and GDF-5 are pro-angiogenic, while BMP-9 and BMP-10 are anti-angiogenic; thus, to inhibit angiogenesis, natural BMP antagonists like noggin can be used to target pro-angiogenic BMPs, and recombinant BMP-9 and BMP-10 can be used to suppress angiogenesis [142, 143]. However, in certain cancers, the attenuation of BMP-9-

induced ALK-1, a BMP type I receptor, signaling with neutralizing antibody and small molecule was able to inhibit endothelial cell sprouting [144–146]. PF-03446962, an antibody against ALK-1 (Pfizer), and dalantercept, a soluble chimeric protein (ALK1-Fc) which displays high-affinity binding with BMP-9 and BMP-10, have been shown as potent inhibitors for blocking the development of blood vessels [147, 148]. An endoglin antibody, also known as CD105, a co-receptor of BMP-9 and TGF-β that mediates a transition of endothelial cells from quiescent to active status during angiogenesis through preferential phosphorylation of SMAD 1/5/8, has also exhibited anti-angiogenic potential [149, 150]. Overall, BMPs and their signaling pathways play critical roles in the development, progression, and metastasis of various cancers in part by governing with their involvement in angiogenesis, inflammation, and immunosuppression and thus may serve as promising targets for therapeutic potential. Taken together, it remains to be seen that targeting one specific receptor with small molecule or an antibody or Fc conjugates could render the required outcome, as tumorigenesis is a result of a disturbed cascade of several biological events. For more details of the role of BMP signaling in mammary tumor growth and regulation, refer to the chapter on "Bone Morphogenetic Proteins in the Initiation and Progression of Breast Cancer".

13 Conclusion

BMPs are highly conserved from fly to man. The systems biology of BMP is a prerequisite for most of tissue induction during development and recapitulates it in adult tissue repair, regeneration, and homeostasis. The outcome of tissue induction/responsiveness is dictated by the responding cell than by BMP signal. BMP governs its function through a concentration gradient and is context-dependent in a permissive microenvironment. There are several BMPs, BMP antagonists, and receptors to govern its function as and when needed and to govern the inductive events in control fashion. Extracellular matrices and various BMP-specific antagonists that interact with BMP ligands add to that regulation. An aberrant expression in either ligand or receptor or antagonist can dictate unwanted cell growth and differentiation than required for normalcy. Thus far, BMP-based biologics have been approved for use only for local bone formation. There are several BMP-based therapeutics that are being evaluated in the clinic as drugs and/or biologics to improve tissue function against parenchymal fibrosis and to curtail angiogenesis in certain rare genetic disorders like FOP and anemia. Overall, the systems biology of BMP is promising, but the challenges are abundant as it comes to applying safely to achieve the required outcome in the clinic.

Acknowledgments I thank all of my past colleagues and collaborators with whom I have had the privilege to work with over 25 plus years; they made this chapter possible. Because of page constraints, I have included selective references; a lot more of them are available in respective chapters by other authors of the book.

References

1. Cunningham NS, Paralkar V, Reddi AH (1992) Osteogenin and recombinant bone morphogenetic protein 2b are chemotactic for human monocytes and stimulate transforming growth factor β1 mRNA expression. Proc Natl Acad Sci U S A 89:11740–11744
2. Zhang W, Zhu C, Wu Y, Ye D, Wang S, Zou D, Zhang X, Kaplan DL, Jiang X (2014) VEGF and BMP-2 promote bone regeneration by facilitating bone marrow stem cell homing and differentiation. Eur Cell Mater 27:1–11
3. Kann S, Chiu R, T M, SB G (2010) OP-1 (BMP-7) stimulates osteoprogenitor cell differentiation in the presence of polymethylmethacrylate particles. J Biomed Mater Res A 94(2):485–488
4. Reddi AH, Reddi A (2009) Bone morphogenetic proteins (BMPs): from morphogens to metabologens. Cytokine Growth Factor Rev 20(5–6):341–342
5. Zhang J, Li L (2005) BMP signaling and stem cell regulation. Dev Biol 284(1):1–11
6. Matsuda S, Harmansa S, Affolter M (2016) BMP morphogen gradients in flies. Cytokine Growth F Rev 27:119–127
7. Bier E, De Robertis EM (2015) EMBRYO DEVELOPMENT. BMP gradients: a paradigm for morphogen-mediated developmental patterning. Science 348(6242):aaa5838. doi:10.1126/science.aaa5838
8. Hogan BL (1996) Bone morphogenetic proteins in development. Curr Opin Genet Dev 6:432–443
9. Dudley AT, Lyons KM, Robertson EJ (1995) A requirement for bone morphogenetic protein-7 during development of the mammalian kidney and eye. Genes Dev 9:2795–2807
10. Luo G, Hofmann C, Bronckers AL, Sohocki M, Bradley A, Karsenty G (1995) BMP-7 is an inducer of nephrogenesis, and is also required for eye development and skeletal patterning. Genes Dev 9:2808–2820
11. Zhang H, Bradley A (1996) Mice deficient for BMP2 are nonviable and have defects in amnion/chorion and cardiac development. Development 122:2977–2986
12. Bellusci S, Henderson R, Winnier G, Oikawa T, BL H (1996) Evidence from normal expression and targeted misexpression that bone morphogenetic protein (Bmp-4) plays a role in mouse embryonic lung morphogenesis. Development 122(6):1693–1702
13. Kishigami S, Mishina Y (2005) BMP signaling and early embryonic patterning. Cytokine Growth Factor Rev 16:265–278
14. Reddi AH, Huggins CB (1972) Biochemical sequence in the transformation of fibroblasts into cartilage and bone. Proc Natl Acad Sci U S A 69:1601–1605
15. Sampath TK, Reddi AH (1981) Dissociative extraction and reconstitution of extracellular matrix components involved in local bone differentiation. Proc Natl Acad Sci U S A 78:7599–7602
16. Sampath TK, Maliakal JC, Hauschka PV, Jones WK, Sasak H, Tucker RF, White KH, Coughlin JE, Tucker MM, Pang RH et al (1992) Recombinant human osteogenic protein-1 (hOP-1) induces new bone formation in vivo with a specific activity comparable with natural bovine osteogenic protein and stimulates osteoblast proliferation and differentiation in vitro. J Biol Chem 267:20352–20362
17. Sampath TK, Rueger DC (1994) Structure, function and orthopedic application of osteogenic protein-1 (OP-1). Complicat Orthopedics 9:101–107
18. Massague J (1990) The transforming growth factor-β family. Annu Rev Cell Biol 6:597–641
19. Griffith DL, Keck PC, Sampath TK, Rueger DC, Carlson WD (1996) Three-dimensional structure of recombinant human Osteogenic Protein-1: structure paradigm for the transforming growth factor β superfamily. Proc Natl Acad Sci U S A 93:878–883
20. Massagué J (1998) TGF-β signal transduction. Annu Rev Biochem 67:753–791
21. ten Dijke P, Yamashita H, Sampath TK, Reddi AH, Estevez M, Riddle DL, Ichijo H, Heldin C-H, Miyazono K (1994) Identification of type I receptors for osteogenic protein-1 and bone morphogenetic protein-4. J Biol Chem 269:16985–16988

22. Heldin C-H, Miyazono K, ten Dijke P (1997) TGF-β signaling from cell membrane to nucleus via Smad proteins. Nature 390:465–471
23. Yanagita M (2008) Bone morphogenetic protein antagonists and kidney. In: Vukicevic S, Sampath TK (eds) Bone morphogenetic proteins: from local to systemic therapeutics. Birkhauser, Basel, pp. 213–232
24. Vukicevic S, Sampath TK (eds) Bone morphogenetic proteins: from local to systemic therapeutics. Birkhauser Verlag, Basel
25. Macias D, Gañan Y, Sampath TK, Piedra ME, Ros MA, Hurle JM (1997) Role of BMP-2 and OP-1 (BMP-7) in programmed cell death and skeletogenesis during chick limb development. Development 124(6):1109–1117
26. Rosen V, Nove J, Song JJ, Thies RS, Cox K, Wozney JM (1994) Responsiveness of clonal limb bud cell lines to bone morphogenetic protein 2 reveals a sequential relationship between cartilage and bone cell phenotypes. J Bone Miner Res 9(11):1759–1768
27. Sato K, Miura T, Iwata H (1988) Cartilaginous transdifferentiation of rat synovial cells under the influence of bone morphogenetic protein in tissue culture. Clin Orthop Relat Res 236:233–239
28. Olnot C (2009) Skeletal cell fate decisions within periosteum and bone marrow during bone regeneration. J Bone Miner Res 24:274–282
29. Lietman SA, Yamagishita M, Sampath TK, Reddi AH (1997) Stimulation of proteoglycan synthesis in porcine articular cartilage explants by recombinant Osteogenic Protein-1 (BMP-7*)*. J Bone Joint Surg 79:1132–1136
30. Maliakal JC, Asahina I, Hauschka PV, Sampath TK (1994) Osteogenic protein-1 (BMP-7) inhibits cell proliferation and stimulates the expression of markers characteristic of osteoblast phenotype in rat osteosarcoma (17/2.8) cells. Growth Factors 11(3):227–234
31. Katagiri T, Yamaguchi A, Komaki M, Abe E, Takahashi N, Ikeda T, Rosen V, Wozney JM, Fujisawa-Sehara A, Suda T (1994) Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. J Cell Biol 127(6 Pt 1):1755–1766
32. Thies RS, Bauduy M, Ashton BA, Kurtzberg L, Wozney JM, Rosen V (1992) Recombinant human bone morphogenetic protein-2 induces osteoblastic differentiation in W-20-17 stromal cells. Endocrinology 130(3):1318–1324
33. Hollnagel A, Oehlmann V, Heymer J, Rüther U, Nordheim A (1999) Id genes are direct targets of bone morphogenetic protein induction in embryonic stem cells. J Biol Chem 274(28):19838–19845
34. Zehentner BK, Dony C, Burtscher H (1999) The transcription factor Sox9 is involved in BMP-2 signaling. J Bone Miner Res 14(10):1734–1741
35. Ito Y, Miyazono K (2003) RUNX transcription factors as key targets of TGF-beta superfamily signaling. Curr Opin Genet Dev 13(1):43–47
36. Huang L, Teng XY, Cheng YY, Lee KM, Kumta SM (2004) Expression of preosteoblast markers and Cbfa-1 and Osterix gene transcripts in stromal tumour cells of giant cell tumour of bone. Bone 34(3):393–401
37. Flechtenmacher J, Huch K, Thonar EJ-MA, Mollenhauer JA, Davies SR, Schmid TM, Puhl W, Sampath TK, Adelotte MB, Kuettner KE (1996) Recombinant human osteogenic protein-1is a potent stimulator of the synthesis of cartilage proteoglycans and collagens by human articular chondrocytes. Arthritis Rheum 39:1896–1904
38. Logeart-Avramoglou D, Bourguignon M, Oudina K, Ten Dijke P, Petite H (2006) An assay for the determination of biologically active bone morphogenetic proteins using cells transfected with an inhibitor of differentiation promoter-luciferase construct. Anal Biochem 349(1):78–86
39. Matsumoto Y, Ikeya M, Hino K, Horigome K, Fukuta M, Watanabe M, Nagata S, Yamamoto T, Otsuka T, Toguchida J (2015) New protocol to optimize iPS cells for genome analysis of fibrodysplasia ossificans progressiva. Stem Cells 33:1730–1742
40. Reddi AH (1998) Role of morphogenetic proteins in skeletal tissue engineering and regeneration. Nat Biotechnol 16:247–252

41. Minas T, Chiu R (2000) Autologous chondrocyte implantation. Am J Knee Surg 13(1):41–50
42. Rueger DC, Chubinskaya S (2004) Bone morphogenetic proteins in articular cartilage repair. In: Vukicevic S, Sampath TK (eds) Bone morphogenetic proteins: regeneration of bone and beyond. Birkhauser Verlag, Basel
43. Flechtenmacher J, Huch K, Thonar EJ-MA, Mollenhauer JA, Davies SR, Schmid TM, Puhl W, Sampath TK, Adelotte MB, Kuettner KE (1996) Recombinant human osteogenic protein-1is a potent stimulator of the synthesis of cartilage proteoglycans and collagens by human articular chondrocytes. Arthritis Rheum 39:1896–1904
44. Merrihew K, Kumar B, Heretis K, Rueger DC, Kuettner KE, Chubinskaya S (2003) Alterations in endogenous osteogenic protein-1 with degeneration of human articular cartilage. J Orthop Res 21:899–907
45. Jelic M, Pecina M, Haspl M, Kos J, Taylor K, Marticic D, McCartney J, Yin s, Rueger D, Vukicevic S (2001) Regeneration of articular cartilage chondral defects by osteogenic protein-1 (bone morphogenetic protein-7) in sheep. Growth Factors 19:101–113
46. Hayashi M, Muneta T, Takahashi T, Ju YJ, Tsuji K, Sekiya I (2010) Intra-articular injections of bone morphogenetic protein-7 retard progression of existing cartilage degeneration. J Orthop Res 28:1502–1506
47. Hunter DJ, Pike MC, Jonas BL, Kissin E, Krop J, McAlindon T (2010) Phase 1 safety and tolerability study of BMP-7 in symptomatic knee osteoarthritis. BMC Musculoskelet Disord 11:232
48. Medtronic Sofamor Danek USA, Inc. (2006) INFUSE bone graft product information: oral/facial. Memphis. Available online at www.accessdata.fda.gov/cdrh_docs/pdf5/P050053c.pdf. Last accessed Feb 2010
49. Stryker Biotech (2009) OP-1 Implant® product information. Hopkinton. Available online at www.stryker.com/stellent/groups/public/documents/webprod/126737.pdf. Last accessed Feb 2010
50. Stryker Biotech (2009) OP-1 Putty® product information. Hopkinton. Available online at www.stryker.com/stellent/groups/public/documents/webprod/127024.pdf. Last accessed Feb 2010
51. Friedlaender GE, Perry CR, Cole JD, Cook SD, Clerny G, Muschler GF, Zych GA, Calhoun JH, LaForte AJ, Yin S (2001) Osteogenic protein-1 (bone morphogenetic protein-7) in the treatment of tibial non-unions: a prospective randomized clinical trial comparing rhOP-1 with fresh bone autograft. J Bone Joint Surg Am 83(Suppl 1):S151–S158
52. Vaccaro AR, Patel T, Fischgrund J et al (2004) A pilot study evaluating the safety and efficacy of OP-1 Putty (rhBMP-7) as a replacement for iliac crest autograft in posterolateral lumbar arthrodesis for degenerative spondylolisthesis. Spine 29:1885–1892
53. Vaccaro AR, Lawrence JP, Patel T et al (2008) The safety and efficacy of OP-1 (rhBMP-7) as a replacement for iliac crest autograft in posterolateral lumbar arthrodesis: a long-term (>4 years) pivotal study. Spine 33:2850–2862
54. Boden SD, Zdeblick TA, Sandhu HS et al (2000) The use of rhBMP-2 in interbody fusion cages. Definitive evidence of osteoinduction in humans: a preliminary report. Spine 25:376–381
55. Burkus JK, Gornet MF, Dickman CA et al (2002) Anterior lumbar interbody fusion using rhBMP-2 with tapered interbody cages. J Spinal Disord Tech 15:337–349
56. Burkus JK, Dorchak JD, Sanders DL (2003) Radiographic assessment of interbody fusion using recombinant human bone morphogenetic protein type 2. Spine 28:372–377
57. Dawson E, Bae HW, Burkus JK et al (2009) Recombinant human bone morphogenetic protein-2 on an absorbable collagen sponge with an osteoconductive-bulking agent in posterolateral arthrodesis with instrumentation. A prospective randomized trial. J Bone Joint Surg Am 91:1604–1613
58. Dimar JR 2nd, Glassman SD, Burkus JK et al (2009) Clinical and radiographic analysis of an optimized rhBMP-2 formulation as an autograft replacement in posterolateral lumbar spine arthrodesis. J Bone Joint Surg Am 91:1377–1386

59. Crawford CH 3rd, Carreon LY et al (2009) Perioperative complications of recombinant human bone morphogenetic protein-2 on an absorbable collagen sponge versus iliac crest bone graft for posterior cervical arthrodesis. Spine 34:1390–1394
60. Wong DA, Kumar A, Jatana S et al (2008) Neurologic impairment from ectopic bone in the lumbar canal: a potential complication of off-label PLIF/TLIF use of bone morphogenetic protein-2 (BMP-2). Spine J 8:1011–1018
61. Song K, Krause C, Shi S et al (2010) Identification of a key residue mediating bone morphogenetic Protein (BMP)-6 resistance to noggin inhibition allows for engineered BMPs with superior agonist activity. J Biol Chem 285:12169–12180
62. Wikesjö UME, Qahash M, Huang Y-H, Xiropaidis AV, Polimeni G, Susin C (2009) Bone morphogenetic proteins for periodontal and alveolar indications; Biological observations – clinical implications. Orthod Craniofac Res 12:263–270
63. Ripamonti U, Herbst NN, Ramoshebi LN (2005) Bone morphogenetic proteins in craniofacial and periodontal tissue engineering: experimental studies in the non-human primate Papio ursinus. Cytokine Growth Factor Rev 16(3):357–368
64. Weng D, Pöhling S, Pippig S, Bell M, Richter EJ, Zuhr O, Hürzeler MB (2009) The effects of recombinant human growth/differentiation factor-5 (rhGDF-5) on bone regeneration around titanium dental implants in barrier membrane-protected defects: a pilot study in the mandible of beagle dogs. Int J Oral Maxillofac Implants 24:31–37
65. Roldán JC, Jepsen S, Schmidt C, Knüppel H, Rueger DC, Açil Y, Terheyden H (2004) Sinus floor augmentation with simultaneous placement of dental implants in the presence of platelet-rich plasma or recombinant human bone morphogenetic protein-7. Clin Oral Implants Res 15:716–723
66. Koch FP, Becker J, Terheyden H, Capsius B, Wagner W (2010) A prospective, randomized pilot study on the safety and efficacy of recombinant human growth and differentiation factor-5 coated onto β -tricalcium phosphate for sinus lift augmentation. Clin Oral Implants Res 21:1301–1308
67. Nakashima M, Reddi AH (2003) The application of bone morphogenetic proteins to dental tissue engineering. Nat Biotechnol 21(9):1025–1032
68. Ozkaynak E, Schnegelsberg PN, Oppermann H (1991) Murine osteogenic protein-1 (OP-1): high levels of mRNA in kidney. Biochem Biophys Res Commun 179:116–123
69. Vukicevic S, Kopp JB, Luyten FB, Sampath TK (1996) Induction of nephrogenic mesenchyme by osteogenic protein-1 (bone morphogenetic protein 7). Proc Natl Acad Sci U S A 93:9021–9026
70. Vukicevic S, Basic V, Rogic D, Basic N, Shih M, Shepard A, Jin D, Dattatreyamurty B, Jones W, Dorai H et al (1998) Osteogenic protein-1 (bone morphogenetic protein-7) reduces severity of injury after ischemic acute renal failure in rat. J Clin Invest 102:202–214
71. Zeisberg M, Hanai J, Sugimoto H, Mammoto T, Charytan D, Strutz F, Kalluri R (2003) BMP-7 counteracts TGF-beta1-induced epithelial-to-mesenchymal transition and reverses chronic renal injury. Nat Med 9:964–968
72. Zeisberg M, Bottiglio C, Kumar N, Maeshima Y, Strutz F, Müller GA, Kalluri R (2003) Bone morphogenic protein-7 inhibits progression of chronic renal fibrosis associated with two genetic mouse models. Am J Physiol Renal Physiol 285:F1060
73. Chen C, Grzegorzewski KJ, Barash S, Zhao Q, Schneider H, Wang Q, Singh M, Pukac L, Bell AC, Duan R, Coleman T, Duttaroy A, Cheng S, Hirsch J, Zhang L, Lazard Y, Fischer C, Barber MC, Ma ZD, Zhang YQ, Reavey P, Zhong L, Teng B, Sanyal I, Ruben SM, Blondel O, Birse CE (2003) An integrated functional genomics screening program reveals a role for BMP-9 in glucose homeostasis. Nat Biotechnol 21(3):294–301
74. Mitu GM, Wang S, Hirschberg R (2007) BMP7 is a podocyte survival factor and rescues podocytes from diabetic injury. Am J Physiol Renal Physiol 293(5):F1641–F1648
75. Wang SN, Lapage J, Hirschberg R (2001) Loss of tubular bone morphogenetic protein 7 in diabetic nephropathy. J Am Soc Nephrol 12:2392–2399
76. Henley KD, Gooding KA, Economides AN, Gannon M (2012) Inactivation of the dual Bmp/Wnt inhibitor Sostdc1 enhances pancreatic islet function. Am J Physiol Endocrinol Metab 303(6):E752–E761

77. Tseng YH et al (2008) New role of bone morphogenetic protein 7 in brown adipogenesis and energy expenditure. Nature 454:1000–1004
78. Boon MR, van den Berg SA, Wang Y, van den Bossche J, Karkampouna S, Bauwens M, De Saint-Hubert M, van der Horst G, Vukicevic S, de Winther MP, Havekes LM, Jukema JW, Tamsma JT, van der Pluijm G, van Dijk KW, Rensen PC (2013) BMP7 activates brown adipose tissue and reduces diet-induced obesity only at sub-thermoneutrality. PLoS One 8(9):e74083
79. González EA, Lund RJ, Martin KJ, McCartney JE, Tondravi MM, Sampath TK, Hruska KA (2002) Treatment of a murine model of high-turnover renal osteodystrophy by exogenous BMP-7. Kidney Int 61(4):1322–1331
80. Lund RJ, Davies MR, Hruska KA (2002) Bone morphogenetic protein-7: an anti-fibrotic morphogenetic protein with therapeutic importance in renal disease. Curr Opin Nephrol Hypertens 11(1):31–36
81. Davies MR, Lund RJ, Hruska KA (2003) BMP-7 is an efficacious treatment of vascular calcification in a murine model of atherosclerosis and chronic renal failure. Am Soc Nephrol 14(6):1559–1567
82. Nicolas G et al (2002) The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. J Clin Invest 110:1037–1044. doi:10.1172/JCI200215686
83. Ganz T (2003) Hepcidin, a key regulator of iron metabolism and mediator of anemia of inflammation. Blood 102:783–788
84. Donovan A, Brownlie A, Zhou Y et al (2000) Positional cloning of zebrafish ferroportin1 identifies a conserved vertebrate iron exporter. Nature 403:776–781
85. Nemeth E, Tuttle MS, Powelson J (2004) Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science 306:2090–2093
86. Babitt JL, Huang FW, Xia Y, Sidis Y, Andrews NC, Lin HY (2007) Modulation of bone morphogenetic protein signaling in vivo regulates systemic iron balance. J Clin Invest 117(7):1933–1939
87. Babitt JL, Huang FW, Wrighting DM, Xia Y, Sidis Y, Samad TA, Campagna JA, Chung RT, Schneyer AL, Woolf CJ et al (2006) Bone morphogenetic protein signaling by hemojuvelin regulates hepcidin expression. Nat Genet 38:531–553
88. Andriopoulos B Jr, Corradini E, Xia Y, Faasse SA, Chen S, Grgurevic L, Knutson MD, Pietrangelo A, Vukicevic S, Lin HY, Babitt JL (2009) BMP6 is a key endogenous regulator of hepcidin expression and iron metabolism. Nat Genet 41:482–487
89. Meynard D, Kautz L, Darnaud V, Canonne-Hergaux F, Coppin H, Roth MP (2009) Lack of the bone morphogenetic protein BMP6 induces massive iron overload. Nat Genet 41:478–481
90. Bartnikas TB, Fleming MD (2012) Hemojuvelin is essential for transferrin-dependent and transferrin-independent hepcidin expression in mice. Haematologica 97(2):189–192
91. Daher R, Kannengiesser C, Houamel D, Lefebvre T, Bardou-Jacquet E, Ducrot N, de Kerguenec C, Jouanolle AM, Robreau AM, Oudin C, Le Gac G, Moulouel B, Loustaud-Ratti V, Bedossa P, Valla D, Gouya L, Beaumont C, Brissot P, Puy H, Karim Z, Tchernitchko D (2016) Heterozygous mutations in BMP6 pro-peptide lead to inappropriate hepcidin synthesis and moderate iron overload in humans. Gastroenterol 150(3):672–683
92. Morrell NW et al (2009) Cellular and molecular basis of pulmonary arterial hypertension. J Am Coll Cardiol 54:S20–S31
93. Masri FA et al (2007) Hyperproliferative apoptosis-resistant endothelial cells in idiopathic pulmonary arterial hypertension. Am J Physiol Lung Cell Mol Physiol 293:L548–L554
94. McLaughlin VV, Archer SL, Badesch DB, Barst HW, Linder JR et al (2009) ACCF/AHA 2009 expert consensus document on Pulmonary hypertension: a report of the American College of Cardiology Foundation Task Force on Expert Consensus Documents and the American Heart Association developed in collaboration with the American College of Chest Physicians; American Thoracic Society Inc.' and the Pulmonary Hypertension Association. J Am Coll Cardiol 53:1573–619
95. Christman BW et al (1992) An imbalance between the excretion of thromboxane and prostacyclin metabolites in pulmonary hypertension. N Engl J Med 327:70–75

96. Yu PB, Deng DY, Beppu H, Hong CC, Lai C, Hoyng SA et al (2008) Bone morphogenetic protein (BMP) type II receptor is required for BMP-mediated growth arrest and differentiation in pulmonary artery smooth muscle cells. J Biol Chem 283(7):3877–3888
97. Lane KB et al (2000) Heterozygous germline mutations in BMPR2, encoding a TGF-beta receptor, cause familial primary pulmonary hypertension. Inter PPH Consortium Nat Genet 26:81–84
98. Hong KH et al (2008) Genetic ablation of the BMPR2 gene in pulmonary endothelium is sufficient to predispose to pulmonary arterial hypertension. Circulation 118:722–730
99. Yang J et al (2008) Mutations in bone morphogenetic protein type II receptor cause dysregulation of Id gene expression in pulmonary artery smooth muscle cells: implications for familial pulmonary arterial hypertension. Circ Res 102:1212–1221
100. Trembath RC et al (2001) Clinical and molecular genetic features of pulmonary hypertension in patients with hereditary hemorrhagic telangiectasia. N Engl J Med 345:325–334
101. Yang J et al (2008) Mutations in bone morphogenetic protein type II receptor cause dysregulation of Id gene expression in pulmonary artery smooth muscle cells: implications for familial pulmonary arterial hypertension. Circ Res 102:1212–1221
102. Harrison RE et al (2003) Molecular and functional analysis identifies ALK-1 as the predominant cause of pulmonary hypertension related to hereditary haemorrhagic telangiectasia. J Med Genet 40:865–871
103. Powell LW, Seckington RC, Deugnier Y (2016). Haemochromatosis. Lancet 388(10045):706–16. doi:10.1016/S0140–6736(15)01315-X. [Epub ahead of print]
104. Wallace DF, Summerville L, Crampton EM, Frazer DM, Anderson GJ, Subramaniam VN (2009) Combined deletion of Hfe and transferrin receptor 2 in mice leads to marked dysregulation of hepcidin and iron overload. Hepatology 50:1992–2000
105. Ahmad KA, Ahmann JR, Migas MC et al (2002) Decreased liver hepcidin expression in the Hfe knockout mouse. Blood Cells Mol Dis 29(3):361–366
106. Corradini E, Garuti C, Montosi G, Ventura P, Andriopoulos B Jr, Lin HY, Pietrangelo A, Babitt JL (2009) Bone morphogenetic protein signaling is impaired in an HFE knockout mouse model of hemochromatosis. Gastroenterology 137:1489–1497
107. Feder JN, Gnirke A, Thomas W et al (1996) A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis. Nat Genet 13(4):399–408
108. Goswami T, Andrews NC (2006) Hereditary hemochromatosis protein, HFE, interaction with transferrin receptor 2 suggests a molecular mechanism for mammalian iron sensing. J Biol Chem 281:28494–28498
109. Gao J, Chen J, Kramer M, Tsukamoto H, Zhang AS, Enns CA (2009) Interaction of the hereditary hemochromatosis protein HFE with transferrin receptor 2 is required for transferrin-induced hepcidin expression. Cell Metab 9:217–227
110. Cohen RB, Hahn GV, Tabas JA, Peeper J, Levitz CL, Sando A, Sando N, Zasloff M, Kaplan FS (1993) The natural history of heterotopic ossification in patients who have fibrodysplasia ossificans progressiva. A study of forty-four patients. J Bone Joint Surg Am 75(2):215–219
111. Kaplan FS, Tabas JA, Gannon FH, Finkel G, Hahn GV, Zasloff MA (1993) The histopathology of fibrodysplasia ossificans progressiva. An endochondral process. J BoneJoint Surg Am 75(2):220–230
112. Shore EM, Xu M, Feldman GJ, Fenstermacher DA, Cho TJ, Choi IH, Connor JM, Delai P, Glaser DL, LeMerrer M, Morhart R, Rogers JG, Smith R, Triffitt JT, Urtizberea JA, Zasloff M, Brown MA, Kaplan FS (2006) A recurrent mutation in the BMP type I receptor ACVR1 causes inherited and sporadic fibrodysplasia ossificans progressive. Nat Genet 38:525–527
113. Kaplan FS, Xu M, Seemann P, Connor JM, Glaser DL, Carroll L, Delai P, Fastnacht-Urban E, Forman SJ, Gillessen-Kaesbach G, Hoover-Fong J, Koster B, Pauli RM, Reardon W, Zaidi SA, Zasloff M, Morhart R, Mundlos S, Groppe J, Shore EM (2009) Classic and atypical fibrodysplasia ossificans progressiva (FOP) phenotypes are caused by mutations in the bone morphogenetic protein (BMP) type I receptor ACVR1. Hum Mutat 30:379–390
114. Shen Q, Little SC, Xu M, Haupt J, Ast C, Katagiri T, Mundlos S, Seemann P, Kaplan FS, Mullins MC, Shore EM (2009) The fibrodysplasia ossificans progressiva R206H ACVR1

mutation activates BMP-independent chondrogenesis and zebrafish embryo ventralization. J Clin Invest 119:3462–3472
115. Bagarova J, Vonner AJ, Armstrong KA, Börgermann J, Lai CS, Deng DY, Beppu H, Alfano I, Filippakopoulos P, Morrell NW, Bullock AN, Knaus P, Mishina Y, Yu PB (2013) Constitutively active ALK2 receptor mutants require type II receptor cooperation. Mol Cell Biol 33(12):2413–2424
116. Fukuda T, Kohda M, Kanomata K, Nojima J, Nakamura A, Kamizono J, Noguchi Y, Iwakiri K, Kondo T, Kurose J, Endo K, Awakura T, Fukushi J, Nakashima Y, Chiyonobu T, Kawara A, Nishida Y, Wada I, Akita M, Komori T, Nakayama K, Nanba A, Maruki Y, Yoda T, Tomoda H, Yu PB, Shore EM, Kaplan FS, Miyazono K, Matsuoka M, Ikebuchi K, Ohtake A, Oda H, Jimi E, Owan I, Okazaki Y, Katagiri T (2009) Constitutively activated ALK2 and increased SMAD1/5 cooperatively induce bone morphogenetic protein signaling in fibrodysplasia ossificans progressiva. J Biol Chem 284(11):7149–7156
117. Chaikuad A, Alfano I, Kerr G, Sanvitale CE, Boergermann JH, Triffitt JT, von Delft F, Knapp S, Knaus P, Bullock AN (2012) Structure of the bone morphogenetic protein receptor ALK2 and implications for fibrodysplasia ossificans progressiva. J Biol Chem 287(44): 36990–36998
118. Yu PB, Deng DY, Lai CS, Hong CC, Cuny GD, Bouxsein ML, Hong DW, McManus PM, Katagiri T, Sachidanandan C, Kamiya N, Fukuda T, Mishina Y, Peterson RT, Bloch KD (2008) BMP type I receptor inhibition reduces heterotopic ossification. Nat Med 14: 1363–1369
119. Cai J, Orlova VV, Cai X, Eekhoff EM, Zhang K, Pei D, Pan G, Mummery CL, Ten Dijke P (2015) Induced pluripotent stem cells to model human fibrodysplasia ossificans progressiva. Stem Cell Reports 5(6):963–970. doi: 10.1016/j.stemcr.2015.10.020. Epub 2015 Nov 26
120. Hao J, Daleo MA, Murphy CK, Yu PB, Ho JN, Hu J, Peterson RT, Hatzopoulos AK, Hong CC (2008) Dorsomorphin, a selective small molecule inhibitor of BMP signaling, promotes cardiomyogenesis in embryonic stem cells. PLoS One 3(8):e2904. doi:10.1371/journal.pone.0002904
121. Sanvitale CE, Kerr G, Chaikuad A, Ramel M-C, Mohedas AH, Reichert S, Wang Y, Triffitt JT, Cuny GD, Paul YB, Hill CS, Bullock AN (2013) A new class of small molecule inhibitor of BMP signaling. PLoS One 8:e62721
122. Chakkalakal SA, Uchibe K, Convente MR, Zhang D, Economides AN, Kaplan FS, Pacifici M, Iwamoto M, Shore EM 2016. Palovarotene inhibits heterotopic ossification and maintains limb mobility and growth in mice with the human ACVR1R206H Fibrodysplasia Ossificans Progressiva (FOP) mutation. J Bone Miner Res. doi: 10.1002/jbmr.2820. [Epub ahead of print]
123. Hatsell SJ, Idone V, Wolken DM, Huang L, Kim HJ, Wang L, Wen X, Nannuru KC, Jimenez J, Xie L, Das N, Makhoul G, Chernomorsky R, D'Ambrosio D, Corpina RA, Schoenherr CJ, Feeley K, Yu PB, Yancopoulos GD, Murphy AJ, Economides AN (2015) ACVR1R206H receptor mutation causes fibrodysplasia ossificans progressiva by imparting responsiveness to activin A. Sci Transl Med 7(303):303ra137. doi:10.1126/scitranslmed.aac4358
124. Forlino A, Marini JC (2016) Osteogenesis imperfecta. Lancet 387(10028):1657–1671
125. Palomo T, Fassier F, Ouellet J, Sato A, Montpetit K, Glorieux FH, Rauch F (2015) Intravenous bisphosphonate therapy of young children with osteogenesis imperfecta: skeletal findings during follow up throughout the growing years. J Bone Miner Res 30(12):2150–2157. doi:10.1002/jbmr.2567. Epub 2015 Jun 30
126. Gebken J, Brenner R, Feydt A, Notbohm H, Brinckmann J, Müller PK, Bätge B (2000) Increased cell surface expression of receptors for transforming growth factor-beta on osteoblasts from patients with Osteogenesis imperfecta. Pathobiol 68(3):106–112
127. Grafe I, Yang T, Alexander S, Homan EP, Lietman C, Jiang MM, Bertin T, Munivez E, Chen Y, Dawson B, Ishikawa Y, Weis MA, Sampath TK, Ambrose C, Eyre D, HP B, Lee B (2014) Excessive transforming growth factor-β signaling is a common mechanism in osteogenesis imperfecta. Nat Med 20:670–675
128. Grafe I, Alexander S, Yang T, Lietman C, EP H, Munivez E, Chen Y, MM J, Bertin T, Dawson B, Asuncion F, HZ K, MS O, Lee B (2016) Sclerostin antibody treatment improves the bone

phenotype of Crtap (−/−) mice, a model of recessive osteogenesis imperfecta. J Bone Miner Res 31:1030–1040
129. Cho SY, Asharani PV, Kim OH, Iida A, Miyake N, Matsumoto N, Nishimura G, Ki CS, Hong G, Kim SJ, Sohn YB, Park SW, Lee J, Kwun Y, Carney TJ, Huh R, Ikegawa S, Jin DK (2015) Identification and in vivo functional characterization of novel compound heterozygous BMP1 variants in osteogenesis imperfecta. Hum Mutat 36(2):191–195
130. Vadon-Le Goff S, DJ H, Moali C (2015) BMP-1/tolloid-like proteinases synchronize matrix assembly with growth factor activation to promote morphogenesis and tissue remodeling. Matrix Biol 44–46:14–23
131. Sánchez-Duffhues G, Hiepen C, Knaus P, Ten Dijke P (2015) Bone morphogenetic protein signaling in bone homeostasis. Bone 80:43–59
132. McLean K, Gong Y, Choi Y, Deng N, Yang K, Bai S, Cabrera L, Keller E, McCauley L, Cho KR, RJ B (2011) Human ovarian carcinoma–associated mesenchymal stem cells regulate cancer stem cells and tumorigenesis via altered BMP production. J Clin Invest 121:3206–3219
133. Buijs JT, Henriquez NV, van Overveld PG, van der Horst G, ten Dijke P, van der Pluijm G (2007) TGF-β and BMP7 interactions in tumour progression and bone metastasis. Clin Exp Metastasis 24:609–617
134. Langenfeld E, Deen M, Zachariah E, Langenfeld J (2013) Small molecule antagonist of the bone morphogenetic protein type I receptors suppresses growth and expression of Id1 and Id3 in lung cancer cells expressing Oct4 or nestin. Mol Cancer 12(1):129
135. Liu H, Jia D, Li A, Chau J, He D, Ruan X, Liu F, Li J, He L, Li B (2013) p53 regulates neural stem cell proliferation and differentiation via BMP-Smad1 signaling and Id1. Stem Cells Dev 22(6):913–927. doi:10.1089/scd.2012.0370. Epub 2013 Jan 30
136. Yan W, Chen X (2007) Targeted repression of bone morphogenetic protein 7, a novel target of the p53 family, triggers proliferative defect in p53-deficient breast cancer cells. Cancer Res 67:9117–9124
137. Zheng X, Carstens JL, Kim J, Scheible M, Kaye J, Sugimoto H, Wu CC, LeBleu VS, Kalluri R (2015) Epithelial-to-mesenchymal transition is dispensable for metastasis but induces chemoresistance in pancreatic cancer. Nature 527(7579):525–530
138. Ye L, Jiang WG (2016) Bone morphogenetic proteins in tumour associated angiogenesis and implication in cancer therapies. Cancer Lett 380(2):586–597
139. Ye L, Mason MD, Jiang WG (2011) Bone morphogenetic protein and bone metastasis, implication and therapeutic potential. Front Biosci (Landmark Ed) 16:865–897
140. Morrissey C, Brown LG, Pitts TE, Vessella RL, Corey E (2010) Bone morphogenetic protein 7 is expressed in prostate cancer metastases and its effects on prostate tumor cells depend on cell phenotype and the tumor microenvironment. Neoplasia 12(2):192–205
141. Katsuno Y, Hanyu A, Kanda H, Ishikawa Y, Akiyama F, Iwase T, Ogata E, Ehata S, Miyazono K, Imamura T (2008) Bone morphogenetic protein signaling enhances invasion and bone metastasis of breast cancer cells through Smad pathway. Oncogene 27:6322–6333
142. Yoshimatsu Y, Lee YG, Akatsu Y, Taguchi L, Suzuki HI, Cunha SI, Maruyama K, Suzuki Y, Yamazaki T, Katsura A, Oh SP, Zimmers TA, Lee SJ, Pietras K, Koh GY, Miyazono K, Watabe T (2013) Bone morphogenetic protein-9 inhibits lymphatic vessel formation via activin receptor-like kinase 1 during development and cancer progression. Proc Natl Acad Sci U S A 110(47):18940–18945
143. Hawinkels LJ, Garcia de Vinuesa A, Ten Dijke P (2013) Activin receptor-like kinase 1 as a target for anti-angiogenesis therapy. Expert Opin Investig Drugs 22(11):1371–1383
144. Kerr G, Sheldon H, Chaikuad A, Alfano I, von Delft F, Bullock AN, Harris AL (2015) A small molecule targeting ALK1 prevents Notch cooperativity and inhibits functional angiogenesis. Angiogenesis 18(2):209–217
145. Simonelli M, Zucali P, Santoro A, Thomas MB, de Braud FG, Borghaei H, Berlin J, Denlinger CS, Noberasco C, Rimassa L, Kim TY, English PA, Abbattista A, Gallo Stampino C, Carpentieri M, Williams JA (2016) Phase I study of PF-03446962, a fully human monoclonal antibody against activin receptor-like kinase 1 in patients with hepatocellular carcinoma Ann Oncol. pii: mdw240. [Epub ahead of print]

146. Doi T, Lee KH, Kim TM, Ohtsu A, Kim TY, Ikeda M, Yoh K, Gallo Stampino C, Hirohashi T, Suzuki A, Fujii Y, Andrew Williams J, Bang YJ (2016) A phase I study of the human anti-activin receptor-like kinase 1 antibody PF-03446962 in Asian patients with advanced solid tumors Cancer Med. doi:10.1002/cam4.724. [Epub ahead of print]
147. Hawinkels LJ, de Vinuesa AG, Paauwe M, Kruithof-de Julio M, Wiercinska E, Pardali E, Mezzanotte L, Keereweer S, Braumuller TM, Heijkants RC, Jonkers J, Löwik CW, Goumans MJ, ten Hagen TL, ten Dijke P (2016) Activin receptor-like Kinase 1 ligand trap reduces microvascular density and improves chemotherapy efficiency to various solid tumors. Clin Cancer Res 22(1):96–106
148. Makker V, Filiaci VL, Chen LM, Darus CJ, Kendrick JE, Sutton G, Moxley K, Aghajanian C (2015) Phase II evaluation of dalantercept, a soluble recombinant activin receptor-like kinase 1 (ALK1) receptor fusion protein, for the treatment of recurrent or persistent endometrial cancer: an NRG Oncology/Gynecologic Oncology Group Study 0229N. Gynecol Oncol 138(1):24–29
149. Apolo AB, Karzai FH, Trepel JB, Alarcon S, Lee S, Lee MJ, Tomita Y, Cao L, Yu Y, Merino MJ, Madan RA, Parnes HL, Steinberg SM, Rodriguez BW, Seon BK, Gulley JL, Arlen PM, Dawson NA, Figg WD, Dahut WL (2016) A phase II clinical trial of TRC105 (anti-endoglin antibody) in adults with advanced/metastatic urothelial carcinoma. Clin Genitourin Cancer. pii: S1558–7673(16)30136–7
150. Duffy AG, Ulahannan SV, Cao L, Rahma OE, Makarova-Rusher OV, Kleiner DE, Fioravanti S, Walker M, Carey S, Yu Y, Venkatesan AM, Turkbey B, Choyke P, Trepel J, Bollen KC, Steinberg SM, Figg WD, Greten TF (2015) A phase II study of TRC105 in patients with hepatocellular carcinoma who have progressed on sorafenib United. Eur Gastroenterol J 3(5):453–461

Embryonic Skeletogenesis and Craniofacial Development

Yuji Mishina and Nobuhiro Kamiya

Abstract Bone morphogenetic proteins (BMPs) are originally identified with their ability to induce heterotopic ossification. Several decades of studies have demonstrated that BMPs have pleiotropic functions in numbers of tissues for many different aspects. This review focuses on the effects of BMP signaling on skeletogenesis and craniofacial development. We will summarize recent progresses on in vitro studies, animal models, and human genetics to uncover highly context-dependent functions of BMP signaling, including unexpected outcomes, and the mechanisms of how BMP signaling regulates bone mass. We will also summarize reported findings about BMP signaling-related genes identified as causes of human diseases in skeletal system such as chondrodysplasia, facial cleft, and craniosynostosis.

Keywords Osteoblast • Chondrocyte • Osteocyte • Osteoclast • Mesenchyme • Neural crest • BMP signaling • Wnt signaling • Hedgehog • FGF • Facial process • Cleft palate • Cleft lip • Craniosynostosis • Chondrodysplasia • Temporomandibular joint

1 Introduction

Bone morphogenetic proteins (BMPs) were discovered and named in 1965 by Marshall Urist, who initially identified the ability of a then unknown factor in the bone to induce ectopic bones in muscle [204]. In the past 50 years, the osteogenic function of BMPs has been extensively examined [188]. The US Food and Drug

Y. Mishina, PhD (✉)
University of Michigan, School of Dentistry, Department of Biologic and Materials Sciences, 1011 N. University Avenue, Ann Arbor, MI 48109-1078, USA
e-mail: mishina@umich.edu

N. Kamiya, MD, PhD
Department of Sports Medicine, Tenri University, Tenri, Nara 632-0071, Japan
e-mail: nkamiya1@sta.tenri-u.ac.jp

S. Vukicevic, K.T. Sampath (eds.), *Bone Morphogenetic Proteins: Systems Biology Regulators*, Progress in Inflammation Research,
DOI 10.1007/978-3-319-47507-3_3

Administration (FDA) has approved BMP2 and BMP7 for clinical use in long bone open fractures, nonunion fractures, and spinal fusion. Therefore, the exogenous role of BMPs in the bone is well known in orthopedics. However, it is crucial to understand endogenous or physiological roles of BMPs during skeletogenesis and bone remodeling.

BMP signaling plays important roles in a variety of cell types in the skeleton including osteoblasts, chondrocytes, and osteoclasts. The osteogenic function of BMPs and BMP signaling has been further investigated over the last decade using gene-targeting technology in animals. This chapter focuses on the physiological roles of BMP signaling on bone formation, bone resorption, and bone mass control, specifically via its action on osteoblasts or chondrocytes by reviewing mouse genetic studies of skeletal development and bone remodeling. This chapter also focuses on roles of BMP during craniofacial development including formation of calvaria and mandible.

2 Embryonic Skeletogenesis

2.1 Developmental Stages of Ossification

One of the key components derived from the paraxial mesoderm is the bone. The skeleton which includes the bone is generated from three distinct lineages: (1) the somites which generate the axial skeleton, (2) the lateral plate mesoderm which generates the limb skeleton, and (3) the cranial neural crest which generates the branchial and craniofacial bones and cartilage. The skeleton in mammals is formed through two distinct processes during embryogenesis: intramembranous ossification and endochondral ossification [51, 110]. Both processes involve the transformation of a preexisting mesenchymal tissue into the bone tissue as they are called "bone formation" or "osteogenesis." The intramembranous ossification is a direct conversion of mesenchymal tissue into the bone, which primarily occurs in flat bones including the skull, the mandible, and the clavicle. On the other hand, endochondral ossification, which occurs in long bones, is an indirect conversion of mesenchymal tissue into the bone; i.e., the mesenchymal tissue differentiates into cartilage and this cartilage is later replaced by the bone.

2.2 BMP and Osteogenesis

At cellular levels, the in vivo physiological process of "osteogenesis" or "bone formation" can be described as two distinct processes: (1) intramembranous ossification through osteoblastogenesis that is direct differentiation of mesenchymal cells into bone cells (i.e., osteoblasts) and (2) the endochondral ossification, which includes an initial chondrogenesis that is differentiation from mesenchymal cells into cartilage

cells (i.e., chondrocytes) followed by the apoptosis of chondrocyte secondary differentiation from osteoblast precursor to osteoblasts via osteogenesis. Therefore, "osteogenesis" encompasses osteoblastogenesis and chondrogenesis. The key molecules of BMP pathway involved in osteogenesis are listed (Table 1). Note that BMPRIA is a potent receptor of BMP2 and BMP4 [66], as is ACVRI for BMP7 [136].

In mice, BMP2 is expressed in a variety of sites including the developing limb buds [134], mesenchymal derivatives of which undergo endochondral ossification. The osteogenic (i.e., anabolic) roles of BMPs have been extensively examined over 50 years, and human recombinant BMP2, BMP4, BMP6, and BMP7 proteins have been vigorously used for mammalian cells to induce their differentiation in culture. To induce chondrogenesis or osteogenesis, primary cells or pluripotent mesenchymal cell lines such as C3H10T1/C3H10T2 [43], C2C12 [99], ATDC5 [186], N1511 [90], MC3T3 [13], and ST2 [219] have been treated with BMPs. In these cells, BMPs directly activate *Sox9* and *Cbfa1*, transcriptional master genes required for chondrogenesis and osteoblastogenesis, respectively [114, 160, 232], to secondarily induce expression of chondrogenic (i.e., aggrecan, ColII, ColIX, ColX, etc.) or osteogenic (i.e., ALP, osteocalcin, BSP, Col1, etc.) markers. Based on the accumulated evidence of anabolic actions of BMPs, BMP2 and BMP7 have been approved by the US FDA for clinical application [56, 62]. It is noted that average circulating serum levels of BMPs are around 300~600 pg/ml [102, 206] while a typical dosage range of BMPs in culture experiments is 0~300 ng/ml. Also expression levels of BMPs by primary osteoblasts and pluripotent mesenchymal cell lines are quite low demonstrating a significant discrepancy between levels of BMPs found in tissues and those used for pharmacological experiments.

In addition to BMP signaling, the impacts of Wnt signaling on skeletogenesis and bone formation have been investigated for a decade [16, 57, 63, 64, 109]. The relationship of BMP signal with Wnt signal in the skeletal system is of interest. In vitro experiments using pluripotent mesenchymal cell lines or primary osteoblasts to test the interaction between BMP and Wnt signaling in osteoblasts have yielded both synergistic and antagonistic results: C2C12 cells and primary osteoblasts induce *Wnt3a* expression and stabilize Wnt/β-catenin signaling upon BMP2 treatment [7, 33, 141]. Alternatively, C3H10T1/2 cells treated with Wnt3a induce BMP4 expression [215]. These facts suggest the presence of a positive autocrine loop

Table 1 The key molecules in BMPs' signaling cascade regarding osteogenesis

Function	Key molecules
Antagonists	Noggin, Chordin, Gremlin
Ligands	BMP2, BMP4, BMP6, BMP7
Type I receptors	BMPRIA/ALK3, ACVRI/ALK2, BMPRIB/ALK6
Type II receptors	BMPRII, ActRIIA, ActRIIB
R-Smad	Smad1, Smad5, Smad8
Co-Smad	Smad4
I-Smad	Smad6, Smad7
Non-Smad pathways	p38 MAPK, TAK1

between BMP and Wnt signaling pathways [33, 171]. In contrast, primary osteoblasts show increased Wnt canonical signaling when BMP signaling is inhibited upon treatment with Dorsomorphin, an inhibitor for BMP type I receptors [92]. Wnt3a treatment represses BMP2-dependent *Id1* expression in C2C12 cells [152]. Similarly, treatment of cultured skull bone with a BMP antagonist *Noggin* increases Wnt canonical signaling [95]. Moreover, one study investigated intracellular cross talk between BMP and Wnt pathways using uncommitted bone marrow stromal cells [127]. Dishevelled homolog 1 (Dvl1) is a cytoplasmic protein known to act as a signaling molecule for Wnt pathway. This study found that BMP2 antagonizes Wnt3a-induced proliferation and Wnt/β-catenin activation through an interaction between Smad1 and Dvl1. Another intracellular interaction via Pten/Akt pathway has been reported in hair follicle stem/progenitor cells [234]; however, this pathway is less likely functional in osteoblasts [68]. Taken together, these facts suggest that both positive and negative feedback loops are present between the two signaling pathways, BMP and Wnt, in a context-dependent manner.

2.3 Functional Studies in Animal Models

As detailed in Chap. 4, the BMP family members are involved with early patterning of the mouse embryo. Conventional knockout mice for the key genes (i.e., BMP2, BMP4, and BMP7 and their receptors BMPRIA and ACVRI) are lethal, and, thus, it is not possible to investigate bone development and remodeling using these mouse models [49, 61, 132, 143, 146, 216, 233]. To avoid the embryonic lethality, a strategy of conditional knockout mice using a Cre-loxP system has been employed.

Both osteoblasts and chondrocytes are derived from mesenchymal cells and are responsible for the bone and cartilage, respectively. Recent animal studies have been designed to investigate the physiologic function of BMP signaling in these different cell types (mesenchymal cells, chondrocytes, and osteoblasts) independently (Table 2). Interestingly, BMP signaling both in chondrocytes and mesenchymal cells positively controls bone size and mass while negatively controls the same in osteoblasts. Accumulated evidence has revealed similarities between mesenchymal cells and chondrocytes and differences between these cells and osteoblasts regarding how BMP signaling affects their behavior (i.e., bone size).

2.3.1 BMP and Osteoblasts

An osteoblast-specific conditional deletion of *Bmpr1a* using the *Og2-Cre* mouse line, in which Cre recombination is restricted in differentiated osteoblasts under the osteocalcin promoter, was first reported in 2004 [145]. The co-Smad, Smad4, was also conditionally deleted in osteoblasts using another *Og2-Cre* mouse line [197]. Interestingly, these two studies demonstrated that the response of osteoblasts after loss of BMP signaling is age dependent; trabecular bone volume is lower in young

Table 2 Mouse studies of BMP signaling in different cell type

	Promoter to drive transgene or Cre	BMP signal	Stage	Bone mass	Ref.
Chondrocyte					
Bmpr1a cKO	Gdf5-Cre	Down	E12.5–E16.5, 7w, 9M	Reduced	[176]
Double knockout of *Bmpr1a* and *Bmpr1b*	Col2-Cre	Down	E12.5–E16.5	Reduced	[227]
Bmp4 overexpression	Col11a2	Up	E18.5	Increased	[202]
Noggin overexpression	Col11a2	Down	E18.5	Reduced	[202]
Double cKO of Smad1 and Smad5	Col2-Cre	Down	E12.5–newborn	Reduced	[172]
Bmpr1a cKO	Aggrecan-CreER	Down	2, 4, 8, 20w	Reduced	[86]
Acvr1 cKO	Col11a2-Cre	Slightly down	E17.5, P0	Not reported	[174]
Mesenchymal cell					
Double cKO of *Bmp2* and *Bmp4*	Prx1-Cre	Down	E10.5–newborn, 3w	Reduced	[11]
Bmp2 cKO	Prx1-Cre	Down	5M	Reduced	[201]
Bmpr2 cKO	Prx1-Cre	Normal[a]	2 M	Increased	[130]
Osteoblast					
Bmpr1a cKO	Ogl2-Cre	Down	3M 10M	Reduced Increased	[145]
Smad4 cKO	Ogl2-Cre	Down	3~12w 11M	Reduced Increased	[197]
Bmp4 overexpression	2.3 kb Col1	Up	E18.5	Reduced	[158]
Noggin overexpression	2.3 kb Col1	Down	E17.5, 3w	Increased	[158]
Bmpr1a cKO	3.2 kb Col1-CreER	Down	E18.5, 3w, 5M	Increased	[92, 94, 95]
Acvr1 cKO	3.2 kb Col1-CreER	Down	E18.5, 3w, 5M	Increased	[91]
Osteoclast					
Bmpr1a cKO	Ctsk-Cre	Down	8w	Increased	[157]
Osteocyte					
Bmpr1a cKO	Dmp1-Cre	Down	1M, 2M, 4M	Increased	[93, 124]

[a]Activin signal is increased while BMP signal is unchanged

mutant mice but higher in aged mutant mice. In addition, the activity of osteoclasts is reduced in aged osteoblast-specific *Bmpr1a*-deficient mice, which may have led to the complex skeletal phenotype [145, 197]. These facts suggest that BMP signaling in differentiated osteoblasts controls the balance between bone formation by osteoblasts and resorption by osteoclasts, thereby affecting the final outcome of the amount of bone mass in an age-dependent manner. Increased bone mass in *Bmpr1a*-deficient mice appeared to be challenging to the general concept of BMPs as osteogenic inducers.

Comprehensive functions of BMP signaling in skeletogenesis have been further investigated and led to a new paradigm that alternation of Wnt signal by BMP is the key modulator of skeletal development. The loss of function of BMP signaling via BMPRIA in osteoblasts upregulates Wnt canonical signaling during embryonic and postnatal bone development, suggesting a negative regulation of Wnt signaling by BMP [92, 95]. These studies show that the upregulation of Wnt signaling is at least in part mediated by suppression of Wnt inhibitors including Sost/sclerostin and Dkk1 because both Sost/sclerostin and Dkk1 are direct targets of BMP signaling (Fig. 1). In addition, *Sost* expression was severely downregulated in *Bmpr1a*-deficient bones as assessed by microarray analysis [92, 95]. Interestingly, both Smad-dependent and Smad-independent pathways appear to contribute to Dkk1 expression, whereas Sost/sclerostin requires only Smad-dependent signaling, suggesting differential regulation of these genes by BMP signaling via BMPRIA [92]. BMP and Wnt signaling regulate the development and remodeling of many tissues and interact synergistically or antagonistically in a context- and age-dependent manner in vivo [17, 77]. Lastly, the role of BMPR1A in osteocytes was recently investigated by conditional disruption of *Bmpr1a* using *Dmp1-Cre* mouse line from two independent groups [93, 124]. The resulting mutant mice demonstrated an increased bone mass concomitant with accelerated cell proliferation and SOST reduction [93, 124]. It is interesting that the increased bone phenotype was much stronger in the osteocyte-specific condition (i.e. Dmp1Cre:Bmpr1a mice) compared

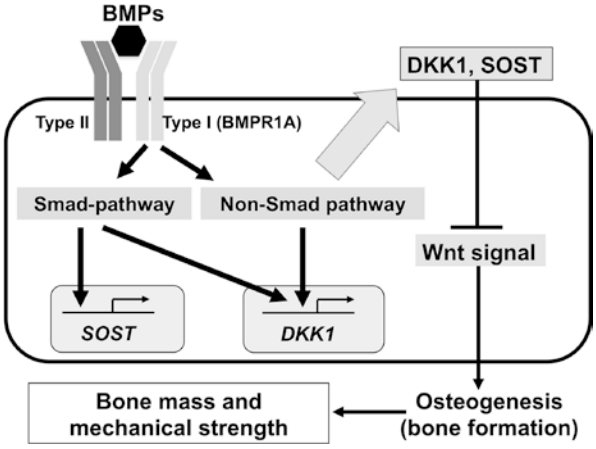


Fig. 1 A proposed model of the relationship between the BMP signaling via BMPRIA and the canonical Wnt signaling in osteoblasts. Both Dkk1 and Sost/sclerostin are downstream targets of the BMP signaling. The BMP signaling upregulates *Sost* expression primarily through the Smad-dependent signaling while it upregulates *Dkk1* expression through both the Smad and non-Smad signaling pathways (p38 MAPK). As DKK1 and SOST/sclerostin act as Wnt signaling inhibitors, BMP signaling in osteoblasts, in turn, inhibits osteogenesis and decreases bone mass. DKK1 and Sost/sclerostin play an important role in regulating bone mass and mechanical strength as downstream effectors of BMPR1A signaling in bone by taking balances between BMP signaling and Wnt signaling

with osteoblast-specific condition (i.e. Col1Cre:Bmpr1a mice). In addition, similar to the Col1Cre:Bmpr1a mice, Wnt signal is activated while RANKL is suppressed in the Dmp1Cre:Bmpr1a mice [93]. This fact is very intriguing because recent reports show osteocytes as a primary source of RANKL production [153, 219] and therefore BMPR1A can be a key molecule in osteocytes by regulating RANKL production.

Similarly, the loss of function of BMP signaling in osteoblasts via ACVR1, another type I receptor, results in increased bone mass [91]. In this mouse model, upregulation of Wnt canonical signaling is observed concomitant with reduction in *Dkk1* and *Sost* expression during embryonic and postnatal bone development [91]. Because the resulting *Acvr1* mutant mice show similar bone phenotypes to those found in *Bmpr1a* mutant mice, despite structural and functional similarities between two receptors, the other does not compensate loss of one receptor.

Sost/sclerostin was originally reported as a member of the BMP antagonist DAN family [111, 214]. Although DAN family members modulate both BMP and Wnt signaling in *Xenopus* [19, 79, 167], recent studies suggest a primary role of Sost/sclerostin in Wnt signaling in mouse and humans: Sost/sclerostin is not a BMP antagonist [207] but rather a Wnt inhibitor [208] that binds the Wnt co-receptors low-density lipoprotein receptor-related proteins 5 and 6 (LRP5 and LRP6) [123, 183]. It is known that both DKK1 and Sost/sclerostin inhibit Wnt/β-catenin signaling by binding to co-receptors. As both Dkk1 and Sost/sclerostin are secreted proteins expressed by osteoblasts, their role in regulating bone mass has been investigated using human and mouse genetic approaches. Although conventional knockouts of *Dkk1* die in utero from defective head induction and limb formation [151], mice heterozygous for *Dkk1* (*Dkk1*$^{+/-}$ mice) exhibit a high bone mass (HBM) phenotype [150], while overexpression of *Dkk1* in osteoblasts causes osteopenia [118]. In addition, increased *DKK1* expression in bone marrow has also been associated with lytic bone lesions in patients with multiple myeloma [199].

Similar to *Dkk1*$^{+/-}$ mice, conventional knockouts of *Sost* are viable and exhibit increased bone mass [122]. In humans, the loss of function and hypomorphic mutations in *SOST* cause sclerosteosis [9, 30] and van Buchem disease [10, 191], respectively, with a high bone mass (HBM) phenotype. These mutants share the HBM phenotypes with other gain of function of LRP5 mutation effects, due to defect in Dkk1-mediated regulation of LRP5 in humans [26, 125, 209] and overexpression of *Lrp5* in mice [6]. In contrast, the loss of function of LRP5 leads to OPPG with low bone mass [59], which is similar to the bone phenotype of mice overexpressing *Sost* [214]. In addition, recent genome-wide SNP-based analyses identified a significant association between bone mineral density and the *SOST* gene locus [76, 194, 226]. Consistent with these observations, conditional knockouts of *Bmpr1a*, which show reductions in expressions of *Dkk1* and *Sost*, show an HBM phenotype [92–95]. Furthermore, increased expression of *Dkk1* and *Sost* in osteoblasts by constitutive activation of BMPRIA signaling is associated with a partial rescue of the bone phenotype of *Bmpr1a*-deficient mice [92]. These facts support the interpretation that Dkk1 and Sost/sclerostin act physiologically as inhibitors of Wnt canonical signaling and therefore as negative regulators of bone mass.

2.3.2 BMP and Chondrocytes

When BMP signaling was enhanced by overexpression of *Bmp4* in chondrocytes using a chondrocyte-specific Cre mouse line, the mutant mice demonstrated an increase in bone mass [202]. By contrast, when the BMP signaling was attenuated by overexpression of *Noggin*, an antagonist for BMPs (BMP2, BMP4, BMP5, BMP6, and BMP7) [236], in chondrocytes, the mutant mice showed a decrease in bone mass [202]. Similarly, the loss of function of BMP signaling via BMPRIA in chondrocytes, which is a potent receptor for BMP2 and BMP4, demonstrated impairment of articular cartilage and growth plate cartilage, resulting in decreased bone size [86, 176, 227]. Mice deficient for *Bmpr1a* or *Bmpr1b* in chondrocytes can form intact cartilage during skeletal development, while double mutant embryos deficient for both *Bmpr1a* and *Bmpr1b* exhibit a severe defect in cartilage (i.e., chondrodysplasia) around embryonic day 12.5 (E12.5) to E16.5 [227]. These facts suggest a possible functional compensation mechanism between BMPR1A and BMPR1B in chondrocytes during early cartilage development in growth plates [227]. Mice deficient in *Acvr1* in chondrocytes using a *Col2-Cre*-driven conditional deletion are viable but exhibit defects in the development of cranial and axial structures [174]. The mutant mice exhibit shortened cranial base, and cervical vertebrae are hypoplastic. Unlike compound mutant mice for *Bmpr1a* and *Bmpr1b*, compound mutant mice for *Avcr1* and *Bmpr1b* can develop cartilage primordia and subsequent bones through endochondral ossification [174], suggesting that BMP signaling through ACVR1 plays a relatively minor role compared with other type 1 receptors during chondrogenesis.

Recent study using *aggrecan CreERT2-Cre* mice to conditionally disrupt *Bmpr1a* in chondrocytes demonstrated a severe reduction in bone length and bone mass in the mutant femur at the age of 1 month [86], indicating a more distinct role of BMPR1A in chondrocytes postnatally which is not redundant with other receptors. Note that cell proliferation assessed by BrdU incorporation was strikingly reduced in the mutant mice at 2 weeks of age, which may reduce the size of cartilaginous foundation during the process of endochondral bone formation, leading finally to reduced bone length and mass. Taken together, these facts strongly demonstrate that BMP signal in chondrocytes plays a positive and potent role in regulating bone mass.

2.3.3 BMP and Mesenchymal Cells

Similar to chondrocytes, BMP signaling in mesenchymal cells contributes to an increase in bone mass (Table 2). A mesenchymal cell-specific Cre mouse line, *Prx1-Cre*, is used for these studies since Cre is active in mesenchymal cells as early as E9.5 in this line [128]. The simultaneous disruption of *Bmp2* and *Bmp4* in mesenchymal cells resulted in impairment of osteogenesis with reduced bone size [11]. Disruption of *Bmp2* in mesenchymal cells impaired the initiation of fracture healing, presumably due to a defect in endochondral bone formation after a bone

fracture, in which chondrocytes derived from mesenchymal cells play an important role [201]. These facts demonstrate the necessity of BMP signaling in mesenchymal cells for proper bone mass during development and remodeling. Recently, the role of type 2 receptor, BMPRII, in the skeleton was investigated using the *Prx1-Cre* mouse line. The resulting mutant mice are expectedly normal probably due to the compensation mechanism by other type 2 receptors, ACVR2A and ACVR2B, suggesting BMPRII is not required for endochondral ossification in the limb [54]. The same group further investigated the mutant mice and found increased bone mass at 2 months after birth [130]. While BMP signal is unchanged, activin signal is impaired in mutant mice, leading to increased osteoblast activity. This study raises the possibility that type 2 receptor segregation and/or competition could be a generalized mechanism by which BMP and activin signaling interact.

2.3.4 BMP and Osteoclasts

A putative coupling theory in bone metabolism states that in general, bone anabolism is locally induced by bone catabolism [71]. Osteoblasts control bone resorption by expressing RANK ligand (RANKL) and its decoy receptor, osteoprotegerin (OPG) [112, 187]. BMPs induce osteoclastogenesis via the RANKL-OPG pathway in an osteoblast-dependent manner. Exogenous treatment of BMP2 in vitro induces osteoclastogenesis by upregulating RANKL while treatment with BMP antagonist Noggin blocks osteoclastogenesis [1, 80, 159, 163]. In vivo studies using genetically engineered mutant mice demonstrated similar results (Table 2). Gain of function of BMP signaling by *Bmp4* overexpression in osteoblasts results in an increase of osteoclastogenesis and reduced bone mass [158]. In contrast, the loss of function of BMP signaling by disruption of *Bmpr1a* or *Noggin* overexpression results in reduction of osteoclastogenesis, leading to an increase of bone mass [145, 158] due to a decrease in the RANKL-OPG ratio [94, 95]. Taken together, these facts indicate that BMP signal has an indirect positive role in osteoclast function through osteoblast as a secondary effect. In a nonhuman primate bone defect model, treatment with BMP2 increases the size of the defect in association with increased osteoclast number and bone resorption, which is followed by bone formation [182].

In addition, it is also possible that BMPs directly control osteoclasts since *Bmp2* and its receptor *Bmpr1a* both are expressed in osteoclasts [55, 98]. When BMP signaling through BMPR1A is conditionally ablated in osteoclasts using a *cathepsin K* promoter (*CtsK*) to drive Cre, bone mass increased in association with reduced osteoclast number in the bone as expected [157] (Table 2). Interestingly, both bone formation rate and osteoblast number assessed by bone histomorphometric analysis are greater in the mutant mice compared to their control littermates. This evidence suggests a possibility that BMPR1A signaling in osteoclasts negatively regulates osteoblast function though its downstream target genes within osteoclasts.

Several recent reports have emerged revealing factors secreted by osteoclasts such as sphingosine-1-phosphate regulate osteogenesis [164]. It is an interesting future direction how BMP signaling involves osteoclast-mediated osteoblast differentiation.

2.3.5 BMP and Other Cell Types in Skeletal System

Angiogenesis is another necessary step in new bone formation in skeletal development as well as in bone remodeling after fracture [31, 97]. Both BMP2 and BMP7 are known to induce angiogenesis by associating with other growth factors such as VEGF (vascular endothelial growth factor), bFGF (basic fibroblast growth factor), and TGF-β1 [40]. Overexpression of BMP9 in muscle induces heterotopic bone formation similar to BMP2 [34, 166]. As BMP9 is abundantly expressed in endothelial cells that are a primary cell type for angiogenesis [38], it is possible that BMP signaling in endothelial cells synergizes anabolic bone formation. The mechanism and origin of precursor cells for heterotopic bone formation, which is pathologically observed in fibrodysplasia ossificans progressiva (FOP) patients, is under investigation [96, 129, 229]. Taken together, the fact that BMPs implanted subcutaneously induce ectopic bone and increase bone mass [204] is likely due to the primary effects of BMP signaling on cells that are positive regulators for bone mass, including mesenchymal cells, chondrocytes, and endothelial cells (Table 3).

The current application of BMP therapy via systemic and local treatment can affect multiple cell types simultaneously in bone tissue including mesenchymal cells, chondrocytes, osteocytes, osteoblasts, osteoclasts, and endothelial cells, because typically a BMP2-soaked collagen sponge is applied around bone defects in orthopedic surgeries and the BMP2 diffuses to other tissues around the bone. Thus, it is important to evaluate the effects of BMPs on more than just osteoblasts. In addition to these cell types, we recently investigated the effects of high-dose BMP2 on periosteum and found that high concentration of BMP2 can reduce cell proliferation and increase apoptosis via DKK1 and SOST by inhibiting Wnt activity in human primary periosteal cells [102]. Interestingly, a lower concentration of BMP2 (i.e., 50–200 ng/ml) shows a trend of decreased caspase activity which is

Table 3 A variety of cell types possibly affected by BMP therapy in the bone

Cell types that can increase bone mass	Cell types that can reduce bone mass
Mesenchymal cells	Osteoclasts
Chondrocytes	Osteoblasts · osteocytes[a]
Osteoblasts · osteocytes	Periosteal cells
Endothelial cells	

[a]Note that both osteoblast and osteocyte may have an indirect effect on bone mass through osteoclast activation via RANKL

opposite to the effect of higher concentrations of BMP2 (500–2000 ng/ml) that shows an increased caspase activity, suggesting a "biphasic nature" of BMP2 depending on its concentration. Note that BMP2 belongs to the TGF-beta superfamily and TGF-beta also has biphasic effects in a concentration-dependent manner with distinct molecular mechanisms [218]. This study is clinically significant because BMP2 is generally applied around the periosteum in orthopedic surgeries for fracture repair and spinal fusion and, therefore, it is important to delineate the effects of the BMP2 concentration on human periosteum-derived cells. In addition, the BMP2 concentration of clinical applications is extremely high (i.e., 1.5 mg/ml [InFUSE Bone Graft/LT-CAGE Lumbar Tapered Fusion Device. Summary of safety and effective data premarket approval application P000058, 2002, US Food and Drug Administration, Sliver Spring, MD, http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cftopic/pma/pma.cfm?num=P000058]), compared with the BMP2 concentration of cell basis studies (i.e., 0~300 ng/ml) as described before. It is possible that the negative role of BMP2 on cell proliferation leads to a reduction in bone mass because the cell proliferation is an initial phase prior to the cell differentiation phase that is required for new bone formation (Table 3).

The potential effects of BMP signal on mesenchymal cells, chondrocytes, and osteoblasts have been discussed. It is possible that chondrocytes or mesenchymal cells increase bone mass by responding to BMPs while osteoblasts or osteocytes reduce net bone mass (Fig. 2). This possibility supports a physiological role of BMPs in endogenous bone formation and remodeling, while the current view that BMPs enhances bone formation reflects a pharmacological role. Apparently, BMP signal has a different function depending on each context (i.e., endogenous vs. exogenous, low dose vs. high dose, chondrocyte vs. osteoblast).

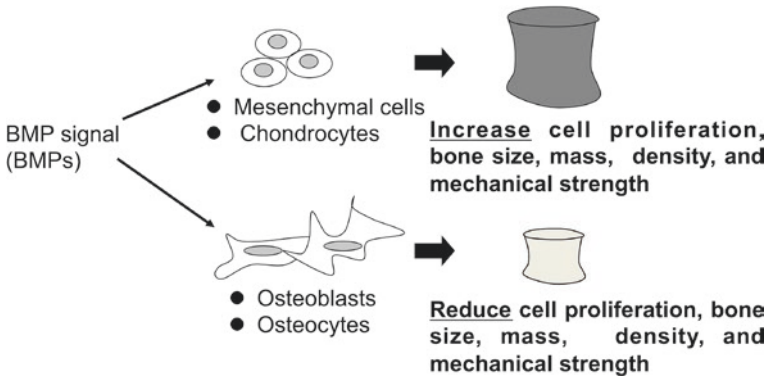


Fig. 2 Possible effects of BMP signal induced by BMPs on mesenchymal cells, chondrocytes, osteoblasts, and osteocytes. Based on the recent progresses shown in Tables 2 and 3, it is possible that BMP signaling in chondrocytes or mesenchymal cells can function to increase cell proliferation, bone size, mass, density, and mechanical strength while BMP signaling in osteoblasts or osteocytes may have opposite outcomes through regulating balance between bone formation and resorption

Table 4 Skeletal abnormalities associated with the molecules in BMP signaling

Gene	Disease	Ref.
BMP2 regulatory element	Brachydactyly type A2	[37]
BMP4	Poly/syndactyly	[8]
CDMP1/GDF5	Acromesomelic chondrodysplasia	[198]
	Brachydactyly type A1	[41]
	Brachydactyly type C	[168]
GDF6	Hemi-vertebrae, polydactyly, Klippel–Feil, rib malformation, spondylothoracic dysostosis	[5]
GDF3	Scoliosis, Klippel–Feil, vertebral fusion	[225]
SOST	Sclerosteosis	[9, 30]
	Van Buchem disease	[10, 191]
BMPR1B	Brachydactyly type A2	[116]
	Acromesomelic chondrodysplasia	[42]
ALK2	Fibrodysplasia ossificans progressiva	[185]
NOGGIN	Brachydactyly type B	[115]

2.4 BMP and Bone-Related Diseases

Studies of human mutations also suggest the importance of BMP signaling for skeletogenesis and bone-related diseases such as chondrodysplasia and fibrodysplasia ossificans progressiva [185, 198]. Mutations in genes involving BMP signaling associated with skeletal abnormalities in humans are summarized in Table 4 [5, 8–10, 30, 37, 41, 42, 115, 116, 168, 191, 225]. While the association of each molecule with its skeletal abnormality is known (Table 4), precise molecular mechanisms including tissue source and cell type responsible for the pathogenesis are still under investigation.

3 Craniofacial Development

3.1 Head Induction

Soon after implantation and before gastrulation, one group of cells formed at the distal tip of the visceral endoderm moves along one direction to form the anterior visceral endoderm (AVE). The AVE acts as a signaling center to instruct underneath epiblast (embryonic ectoderm) to form the future head [103, 193]. Nodal signaling plays a critical role for migration of the AVE [44, 222]. BMP signaling mediated by BMPR1A is critical to orient migration of the AVE [147, 221]. Similarly, BMPR1A signaling in epiblast regulates functions in the AVE for head induction [39]. The loss of *Bmp4* and *Bmp2* affect normal head formation; however, usage of different

receptors in this context is not fully understood [32, 216, 233]. These facts suggest that BMP signaling is critical for induction of the head structure around the gastrulation stage and causes of some of craniofacial abnormalities may be traced back to such early stages.

3.2 Facial Development and Abnormalities

Fetuses (by the end of 5 weeks for humans and at 10.5 days of mice) develop the frontonasal prominence (FNP) [154] (Fig. 3). Neural crest cells (NCCs) formed at the dorsal ectodermal midline in vertebrate embryos migrate laterally and ventrally on all axial levels [24]. Cranial neural crest cells (CNCCs) migrate into the FNP and branchial arches and differentiate to most of the facial tissues. The FNP further splits into four processes, a pair of the medial nasal process and a pair of the lateral nasal processes [195, 200] (Fig. 3). The maxillary and mandibular processes are derived from first branchial arch. The face is formed by fusion of these primordial structures, namely, four processes developed from the FNP and the paired maxillary and mandibular processes. Fusion of the two medial nasal processes at the midline provides the continuity of the nose, the middle upper lip, and the primary palate. Fusion of the medial nasal and maxillary prominences provides the continuity of the upper lip and jaw.

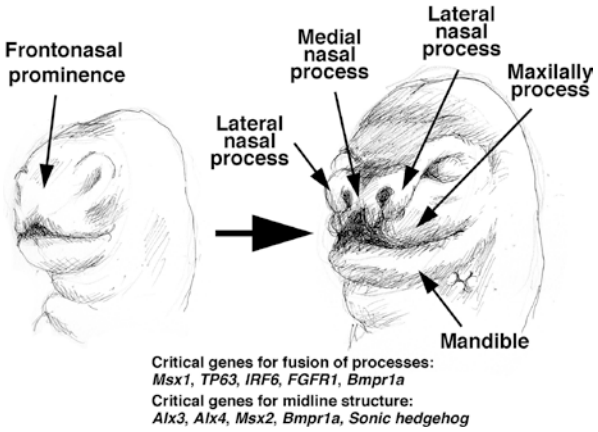


Fig. 3 Facial development and fusion of facial processes. During early facial development, pairs of the medial nasal processes and the lateral nasal processes are developed from the frontonasal prominences. Pairs of the maxillary process and the mandibular processes are developed from the first and second pharyngeal arches, respectively. Failure of the fusion of these processes causes facial clefts as detailed in the text. BMPs and related molecules play a critical role in the fusion process

3.2.1 BMP and Cleft Lip

Failure of fusions of any processes will develop facial cleft. For examples, failure of fusion between the medial nasal and the maxillary processes results in uni- or bilateral cleft lip and that between the lateral nasal and the maxillary processes results in oblique facial cleft. The fusion of these processes is critical for formation of the lip and the alveolar ridge in the primary palate. Following closure of the primary palate, closure of the secondary palate takes place by elevation of the palatal shelves. In some cases, these facial clefts occur alone (cleft lip without cleft palate), while other cases, these clefts accompany cleft palate (cleft lip with cleft palate) [45]. Studies in human genetics and animal models reveal several genes involved in development of cleft lips such as mutations in *MSX1*, tumor protein 63 (*TP63*), interferon regulatory factor 6 (*IRF6*), and fibroblast growth factor receptor 1 (*FGFR1*) [27, 28, 45]. Since MSX1 is one of the established downstream targets of BMP signaling, involvement of BMP signaling during fusion process for lip formation has been speculated. Disruption of *Bmpr1a* in a dental epithelial-specific manner using *Nestin-Cre* results in bilateral cleft lip in association with increased apoptosis in the medial nasal processes [126] (Fig. 3).

3.2.2 BMP, Facial Cleft, and Midline Structure

Craniofacial syndromes that include median facial cleft are believed to be caused by dysplasia of the frontonasal prominence [181]. When the fusion between left and right medial nasal processes fails, that likely results in midface clefting [23]. In humans, it is reported that mutations in aristaless-related homeobox transcription factor 3 and 4 (*Alx3* and *Alx4*) are identified in frontonasal dysplasia patients (FND OMIM ID, 136760; FND2 OMIM ID, 613451) [22, 203, 205]. FND is characterized by hypertelorism, severely depressed nasal bridge and ridge, and bifid nasal tip. In the mouse, similar phenotypes are seen in *Alx3/Alx4 or Alx1/Alx4* compound mutant mice [23, 169]. A significant increase of apoptosis is detected in the outgrowing frontonasal prominence at E10, which is proposed to be the underlying cause of the subsequent nasal cleft [23]. Potential involvement of BMP signaling in FND is poorly understood. However, it is reported that a gain-of-function mutation in *Msx2* causes midface clefting [217]. Neural crest-specific expression of ca*Bmpr1a* results in short nasal septum due to increased cell death [67] (Fig. 3). The amount of Hedgehog signaling is known to be strongly associated with alterations in midline facial structures [29, 231]. Since BMP signaling and Hedgehog signaling regulate each other in highly context-dependent manner, it is possible to speculate that BMP signaling may also play a critical role in midline development and failure of precise control of signaling activity may result in medial facial cleft and FND.

There are several evidences indicating that increased BMP signaling leads to a reduction or loss of the midline structure. Noggin mutant mice develop a microform of holoprosencephaly (HPE) [113]. The fact that compound mutations of Noggin and Chordin results in variable forms of HPE [104] suggests that levels of BMP

signaling are associated with severity of HPE. It is reported that BMP ligands interact with NODAL, another TGF-beta superfamily ligand, and it is possible that increased availability of BMP ligands because of the loss of their binding antagonists (Noggin and Chordin) secondarily influences NODAL signaling activity that plays a critical role in head formation soon after gastrulation [223, 224]. Alternatively, but not exclusively, it is also possible to speculate that increased BMP signaling activity may suppress Hedgehog activity. In tooth development, BMP signaling has been shown to negatively regulate Hedgehog signaling activity [117]. It is reported that disruption of *Shh* in mice results in holoprosencephaly and cyclopia [35] and mutations in SHH in human are associated with holoprosencephaly [20, 175, 190]. Thus, there exists a possibility of cross talk with increased BMP signaling activity suppressing Hedgehog signaling leading to midline hypoplasia.

3.2.3 BMP and Cleft Palate

During palatogenesis, first a pair of palatal shelves is formed downward around 7 weeks of gestation in humans and E11.5 in mice with interposition of the tongue. Fetal growth allows downward movement of the tongue to reorient palatal shelves to medial direction around 8 weeks in human and E13–14 in mice. These shelves grow, come closer, and then fuse to separate the oral and nasal cavity by 9 weeks in human and E15.5 in mice [45, 107] (Fig. 4). Thus, failure of fetal growth, movement of tongue reorientation of the palatal shelves, and/or growth of the palatal shelves may result in cleft palate. The final step of palatogenesis is dissolution of the medial edge epithelium (MEE) likely due to the cell death of this population. Persistence of the MEE results in submucosal cleft, i.e., the soft tissue has fused, while underlying palatal bone and muscle layer remain unfused.

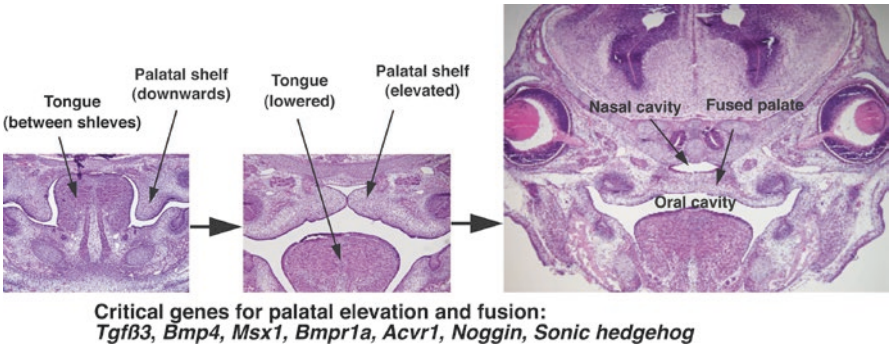


Fig. 4 Development of the palatal shelf and formation of the secondary palate. During midgestation, a pair of palatal shelves are formed from the mandibular processes and grow downwards. Along with the growth of the mandibular, the position of the tongue lowers allowing the shelves elevate. The elevated shelves further grow to reach each other and then fuse together to form the secondary palate. The primary palate is formed anterior to the secondary palate as a derivative of the medial nasal process and the frontonasal prominence

Mice homozygous for *Tgfß3* null mutation develop cleft palate demonstrating for the first time that TGFß superfamily signaling plays a critical role in palatogenesis [89]. Tissue-specific inactivation of *Tgfbr1*, a type 1 receptor for TGFß, using *Wnt1*-Cre or *K14*-Cre also results in cleft palate [46, 47]. Involvement of BMP signaling in palatogenesis was initially suggested in a retinoic acid-induced cleft palate model [74, 131], where pathogenesis coincided with downregulation of BMP ligands such as *Bmp2*, *Bmp3*, *Bmp4*, *Bmp5*, and *Bmp7*. *Msx1*-null mice also develop cleft palate [180]. *Msx1* is expressed in mesenchymal tissues in anterior palatal shelves, and the loss of *Msx1* results in downregulation of *Bmp4* [4, 235]. Detailed analyses suggest that in the palatal shelves, BMP4 induces *Shh* expression that in turn induces *Bmp2* expression that positively regulates cell proliferation [4].

Neural crest-specific disruption of *Acvr1*, one of the type 1 receptors for BMPs, results in cleft palate along with multiple craniofacial defects including a hypomorphic mandible [48]. Neural crest-specific disruption of *Bmpr1a* results in mid-gestation lethality due to cardiac malfunctions [156, 192]. When the said cardiac malfunction is compensated by administration of isoproterenol, a beta-adrenergic agonist, the mutant embryos can survive until term and develop reduced projection of facial structures [148] and cleft palate [119]. In addition to the cleft lip mentioned above, deletion of *Bmpr1a* using Nestin-Cre resulted in cleft palate [126]. However, deletion of *Bmpr1a* in a neural crest-specific manner using *Wnt1*-Cre resulted in anterior clefting only [119], suggesting that BMP signaling mediated by ACVR1 and BMPR1A positively regulates proliferation of the cells in the anterior palatal shelve mesenchyme.

A BMP antagonist Noggin is highly expressed in the palatal shelf epithelium [138]. Disruption of Noggin results in cleft palate [70] suggesting that increased BMP signaling activity also affect normal palatogenesis. In the anterior regions of the secondary palate, the loss of *Noggin* results in upregulation of *Bmp2* expression leading to an increase of cell proliferation. In the posterior regions of the secondary palate, in contrast, the loss of *Noggin* induces ectopic expression of *Tgfß3* that coincides to ectopic fusion of palatal shelves to epithelia of the oral cavity and tongue [70]. Expression of a constitutively active form of *Bmpr1a* in the oral epithelium also leads to the similar phenotype [70]. Taken together, these facts suggest that suppression of BMP signaling is critical to prevent premature or ectopic fusions of palatal shelves to maintain structural integrity within the oral cavity. In contrast, expression of a constitutively active form of *Acvr1* in the oral epithelium using K14-Cre results in submucosal cleft in association with a reduced cell death in the MEE [155]. These results might suggest that BMP signaling mediated by different receptors plays distinct roles during palatogenesis. Further investigation is required to address this exciting hypothesis.

3.3 Calvarial Vault and Cranial Base

Mammalian craniofacial skeleton consists of a little more than 20 bones. Bones comprising the cranial vault are generated through intramembranous ossification. In contrast, bones in cranial base are generated through endochondral ossification. The

majority of cranial bones and cartilage residing in the anterior part of the head are derived from cranial neural crest cells (CNCCs), whereas the posterior part of elements is from paraxial mesoderm [137, 144, 177, 189, 212]. BMP signaling components are highly expressed in the migrating cranial neural crest cells and later in the cranial cartilage and bone [135]. These reports suggest that BMP signaling regulates skeletal development by organizing neural crest cell proliferation and cell death [36]. Both CNCC-derived and paraxial mesoderm-derived osteoprogenitor cells undergo intramembranous ossification to generate corresponding skull elements. Interestingly, osteoblasts from neural crest-derived bones show a higher level of activation of FGF signaling pathways compared with osteoblasts from paraxial mesoderm-derived bones [121, 170]. Osteoblasts from neural crest-derived bones also show lower apoptotic response when stimulated by TGFß signaling [120]. Regenerative ability of skull defects in the frontal bone is higher than that in parietal bones [18]. Taken together, these results suggest that neural crest-derived bones are more proliferative and less apoptotic than paraxial-derived bones due to enhanced signaling of FGF, BMP, and Wnt signaling pathways with a reduction in the TGF-beta pathway [184].

Sutures are a fibrous connective tissue found between bones in the cranial vault and cranial base. Sutures are critical growth sites in the skull. Mesenchymal cells proliferate and differentiate into osteoblasts that deposit collagen fibers and minerals to the bony plates to increase their size. Genetic studies in mice demonstrate that nasal and metopic sutures, which connect nasal bones and frontal bones, are of neural crest origin [83]. Coronal sutures are of mesodermal origin and are formed between the neural crest-derived frontal bones and the mesoderm-derived parietal bones. The sagittal suture is formed between the two mesoderm-derived parietal bones and is of neural crest origin. Since sutures are critical for growth of the skull, premature fusion of sutures results in cessation of skull growth at the site of fusion causing a pathological condition called craniosynostosis resulting in increased intracranial pressure and skull deformity [144, 149, 173].

3.3.1 BMP and Skull Formation

BMP signaling alters the homeobox *Msx* genes which are important for normal skull development [15, 21, 140]. A conventional gain-of-function mutation in *Msx2* results in skeletal defects such as mandibular hypoplasia and aplasia of interparietal bone [217]. BMP signaling plays crucial roles in regulation of cranial suture morphogenesis [101]. BMP signaling components such as *Bmp2*, *Bmp4*, *Msx1*, and *Msx2* are expressed in sagittal suture during its development [101]. Local application of BMP4 protein into mouse calvarial explants induces expression of *Msx* genes and obliteration of the mid-sutural space [101], which is probably through a BMP-responsive element located proximal to the *Mxs2* promoter [12]. Both *Msx1* and *Msx2* mutant mice develop persistent calvarial foramina [78, 179, 180]. Compound heterozygous mutant mice for Msx1 and Msx2 lack formation of the frontal and parietal bones [78]. These results suggest that BMP signaling plays a critical role through expression of *Msx1* and *Msx2* on osteoblast differentiation for normal skull vault formation.

3.3.2 BMP and Suture Formation

Fibroblast growth factor (FGF) family is known to play critical role during facial development and cranial vault formation [65, 142, 144]. Gain-of-function mutations in FGF signaling are known to cause some types of craniosynostosis [162, 213]. For example, two missense mutations (S252W and P253R) have been found in the IgII-LgIII linker region of FGFR2 and are associated with Apert syndrome [149, 162]. Gain-of-function mutations in *MSX2* also result in Boston-type craniosynostosis in human (OMIM ID: 604757) by inducing premature fusion in cranial sutures [82]. Noggin is present in postnatal sutures, and its expression is under negative regulation of FGF signaling. *Fgf* gain-of-function mutations in syndromic forms of craniosynostosis might inappropriately reduce Noggin expression such that the suture loses its patency [211]. Direct involvement of BMP signaling in skull deformity and craniosynostosis was recently demonstrated. Enhanced BMP signaling through constitutively active form of *Bmpr1a* (*caBmpr1a*) in neural crest cells results in craniosynostosis through premature fusion of the anterior frontal suture in mice [95, 105]. Increased BMP signaling in neural crest cells also leads to craniofacial skeletal defects. Constitutive activation of *Bmpr1a* in neural crest cell linage using *P0-Cre* or *Wnt1-Cre* leads to increased level of cell death in skeletal primordia. These mutant mice exhibited bone and cartilage defects of nasomaxillary complex such as nasal bone and nasal septum [60, 67, 105].

In contrasting to neural crest-specific augmentation of BMP signaling activity, osteoblast-specific augmentation of BMPR1A signaling does not cause overt skull deformity [105]. Increased apoptosis is found in the skull vault in this animal model, and the skull deformity is rescued by prevention of cell death by inhibition of p53 together suggesting that augmented BMP signaling increases p53-dependent cell death resulting in depletion of osteogenic progenitor cells leading to premature suture fusion [67, 105]. This is an interesting finding since it is believed that premature fusion of cranial sutures is a result of increased bone formation within the cranial suture [52].

In the craniosynostosis mouse model caused by neural crest-specific augmentation of BMPR1A signaling, it is shown that only a small increase in BMP signaling (50 %) is enough to result in skull deformity and craniosynostosis [105]. It is reasonable to speculate that several folds of changes in BMP signaling may result in early embryonic lethality [53, 75]. Recent genome-wide association studies find single-nucleotide polymorphisms (SNPs) located in proximity to BMP-related genes that are associated with skull morphology such as sagittal suture craniosynostosis [88]. Direct connection between mutations in BMP-related genes and human skull deformity has not been demonstrated; however, gain-of-function mutation in *MSX2*, a known downstream target gene of BMP signaling, results in Boston-type craniosynostosis as mentioned earlier [82]. Suture mesenchymal cells isolated from craniosynostosis patients show mutations in glypican-1 and glypican-3 (*GPC1* and *GPC3*) that negatively regulate BMP signaling [50]. It is possible to speculate that SNIPs found in proximity of *BMP2* may alter enhancer activity to increase BMP signaling in the sagittal suture [88]. Taken together, these circumstantial evidences imply that some human cases may be caused by augmented BMP signaling in suture mesenchymal cells.

3.3.3 BMP and Cranial Base

Unlike calvarial vault, bones in the calvarial base (ethmoid, presphenoid, and basisphenoid) are formed through endochondral ossification. Very little is known about involvement of BMP signaling in the calvarial base. Cartilage structures called synchondrosis connect between the bones in the skull base. The ethmoid bone and the basisphenoid bone are articulated to the frontal and the basioccipital bones, respectively, through synchondrosis. Bmp2, 3, 4, 5, and 6 are expressed in cranial base with a temporally dynamic manner [100]. Development of growth plates in synchondrosis are tightly regulated by SHH and FGF signaling like the ones in long bones [139, 228]. Expression of inhibitor of differentiation 2 (*Id2*) is regulated in part by BMP-Smad signaling. The mutant mice for Id2 are born without overt abnormalities; however, they show a narrower hypertrophic zone in the synchondrosis postnatally [178].

3.4 Mandibular Development and Temporomandibular Joint Formation

The mandible that forms the lower jaw is unique among bones in the body because it is formed through both intramembranous ossification and endochondral ossification [161] (Fig. 5). The body (or base) of the mandible and the ramus undergo

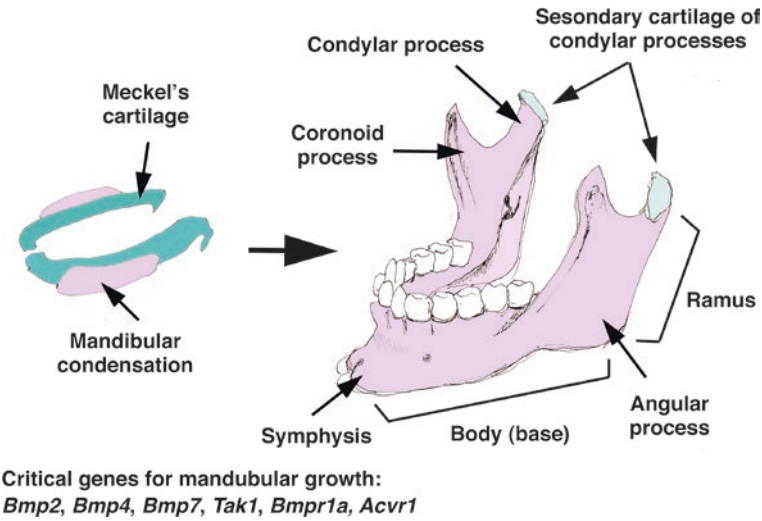


Fig. 5 Mandibular development. A pair of mandibular condensation occurs along with Meckel's cartilage that forms the body of mandibular. Another pair of condensation forms posteriorly to give rise ramus of mandibular that eventually fused with the body of the mandibular. Secondary cartilage is developed at the tip of the condylar process and participates formation of the temporomandibular joint

intramembranous ossification; however, processes from these bones such as condyle, coronoid, and symphysis undergo endochondral ossification. The body of the mandible forms along with the Meckel's cartilage; however, cells in the Meckel's cartilage do not contribute the body. The body and the ramus form separately then fused together [45]. Cartilage is formed between 10 and 14 weeks in the human fetus at the head of condyle, coronoid, and symphysis. These cartilages are called as secondary cartilage since the cartilage primordia for endochondral ossification are formed at 5 weeks. The endochondral bone growth driven by the condylar cartilage is the most significant contributor to mandibular growth. The condylar process articulates to the temporal bone to form the temporomandibular joint (TMJ) [72, 73].

3.4.1 BMP and Mandible

Bmp2 and *Bmp7* are expressed at early stages of the developing Meckel's cartilage, while Noggin expression persists and is continuous [210]. Noggin-deficient mice that result in increased pSmad1/pSmad5/pSmad9 develop a significantly thicker Meckel's cartilage that is later ossified instead of degenerating [210]. In contrast, the growth of Meckel's cartilage is reduced in *Bmp7*-deficient mice [108]. This animal model develops small mandible (micrognathia) leading to cleft palate since the palatal shelves can fuse when whole upper jaws are cultured in vitro [108, 237]. Similar skeletal defects are observed when *Tak1*, a downstream component critical for non-Smad signaling pathway, is disrupted in a neural crest-specific manner and the cleft palate phenotype is rescued when the whole upper jaws are cultured [230]. These suggest that compromised BMP signaling during mandibular development may be one of the causes of the Pierre Robin syndrome [196].

Neural crest-specific disruption of both *Bmp2* and *Bmp4* using *Wnt1*-Cre results in mandibular and cranial bone defects in mice [25]. Subsequent analyses demonstrate that BMP signaling is required for self-renewal of cranial neural crest cells, and thus the loss of BMP signaling results in micrognathia and enlarged frontal fontanelle phenotype [25]. Similar skeletal phenotypes are reported in neural crest-specific mutant mice for *Acvr1* [48]. In contrast, overexpression of *Bmp4* in neural crest cells leads to syngnathia, a rare human bony birth defect manifested by a bony connection between maxilla and mandible [69].

3.4.2 BMP and the Temporomandibular Joint

The temporomandibular joint (TMJ) forms between the condyle process and the temporal bone in the calvarial vault and plays a critical role in jaw movement during chewing and articulating sound while speaking. The secondary cartilage found in the TMJ is different from primary cartilages by the fact that cells in the prechondroblastic layer produce type 1 collagens rather than type 2 collagens [73]. Cells in the prechondroblastic layer are dual potent, i.e., they can differentiate into either

cartilage or bone depending on their mechanical environment [58, 133]. Direct transformation of chondrocytes in condylar cartilage into osteoblasts is recently demonstrated in vivo using a lineage tracing technique [87]. Genes affecting growth and differentiation of primary cartilages such as *Sox9*, *Shh*, and *Pthrp* play important roles in normal TMJ development [72, 84]. Neural crest-specific disruption of *Bmpr1a* results in malformation of TMJ including failure of articular disc separation from a hypoplastic condyle [60]. Similarly, cartilage-specific removal of *Bmpr1a* also develops chondrodysplastic phenotypes in TMJ, and mandibular condyle growth is significantly compromised [85]. In the global *Bmp7* mutant mice, the secondary cartilage does not form at the anterior end of the mandible (symphysis) [106]. In this animal model, condylar cartilage however seems to be developed suggesting that requirement of BMP signaling activity in the secondary cartilage may be different depending on anatomical sites.

4 Perspective and Conclusions

The current review elucidates how BMP signal has multifaceted functions in different cell types, ages, and anatomical sites of bones. Knowledge gained from studies on genetically altered animal models and human genetics demonstrates that functions of BMP signaling are highly context dependent and that alterations of BMP signaling in one tissue type secondarily affect behavior of other tissues. It is noteworthy that levels of BMP2 or BMP7 clinically used for fracture healing are very high compared with endogenous levels of BMPs. The functions of BMPs that we have learned from clinical applications may be better applied to understand pathogenesis of genetically induced and trauma-induced heterotopic ossifications [2, 3, 165, 185]. It is now an established concept that both bone mass and bone quality such as collagen cross-linking and mineral crystallinity are important factors contributing to biomechanical properties of bones [14, 81]. How BMP signaling influences bone quality in addition to bone mass in a physiological condition is an interesting future direction.

Acknowledgment We thank Dr. Sudha Rajderkar for critical reading and Yoshiko Mishina for her artwork. We are sorry for not including all critical references due to the space limitation. Y.M. is supported by the National Institutes of Health (R01DE020843) and the Department of Defense (W81XWH-11-2-0073).

References

1. Abe E, Yamamoto M, Taguchi Y, Lecka-Czernik B, O'Brien CA, Economides AN, Stahl N, Jilka RL, Manolagas SC (2000) Essential requirement of BMPs-2/4 for both osteoblast and osteoclast formation in murine bone marrow cultures from adult mice: antagonism by noggin. J Bone Miner Res 15:663–673

2. Agarwal S, Loder S, Brownley C, Cholok D, Mangiavini L, Li J, Breuler C, Sung HH, Li S, Ranganathan K et al (2016) Inhibition of Hif1alpha prevents both trauma-induced and genetic heterotopic ossification. Proc Natl Acad Sci U S A 113(3):E338–E347
3. Agarwal S, Loder SJ, Brownley C, Eboda O, Peterson JR, Hayano S, Wu B, Zhao B, Kaartinen V, Wong VC et al (2015) BMP signaling mediated by constitutively active Activin type 1 receptor (ACVR1) results in ectopic bone formation localized to distal extremity joints. Dev Biol 400:202–209
4. Alappat S, Zhang ZY, Chen YP (2003) Msx homeobox gene family and craniofacial development. Cell Res 13:429–442
5. Asai-Coakwell M, French CR, Ye M, Garcha K, Bigot K, Perera AG, Staehling-Hampton K, Mema SC, Chanda B, Mushegian A et al (2009) Incomplete penetrance and phenotypic variability characterize Gdf6-attributable oculo-skeletal phenotypes. Hum Mol Genet 18:1110–1121
6. Babij P, Zhao W, Small C, Kharode Y, Yaworsky PJ, Bouxsein ML, Reddy PS, Bodine PV, Robinson JA, Bhat B et al (2003) High bone mass in mice expressing a mutant LRP5 gene. J Bone Miner Res 18:960–974
7. Bain G, Muller T, Wang X, Papkoff J (2003) Activated beta-catenin induces osteoblast differentiation of C3H10T1/2 cells and participates in BMP2 mediated signal transduction. Biochem Biophys Res Commun 301:84–91
8. Bakrania P, Efthymiou M, Klein JC, Salt A, Bunyan DJ, Wyatt A, Ponting CP, Martin A, Williams S, Lindley V et al (2008) Mutations in BMP4 cause eye, brain, and digit developmental anomalies: overlap between the BMP4 and hedgehog signaling pathways. Am J Hum Genet 82:304–319
9. Balemans W, Ebeling M, Patel N, Van Hul E, Olson P, Dioszegi M, Lacza C, Wuyts W, Van Den Ende J, Willems P et al (2001) Increased bone density in sclerosteosis is due to the deficiency of a novel secreted protein (SOST). Hum Mol Genet 10:537–543
10. Balemans W, Patel N, Ebeling M, Van Hul E, Wuyts W, Lacza C, Dioszegi M, Dikkers FG, Hildering P, Willems PJ et al (2002) Identification of a 52 kb deletion downstream of the SOST gene in patients with van Buchem disease. J Med Genet 39:91–97
11. Bandyopadhyay A, Tsuji K, Cox K, Harfe BD, Rosen V, Tabin CJ (2006) Genetic analysis of the roles of BMP2, BMP4, and BMP7 in limb patterning and skeletogenesis. PLoS Genet 2:e216
12. Bandyopadhyay A, Yadav PS, Prashar P (2013) BMP signaling in development and diseases: a pharmacological perspective. Biochem Pharmacol 85:857–864
13. Banerjee C, Javed A, Choi JY, Green J, Rosen V, van Wijnen AJ, Stein JL, Lian JB, Stein GS (2001) Differential regulation of the two principal Runx2/Cbfa1 n-terminal isoforms in response to bone morphogenetic protein-2 during development of the osteoblast phenotype. Endocrinology 142:4026–4039
14. Banse X (2002) When density fails to predict bone strength. Acta Orthop Scand Suppl 73:1–57
15. Barlow AJ, Francis-West PH (1997) Ectopic application of recombinant BMP-2 and BMP-4 can change patterning of developing chick facial primordia. Development 124: 391–398
16. Baron R, Rawadi G, Roman-Roman S (2006) Wnt signaling: a key regulator of bone mass. Curr Top Dev Biol 76:103–127
17. Barrow JR, Thomas KR, Boussadia-Zahui O, Moore R, Kemler R, Capecchi MR, McMahon AP (2003) Ectodermal Wnt3/beta-catenin signaling is required for the establishment and maintenance of the apical ectodermal ridge. Genes Dev 17:394–409
18. Behr B, Panetta NJ, Longaker MT, Quarto N (2010) Different endogenous threshold levels of Fibroblast Growth Factor-ligands determine the healing potential of frontal and parietal bones. Bone 47:281–294
19. Bell E, Munoz-Sanjuan I, Altmann CR, Vonica A, Brivanlou AH (2003) Cell fate specification and competence by Coco, a maternal BMP, TGFbeta and Wnt inhibitor. Development 130:1381–1389

20. Belloni E, Muenke M, Roessler E, Traverso G, Siegel-Bartelt J, Frumkin A, Mitchell HF, Donis-Keller H, Helms C, Hing AV et al (1996) Identification of Sonic hedgehog as a candidate gene responsible for holoprosencephaly. Nat Genet 14:353–356
21. Bennett JH, Hunt P, Thorogood P (1995) Bone morphogenetic protein-2 and −4 expression during murine orofacial development. Arch Oral Biol 40:847–854
22. Bertola DR, Rodrigues MG, Quaio CR, Kim CA, Passos-Bueno MR (2013) Vertical transmission of a frontonasal phenotype caused by a novel ALX4 mutation. Am J Med Genet A 161A:600–604
23. Beverdam A, Brouwer A, Reijnen M, Korving J, Meijlink F (2001) Severe nasal clefting and abnormal embryonic apoptosis in Alx3/Alx4 double mutant mice. Development 128:3975–3986
24. Bhatt S, Diaz R, Trainor PA (2013) Signals and switches in Mammalian neural crest cell differentiation. Cold Spring Harb Perspect Biol 5(2)
25. Bonilla-Claudio M, Wang J, Bai Y, Klysik E, Selever J, Martin JF (2012) Bmp signaling regulates a dose-dependent transcriptional program to control facial skeletal development. Development 139:709–719
26. Boyden LM, Mao J, Belsky J, Mitzner L, Farhi A, Mitnick MA, Wu D, Insogna K, Lifton RP (2002) High bone density due to a mutation in LDL-receptor-related protein 5. N Engl J Med 346:1513–1521
27. Brown JM, Robertson KE, Wedden SE, Tickle C (1997) Alterations in Msx 1 and Msx 2 expression correlate with inhibition of outgrowth of chick facial primordia induced by retinoic acid. Anat Embryol (Berl) 195:203–207
28. Brown JM, Wedden SE, Millburn GH, Robson LG, Hill RE, Davidson DR, Tickle C (1993) Experimental analysis of the control of expression of the homeobox-gene Msx-1 in the developing limb and face. Development 119:41–48
29. Brugmann SA, Allen NC, James AW, Mekonnen Z, Madan E, Helms JA (2010) A primary cilia-dependent etiology for midline facial disorders. Hum Mol Genet 19:1577–1592
30. Brunkow ME, Gardner JC, Van Ness J, Paeper BW, Kovacevich BR, Proll S, Skonier JE, Zhao L, Sabo PJ, Fu Y et al (2001) Bone dysplasia sclerosteosis results from loss of the SOST gene product, a novel cystine knot-containing protein. Am J Hum Genet 68:577–589
31. Carano RA, Filvaroff EH (2003) Angiogenesis and bone repair. Drug Discov Today 8:980–989
32. Castranio T, Mishina Y (2009) Bmp2 is required for cephalic neural tube closure in the mouse. Dev Dyn 238:110–122
33. Chen Y, Whetstone HC, Youn A, Nadesan P, Chow EC, Lin AC, Alman BA (2007) Beta-catenin signaling pathway is crucial for bone morphogenetic protein 2 to induce new bone formation. J Biol Chem 282:526–533
34. Cheng H, Jiang W, Phillips FM, Haydon RC, Peng Y, Zhou L, Luu HH, An N, Breyer B, Vanichakarn P et al (2003) Osteogenic activity of the fourteen types of human bone morphogenetic proteins (BMPs). J Bone Joint Surg Am 85-A:1544–1552
35. Chiang C, Litingtung Y, Lee E, Young KE, Corden JL, Westphal H, Beachy PA (1996) Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. Nature 383:407–413
36. Creuzet S, Schuler B, Couly G, Le Douarin NM (2004) Reciprocal relationships between Fgf8 and neural crest cells in facial and forebrain development. Proc Natl Acad Sci U S A 101:4843–4847
37. Dathe K, Kjaer KW, Brehm A, Meinecke P, Nurnberg P, Neto JC, Brunoni D, Tommerup N, Ott CE, Klopocki E et al (2009) Duplications involving a conserved regulatory element downstream of BMP2 are associated with brachydactyly type A2. Am J Hum Genet 84:483–492
38. David L, Feige JJ, Bailly S (2009) Emerging role of bone morphogenetic proteins in angiogenesis. Cytokine Growth Factor Rev 20:203–212
39. Davis S, Miura S, Hill C, Mishina Y, Klingensmith J (2004) BMP receptor IA is required in the mammalian embryo for endodermal morphogenesis and ectodermal patterning. Dev Biol 270:47–63

40. Deckers MM, van Bezooijen RL, van der Horst G, Hoogendam J, van Der Bent C, Papapoulos SE, Lowik CW (2002) Bone morphogenetic proteins stimulate angiogenesis through osteoblast-derived vascular endothelial growth factor A. Endocrinology 143: 1545–1553
41. Degenkolbe E, Konig J, Zimmer J, Walther M, Reissner C, Nickel J, Ploger F, Raspopovic J, Sharpe J, Dathe K et al (2014) A GDF5 point mutation strikes twice--causing BDA1 and SYNS2. PLoS Genet 9:e1003846
42. Demirhan O, Turkmen S, Schwabe GC, Soyupak S, Akgul E, Tastemir D, Karahan D, Mundlos S, Lehmann K (2005) A homozygous BMPR1B mutation causes a new subtype of acromesomelic chondrodysplasia with genital anomalies. J Med Genet 42:314–317
43. Denker AE, Haas AR, Nicoll SB, Tuan RS (1999) Chondrogenic differentiation of murine C3H10T1/2 multipotential mesenchymal cells: I. Stimulation by bone morphogenetic protein-2 in high-density micromass cultures. Differentiation 64:67–76
44. Ding J, Yang L, Yan YT, Chen A, Desai N, Wynshaw-Boris A, Shen MM (1998) Cripto is required for correct orientation of the anterior-posterior axis in the mouse embryo. Nature 395:702–707
45. Dixon MJ, Marazita ML, Beaty TH, Murray JC (2011) Cleft lip and palate: understanding genetic and environmental influences. Nat Rev Genet 12:167–178
46. Dudas M, Kim J, Li WY, Nagy A, Larsson J, Karlsson S, Chai Y, Kaartinen V (2006) Epithelial and ectomesenchymal role of the type I TGF-beta receptor ALK5 during facial morphogenesis and palatal fusion. Dev Biol 296:298–314
47. Dudas M, Nagy A, Laping NJ, Moustakas A, Kaartinen V (2004) Tgf-beta3-induced palatal fusion is mediated by Alk-5/Smad pathway. Dev Biol 266:96–108
48. Dudas M, Sridurongrit S, Nagy A, Okazaki K, Kaartinen V (2004) Craniofacial defects in mice lacking BMP type I receptor Alk2 in neural crest cells. Mech Dev 121:173–182
49. Dudley AT, Lyons KM, Robertson EJ (1995) A requirement for bone morphogenetic protein-7 during development of the mammalian kidney and eye. Genes Dev 9:2795–2807
50. Dwivedi PP, Grose RH, Filmus J, Hii CS, Xian CJ, Anderson PJ, Powell BC (2013) Regulation of bone morphogenetic protein signalling and cranial osteogenesis by Gpc1 and Gpc3. Bone 55:367–376
51. Franz-Odendaal TA (2011) Induction and patterning of intramembranous bone. Front Biosci (Landmark Ed) 16:2734–2746
52. Fromigue O, Modrowski D, Marie PJ (2005) Apoptosis in membranous bone formation: role of fibroblast growth factor and bone morphogenetic protein signaling. Crit Rev Eukaryot Gene Expr 15:75–92
53. Fukuda T, Scott G, Komatsu Y, Araya R, Kawano M, Ray MK, Yamada M, Mishina Y (2006) Generation of a mouse with conditionally activated signaling through the BMP receptor, ALK2. Genesis 44:159–167
54. Gamer LW, Tsuji K, Cox K, Capelo LP, Lowery J, Beppu H, Rosen V (2011) BMPR-II is dispensable for formation of the limb skeleton. Genesis 49:719–724
55. Garimella R, Tague SE, Zhang J, Belibi F, Nahar N, Sun BH, Insogna K, Wang J, Anderson HC (2008) Expression and synthesis of bone morphogenetic proteins by osteoclasts: a possible path to anabolic bone remodeling. J Histochem Cytochem 56:569–577
56. Garrison KR, Donell S, Ryder J, Shemilt I, Mugford M, Harvey I, Song F (2007) Clinical effectiveness and cost-effectiveness of bone morphogenetic proteins in the non-healing of fractures and spinal fusion: a systematic review. Health Technol Assess 11:1–168
57. Glass DA 2nd, Karsenty G (2006) Molecular bases of the regulation of bone remodeling by the canonical Wnt signaling pathway. Curr Top Dev Biol 73:43–84
58. Glineburg RW, Laskin DM, Blaustein DI (1982) The effects of immobilization on the primate temporomandibular joint: a histologic and histochemical study. J Oral Maxillofac Surg 40:3–8
59. Gong Y, Slee RB, Fukai N, Rawadi G, Roman-Roman S, Reginato AM, Wang H, Cundy T, Glorieux FH, Lev D et al (2001) LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development. Cell 107:513–523

60. Gu S, Wu W, Liu C, Yang L, Sun C, Ye W, Li X, Chen J, Long F, Chen Y (2014) BMPRIA mediated signaling is essential for temporomandibular joint development in mice. PLoS One 9:e101000
61. Gu Z, Reynolds EM, Song J, Lei H, Feijen A, Yu L, He W, MacLaughlin DT, van den Eijnden-van Raaij J, Donahoe PK et al (1999) The type I serine/threonine kinase receptor ActRIA (ALK2) is required for gastrulation of the mouse embryo. Development 126: 2551–2561
62. Gupta MC, Khan SN (2005) Application of bone morphogenetic proteins in spinal fusion. Cytokine Growth Factor Rev 16:347–355
63. Harada S, Rodan GA (2003) Control of osteoblast function and regulation of bone mass. Nature 423:349–355
64. Hartmann C (2006) A Wnt canon orchestrating osteoblastogenesis. Trends Cell Biol 16:151–158
65. Hatch NE (2010) FGF signaling in craniofacial biological control and pathological craniofacial development. Crit Rev Eukaryot Gene Expr 20:295–311
66. Hatta T, Konishi H, Katoh E, Natsume T, Ueno N, Kobayashi Y, Yamazaki T (2000) Identification of the ligand-binding site of the BMP type IA receptor for BMP-4. Biopolymers 55:399–406
67. Hayano S, Komatsu Y, Pan H, Mishina Y (2015) Augmented BMP signaling in the neural crest inhibits nasal cartilage morphogenesis by inducing p53-mediated apoptosis. Development 142:1357–1367
68. Hays E, Schmidt J, Chandar N (2009) Beta-catenin is not activated by downregulation of PTEN in osteoblasts. In Vitro Cell Dev Biol Anim 45:361–370
69. He F, Hu X, Xiong W, Li L, Lin L, Shen B, Yang L, Gu S, Zhang Y, Chen Y (2014) Directed Bmp4 expression in neural crest cells generates a genetic model for the rare human bony syngnathia birth defect. Dev Biol 391:170–181
70. He F, Xiong W, Wang Y, Matsui M, Yu X, Chai Y, Klingensmith J, Chen Y (2010) Modulation of BMP signaling by Noggin is required for the maintenance of palatal epithelial integrity during palatogenesis. Dev Biol 347:109–121
71. Henriksen K, Neutzsky-Wulff AV, Bonewald LF, Karsdal MA (2009) Local communication on and within bone controls bone remodeling. Bone 44:1026–1033
72. Hinton RJ (2014) Genes that regulate morphogenesis and growth of the temporomandibular joint: a review. Dev Dyn 243:864–874
73. Hinton RJ, Jing J, Feng JQ (2015) Genetic influences on temporomandibular joint development and growth. Curr Top Dev Biol 115:85–109
74. Ho CT, Lau TY, Jin Y, Lu HB, Liong E, Leung KM, Tipoe GL (2004) Overexpression of iNOS and down-regulation of BMPs-2, 4 and 7 in retinoic acid induced cleft palate formation. Histol Histopathol 19:95–104
75. Hu Q, Ueno N, Behringer RR (2004) Restriction of BMP4 activity domains in the developing neural tube of the mouse embryo. EMBO Rep 5:734–739
76. Huang QY, Li GH, Kung AW (2009) The −9247 T/C polymorphism in the SOST upstream regulatory region that potentially affects C/EBPalpha and FOXA1 binding is associated with osteoporosis. Bone 45:289–294
77. Huelsken J, Vogel R, Erdmann B, Cotsarelis G, Birchmeier W (2001) beta-Catenin controls hair follicle morphogenesis and stem cell differentiation in the skin. Cell 105:533–545
78. Ishii M, Merrill AE, Chan YS, Gitelman I, Rice DP, Sucov HM, Maxson RE Jr (2003) Msx2 and Twist cooperatively control the development of the neural crest-derived skeletogenic mesenchyme of the murine skull vault. Development 130:6131–6142
79. Itasaki N, Jones CM, Mercurio S, Rowe A, Domingos PM, Smith JC, Krumlauf R (2003) Wise, a context-dependent activator and inhibitor of Wnt signalling. Development 130:4295–4305
80. Itoh K, Udagawa N, Katagiri T, Iemura S, Ueno N, Yasuda H, Higashio K, Quinn JM, Gillespie MT, Martin TJ et al (2001) Bone morphogenetic protein 2 stimulates osteoclast differentiation and survival supported by receptor activator of nuclear factor-kappaB ligand. Endocrinology 142:3656–3662

81. Iura A, McNerny EG, Zhang Y, Kamiya N, Tantillo M, Lynch M, Kohn DH, Mishina Y (2015) Mechanical Loading Synergistically Increases Trabecular Bone Volume and Improves Mechanical Properties in the Mouse when BMP Signaling Is Specifically Ablated in Osteoblasts. PLoS One 10:e0141345
82. Jabs EW, Muller U, Li X, Ma L, Luo W, Haworth IS, Klisak I, Sparkes R, Warman ML, Mulliken JB et al (1993) A mutation in the homeodomain of the human MSX2 gene in a family affected with autosomal dominant craniosynostosis. Cell 75:443–450
83. Jiang X, Iseki S, Maxson RE, Sucov HM, Morriss-Kay GM (2002) Tissue origins and interactions in the mammalian skull vault. Dev Biol 241:106–116
84. Jing J, Hinton RJ, Feng JQ (2015) Bmpr1a signaling in cartilage development and endochondral bone formation. Vitam Horm 99:273–291
85. Jing J, Hinton RJ, Mishina Y, Liu Y, Zhou X, Feng JQ (2014) Critical role of Bmpr1a in mandibular condyle growth. Connect Tissue Res 55(Suppl 1):73–78
86. Jing J, Ren Y, Zong Z, Liu C, Kamiya N, Mishina Y, Liu Y, Zhou X, Feng JQ (2013) BMP receptor 1 A determines the cell fate of the postnatal growth plate. Int J Biol Sci 9:895–906
87. Jing Y, Zhou X, Han X, Jing J, von der Mark K, Wang J, de Crombrugghe B, Hinton RJ, Feng JQ (2015) Chondrocytes directly transform into bone cells in mandibular condyle growth. J Dent Res 94:1668–1675
88. Justice CM, Yagnik G, Kim Y, Peter I, Jabs EW, Erazo M, Ye X, Ainehsazan E, Shi L, Cunningham ML et al (2012) A genome-wide association study identifies susceptibility loci for nonsyndromic sagittal craniosynostosis near BMP2 and within BBS9. Nat Genet 44:1360–1364
89. Kaartinen V, Voncken JW, Shuler C, Warburton D, Bu D, Heisterkamp N, Groffen J (1995) Abnormal lung development and cleft palate in mice lacking TGF-beta 3 indicates defects of epithelial-mesenchymal interaction. Nat Genet 11:415–421
90. Kamiya N, Jikko A, Kimata K, Damsky C, Shimizu K, Watanabe H (2002) Establishment of a novel chondrocytic cell line N1511 derived from p53-null mice. J Bone Miner Res 17:1832–1842
91. Kamiya N, Kaartinen VM, Mishina Y (2011) Loss-of-function of ACVR1 in osteoblasts increases bone mass and activates canonical Wnt signaling through suppression of Wnt inhibitors SOST and DKK1. Biochem Biophys Res Commun 414:326–330
92. Kamiya N, Kobayashi T, Mochida Y, Yu PB, Yamauchi M, Kronenberg HM, Mishina Y (2010) Wnt inhibitors Dkk1 and Sost are downstream targets of BMP signaling through the type IA receptor (BMPRIA) in osteoblasts. J Bone Miner Res 25:200–210
93. Kamiya N, Shuxian L, Yamaguchi R, Phipps M, Aruwajoye O, Adapala NS, Yuan H, Kim HK, Feng JQ (2016) Targeted disruption of BMP signaling through type IA receptor (BMPR1A) in osteocyte suppresses SOST and RANKL, leading to dramatic increase in bone mass, bone mineral density and mechanical strength. Bone 91:53–63. doi: 10.1016/j.bone.2016.07.002. Epub 2016 Jul 8. PMID: 27402532
94. Kamiya N, Ye L, Kobayashi T, Lucas DJ, Mochida Y, Yamauchi M, Kronenberg HM, Feng JQ, Mishina Y (2008) Disruption of BMP signaling in osteoblasts through type IA receptor (BMPRIA) increases bone mass. J Bone Miner Res 23:2007–2017
95. Kamiya N, Ye L, Kobayashi T, Mochida Y, Yamauchi M, Kronenberg HM, Feng JQ, Mishina Y (2008) BMP signaling negatively regulates bone mass through sclerostin by inhibiting the canonical Wnt pathway. Development 135:3801–3811
96. Kan L, Hu M, Gomes WA, Kessler JA (2004) Transgenic mice overexpressing BMP4 develop a fibrodysplasia ossificans progressiva (FOP)-like phenotype. Am J Pathol 165:1107–1115
97. Kanczler JM, Oreffo RO (2008) Osteogenesis and angiogenesis: the potential for engineering bone. Eur Cell Mater 15:100–114
98. Kaneko H, Arakawa T, Mano H, Kaneda T, Ogasawara A, Nakagawa M, Toyama Y, Yabe Y, Kumegawa M, Hakeda Y (2000) Direct stimulation of osteoclastic bone resorption by bone morphogenetic protein (BMP)-2 and expression of BMP receptors in mature osteoclasts. Bone 27:479–486

99. Katagiri T, Yamaguchi A, Komaki M, Abe E, Takahashi N, Ikeda T, Rosen V, Wozney JM, Fujisawa-Sehara A, Suda T (1994) Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. J Cell Biol 127:1755–1766
100. Kettunen P, Nie X, Kvinnsland IH, Luukko K (2006) Histological development and dynamic expression of Bmp2-6 mRNAs in the embryonic and postnatal mouse cranial base. Anat Rec A Discov Mol Cell Evol Biol 288:1250–1258
101. Kim HJ, Rice DP, Kettunen PJ, Thesleff I (1998) FGF-, BMP- and Shh-mediated signalling pathways in the regulation of cranial suture morphogenesis and calvarial bone development. Development 125:1241–1251
102. Kim HK, Oxendine I, Kamiya N (2013) High-concentration of BMP2 reduces cell proliferation and increases apoptosis via DKK1 and SOST in human primary periosteal cells. Bone 54:141–150
103. Kishigami S, Mishina Y (2005) BMP signaling and early embryonic patterning. Cytokine Growth Factor Rev 16:265–278
104. Klingensmith J, Matsui M, Yang YP, Anderson RM (2010) Roles of bone morphogenetic protein signaling and its antagonism in holoprosencephaly. Am J Med Genet C Semin Med Genet 154C:43–51
105. Komatsu Y, Yu PB, Kamiya N, Pan H, Fukuda T, Scott GJ, Ray MK, Yamamura K, Mishina Y (2013) Augmentation of Smad-dependent BMP signaling in neural crest cells causes craniosynostosis in mice. J Bone Miner Res 28:1422–1433
106. Kouskoura T, El Fersioui Y, Angelini M, Graf D, Katsaros C, Chiquet M (2016) Dislocated tongue muscle attachment and cleft palate formation. J Dent Res 95(4):453–459
107. Kouskoura T, Fragou N, Alexiou M, John N, Sommer L, Graf D, Katsaros C, Mitsiadis TA (2011) The genetic basis of craniofacial and dental abnormalities. Schweiz Monatsschr Zahnmed 121:636–646
108. Kouskoura T, Kozlova A, Alexiou M, Blumer S, Zouvelou V, Katsaros C, Chiquet M, Mitsiadis TA, Graf D (2013) The etiology of cleft palate formation in BMP7-deficient mice. PLoS One 8:e59463
109. Krishnan V, Bryant HU, Macdougald OA (2006) Regulation of bone mass by Wnt signaling. J Clin Invest 116:1202–1209
110. Kronenberg HM (2003) Developmental regulation of the growth plate. Nature 423:332–336
111. Kusu N, Laurikkala J, Imanishi M, Usui H, Konishi M, Miyake A, Thesleff I, Itoh N (2003) Sclerostin is a novel secreted osteoclast-derived bone morphogenetic protein antagonist with unique ligand specificity. J Biol Chem 278:24113–24117
112. Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S et al (1998) Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 93:165–176
113. Lana-Elola E, Tylzanowski P, Takatalo M, Alakurtti K, Veistinen L, Mitsiadis TA, Graf D, Rice R, Luyten FP, Rice DP (2011) Noggin null allele mice exhibit a microform of holoprosencephaly. Hum Mol Genet 20:4005–4015
114. Lee MH, Javed A, Kim HJ, Shin HI, Gutierrez S, Choi JY, Rosen V, Stein JL, van Wijnen AJ, Stein GS et al (1999) Transient upregulation of CBFA1 in response to bone morphogenetic protein-2 and transforming growth factor beta1 in C2C12 myogenic cells coincides with suppression of the myogenic phenotype but is not sufficient for osteoblast differentiation. J Cell Biochem 73:114–125
115. Lehmann K, Seemann P, Silan F, Goecke TO, Irgang S, Kjaer KW, Kjaergaard S, Mahoney MJ, Morlot S, Reissner C et al (2007) A new subtype of brachydactyly type B caused by point mutations in the bone morphogenetic protein antagonist NOGGIN. Am J Hum Genet 81:388–396
116. Lehmann K, Seemann P, Stricker S, Sammar M, Meyer B, Suring K, Majewski F, Tinschert S, Grzeschik KH, Muller D et al (2003) Mutations in bone morphogenetic protein receptor 1B cause brachydactyly type A2. Proc Natl Acad Sci U S A 100:12277–12282
117. Li J, Feng J, Liu Y, Ho TV, Grimes W, Ho HA, Park S, Wang S, Chai Y (2015) BMP-SHH signaling network controls epithelial stem cell fate via regulation of its niche in the developing tooth. Dev Cell 33:125–135

118. Li J, Sarosi I, Cattley RC, Pretorius J, Asuncion F, Grisanti M, Morony S, Adamu S, Geng Z, Qiu W et al (2006) Dkk1-mediated inhibition of Wnt signaling in bone results in osteopenia. Bone 39:754–766
119. Li L, Lin M, Wang Y, Cserjesi P, Chen Z, Chen Y (2011) BmprIa is required in mesenchymal tissue and has limited redundant function with BmprIb in tooth and palate development. Dev Biol 349:451–461
120. Li S, Meyer NP, Quarto N, Longaker MT (2013) Integration of multiple signaling regulates through apoptosis the differential osteogenic potential of neural crest-derived and mesoderm-derived Osteoblasts. PLoS One 8:e58610
121. Li S, Quarto N, Longaker MT (2010) Activation of FGF signaling mediates proliferative and osteogenic differences between neural crest derived frontal and mesoderm parietal derived bone. PLoS One 5:e14033
122. Li X, Ominsky MS, Niu QT, Sun N, Daugherty B, D'Agostin D, Kurahara C, Gao Y, Cao J, Gong J et al (2008) Targeted deletion of the sclerostin gene in mice results in increased bone formation and bone strength. J Bone Miner Res 23:860–869
123. Li X, Zhang Y, Kang H, Liu W, Liu P, Zhang J, Harris SE, Wu D (2005) Sclerostin binds to LRP5/6 and antagonizes canonical Wnt signaling. J Biol Chem 280:19883–19887
124. Lim J, Shi Y, Karner CM, Lee SY, Lee WC, He G, Long F (2016) Dual function of Bmpr1a signaling in restricting preosteoblast proliferation and stimulating osteoblast activity in the mouse. Development 143(2):339–347
125. Little RD, Carulli JP, Del Mastro RG, Dupuis J, Osborne M, Folz C, Manning SP, Swain PM, Zhao SC, Eustace B et al (2002) A mutation in the LDL receptor-related protein 5 gene results in the autosomal dominant high-bone-mass trait. Am J Hum Genet 70:11–19
126. Liu W, Sun X, Braut A, Mishina Y, Behringer RR, Mina M, Martin JF (2005) Distinct functions for Bmp signaling in lip and palate fusion in mice. Development 132:1453–1461
127. Liu Z, Tang Y, Qiu T, Cao X, Clemens TL (2006) A dishevelled-1/Smad1 interaction couples WNT and bone morphogenetic protein signaling pathways in uncommitted bone marrow stromal cells. J Biol Chem 281:17156–17163
128. Logan M, Martin JF, Nagy A, Lobe C, Olson EN, Tabin CJ (2002) Expression of Cre Recombinase in the developing mouse limb bud driven by a Prxl enhancer. Genesis 33:77–80
129. Lounev VY, Ramachandran R, Wosczyna MN, Yamamoto M, Maidment AD, Shore EM, Glaser DL, Goldhamer DJ, Kaplan FS (2009) Identification of progenitor cells that contribute to heterotopic skeletogenesis. J Bone Joint Surg Am 91:652–663
130. Lowery JW, Intini G, Gamer L, Lotinun S, Salazar VS, Ote S, Cox K, Baron R, Rosen V (2015) Loss of BMPR2 leads to high bone mass due to increased osteoblast activity. J Cell Sci 128:1308–1315
131. Lu H, Jin Y, Tipoe GL (2000) Alteration in the expression of bone morphogenetic protein-2,3,4,5 mRNA during pathogenesis of cleft palate in BALB/c mice. Arch Oral Biol 45:133–140
132. Luo G, Hofmann C, Bronckers AL, Sohocki M, Bradley A, Karsenty G (1995) BMP-7 is an inducer of nephrogenesis, and is also required for eye development and skeletal patterning. Genes Dev 9:2808–2820
133. Lydiatt DD, Davis LF (1985) The effects of immobilization on the rabbit temporomandibular joint. J Oral Maxillofac Surg 43:188–193
134. Lyons KM, Pelton RW, Hogan BL (1990) Organogenesis and pattern formation in the mouse: RNA distribution patterns suggest a role for bone morphogenetic protein-2 A (BMP-2 A). Development 109:833–844
135. Macatee TL, Hammond BP, Arenkiel BR, Francis L, Frank DU, Moon AM (2003) Ablation of specific expression domains reveals discrete functions of ectoderm- and endoderm-derived FGF8 during cardiovascular and pharyngeal development. Development 130:6361–6374
136. Macias-Silva M, Hoodless PA, Tang SJ, Buchwald M, Wrana JL (1998) Specific activation of Smad1 signaling pathways by the BMP7 type I receptor, ALK2. J Biol Chem 273:25628–25636
137. Manzanares MC, Goret-Nicaise M, Dhem A (1988) Metopic sutural closure in the human skull. J Anat 161:203–215

138. Matsui M, Klingensmith J (2014) Multiple tissue-specific requirements for the BMP antagonist Noggin in development of the mammalian craniofacial skeleton. Dev Biol 392:168–181
139. Matsushita T, Wilcox WR, Chan YY, Kawanami A, Bukulmez H, Balmes G, Krejci P, Mekikian PB, Otani K, Yamaura I et al (2009) FGFR3 promotes synchondrosis closure and fusion of ossification centers through the MAPK pathway. Hum Mol Genet 18:227–240
140. Maxson R, Ishii M (2008) The Bmp pathway in skull vault development. Front Oral Biol 12:197–208
141. Mbalaviele G, Sheikh S, Stains JP, Salazar VS, Cheng SL, Chen D, Civitelli R (2005) Beta-catenin and BMP-2 synergize to promote osteoblast differentiation and new bone formation. J Cell Biochem 94:403–418
142. Miraoui H, Ringe J, Haupl T, Marie PJ (2010) Increased EFG- and PDGFalpha-receptor signaling by mutant FGF-receptor 2 contributes to osteoblast dysfunction in Apert craniosynostosis. Hum Mol Genet 19:1678–1689
143. Mishina Y, Crombie R, Bradley A, Behringer RR (1999) Multiple roles for activin-like kinase-2 signaling during mouse embryogenesis. Dev Biol 213:314–326
144. Mishina Y, Snider TN (2014) Neural crest cell signaling pathways critical to cranial bone development and pathology. Exp Cell Res 325:138–147
145. Mishina Y, Starbuck MW, Gentile MA, Fukuda T, Kasparcova V, Seedor JG, Hanks MC, Amling M, Pinero GJ, Harada S et al (2004) Bone morphogenetic protein type IA receptor signaling regulates postnatal osteoblast function and bone remodeling. J Biol Chem 279:27560–27566
146. Mishina Y, Suzuki A, Ueno N, Behringer RR (1995) Bmpr encodes a type I bone morphogenetic protein receptor that is essential for gastrulation during mouse embryogenesis. Genes Dev 9:3027–3037
147. Miura S, Singh AP, Mishina Y (2010) Bmpr1a is required for proper migration of the AVE through regulation of Dkk1 expression in the pre-streak mouse embryo. Dev Biol 341:246–254
148. Morikawa Y, Zehir A, Maska E, Deng C, Schneider MD, Mishina Y, Cserjesi P (2009) BMP signaling regulates sympathetic nervous system development through Smad4-dependent and -independent pathways. Development 136:3575–3584
149. Morriss-Kay GM, Wilkie AO (2005) Growth of the normal skull vault and its alteration in craniosynostosis: insights from human genetics and experimental studies. J Anat 207: 637–653
150. Morvan F, Boulukos K, Clement-Lacroix P, Roman Roman S, Suc-Royer I, Vayssiere B, Ammann P, Martin P, Pinho S, Pognonec P et al (2006) Deletion of a single allele of the Dkk1 gene leads to an increase in bone formation and bone mass. J Bone Miner Res 21:934–945
151. Mukhopadhyay M, Shtrom S, Rodriguez-Esteban C, Chen L, Tsukui T, Gomer L, Dorward DW, Glinka A, Grinberg A, Huang SP et al (2001) Dickkopf1 is required for embryonic head induction and limb morphogenesis in the mouse. Dev Cell 1:423–434
152. Nakashima A, Katagiri T, Tamura M (2005) Cross-talk between Wnt and bone morphogenetic protein 2 (BMP-2) signaling in differentiation pathway of C2C12 myoblasts. J Biol Chem 280:37660–37668
153. Nakashima T1, Hayashi M, Fukunaga T, Kurata K, Oh-Hora M, Feng JQ, Bonewald LF, Kodama T, Wutz A, Wagner EF, Penninger JM, Takayanagi H (2011) Evidence for osteocyte regulation of bone homeostasis through RANKL expression. Nat Med 17(10):1231–4. doi: 10.1038/nm.2452
154. Neskey D, Eloy JA, Casiano RR (2009) Nasal, septal, and turbinate anatomy and embryology. Otolaryngol Clin North Am 42(193–205):vii
155. Noda K, Mishina Y, Komatsu Y (2016) Constitutively active mutation of ACVR1 in oral epithelium causes submucous cleft palate in mice. Dev Biol 415(2):306–313
156. Nomura-Kitabayashi A, Phoon CK, Kishigami S, Rosenthal J, Yamauchi Y, Abe K, Yamamura K, Samtani R, Lo CW, Mishina Y (2009) Outflow tract cushions perform a critical valve-like function in the early embryonic heart requiring BMPRIA-mediated signaling in cardiac neural crest. Am J Physiol Heart Circ Physiol 297:H1617–H1628

157. Okamoto M, Murai J, Imai Y, Ikegami D, Kamiya N, Kato S, Mishina Y, Yoshikawa H, Tsumaki N (2011) Conditional deletion of Bmpr1a in differentiated osteoclasts increases osteoblastic bone formation, increasing volume of remodeling bone in mice. J Bone Miner Res 26:2511–2522
158. Okamoto M, Murai J, Yoshikawa H, Tsumaki N (2006) Bone morphogenetic proteins in bone stimulate osteoclasts and osteoblasts during bone development. J Bone Miner Res 21:1022–1033
159. Otsuka E, Notoya M, Hagiwara H (2003) Treatment of myoblastic C2C12 cells with BMP-2 stimulates vitamin D-induced formation of osteoclasts. Calcif Tissue Int 73:72–77
160. Pan Q, Yu Y, Chen Q, Li C, Wu H, Wan Y, Ma J, Sun F (2008) Sox9, a key transcription factor of bone morphogenetic protein-2-induced chondrogenesis, is activated through BMP pathway and a CCAAT box in the proximal promoter. J Cell Physiol 217:228–241
161. Parada C, Chai Y (2015) Mandible and tongue development. Curr Top Dev Biol 115:31–58
162. Passos-Bueno MR, Serti Eacute AE, Jehee FS, Fanganiello R, Yeh E (2008) Genetics of craniosynostosis: genes, syndromes, mutations and genotype-phenotype correlations. Front Oral Biol 12:107–143
163. Paul S, Lee JC, Yeh LC (2009) A comparative study on BMP-induced osteoclastogenesis and osteoblastogenesis in primary cultures of adult rat bone marrow cells. Growth Factors 27:121–131
164. Pederson L, Ruan M, Westendorf JJ, Khosla S, Oursler MJ (2008) Regulation of bone formation by osteoclasts involves Wnt/BMP signaling and the chemokine sphingosine-1-phosphate. Proc Natl Acad Sci U S A 105:20764–20769
165. Peterson JR, De La Rosa S, Eboda O, Cilwa KE, Agarwal S, Buchman SR, Cederna PS, Xi C, Morris MD, Herndon DN et al (2014) Treatment of heterotopic ossification through remote ATP hydrolysis. Sci Transl Med 6:255ra132
166. Phillips FM, Bolt PM, He TC, Haydon RC (2005) Gene therapy for spinal fusion. Spine J 5:250S–258S
167. Piccolo S, Agius E, Leyns L, Bhattacharyya S, Grunz H, Bouwmeester T, De Robertis EM (1999) The head inducer Cerberus is a multifunctional antagonist of Nodal, BMP and Wnt signals. Nature 397:707–710
168. Polinkovsky A, Robin NH, Thomas JT, Irons M, Lynn A, Goodman FR, Reardon W, Kant SG, Brunner HG, van der Burgt I et al (1997) Mutations in CDMP1 cause autosomal dominant brachydactyly type C. Nat Genet 17:18–19
169. Qu S, Tucker SC, Zhao Q, deCrombrugghe B, Wisdom R (1999) Physical and genetic interactions between Alx4 and Cart1. Development 126:359–369
170. Quarto N, Wan DC, Kwan MD, Panetta NJ, Li S, Longaker MT (2010) Origin matters: differences in embryonic tissue origin and Wnt signaling determine the osteogenic potential and healing capacity of frontal and parietal calvarial bones. J Bone Miner Res 25:1680–1694
171. Rawadi G, Vayssiere B, Dunn F, Baron R, Roman-Roman S (2003) BMP-2 controls alkaline phosphatase expression and osteoblast mineralization by a Wnt autocrine loop. J Bone Miner Res 18:1842–1853
172. Retting KN, Song B, Yoon BS, Lyons KM (2009) BMP canonical Smad signaling through Smad1 and Smad5 is required for endochondral bone formation. Development 136:1093–1104
173. Rice DP, Rice R, Thesleff I (2003) Molecular mechanisms in calvarial bone and suture development, and their relation to craniosynostosis. Eur J Orthod 25:139–148
174. Rigueur D, Brugger S, Anbarchian T, Kim JK, Lee YJ, Lyons K (2015) The type I BMP receptor ACVR1/ALK2 is required for chondrogenesis during development. J Bone Miner Res 30(4):733–741
175. Roessler E, Belloni E, Gaudenz K, Jay P, Berta P, Scherer SW, Tsui LC, Muenke M (1996) Mutations in the human Sonic Hedgehog gene cause holoprosencephaly. Nat Genet 14:357–360
176. Rountree RB, Schoor M, Chen H, Marks ME, Harley V, Mishina Y, Kingsley DM (2004) BMP receptor signaling is required for postnatal maintenance of articular cartilage. PLoS Biol 2:e355

177. Sahar DE, Longaker MT, Quarto N (2005) Sox9 neural crest determinant gene controls patterning and closure of the posterior frontal cranial suture. Dev Biol 280:344–361
178. Sakata-Goto T, Takahashi K, Kiso H, Huang B, Tsukamoto H, Takemoto M, Hayashi T, Sugai M, Nakamura T, Yokota Y et al (2012) Id2 controls chondrogenesis acting downstream of BMP signaling during maxillary morphogenesis. Bone 50:69–78
179. Satokata I, Ma L, Ohshima H, Bei M, Woo I, Nishizawa K, Maeda T, Takano Y, Uchiyama M, Heaney S et al (2000) Msx2 deficiency in mice causes pleiotropic defects in bone growth and ectodermal organ formation. Nat Genet 24:391–395
180. Satokata I, Maas R (1994) Msx1 deficient mice exhibit cleft palate and abnormalities of craniofacial and tooth development. Nat Genet 6:348–356
181. Sedano HO, Cohen MM Jr, Jirasek J, Gorlin RJ (1970) Frontonasal dysplasia. J Pediatr 76:906–913
182. Seeherman HJ, Li XJ, Bouxsein ML, Wozney JM (2010) rhBMP-2 induces transient bone resorption followed by bone formation in a nonhuman primate core-defect model. J Bone Joint Surg Am 92:411–426
183. Semenov M, Tamai K, He X (2005) SOST is a ligand for LRP5/LRP6 and a Wnt signaling inhibitor. J Biol Chem 280:26770–26775
184. Senarath-Yapa K, Li S, Meyer NP, Longaker MT, Quarto N (2013) Integration of multiple signaling pathways determines differences in the osteogenic potential and tissue regeneration of neural crest-derived and mesoderm-derived calvarial bones. Int J Mol Sci 14:5978–5997
185. Shore EM, Xu M, Feldman GJ, Fenstermacher DA, Cho TJ, Choi IH, Connor JM, Delai P, Glaser DL, LeMerrer M et al (2006) A recurrent mutation in the BMP type I receptor ACVR1 causes inherited and sporadic fibrodysplasia ossificans progressiva. Nat Genet 38:525–527
186. Shukunami C, Ohta Y, Sakuda M, Hiraki Y (1998) Sequential progression of the differentiation program by bone morphogenetic protein-2 in chondrogenic cell line ATDC5. Exp Cell Res 241:1–11
187. Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Luthy R, Nguyen HQ, Wooden S, Bennett L, Boone T et al (1997) Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. Cell 89:309–319
188. Simpson AH, Mills L, Noble B (2006) The role of growth factors and related agents in accelerating fracture healing. J Bone Joint Surg Br 88:701–705
189. Snider TN, Mishina Y (2015) Cranial neural crest cell contribution to craniofacial formation, pathology, and future directions in tissue engineering. Birth Defects Res C Embryo Today 102:324–332
190. Solomon BD, Bear KA, Wyllie A, Keaton AA, Dubourg C, David V, Mercier S, Odent S, Hehr U, Paulussen A et al (2012) Genotypic and phenotypic analysis of 396 individuals with mutations in Sonic Hedgehog. J Med Genet 49:473–479
191. Staehling-Hampton K, Proll S, Paeper BW, Zhao L, Charmley P, Brown A, Gardner JC, Galas D, Schatzman RC, Beighton P et al (2002) A 52-kb deletion in the SOST-MEOX1 intergenic region on 17q12-q21 is associated with van Buchem disease in the Dutch population. Am J Med Genet 110:144–152
192. Stottmann RW, Choi M, Mishina Y, Meyers EN, Klingensmith J (2004) BMP receptor IA is required in mammalian neural crest cells for development of the cardiac outflow tract and ventricular myocardium. Development 131:2205–2218
193. Stower MJ, Srinivas S (2014) Heading forwards: anterior visceral endoderm migration in patterning the mouse embryo. Philos Trans R Soc Lond B Biol Sci 369:20130546
194. Styrkarsdottir U, Halldorsson BV, Gretarsdottir S, Gudbjartsson DF, Walters GB, Ingvarsson T, Jonsdottir T, Saemundsdottir J, Snorradottir S, Center JR et al (2009) New sequence variants associated with bone mineral density. Nat Genet 41:15–17
195. Sulik KK, Johnston MC (1982) Embryonic origin of holoprosencephaly: interrelationship of the developing brain and face. Scan Electron Microsc (Pt 1):309–322
196. Tan TY, Kilpatrick N, Farlie PG (2013) Developmental and genetic perspectives on Pierre Robin sequence. Am J Med Genet C Semin Med Genet 163C:295–305

197. Tan X, Weng T, Zhang J, Wang J, Li W, Wan H, Lan Y, Cheng X, Hou N, Liu H et al (2007) Smad4 is required for maintaining normal murine postnatal bone homeostasis. J Cell Sci 120:2162–2170
198. Thomas JT, Lin K, Nandedkar M, Camargo M, Cervenka J, Luyten FP (1996) A human chondrodysplasia due to a mutation in a TGF-beta superfamily member. Nat Genet 12:315–317
199. Tian E, Zhan F, Walker R, Rasmussen E, Ma Y, Barlogie B, Shaughnessy JD Jr (2003) The role of the Wnt-signaling antagonist DKK1 in the development of osteolytic lesions in multiple myeloma. N Engl J Med 349:2483–2494
200. Trumpp A, Depew MJ, Rubenstein JL, Bishop JM, Martin GR (1999) Cre-mediated gene inactivation demonstrates that FGF8 is required for cell survival and patterning of the first branchial arch. Genes Dev 13:3136–3148
201. Tsuji K, Bandyopadhyay A, Harfe BD, Cox K, Kakar S, Gerstenfeld L, Einhorn T, Tabin CJ, Rosen V (2006) BMP2 activity, although dispensable for bone formation, is required for the initiation of fracture healing. Nat Genet 38:1424–1429
202. Tsumaki N, Nakase T, Miyaji T, Kakiuchi M, Kimura T, Ochi T, Yoshikawa H (2002) Bone morphogenetic protein signals are required for cartilage formation and differently regulate joint development during skeletogenesis. J Bone Miner Res 17:898–906
203. Twigg SR, Versnel SL, Nurnberg G, Lees MM, Bhat M, Hammond P, Hennekam RC, Hoogeboom AJ, Hurst JA, Johnson D et al (2009) Frontorhiny, a distinctive presentation of frontonasal dysplasia caused by recessive mutations in the ALX3 homeobox gene. Am J Hum Genet 84:698–705
204. Urist MR (1965) Bone: formation by autoinduction. Science 150:893–899
205. Uz E, Alanay Y, Aktas D, Vargel I, Gucer S, Tuncbilek G, von Eggeling F, Yilmaz E, Deren O, Posorski N et al (2010) Disruption of ALX1 causes extreme microphthalmia and severe facial clefting: expanding the spectrum of autosomal-recessive ALX-related frontonasal dysplasia. Am J Hum Genet 86:789–796
206. van Baardewijk LJ, van der Ende J, Lissenberg-Thunnissen S, Romijn LM, Hawinkels LJ, Sier CF, Schipper IB (2013) Circulating bone morphogenetic protein levels and delayed fracture healing. Int Orthop 37:523–527
207. van Bezooijen RL, Roelen BA, Visser A, van der Wee-Pals L, de Wilt E, Karperien M, Hamersma H, Papapoulos SE, ten Dijke P, Lowik CW (2004) Sclerostin is an osteocyte-expressed negative regulator of bone formation, but not a classical BMP antagonist. J Exp Med 199:805–814
208. van Bezooijen RL, Svensson JP, Eefting D, Visser A, van der Horst G, Karperien M, Quax PH, Vrieling H, Papapoulos SE, ten Dijke P et al (2007) Wnt but not BMP signaling is involved in the inhibitory action of sclerostin on BMP-stimulated bone formation. J Bone Miner Res 22:19–28
209. Van Wesenbeeck L, Cleiren E, Gram J, Beals RK, Benichou O, Scopelliti D, Key L, Renton T, Bartels C, Gong Y et al (2003) Six novel missense mutations in the LDL receptor-related protein 5 (LRP5) gene in different conditions with an increased bone density. Am J Hum Genet 72:763–771
210. Wang Y, Zheng Y, Chen D, Chen Y (2013) Enhanced BMP signaling prevents degeneration and leads to endochondral ossification of Meckel's cartilage in mice. Dev Biol 381:301–311
211. Warren SM, Brunet LJ, Harland RM, Economides AN, Longaker MT (2003) The BMP antagonist noggin regulates cranial suture fusion. Nature 422:625–629
212. Warren SM, Greenwald JA, Spector JA, Bouletreau P, Mehrara BJ, Longaker MT (2001) New developments in cranial suture research. Plast Reconstr Surg 107:523–540
213. Wilkie AO, Morriss-Kay GM (2001) Genetics of craniofacial development and malformation. Nat Rev Genet 2:458–468
214. Winkler DG, Sutherland MK, Geoghegan JC, Yu C, Hayes T, Skonier JE, Shpektor D, Jonas M, Kovacevich BR, Staehling-Hampton K et al (2003) Osteocyte control of bone formation via sclerostin, a novel BMP antagonist. EMBO J 22:6267–6276

215. Winkler DG, Sutherland MS, Ojala E, Turcott E, Geoghegan JC, Shpektor D, Skonier JE, Yu C, Latham JA (2005) Sclerostin inhibition of Wnt-3a-induced C3H10T1/2 cell differentiation is indirect and mediated by bone morphogenetic proteins. J Biol Chem 280:2498–2502
216. Winnier G, Blessing M, Labosky PA, Hogan BL (1995) Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse. Genes Dev 9:2105–2116
217. Winograd J, Reilly MP, Roe R, Lutz J, Laughner E, Xu X, Hu L, Asakura T, vander Kolk C, Strandberg JD et al (1997) Perinatal lethality and multiple craniofacial malformations in MSX2 transgenic mice. Hum Mol Genet 6:369–379
218. Wu DT, Bitzer M, Ju W, Mundel P, Bottinger EP (2005) TGF-beta concentration specifies differential signaling profiles of growth arrest/differentiation and apoptosis in podocytes. J Am Soc Nephrol 16:3211–3221
219. Xiong J, Onal M, Jilka RL, Weinstein RS, Manolagas SC, O'Brien CA (2011) Matrix-embedded cells control osteoclast formation. Nat Med 17(10):1235–41. doi: 10.1038/nm.2448. PMID: 21909103, PMCID: PMC3192296
220. Yamaguchi A, Ishizuya T, Kintou N, Wada Y, Katagiri T, Wozney JM, Rosen V, Yoshiki S (1996) Effects of BMP-2, BMP-4, and BMP-6 on osteoblastic differentiation of bone marrow-derived stromal cell lines, ST2 and MC3T3-G2/PA6. Biochem Biophys Res Commun 220:366–371
221. Yamamoto M, Beppu H, Takaoka K, Meno C, Li E, Miyazono K, Hamada H (2009) Antagonism between Smad1 and Smad2 signaling determines the site of distal visceral endoderm formation in the mouse embryo. J Cell Biol 184:323–334
222. Yamamoto M, Saijoh Y, Perea-Gomez A, Shawlot W, Behringer RR, Ang SL, Hamada H, Meno C (2004) Nodal antagonists regulate formation of the anteroposterior axis of the mouse embryo. Nature 428:387–392
223. Yang YP, Anderson RM, Klingensmith J (2010) BMP antagonism protects Nodal signaling in the gastrula to promote the tissue interactions underlying mammalian forebrain and craniofacial patterning. Hum Mol Genet 19:3030–3042
224. Yang YP, Klingensmith J (2006) Roles of organizer factors and BMP antagonism in mammalian forebrain establishment. Dev Biol 296:458–475
225. Ye M, Berry-Wynne KM, Asai-Coakwell M, Sundaresan P, Footz T, French CR, Abitbol M, Fleisch VC, Corbett N, Allison WT et al (2010) Mutation of the bone morphogenetic protein GDF3 causes ocular and skeletal anomalies. Hum Mol Genet 19:287–298
226. Yerges LM, Klei L, Cauley JA, Roeder K, Kammerer CM, Moffett SP, Ensrud KE, Nestlerode CS, Marshall LM, Hoffman AR et al (2009) High-density association study of 383 candidate genes for volumetric BMD at the femoral neck and lumbar spine among older men. J Bone Miner Res 24:2039–2049
227. Yoon BS, Ovchinnikov DA, Yoshii I, Mishina Y, Behringer RR, Lyons KM (2005) Bmpr1a and Bmpr1b have overlapping functions and are essential for chondrogenesis in vivo. Proc Natl Acad Sci U S A 102:5062–5067
228. Young B, Minugh-Purvis N, Shimo T, St-Jacques B, Iwamoto M, Enomoto-Iwamoto M, Koyama E, Pacifici M (2006) Indian and sonic hedgehogs regulate synchondrosis growth plate and cranial base development and function. Dev Biol 299:272–282
229. Yu PB, Deng DY, Lai CS, Hong CC, Cuny GD, Bouxsein ML, Hong DW, McManus PM, Katagiri T, Sachidanandan C et al (2008) BMP type I receptor inhibition reduces heterotopic [corrected] ossification. Nat Med 14:1363–1369
230. Yumoto K, Thomas PS, Lane J, Matsuzaki K, Inagaki M, Ninomiya-Tsuji J, Scott GJ, Ray MK, Ishii M, Maxson R et al (2013) TGF-beta-activated kinase 1 (Tak1) mediates agonist-induced Smad activation and linker region phosphorylation in embryonic craniofacial neural crest-derived cells. J Biol Chem 288:13467–13480
231. Zaghloul NA, Brugmann SA (2011) The emerging face of primary cilia. Genesis 49:231–246
232. Zehentner BK, Dony C, Burtscher H (1999) The transcription factor Sox9 is involved in BMP-2 signaling. J Bone Miner Res 14:1734–1741

233. Zhang H, Bradley A (1996) Mice deficient for BMP2 are nonviable and have defects in amnion/chorion and cardiac development. Development 122:2977–2986
234. Zhang J, He XC, Tong WG, Johnson T, Wiedemann LM, Mishina Y, Feng JQ, Li L (2006) BMP signaling inhibits hair follicle anagen induction by restricting epithelial stem/progenitor cell activation and expansion. Stem Cells 24(12):2826–2839
235. Zhang Z, Song Y, Zhao X, Zhang X, Fermin C, Chen Y (2002) Rescue of cleft palate in Msx1-deficient mice by transgenic Bmp4 reveals a network of BMP and Shh signaling in the regulation of mammalian palatogenesis. Development 129:4135–4146
236. Zimmerman LB, De Jesus-Escobar JM, Harland RM (1996) The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. Cell 86:599–606
237. Zouvelou V, Luder HU, Mitsiadis TA, Graf D (2009) Deletion of BMP7 affects the development of bones, teeth, and other ectodermal appendages of the orofacial complex. J Exp Zool B Mol Dev Evol 312B:361–374

BMP and BMP Regulation: Structure and Function

Kristof Nolan and Thomas B. Thompson

Abstract Due to their vast roles in human development, differentiation, homeostasis, and disease, bone morphogenetic proteins (BMP) have evolved along with numerous potentiating and inhibitory mechanisms to fine-tune signaling outcomes. As such, this chapter focuses on some of the best-studied and utilized extracellular mechanisms of BMP signal regulation. Due to their inherent binding characteristics, BMP ligands are often found engaged with at least of one of these many interacting partners. From a structural and functional perspective, we discuss our current understanding of how BMP ligands interact with these numerous binding partners, including secreted extracellular antagonists, BMP prodomains, and various co-receptors and noncanonical binding partners. Interestingly, while the BMP ligands themselves exhibit very redundant structural features, the composition and structure of their interacting proteins is quite diverse, lending to different ligand-binding modes and mechanisms, which lead to very different biological outcomes. Collectively, biochemical and structural characterization of these important interactions has provided valuable insight into BMP signal regulation.

Keywords BMP • TGF-beta • Regulation • Structure • DAN family • Follistatin • Chordin • Noggin • Antagonism

The transforming growth factor-β (TGF-β) superfamily represents one of the largest protein families in all of vertebrates with at least 33 known and unique signaling ligands. The bone morphogenetic proteins (BMPs) represent the largest subclass of these ligands within the TGF-β superfamily, with greater than 13 members (reviewed in [1, 2]). For this large family of protein cytokines, a filtering process takes place to drastically reduce the number of molecular signaling schemes, where only five Type I and seven Type II receptor subtypes are available for interaction, ultimately leading to one of two possible outcomes: either SMAD 1/5/8 or SMAD 2/3

K. Nolan • T.B. Thompson (✉)
Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati, Cincinnati, OH 45237, USA
e-mail: nolankt@mail.uc.edu; Tom.Thompson@uc.edu

S. Vukicevic, K.T. Sampath (eds.), *Bone Morphogenetic Proteins: Systems Biology Regulators*, Progress in Inflammation Research,
DOI 10.1007/978-3-319-47507-3_4

activation (reviewed in [3–5]). Because of their powerful influence on cell programming and development, several modes of regulation have evolved to further accommodate BMP signaling and increase the number of possible signaling outcomes at the cell surface through the action of very diverse families of proteins.

1 General Mechanisms for BMP Binding to BMP Type I and Type II Receptors

In general, BMP ligands are processed from their larger precursor forms by furin or furin-like proteases to cleave the prodomain region away from the functional mature domain. Following processing, the prodomain can remain bound to the mature, dimeric ligand, which can either function to maintain the ligand in a latent/inactive state, stay associated but not inhibit signaling, or immediately dissociate (discussed below and reviewed in [6, 7]). Mature BMP ligands most typically signal from a dimeric state, where BMP ligand monomers are covalently linked through a central disulfide bond such that they can form functional homo- or heterodimers. Following secretion, mature BMP ligands can subsequently bind to two of each of their respective Type I and Type II serine/threonine kinase receptors. The signaling complex promotes phosphorylation of the Type I kinase domain by the Type II receptor. This results in the phosphorylation of receptor-regulated or rSMAD proteins by the Type I receptor kinase. Subsequently, the activated rSMAD associates with SMAD4 or co-SMAD leading to higher-order complexes, nuclear localization, and target gene activation and/or deactivation (reviewed in [1, 3, 5]). It should also be mentioned that while this represents the canonical signaling pathway, ligands can activate or inhibit noncanonical signaling pathways that are SMAD independent, such as JNK/p38 [8–10], PI3K/Akt [10–15], RANK/RANKL [16, 17], MAPK/ERK/p38 [9–12, 14, 18], as well as substantial cross talk with the Wnt [19, 20] and VEGF [12, 21, 22] signaling cascades (reviewed in part in [23–27]). While the details underlying BMP signaling were being developed from a biochemical and cellular standpoint, our understanding of these mechanisms was greatly accelerated through the high-resolution structures of the free and bound forms of the BMP ligands and their target receptors.

Despite the vast array of physiological functions that the many BMP ligands play in the developing organism, the structures of these ligands gave an unprecedented view of the striking architectural conservation across this family of proteins. As such, each mature BMP ligand can be very adequately described as two hands coming together and shaking, where one monomer, composed of two fingers and a central wrist helix, “shakes” the hand of the opposing monomer with a disulfide linkage near the wrists that joins the hands together (Fig. 1). Furthermore, the fingers of each monomer point toward the periphery, giving the ligand dimer a propeller-like appearance from the top view or a butterfly appearance from the side view (Fig. 1) [28–32]. Lastly, each BMP monomer contains a characteristic cystine knot, composed of three intramolecular disulfide bonds that assemble into a knot- or ringlike

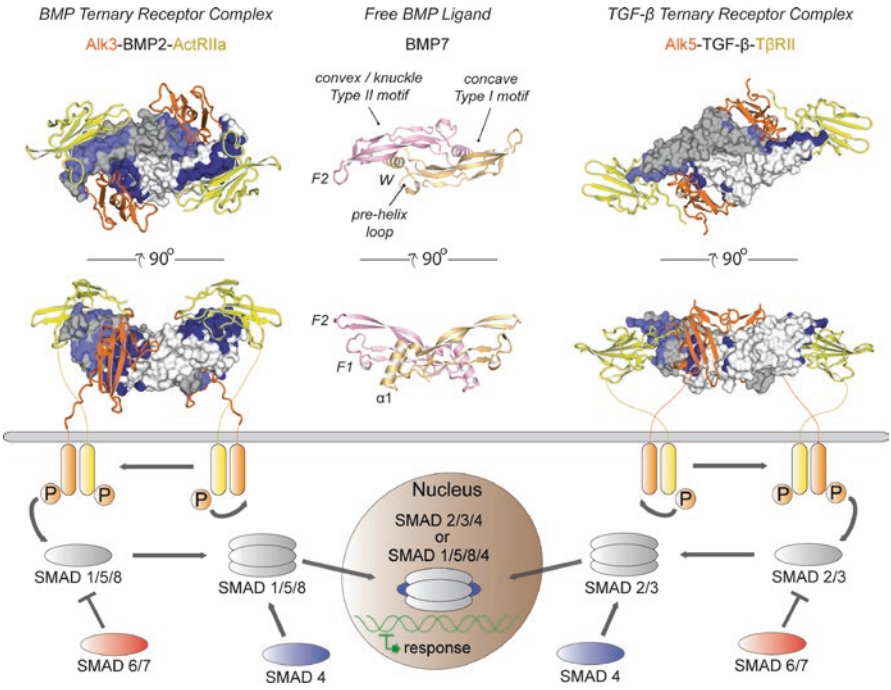


Fig. 1 Canonical BMP/TGF-β signaling. Structures of various BMP signaling components. (Center) Structure of a mature BMP/TGF-β ligand with one monomer colored in pink and the opposing monomer colored in gold (BMP7, PDBID 1BMP). In their mature form, these proteins exist as disulfide-linked homo- or heterodimers. Dimer formation can be described as either two hands shaking or as a propeller. In this sense, each monomer can be described as containing 2 fingers, denoted F1 and F2, and a wrist, denoted W. Dimer formation exposes large hydrophobic surfaces for receptor binding, denoted convex and concave. (Left and right) Active signaling complexes of a respective BMP-ternary receptor complex (left, Alk3-BMP2-ActRIIa, PDBID 2GOO) and a TGF-β-ternary receptor complex (right, TβRI-TGF-β1-TβRII, PDBID 2PJY). Ligands are shown in surface with one monomer in white and the opposing in gray. The receptors are shown in ribbon with the Type I receptors shown in orange and the Type II receptors shown in yellow. Differences in the BMP and TGF-β are apparent, where for BMPs, Type II receptor binding occurs closer to the knuckle region of the ligand while for TGF-β binding occurs near the fingertips, where the Type I and Type II receptors form a synergistic interaction upon ligand binding. Ligand surfaces are colored in dark blue and light blue for opposing monomers to highlight the interfaces utilized on the mature ligand dimers to achieve receptor binding. Upon receptor activation by ligand binding, the intracellular kinase domain of the Type II receptor phosphorylates the Type I receptor, leading to kinase domain activation. This activation allows for the Type I receptor to phosphorylate target SMAD transcription factors (SMAD 2/3 for TGF-β ligands and SMAD 1/5/8 for BMP ligands). Upon SMAD activation, tetrameric complexes form with the aid of SMAD 4 that can subsequently traverse into the nucleus to target specific DNA-binding elements that lead to specific genetic regulatory events. In addition to activation, specific SMADs (SMAD 6/7), known as inhibitory SMADs, can bind to activated SMAD proteins to inhibit their signaling ability

structure in the center of the protein, similar to other well-characterized growth factors, including VEGF, PDGF, and FSH [30, 32–35].

Following the resolution of numerous BMP and TGF-β ligand structures, multiple ligand-receptor complex structures were solved. These studies strengthened our understanding of ligand-receptor stoichiometry and clearly highlighted the epitopes utilized for receptor binding and activation. From these structures, it became evident that BMP ligands utilize their dimer interfaces (or concave surfaces) to bind the Type I receptors, where binding of the Type II receptors occurs away from the dimer interface at the "knuckle" region of the ligand (or convex surface) [36–47]. Clearly, this showed that the BMP surfaces utilized for receptor binding did not promote intermolecular receptor-receptor contacts on the extracellular surface [37]. This is in contrast to the TGF-β ternary receptor structure, where the Type II receptor binds at the fingertip region of the ligand, bringing the Type II receptor into contact with the Type I receptor [43]. Interestingly, these observations are consistent with noted differences in BMP versus TGF-β receptor affinity, where BMPs utilize high-affinity Type I receptor interactions, whereas TGF-βs utilize high-affinity Type II receptor interactions (reviewed in [3, 5, 48]).

In addition, the differences seen in BMP and TGF-β receptor assembly may in part be due to observed differences in mature ligand flexibility. BMP ligands appear more rigid (less flexible) than TGF-β ligands, resulting in a more ordered Type I receptor interface (as observed in the numerous receptor-ligand complexes) [3, 5, 36, 44, 47]. Interestingly, differences within these flexible regions, localized to the ligand wrist, account for the highest sequence divergence across the TGF-β superfamily and likely account for their variable Type I receptor preferences (reviewed in [3, 5, 48]). Supporting this, biochemical studies have shown that swapping ligand wrist regions or using specifically engineered single point mutations can alter their binding preferences or completely abrogate their Type I receptor specificity (e.g., L51P for BMP2) [36, 38, 46, 49–54]. Furthermore, due to the similarities in overall structure, chimeric ligands have been generated, creating novel signaling ligands that have different receptor utilization and designed or enhanced biological properties [53–55]. With this in mind, these structural studies have provided unique insight into how to rationally design novel ligands with engineered and desired receptor-binding affinities and specificities.

2 General Mechanisms for BMP Inhibition and Antagonism

Because of the extreme biological importance of BMP ligands and signaling, numerous mechanisms have evolved to regulate, inhibit, and fine-tune BMP signaling. While mechanisms of regulation have been identified at nearly each stage of the BMP signaling cascade, secreted extracellular antagonists play a major role in regulation, where many of these protein counterparts have been shown to function through direct interactions with the mature ligand dimer, blocking the receptor-binding motifs and inhibiting signal activation (reviewed in [1, 2, 56]).

Extracellular BMP inhibitors span multiple, unique families of proteins, including the follistatin, growth and differentiation factor-associated serum protein (GASP), differential screening-selected gene in neuroblastoma (DAN), noggin, and chordin families of antagonists. Interestingly, while BMP ligands maintain a high level of structural conservation, being nearly identical in architecture from ligand to ligand, the different families of antagonists are extraordinarily diverse, ranging from small single-domain proteins (such as noggin and the DAN family) to large, multidomain proteins (such as GASP, follistatin, and the chordin families) (reviewed in [1–3, 56]). Furthermore, structural architecture and secondary structure elements are highly variable across the various BMP inhibitors, even between proteins of nearly similar size (e.g., noggin and the DAN family) (reviewed in [56]). Not surprisingly, the expression patterns, developmental significance, and pathologies resulting from misregulation of these extracellular inhibitors are highly diverse. Additionally, these antagonists have likely evolved to recognize specific subsets of ligands within the BMP and TGF-β subclasses (e.g., preferred inhibition of activin A and myostatin by follistatin versus preferred inhibition of BMP2, BMP4, and BMP7 by noggin and DAN family proteins). With these details in mind, there is a need for a comprehensive study of these ligand-antagonist interactions, where structural insight provides the clearest details into the features driving these interactions and how they distinguish one BMP ligand from the next.

To date, structures have been solved for the follistatin, noggin, and chordin families of proteins bound to their target ligands, where more recent studies have characterized the unbound forms of the DAN family of antagonists. Collectively, it appears a conserved mechanism arises within these variable antagonists, allowing them to directly compete with the receptor-binding motifs on the mature ligands (Fig. 2a). However, as would be expected from the aforementioned antagonist diversity, vastly different approaches or modes have evolved to achieve this result. The following sections will attempt to describe each of these antagonist families, their structures, and the impact that these works have had on our understanding of BMP signaling, inhibition, and disease.

3 Noggin-Mediated Antagonism

The protein antagonist noggin was originally identified in *Xenopus* and shown to be critical during embryogenesis by negatively regulating BMP signal activation. Furthermore, it was shown that noggin is released from the Spemann organizer, leading to important cell-fate decisions [57–61]. Additionally, noggin has been physiologically linked to successful bone and cartilage development [62–64] as well as limb bud patterning and development [65–71]. Similar to many BMP ligands (e.g., BMP2 and BMP7), noggin also interacts with heparin/heparan oligosaccharides, localizing it to cellular surfaces. While these heparin-/heparan-based interactions do not appear to interfere with noggin-mediated BMP antagonism, this feature is likely critical for establishing anti-BMP gradients through controlled diffusion

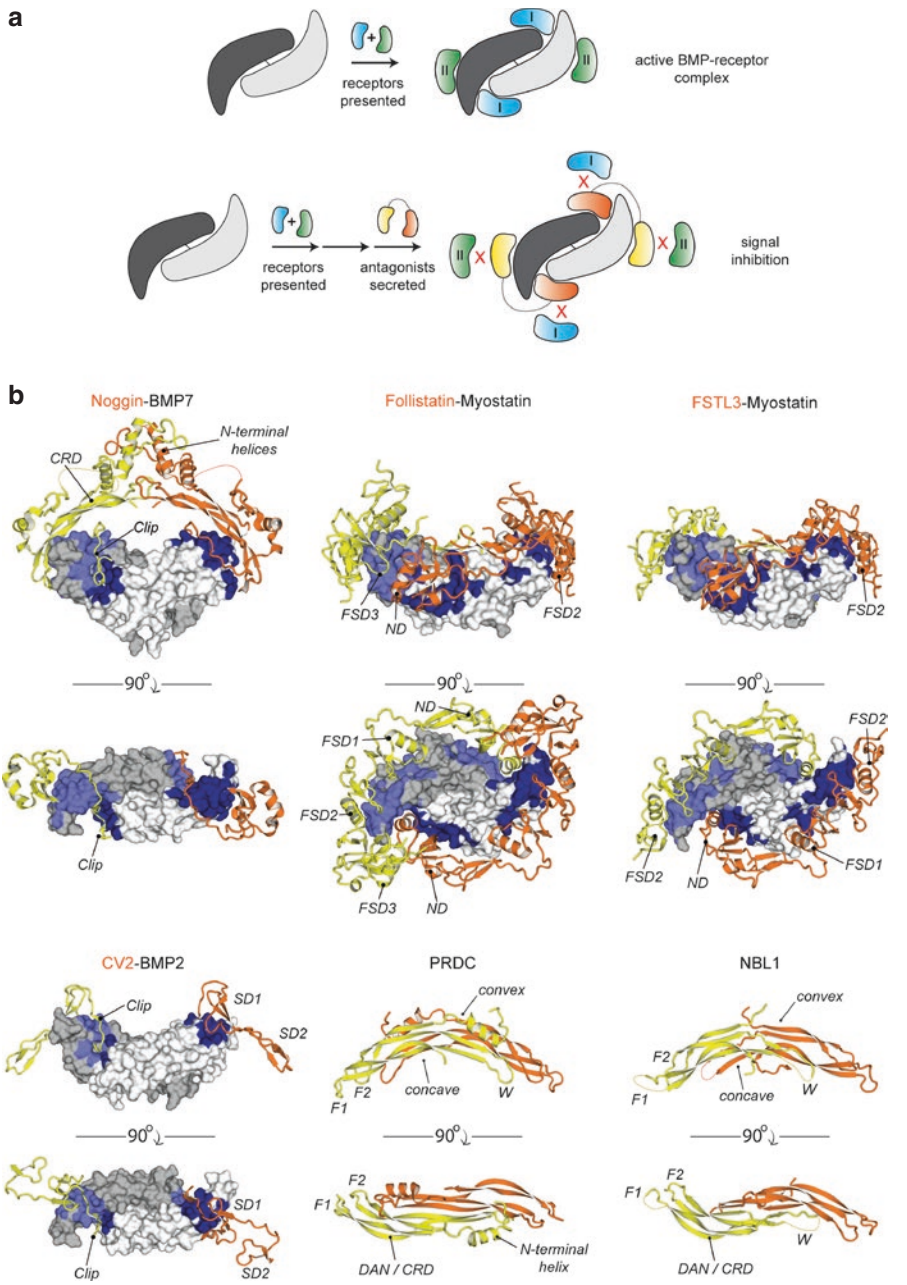


Fig. 2 Extracellular BMP/TGF-β antagonists. (**a**) General mechanism of BMP/TGF-β extracellular antagonism. Under normal conditions (top), the mature ligand can engage and bind to two of each target Type I and Type II receptors, utilizing its hydrophobic convex and concave surfaces, to initiate downstream signaling. Although there are numerous families of extracellular antagonists,

during embryogenesis, which has been shown to be important for proper patterning in and among other growth factor families, including Wnt [72–75].

In 2002, the first structure of a BMP-bound antagonist, noggin-BMP7, was solved through the use of X-ray crystallography. In this seminal work, the mature noggin dimer was shown to form a symmetrical complex with the mature, dimeric BMP ligand, simultaneously binding and sterically blocking both Type I and Type II receptor-binding motifs (Fig. 2b) [76, 77]. Unexpectedly, noggin was shown to adopt a striking growth factor-like fold with a central cystine-knot core, similar to the BMP ligands [42]. Furthermore, both noggin and BMP form symmetric disulfide-linked dimers. Despite these similarities, helical segments in the N-terminus of noggin help orient two opposing monomers in a head-to-head arrangement as opposed to the head-to-tail arrangement of the BMP ligands, resulting in very different dimer architectures. Interestingly, disruption of the noggin disulfide link has little impact on its anti-BMP activity, supporting the extensive nature of the noggin-BMP interface [42]. Despite this, although untested, it is anticipated that a noggin dimer would function as a more potent BMP inhibitor as compared to a noggin monomer due to avidity effects.

The functional region of noggin lies within its extreme N-terminus, lying away from its central cystine core and forming a 'clip' that wraps from the Type II interface, weaving over the apical surface of BMP7 and inserting into the Type I interface of BMP7 (similar to chordin family proteins and likely different from DAN family proteins, discussed in following sections) (Fig. 2b) [77]. This N-terminal region, based upon observed and predicted secondary structure, likely exists in the random coil state prior to binding. Additionally, noggin uses its second finger to form additional contacts with the convex surface of BMP7. Overall, the majority of the noggin-BMP7 interaction overlaps with the Type II receptor-binding motif of the ligand, where mutation of large hydrophobic amino acids within this region of noggin leads to a severe loss in functional inhibitory activity, to a much greater extent than similar mutations within the concave or Type I competing motif (N-terminus) of noggin [77]. Of note, while noggin and the BMP receptors utilize

with very different structures, they have all been determined to utilize conserved mechanisms to inhibit BMP/TGF-β signaling. For inhibition to occur, these antagonists directly bind to their target BMP/TGF-β ligand, where they utilize identical surfaces necessary for Type I and Type II receptor binding, leading to an inability of the ligands to bind to and activate downstream SMAD signaling. (**b**) Known structures of various BMP/TGF-β antagonists and their complexes (noggin-BMP7, PDBID 1M4U; follistatin-myostatin, PDBID 3HH2; FSTL3-myostatin, PDBID 3SEK; CV2-BMP2, PDBID 3BK3; PRDC, PDBID 4JPH; NBL1, PDBID 4X1J). Each structure is labeled with features identified in their corresponding published manuscripts. BMP/TGF-β ligands are shown in surface representation, with one monomer shown in white and the opposing shown in gray. The antagonists are colored in yellow and orange and shown in the ribbon representation. The ligand surfaces are colored dark blue and light blue to show the regions utilized by the antagonists for binding. Compare to the structures in Fig. 1 to compare and see the similarities between the receptor and antagonist-binding surfaces

similar surfaces on BMP, a number of unique amino acids are utilized for each of these interactions. For example, mutation of leucine 333 to proline (L51P in the mature protein, L333P in the full-length sequence) in BMP2 was shown to nearly completely abrogate the ability of BMP2 to signal and bind to its Type I receptor (Alk3 or BMPRIa) [36]. While noggin binds this corresponding amino acid in BMP7 utilizing its N-terminus, this mutation in BMP7 (L367P) did not abrogate noggin binding [77, 78]. With this in mind, amino acids important for discriminating receptor and antagonist binding could provide useful therapeutic options to treat numerous disease states that require controlled selectivity.

Functionally, noggin works by binding to mature BMP ligands, with the highest affinity to BMP2, BMP4, and BMP7 and to a lesser extent BMP5, BMP6, and GDF5. Most interestingly, noggin does not inhibit BMP9 or BMP10 [79–81]. The molecular basis for this difference has recently been identified through analysis of disease causing SNPs. For instance, a SNP in GDF5 (N445 to either T or K) was identified in patients with multiple synostosis syndrome (SYM1). In the structure of noggin-BMP7, this corresponding asparagine is necessary for hydrogen bonding to the N-terminus of noggin, which is disrupted in the N445K and T mutations in GDF5 [77, 79]. Researchers were subsequently able to show that the GDF5 SNP results in resistance to noggin inhibition [79]. Furthermore, BMP9 contains a lysine within this position, where mutation to asparagine leads to noggin susceptibility [79]. More recently, a mutation in GDF6, Y444N, was also found to invoke a similar disease phenotype (SYM4) based upon the likely ability of the protein to evade noggin-based inhibition [82]. Taken together, these studies have greatly improved our understanding of BMP-mediated antagonism, providing evidence of the molecular mechanisms important for imparting antagonistic specificity and disease-state pathologies, and demonstrate that single amino acid differences can dictate ligand-antagonist specificity.

4 Follistatin Family-Mediated Antagonism

Follistatin was originally identified in the follicular fluid of the ovaries, where it was shown to functionally inhibit FSH release through inhibition of the TGF-β ligand activin A [83–87]. Follistatin is a glycoprotein consisting of four modular domains: a unique N-terminal domain (ND) followed by three relatively well-conserved follistatin domains (FSD1–3) [88]. Follistatin has since been shown to have broad ligand specificity, with the highest affinity for activin subclass ligands (e.g., activin A, activin B, myostatin, and GDF11) and low- to mid-level antagonist propensities toward a number of BMP subclass members (e.g., BMP2, BMP4, and especially BMP7) [89]. Interestingly, follistatin can be alternatively spliced, producing either the human 288 or 315 amino acid forms. Functionally, these proteins bind to BMP/TGF-β ligands the same. However, the additional acidic amino acids at the C-terminus in the 315 form confer resistance to heparin/heparan binding as compared to the 288 form, likely suggesting mechanisms for generating a follistatin form with better diffusion or serum availability characteristics [90–92].

In addition to follistatin, a group of related molecules termed follistatin-like proteins (FSTL1–5) also contain at least one conserved FSD domain [93–95]. Furthermore, a group of molecules containing a single follistatin domain, known as the GASP proteins, have been more recently identified [96, 97]. Within this group, FSTL1 and FSTL3 have been implicated in binding and antagonizing specific TGF-β superfamily ligands [98, 99]. FSTL3 is the most similar to follistatin, having a similar domain layout and architecture, but lacking the last (or third) FSD domain when compared to follistatin. In contrast, FSTL1 only contains a single, functional FSD domain. A number of studies have been performed comparing FSTL3 to follistatin and have concluded that FSTL3 is more specific for the activin subclass [89]. Additionally, these two proteins show drastic differences in their bioavailability and diffusion characteristics. This arises from the inability of FSTL3 to bind heparin, thereby making this protein more readily available in serum [89, 100, 101].

Following the resolution of the noggin-BMP7 structure, a number of follistatin structures were resolved (from here on, follistatin will correlate with the follistatin 288 variant), including follistatin-activin A, FSTL3-activin A, follistatin-myostatin, FSTL3-myostatin, isolated domains of follistatin, and follistatin 315-activin A, making the follistatin family of antagonists the most rigorously characterized of all BMP/TGF-β inhibitors (Fig. 2b) [50, 102–107]. As revealed by these structures, two molecules of follistatin or FSTL3 bind symmetrically in a head-to-tail fashion (no follistatin dimers are known to exist), using multiple domains to completely encircle the mature ligand (Fig. 2b). Unlike noggin, which forms a continuous interface with BMP7, follistatin and FSTL3 have two separate and distinct binding epitopes linked by the first FSD domain. Similar to noggin, follistatin binds both receptor-binding motifs on each ligand, where the ND nestles into the ligand concave surface, similar to the Type I receptors, and the second FSD (FSD2) buries the majority of the convex surface of the mature dimer, similar to the Type II receptors [50, 77, 104]. While a structure of follistatin bound to BMP has yet to be solved, binding data suggests that BMP ligands could interact with the Type I receptor in the presence follistatin. This suggests that the ND does not bind BMP ligands significantly [108]. On the other hand, the ND of FSTL3 appears to form a much tighter interaction with activin A and myostatin in comparison to follistatin (Fig. 2b). In this case, superposition of the BMP ligands onto these complexes reveals potentially hindering steric clashes within the ligand wrist regions, possibly explaining why FSTL3 is a poor BMP antagonist [89]. Further contrasting FSTL3 and follistatin, one follistatin molecule, when bound to a mature ligand, is supported by a significant cooperative interaction with the opposing follistatin monomer, where the head of one follistatin (ND) binds the tail of the other (FSD3) (Fig. 2b). This interaction is missing in FSTL3 since it lacks the third FSD domain, not being able to completely wrap around the mature ligand (Fig. 2b). Furthermore, these cooperativity differences have been supported both in vitro and in cellular-based reporter assays [109]. Thus, the ND of follistatin appears more plastic and can likely accommodate changes within the ligand wrist region, which may be further stabilized by cooperative interactions with the adjacent follistatin monomer. Taken together, these findings likely account for the ability of follistatin to target, albeit weakly, specific subsets of BMP ligands [50, 102–104].

While the noggin and follistatin structures represent the most complete antagonist structures to date, it is clear that different strategies have evolved between these very different protein antagonists to inhibit TGF-β ligands. Despite these differences, and very interestingly, direct competition for the ligand-receptor-binding motifs appears to be a common, universal theme in BMP inhibition.

While structures of FSTL1 are not currently available, its function is in stark contrast to that of FSTL3 and follistatin, where FSTL1 shows a preference for BMP2, BMP4, and TGF-β1-based inhibition with no known ability to inhibit activin subclass ligands [99, 110]. For example, it has been demonstrated that FSTL1 knockout mice exhibit severe and pathological defects in both skeletogenesis and lung organogenesis, where the pulmonary effects of FSTL1 could be directly prevented or rescued by introduction of the BMP-specific antagonist, noggin [110, 111]. However, despite its preferences for BMP-based antagonism, FSTL1 exhibits much faster dissociation kinetics in comparison to the slow dissociation kinetics of noggin and follistatin for specific BMP subclass ligands [80, 108, 110]. Taking into account that FSTL1 only contains a single FSD domain, most similar to follistatin FSD1 (the bridging domain in follistatin and FSTL3), it is likely that FSTL1 will utilize a completely different mechanism to achieve ligand binding and inhibition as compared to follistatin and FSTL3, supporting the need for additional structural and biochemical work in this family.

5 Chordin Family-Mediated Antagonism

Chordin was first identified in *Xenopus*, where it was shown to be actively secreted from the developmentally important Spemann organizer, similar to noggin [112]. Furthermore, chordin is crucial for proper embryonic tissue dorsalization [112–114], neural induction [115], skeletogenesis [116], vascular patterning [117], and mesoderm differentiation [118], physiologies specifically resulting from its inhibitory actions on BMP signaling, mainly BMP2, BMP4, and BMP7. Interestingly, during gastrulation in mice, chordin and noggin can be found co-expressed within the node and primitive streak, where their activities appear to be redundant and one can supplement for the other within this specific cellular niche during forebrain development. In contrast, specific regulatory events belonging uniquely to chordin (e.g., antagonism and proteolytic processing) during dorsal-ventral patterning make it uniquely required within specific environments, such as during dorsal-ventral patterning, where its activity cannot be adequately replaced by noggin [119, 120]. Vice versa, noggin has been shown to be critical during somite development, where chordin cannot replace this function [121]. Taken together, this supports that alternative antagonists, although similar in their ability to inhibit BMP ligands, house molecular differences that are required to achieve unique and specific outcomes during development.

Chordin is a large multidomain protein characterized by four Von Willebrand factor type C (VWC) domains [112]. Interestingly, VWC domains are commonly

found in numerous extracellular proteins (e.g., collagen proteins, complement factors, and integrins), exhibiting a vast variety of functions. To date, a number of proteins containing at least one VWC domain capable of modulating BMP signaling have been identified and collectively classified within the chordin-like family of proteins, including chordin-like protein 1, chordin-like protein 2, kielin, and crossveinless-2 (CV2 or its mammalian homologue BMPER) [122–125]. In fact, the VWC domains from a number of these proteins were shown to play important roles in directly binding to BMP ligands, albeit with different affinities [122–126]. Subsequently, it was shown only specific VWC domains within chordin, chordin-like 2, and CV2 were utilized in BMP binding and inhibition/modulation, despite each containing multiple VWC domains. Furthermore, each chordin-like protein had slightly different affinities for their main target ligand, BMP2, where the proteins chordin and chordin-like 2 bind and form a ternary complex in the presence of Tsg to enhance BMP antagonism, while CV2 cannot. This indicates differences in the overall binding mechanisms across this family of proteins and likely different abilities to inhibit signaling in vivo [126]. The chordin family of proteins directly competes for the receptor-binding interfaces on BMP2, similar to both follistatin-activin and noggin-BMP complexes [77, 102, 126]. Interestingly, similar to the structural and functional differences of the FSD, the VWC domains appear to form modular units or scaffolds that have the capacity to evolve the necessary amino acids needed to engage the ligand, as suggested by the variability across this family of proteins as well as other VWC domain-containing proteins. Therefore, function cannot simply be assigned based on the type of domain.

In 2008, the structure of the functional VWC domain (N-terminal domain or VWC1) of CV2 was solved in complex with BMP2, where two VWC1 molecules are found binding to one mature BMP2 dimer (Fig. 2b) [127]. CV2, like chordin, can function as both an inhibitor and enhancer of BMP signaling depending upon the specific cellular context [127–130]. The structure of VWC1 in complex with BMP2 is rather unique, showing a tripartite orientation that can be divided into subdomains based upon disulfide spacing, including subdomains 1 and 2 (SD1 and SD2) (Fig. 2b). These contain two disulfide bonds each and a small two- or three-stranded β-sheet with an N-terminal or 'clip' domain that is unstructured, similar to noggin (Fig. 2b). While bound to BMP2, SD1 provides the majority of the interaction surface for CV2-BMP2, binding to the large convex Type II receptor interface on BMP2. The 'clip' segment wraps around the apical surface of the mature ligand dimer, weaving into the concave Type I receptor-binding interface on the ligand. Together, these interactions functionally block both receptor-binding motifs available on BMP2 through the utilization of a number of hydrophobic residues to directly compete for these epitopes [127]. Taken together, these findings further support the notion that common inhibitory mechanisms have evolved across these variable antagonist families, including the ability to directly block the Type I and Type II receptor-binding motifs on BMP ligands, mediated by strong hydrophobic interactions (Fig. 2a).

Unlike CV-2, chordin binds in a 1:1 stochiometric ratio with the BMP ligand, where at least two of its four VWCs domains are needed to maintain a high-affinity

BMP interaction and effectively achieve signal inhibition [127, 131]. It is proposed that a single molecule of chordin will stretch along the dimer in a rather unique asymmetric manner using different VWC modules to bind corresponding receptor epitopes on each side of the ligand [131]. This arrangement sensitizes chordin to negative regulation by the metalloprotease, tolloid, which acts to cleave chordin between its functional VWC domains, alleviating inhibition and allowing the mature BMP ligand to signal [119, 120]. It remains to be seen if inactivation of other BMP antagonists by proteolysis can also occur. However, this form of antagonist regulation may be more prominent than currently appreciated given that a similar mechanism is used to activate certain ligands from latency (discussed below).

6 DAN Family-Mediated Antagonism

The DAN family of protein antagonists represents one of the largest families of structurally related BMP antagonists. The founding member, NBL1 (or DAN), was identified based upon its upregulation in specific neuroblastoma cell lines, where it was further hypothesized to affect cell cycle progression [132]. Over the years, a number of proteins, including NBL1, were shown to functionally inhibit BMP signaling within developing *Xenopus* organisms [133]. To date, the DAN family consists of seven members: NBL1, gremlin-1 (Grem1), gremlin-2 (Grem2 or PRDC), cerberus (or Cer1), coco (Grem3 or DAND5), sclerostin (or SOST), and USAG-1 [132, 134–139]. Interestingly, each member within this family has been shown to exhibit unique physiological roles, patterns of expression, and signal localization, leading to a wide array of disease-state pathologies upon misregulation (reviewed in [140]). For example, Grem1 has been extensively studied in development, where it has been shown to play crucial roles in limb bud outgrowth and patterning through a signaling relay system with Shh and FGF4 [141–143]. In addition, Grem1 knockout mice fail to develop functional kidneys, supporting that Grem1 plays a critical role in proper kidney development [144, 145]. As such, Grem1 misregulation/upregulated is pivotal in innumerable pathologies, including chronic kidney diseases (CKDs) and fibrosis (as well as USAG-1) [145–151], pulmonary arterial hypertension (PAH) [152–154], as well as numerous and unique pro-cancer phenotypes [155–160]. In contrast, PRDC, which is most closely related to Grem1 (59 % identity), has been implicated in heart development and was shown to regulate atrial-specific cardiomyocyte differentiation during development, where misregulation of PRDC has been linked to atrial hypertrophy in *zebra fish* [161, 162]. Furthermore, the protein sclerostin, identified in the bone disease sclerosteosis, is highly expressed in osteoclasts and osteocytes [163–165], leading to current efforts to target this protein in a number of bone remodeling diseases [166–172]. Beyond their roles in development and disease, Grem1 has been utilized to identify a new population of stem cells (osteochondroreticular) in the bone marrow [173]. The presence of a BMP antagonist is not surprising since ligands are found in several instances to direct stem cell self-renewal or differentiation (reviewed in [174]).

While each member of the family is characterized based upon the spacing of eight conserved cysteines, which form four intramolecular disulfide bonds, the amino acid conservation across the family is rather poor. Similar to noggin, DAN family proteins are single-domain, cysteine-rich proteins. This cysteine-rich domain (CRD), or DAN domain, is composed of a central cystine-knot core and is flanked by highly variable N- and C-terminal extensions. In addition, this lack of conservation likely accounts for the drastically different abilities of each DAN family member to inhibit BMP, where the proteins Grem1, PRDC, and coco can be classified as potent BMP inhibitors, cerberus and NBL1 as moderate BMP inhibitors, and sclerostin and USAG-1 as poor or incapable BMP inhibitors (reviewed in [140]). In terms of specificity, DAN family members have been implicated in antagonizing mainly BMP2, BMP4, and BMP7, with implications for also inhibiting BMP5, BMP6, and GDF5. Similar to noggin, DAN family members do not appear to inhibit BMP9 and BMP10. However, the reason for these discrepancies remains unknown (reviewed in [140]).

To date, no structure of a DAN-BMP complex is available. Rather, and unique to the DAN family of BMP antagonists, four structures have been solved of these proteins in their unbound state, including two separate NMR structures of sclerostin and crystal structures of PRDC and NBL1 [175–178] (Fig. 2b). In each structure, the core DAN domain of these proteins takes on a striking growth factor-like fold, highly similar to both the BMP ligands and noggin. This fold can also be described using the conserved finger-wrist etiology, composed of two fingers and a wrist region, showing the formation of a central cystine-knot core (three disulfide bonds) with an additional intramolecular disulfide bond linking the opposing fingers, similar to noggin [140, 177]. Most apparent in the structures of sclerostin, PRDC, and NBL1 are their noticeable differences in oligomeric state. Sclerostin, on two accounts, has been observed as a monomer in solution, whereas PRDC and NBL1 exist as non-covalent dimers, stabilized by long, intermolecular β-sheets [175–178]. Lastly, as suggested above, are the obvious differences in the terminal extensions among these various protein structures. In sclerostin, these regions take on a completely random coil fold. Similarly for NBL1, its short, punctate N-terminus is void of any secondary structure, which is also predicted to be the case for its nonfunctional C-terminus. In this regard, PRDC is unique as it forms N-terminal helices that intimately lie over the core domain of the protein, likely protecting the hydrophobic convex surface created by dimer formation [177].

Interestingly, in terms of dimerization, only PRDC and NBL1 have been thoroughly tested in this regard, with some level of study being performed on USAG-1, Grem1, and cerberus, supporting the notion of dimerization [179–182]. Historically, members of this family were believed to exist as disulfide-linked dimers, similar to both BMP ligands and noggin [183]. This arises based upon cysteine conservation across the family, where the majority of the members contain nine odd cysteines. Based upon sequence alignments, and now structural data, only eight of these cysteines are required for the DAN family fold, forming cystine knots equal in spacing with those found in the BMP ligands (reviewed in [140]). The placement of the final cysteine is approximately located where the intermolecular disulfide-bonding

cysteine within the BMPs is found. For this reason, Grem1, PRDC, coco, and cerberus were all believed to exist as covalent dimers. Only recently has this concept been resolved, indicating that these proteins in fact exist as non-covalent dimers, supported by various biophysical studies [179, 182, 184]. Furthermore, it was shown for PRDC that this odd cysteine could be mutated away with no functional loss in its ability to form dimers or inhibit BMP signaling [179]. NBL1 uniquely contains ten cysteines and forms an additional fifth disulfide bond, linking this odd cysteine to the one located at its proximal C-terminal extension [178].

Given the recent evidence of dimers within the family, including that for USAG-1, the oligomeric state of sclerostin has been the topic of recent investigation, where it has been shown that sclerostin can exist either in monomeric, dimeric, or higher order oligomeric states depending upon the tissue-specific context [185]. With this in mind, more work is needed to further clarify these concepts and determine the role, if any, that oligomeric states play in BMP-based antagonism.

Functionally, the core domain of DAN family antagonists appears to be important for their ability to inhibit BMP signaling. Initially, studies on Grem1 indicated that its N-terminus was dispensable for BMP4 inhibition [186]. Extending from this, work on PRDC and NBL1 pinpointed several hydrophobic amino acids located on the convex dimer surface of these proteins that are important for BMP-based inhibition, both *in vitro* and *in vivo* [177, 178]. Looking at sequence conservation across these antagonists, the amino acids identified in PRDC as important for BMP-mediated inhibition are conserved in the stronger antagonists while only partially or not conserved in the weaker inhibitors [140, 177, 178].

To study these differences in affinity, comparative studies were performed on PRDC and NBL1. It has been shown, for BMP2, BMP4, and BMP7, that NBL1 is consistently weaker than PRDC, where sclerostin showed no ability to inhibit these ligands via a luciferase reporter assay. For example, PRDC and NBL1 exhibit significantly different potencies toward BMP, where PRDC inhibits BMP2 with an IC50 of ~1 nM and NBL1 is roughly 380-fold less potent [178]. Differences in the BMP-binding epitope between PRDC and NBL1, including Y105 in PRDC and the synonymous S67 in NBL1, are largely responsible for their variation in potency. Introduction of the S67Y mutation into NBL1 increases its activity for BMP2 nearly 40-fold, making it a much more effective antagonist of BMP signaling. In addition, the S67Y mutation in NBL1 makes it functionally equivalent to PRDC for inhibition of BMP7 [178]. Expanding these findings to other DAN family members, these important hydrophobic amino acids show poor conservation in both sclerostin and USAG-1, consistent with weak BMP antagonism.

In terms of a model of inhibition, it has been observed in PRDC that the functional BMP-binding epitope is partially shielded from the solvent by interactions with the N-terminal helices. Therefore, for PRDC, it has been proposed that displacement of this helix might be required to expose the larger, putative BMP-binding epitope [177]. NBL1, on the other hand, has a much shorter N-terminus in comparison, with no helical structure (Fig. 2b). However, in this case it may not be needed since the BMP-binding epitope of NBL1 is significantly less hydrophobic in nature, where, perhaps, the N-terminus of PRDC and others can provide additional contacts

with BMP ligands to enhance binding affinity and possibly specificity (not tested). How DAN family members bind and antagonize BMP ligands, including stoichiometry of the interaction and the receptors they compete with, remains to be determined, though recent studies suggest, at least for cerberus, it blocks both Type I and Type II receptor-binding epitopes [187].

7 Prodomain Interactions and Latent Complex Formation

Each BMP/TGF-β superfamily member contains a long, roughly 300 amino acid long N-terminal prodomain. Within the ER, it is believed that the prodomain ensures proper folding and allows the C-terminal mature domains of BMP/TGF-β ligands to dimerize through a central disulfide linkage, either in homo- or heterodimeric (e.g., GDF9/BMP15, BMP2/BMP7, and Inhibin) forms [188–193]. Once translated, the proteins exist in their unprocessed form as a multidomain, prodomain-mature ligand precursor. Upon secretion, furin or furin-like proteases cleave specific sequences linking the prodomain and mature domain [194]. Following processing, depending upon the specific ligand, a number of different fates can occur. For the TGF-β subclass ligands, including TGF-β1, TGF-β2, and TGF-β3, as well as GDF-8 and GDF-11, ligands are inhibited from receptor binding and signaling by the non-covalent association of the prodomain, which remains bound to the mature ligand, blocking its ability to activate its target receptors [195–207]. This association of the prodomain maintains the mature ligand in a latent form that is bound to the extracellular matrix through specific protein molecules, such as fibrillin-associated proteins and LTBPs. The latent state remains inactive until specific activating cues arise. Examples of these cues include force-based releasing interactions guided by LTBP (often bound to the fibrillin matrix) [195, 208–213] and integrin [214–218] binding (e.g., TGF-β1), binding to other associated extracellular matrix proteins and oligosaccharides such as thrombospondin or perlecan (e.g., TGF-β1, activin A, and myostatin) [219–221], proteolytic processing by tolloid-like proteins (e.g., myostatin and GDF11) [222–225], or direct binding to fibrillin, which potentially mediates trade-off of the mature ligand with the receptors (e.g., BMP7) (Fig. 3a–c) [226–230]. For the ligands BMP2, BMP4, BMP7, BMP9, BMP10, and GDF5, binding and association have been documented between their corresponding prodomains and mature regions, including the ability of these prodomains to associate in several instances with fibrillin [229, 230]. However, for many of these ligands, latent or inhibitory complexes do not appear to form or occlude activity, as is the case for BMP4, BMP5, and BMP7, where transfection of these ligands with their prodomains shows no reduction in mature ligand signaling activity (Fig. 3d) [226, 229, 230]. Although for BMP10, latent complexes were shown to directly occlude functional ligand signaling [230]. While these results begin to address the roles that the prodomain plays in regulating BMP signaling, much more work is needed to better identify which ligands are associated into latent forms, which specific molecules can relieve this latent inhibitory state, and what roles the prodomains play in

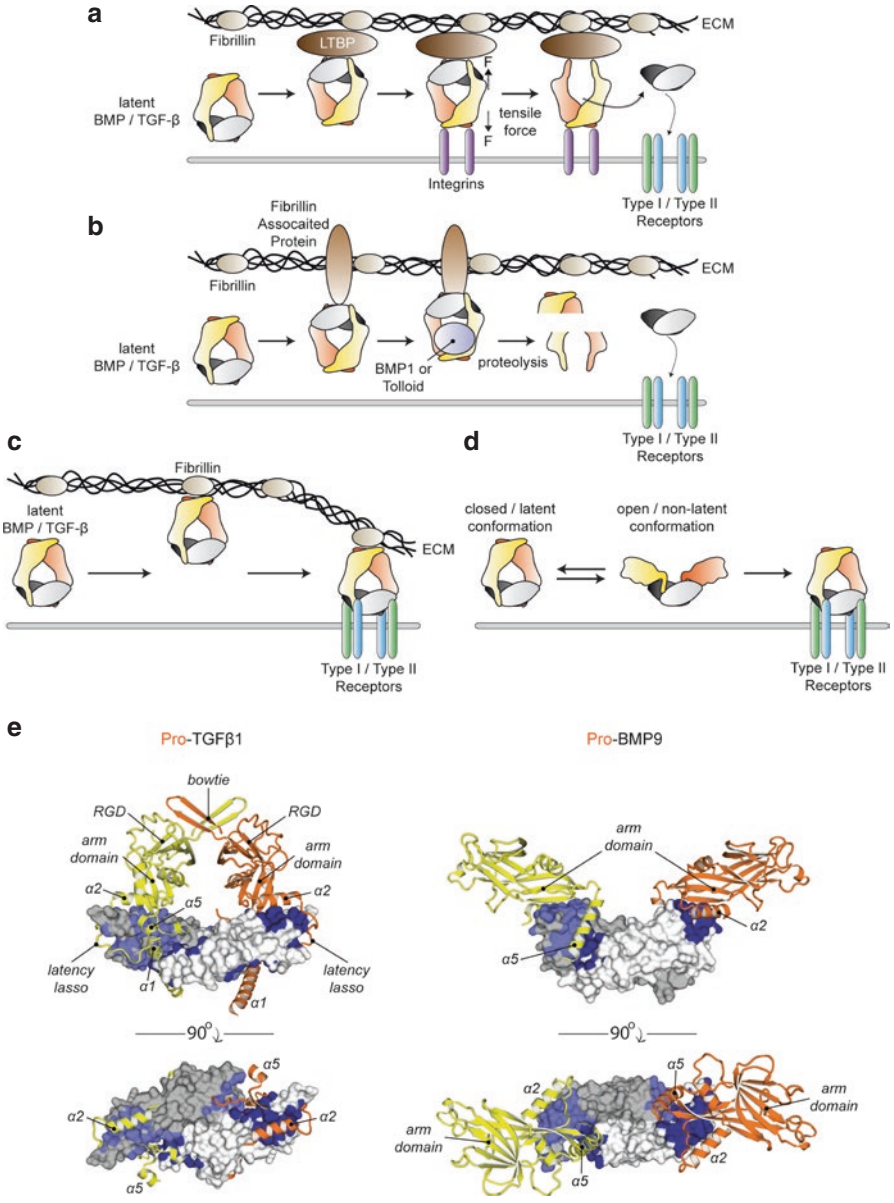


Fig. 3 Prodomain regulation of BMP/TGF-β signaling. Various mechanisms for prodomain-based latency and activation have been suggested. (**a**) As has been seen for the TGF-β subclass of ligands, the mature dimers stay tightly associated with their prodomains. This latent complex stays bound in the ECM, binding to LTBP or other ECM matrix proteins that can bind to fibrillin proteins within the matrix. For activation to occur, the opposing end of the prodomain can bind to integrins associated with the cell surface. Binding to both LTBP and integrins can lead to tensile force generation that is proposed to abrogate the prodomain fold, leading to dissociation of the

inhibiting/promoting receptor or other antagonist binding events. Recent evidence suggests that multiple protease sites may play a role in cleaving the prodomain at alternate locations, creating multiple latent complex forms with unique activities [231–234].

Phenotypically, the prodomains and the formation of latent complexes play a number of important roles in development and disease-state progression. As was suggested above, the prodomains are necessary for proper ligand folding and secretion. As such, numerous SNPs have been identified within the prodomain regions of several TGF-β superfamily members, such as mutations in TGF-β in Camurati-Engelmann disease [189, 235], GDF3 in microphthalmia [236], GDF5 in brachydactyly types A2 and C [237, 238], GDF6 in Klippel-Feil syndrome Type I [239], BMP4 in non-syndromic orofacial cleft type 11 [240], BMP7 with ocular development disorders [241], and BMP15 with premature ovarian failure type 4 [242–244]. Furthermore, mutations in the interacting partners of the prodomains and/or latent complexes, including fibrillin, the LTBPs, and other ECM components, have been linked to numerous pathologies stemming from a lack of ligand processing or release (e.g., mutations if fibrillin being associated in patients with Marfan syndrome) [245–247].

Biochemically, multiple mechanisms have been discovered to relieve prodomain-mature ligand interactions that lead to latency. The best studied of these

promain from the mature ligand domain, allowing the ligand to then initiate downstream signaling. (**b**) Certain ligands, such as myostatin, can remain in a latent complex with their prodomain and can stay within the ECM bound to a number of suggested fibrillin-binding molecules, such as LTBP3 or perlecan. For activation to occur, various proteases are released, such as BMP1 or tolloid, which can bind to the latent complex and proteolyze the prodomain. This leads to the release of the ligand for signaling purposes. (**c**) For certain BMP subclass ligands, prodomain-bound forms, such as for BMP7, have the ability to associate with specific ECM proteins, particularly fibrillin. This interaction, although not shown to be latent in currently explored contexts, can perhaps function to localize ligands to a particular cellular niche or aid in proper formation of ligand signaling gradients. It has been hypothesized that fibrillin, or other proteins, can hand off the mature ligand from the prodomain to achieve receptor binding, possibly forming an intermediate fibrillin-prodomain-BMP-receptor complex. It is believed that the prodomain, in this instance, will not interfere with receptor binding and activation when presented by specific proteins. (**d**) Lastly, many ligands, such as various BMP subclass proteins, including BMP9, stay associated with their prodomain. However, the ability of the prodomain to keep the ligand in a latent state is not well known and may be context dependent. As such, the complex can readily lead to an open arm conformation, where the prodomain is not adequately able to inhibit the mature ligand from signaling. Therefore, signaling likely occurs as it would for the unbound mature ligand form, uninhibited by the presence of the prodomain, but may exhibit altered receptor-binding preferences. (**e**) Structures of the pro-TGF-β1 latent complex (closed arm, PDBID 3RJR) and the non-latent pro-BMP9 complex (open armed, PDBID 4YCG). Differences are readily seen between the two structures, including the helices utilized by the opposing prodomains to achieve binding. The prodomains are shown in ribbon representation and are colored orange and yellow. The mature ligands are shown in surface representation in white and gray, with the interacting surfaces colored in dark blue and light blue

interactions has been the TGF-β subclass of ligands and their latent complexes, which bind to molecules including the LTBPs and integrins (reviewed in [6]). Latent TGF-β complexes bind to LTBP through a free cysteine near the N-terminus of the prodomain that covalently partners with a free cysteine on LTBP [248]. On the opposite end, and closer to the C-terminus of the prodomains, an RGD sequence is exposed (in TGF-β1 and TGF-β3), allowing for the latent complex to bind to specific integrin targets ($\alpha_v\beta_6$ and $\alpha_v\beta_8$) on signal-receiving cells [214–218]. When tethered together at both ends, a tensile force is hypothesized to pass through the latent TGF-β complex [249] (Fig. 3a). This likely stretches the prodomain within the latent complex, relaxing its associated ternary structure and contacts with the mature ligand, thereby releasing the mature TGF-β molecule to signal through its target receptors (Fig. 3a) [249]. Additionally, LTBP within this larger, tethered complex can be further found associated with fibrillin within the ECM, explaining the necessity of fibrillin and its role in TGF-β-related diseases [250–252].

For latent complexes of myostatin, and perhaps other activin subclass molecules such as GDF11, the process is much different. The latent myostatin complex has been shown to directly bind to fibrillin-associated molecules in addition to LTBP3, such as the heparan-modified proteoglycan, perlecan (Fig. 3b) [209, 230]. While latent myostatin maintains some minimal ability to initiate downstream signaling, in order for complete, full-level activation to occur, the myostatin prodomain has to be proteolytically processed by a member of the tolloid-like family of proteases, thereby releasing the mature ligand from the bonds of its prodomain (Fig. 3b) [202, 222, 225, 253]. For the BMP subclass, most ligands do not appear to remain associated with their prodomains and form latent complexes, although certain members, such as BMP7, have been suggested to remain latent under specific cellular contexts, where the prodomain of several BMPs (BMP2, BMP4, BMP7, BMP10, and GDF5) has been shown to directly bind fibrillin proteins through no associated partners, very different from TGF-β and myostatin (Fig. 3c) [226, 229, 230]. Particularly for BMP7, this interaction could potentially keep specific mature BMP ligands within a latent state, securing the ligand within the extracellular matrix. However, this is likely not the case as BMP7, despite binding its prodomain, maintains the ability to signal. With this in mind, fibrillin is likely functioning to localize BMP7 to specific cellular environments and to form proper developmental gradients. In this case, the prodomain-bound form of BMP7 was capable of binding to its Type I and Type II receptors, suggesting a hand-off mechanism for the mature ligand from the prodomain and extracellular matrix to the receptors (Fig. 3c) [228].

Our best insight into mechanisms of prodomain regulation, guiding TGF-β latency, comes from the crystal structures of the latent TGF-β1 and BMP9 complexes (Fig. 3e) [249, 254]. The structure of latent TGF-β1 revealed a very unique and unprecedented protein fold, where the prodomain consists of a straightjacket domain that weaves into both Type I and Type II receptor-binding motifs and a large arm domain positioned distally from the apical side of the ligand (Fig. 3e). The straightjacket forces the TGF-β1 ligand into a distorted or closed confirmation,

breaking away from the classic propeller-like shape of the dimer, where the long helices from the prodomain (α1) insert intimately into the Type I receptor-binding motif of the mature ligand. Furthermore, two small α-helices (α2 and α5) and one strand (β1), as well as the coined latency lasso following α2, interact with the fingers of the TGF-β1 ligand, blocking Type II receptor binding (Fig. 3e) [249]. Interestingly, α1 engages the arm domain to form a fastener or clasp, likely stabilizing the position of α1 in the Type I receptor pocket. The structure also reveals that opposing TGF-β1 monomers interact with the opposing monomer's prodomain (Fig. 3e). This crossover will likely have a profound impact on dimerization of the ligand and might restrict homo- and heterodimerization to certain ligand pairs. Thus it might be possible to direct the formation of certain heterodimers by interchanging the prodomain [249].

Whereas the TGF-β1 structure exists in a closed-arm conformation, supported by an intermolecular disulfide bond between opposing prodomains, the two recent structures of the prodomain-BMP9 complex (one latent and one not) depict a rather striking open-armed conformation, where the two opposing prodomains do not come into contact with one another and lack cysteines necessary to stabilize the closed-arm conformation seen in the latent TGF-β1 structure (Fig. 3e) [254]. In the latent form of the prodomain-BMP9 structure, the ligand conformation is not significantly altered, where BMP9 adopts the standard propeller-like morphology (Fig. 3e). This is consistent with the trend that BMP dimers are more rigid, whereas the more recently evolved ligands, including the TGF-β and activin subclasses, are much more flexible (reviewed in [3, 5, 48]). Furthermore, binding interfaces in the prodomain-BMP9 structure are quite unique, such that the prodomain α1 is not utilized to bind to the Type I receptor interface, but rather α5, much different than that found within latent TGF-β1 (Fig. 3e) [254]. While the Type II receptor interface is relatively similar between the prodomain-ligand complexes, β1 in the prodomain-BMP9 structure is much longer in comparison and there is no discernable latency lasso, suggesting a lack of contact with the mature BMP9 ligand [254].

While the structure shows blockage of at least a portion of the receptor-binding motifs, when tested *in vitro*, the purified prodomain-BMP9 complex maintained its ability to signal, albeit slightly reduced when compared to mature BMP9 alone. Furthermore, the complex was able to maintain reasonable binding to the Type I receptor, ALK1, but, interestingly, lost affinity for ActRIIa when compared to the mature ligand [254]. In addition, ActRIIb and BMPRII maintain equal affinities for the unbound and prodomain-bound forms of BMP9, suggesting a mechanism to alter specific receptor utilization and signaling outcomes [254]. While unexplored, potentially unidentified ECM partners could enhance the prodomain-mature ligand interaction for certain prodomain-BMP complexes, helping to maintain or promote the formation of a more closed-arm, possibly more latent, conformation. As such, events leading to the release of these prodomain-BMP complexes, as suggested for prodomain-BMP9 complex, could result in a shift to an open-armed conformation capable of signaling and modulating receptor utilization (Fig. 3d). As prodomain-BMP9 complexes have been found in significant levels in human blood, determining

the roles for these non-latent BMP complexes could provide useful information for our general understanding of BMP signaling.

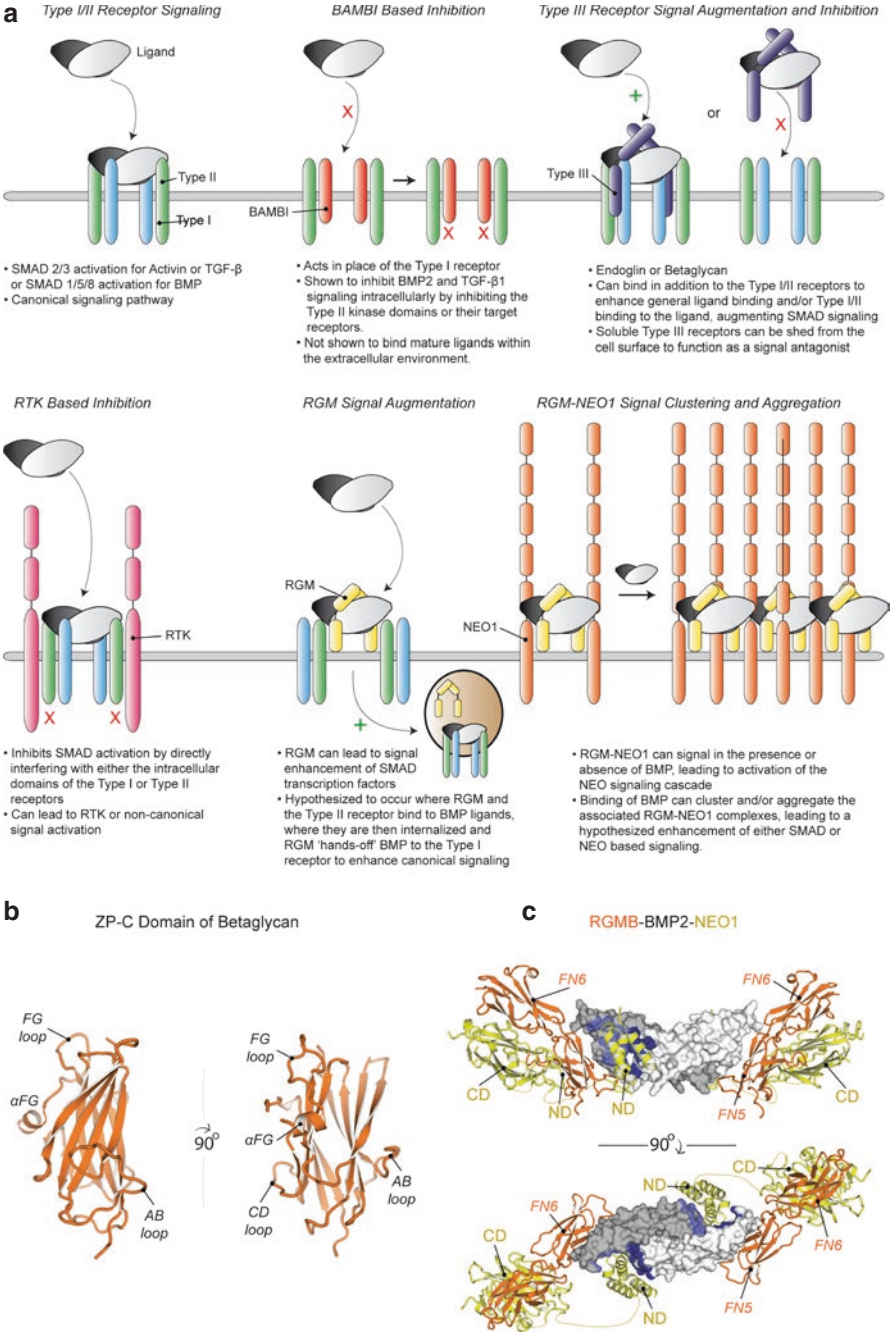
8 Co-receptors, Signal Enhancers, and Other Antagonists

TGF-β family co-receptors and related antagonists perform a number of roles in the regulation of BMP signaling, spanning many different families of proteins similar to the mature ligand-binding antagonists discussed above. Categories of co-receptors include the dragon family of proteins (RGMs), receptor tyrosine kinases (RTKs), TGF-β Type III receptors, and the protein BAMBI, where, depending on the protein and the context, these various proteins can either function to inhibit, enhance, or promote unique cellular signaling outcomes.

In the simplest case, BAMBI represents a negative regulator of TGF-β and BMP signaling. BAMBI can be classified as a pseudo-BMP receptor highly similar to the Type I ligand receptors [255, 256]. Although termed a pseudo-receptor, the functional domain of BAMBI is thought to be the short intracellular domain, which was shown to inhibit receptor activation through direct association with the Type I and/or Type II receptors (Fig. 4a) [255–257]. Curiously, it was determined that the inhibitory action of BAMBI did not depend on the presence of its extracellular domain, which is also incapable of ligand binding [257]. Why BAMBI is structurally homologous to the Type I receptor extracellular domain is not known.

For the RTKs, signal inhibition is achieved very differently from the above extracellular antagonists. Thus far, a number of RTKs have been found to modulate BMP signaling, including TrkC and Ror2. For both of these proteins, direct interactions

Fig. 4 Mechanisms of co-receptor regulation. (**a**) Various general binding schemes for various families of co-receptors, including enhancers and inhibitors. Various families include the BAMBI protein, Type III receptors, RTKs, RGM, and RGM-NEO1 complexes. These various pathways can lead to canonical SMAD signal enhancement, signal inhibition, or activation of independent or noncanonical signaling pathways. Descriptions for each family are given in the text and described in short in the figure below the corresponding cartoon. Ligands are colored gray and white, Type I receptor blue, and the Type II receptor green (for general comparisons). (**b**) Structure of the ZPC domain of betaglycan shown in ribbon representation (PDBID 3QW9). (**c**) Structure of the RGMB-BMP2-NEO1 complex (PDBID 4UI2). BMP2 is shown in surface representation as white and gray, while RGMB is shown in yellow ribbon and NEO1 is shown in orange ribbon. RGMB and NEO1 interact independent of the BMP2 ligand, showing an interaction between the NEO1 FN6 domains and the RGMB CD domain. The ND domain of RGMB binds the Type I interface of BMP2. Although the crystal structure shown NEO1 binding to the convex surface of BMP2, this is a crystallographic artifact. No binding between BMP2 and NEO1 occurs. The surface of BMP2 is colored dark blue and light blue to show the interfaces utilized for this binding event


a
Type I/II Receptor Signaling
Ligand
Type II
Type I
• SMAD 2/3 activation for Activin or TGF-β or SMAD 1/5/8 activation for BMP
• Canonical signaling pathway
BAMBI Based Inhibition
X
BAMBI
X X
• Acts in place of the Type I receptor
• Shown to inhibit BMP2 and TGF-β1 signaling intracellularly by inhibiting the Type II kinase domains or their target receptors.
• Not shown to bind mature ligands within the extracellular environment.
Type III Receptor Signal Augmentation and Inhibition
+
or
X
Type III
• Endoglin or Betaglycan
• Can bind in addition to the Type I/II receptors to enhance general ligand binding and/or Type I/II binding to the ligand, augmenting SMAD signaling
• Soluble Type III receptors can be shed from the cell surface to function as a signal antagonist
RTK Based Inhibition
RTK
X X
• Inhibits SMAD activation by directly interfering with either the intracellular domains of the Type I or Type II receptors
• Can lead to RTK or non-canonical signal activation
RGM Signal Augmentation
RGM
+
• RGM can lead to signal enhancement of SMAD transcription factors
• Hypothesized to occur where RGM and the Type II receptor bind to BMP ligands, where they are then internalized and RGM 'hands-off' BMP to the Type I receptor to enhance canonical signaling
RGM-NEO1 Signal Clustering and Aggregation
NEO1
• RGM-NEO1 can signal in the presence or absence of BMP, leading to activation of the NEO signaling cascade
• Binding of BMP can cluster and/or aggregate the associated RGM-NEO1 complexes, leading to a hypothesized enhancement of either SMAD or NEO based signaling.
b
ZP-C Domain of Betaglycan
FG loop
αFG
AB loop
90°
FG loop
αFG
AB loop
CD loop
c
RGMB-BMP2-NEO1
FN6
FN6
CD
ND
ND
FN5
CD
90°
CD
ND
FN6
CD
FN6
ND
FN5

were observed with the BMP receptors [258–261]. Once bound, the RTKs block or modulate signaling by preventing the Type II receptor from interacting with the Type I receptor, independent of BMP ligand binding, thus inhibiting intracellular phosphorylation events that lead to SMAD activation (Fig. 4a) [258–261]. In the case of Ror2, it is possible that preformed ALK6-Ror2 complexes directly modulate GDF5 signaling. Upon GDF5 binding, ALK6 would phosphorylate Ror2 and not BMPRII. This is believed to be in part due to the strong binding association of Ror2 with ALK6, leading to the displacement of BMPRII and, thus, down regulation of BMP signaling and enhancement of Ror2 signaling [258]. Biochemically, the interaction between ALK6 and Ror2 was determined to be dependent upon the frizzled-like cysteine-rich domain (CRD) within Ror2 [259]. Different from Ror2, TrkC was shown to interact directly with BMPRII or TβRII, but not other Type II receptors, to inhibit BMP2- or TGF-β-dependent SMAD activation [260, 261]. The interaction between TrkC and BMPRII or TβRII requires a functional kinase domain within TrkC that functions to inhibit the Type II receptors from Type I receptor association, thus preventing pathway activation (Fig. 4a) [260, 261]. Interestingly, TrkC has been largely considered an oncoprotein important in a number of different cancers. Since BMP signaling has been shown to maintain cells in a more differentiated state, an increase in TrkC might lead to potential stem cell-like transitions necessary for cancer progression [260, 261].

The most well-studied co-receptor proteins of BMP and TGF-β signaling are the Type III signaling receptors, namely, betaglycan and endoglin. Interestingly, these Type III co-receptors have been implicated in nearly all aspects of TGF-β signaling, where the context of the expression of these proteins can lead to either signal augmentation or inhibition for numerous BMP and TGF-β ligands (Fig. 4a, reviewed in [262]). Furthermore, for both betaglycan and endoglin, direct links have been found associating them to numerous cancer phenotypes directly resulting from aberrant TGF-β signaling [262–268]. On the molecular level, the Type III receptors have been shown to directly bind to and interact with several mature BMP/TGF-β ligands, as well as numerous ECM components, where the preferences for binding between these two related proteins are quite different (reviewed in [262]).

Structurally, both betaglycan and endoglin each contain an extracellular orphan domain (OD), followed by a zona pellucida domain (ZPD), transmembrane region, and a cytoplasmic domain, which contains a PDZ-binding region [269–273]. While both exist as dimers, endoglin dimers are supported through two intermolecular disulfide linkages, while betaglycan is stabilized non-covalently [271]. For both Type III receptors, each has been shown to directly interact with specific subsets of TGF-β superfamily ligands (reviewed in [262]). For betaglycan, the OD and ZPD have been shown to bind to numerous mature proteins, including, mainly, the TGF-β subclass as well as BMP2, BMP4, BMP7, and GDF5, while inhibin can only bind to its ZPD domain and requires this binding to achieve activin signal inhibition [274–277]. For endoglin, binding seems to occur primarily for BMP9 and BMP10 in addition to interactions with TGF-β1 and TGF-β3, excluding

TGF-β2 [275, 278–280]. Functionally, betaglycan has been shown to augment binding of TGF-β subclass ligands to the Type II TGF-β receptor, where binding of the mature ligands can occur independently of the receptors and acting, in a sense, to hand off the mature ligand to the receptors to achieve signal enhancement [281–284]. Endoglin, on the other hand, can only bind the majority of its targets (activin A, BMP2, BMP7, and TGF-β1) within the presence of the Type II receptor [280, 285, 286]. Only BMP9 and BMP10 have been shown to directly bind to and complex with endoglin independent of the canonical signaling receptors [278, 287]. Lastly, the cytoplasmic domains of both co-receptors can be actively phosphorylated by the canonical Type I and Type II receptors, perhaps suggesting alternative signaling mechanisms leading to either SMAD-dependent or SMAD-independent signaling [272, 288–291].

In the antagonistic sense, betaglycan and endoglin can function to inhibit BMP/TGF-β signaling very differently from their ability to augment canonical signaling. Most typically, these proteins are shed from the cellular surface and allowed to diffuse into the extracellular environment (Fig. 4a, reviewed in [262]). Specifically, both proteins are released from the surface by the action of metalloproteases [292, 293]. For soluble endoglin, this inhibitory form has been linked to cancer metastasis, where it functions to bind and sequester specific secreted ligands (e.g., TGF-β1), leading to depressed activation of their target ligands (Fig. 4a) [294–298].

Molecularly, a couple of studies have been performed to clarify structural details of the Type III receptors, leading to important conclusions regarding their functional roles. The structure of the betaglycan ZPC domain was resolved by X-ray crystallography and shows a similar fold to other known ZPC structures, formulated through a concession of several β-sheets (Fig. 4b) [299]. Functionally, the AB loop of this structure is critical for ligand binding. Interestingly, while other ZPC domains are often involved in polymerization through specific proteolytic events, betaglycan and endoglin lack this motif, thus explaining their inability to polymerize [299]. More recently, small-angle X-ray scattering and other biochemical studies of endoglin have provided insight into its role for modulating TGF-β and BMP9 signaling [279]. While endoglin enhances binding for TGF-β to both the Type I and Type II receptors, this is not observed with BMP9 and ALK1 [279]. Furthermore, BMP9 and BMP10 only bind to the endoglin OD, different from TGF-β [278]. With all of these studies in mind, additional work is needed to clarify the details and differences in ligand specificity and signal enhancement or inhibition, where structural work of Type III receptor complexes will prove valuable.

Lastly, the dragon family of proteins, including several repulsive guidance molecule (RGM) proteins, has been mainly implicated as noncanonical enhancers of BMP signaling [300–303]. For signaling to occur within the dragon family, RGMs have to bind to the co-receptor neogenin (NEO1) [304, 305]. Importantly, the dragon family of proteins has been directly linked to a number of severe disease-state pathologies, ranging from cancer (autoimmune encephalomyelitis and colon cancer) [306, 307], inflammatory diseases [308], multiple sclerosis [309], and

hemochromatosis [310]. The RGM proteins were originally identified as BMP-based co-receptors based upon their ability to respond to BMP ligands within BMP-responsive cellular assays, where the receptor NEO1 is modulated in response to this process [303, 304]. Mechanistic studies of the RGM-NEO complexes have determined that the RGM C-terminal domain was responsible for binding to the NEO1 receptor, supported by crystallographic studies [311, 312].

In 2015, the binding epitope in RGM for BMPs was discovered and the crystal structures of the extracellular domains of these various players were solved, including three structures of BMP bound to the N-terminal binding domain of RGM (RGMA-BMP2, RGMB-BMP2, and RGMC-BMP2). Further, the ternary RGMB-BMP2-NEO1 complex has been solved, containing Fn5 and Fn6 of NEO1 and the ND and CD of RGMB (Fig. 4c) [313]. In the RGM-BMP2 structures, the BMP2 ligand, similar to all other BMP subclass structures, resembles the ligand in the unbound conformation. In each structure, the ND of RGM takes on a unique 3-helical bundle fold (α1–α3) that engages the Type I binding motif of BMP2 (Fig. 4c). This is similar to the Type I interaction with BMP and utilizes a combination of hydrophobic and hydrogen bonding amino acids, where the characteristic two tryptophans on the ligand concave surface directly interact with two RGM histidines residing within α3 (where Alk3 uses phenylalanine) [313]. Similar to Alk3, RGMs utilize surfaces on both monomers composing the Type I binding interface to bury this motif on BMP2. Interestingly, the interaction of RGM-ND with BMP2 was shown to be pH dependent and not stable at more acidic pH. In contrast, the Alk3-BMP2 complex appears to be more broadly stable from pH 5.5–7.5. This difference in stability might explain how BMP2 signaling is enhanced even though RGM-ND occupies the Type I receptor site, where the low pH of an endosome might allow RGM to be displaced from this complex, thus enabling the BMP ligand to bind the Type I receptor [313].

In the RGMB-BMP2-NEO1 structure, again, BMP2 maintains its rigid-like propeller fold. NEO1 directly interacts with the Type II, convex surface of BMP2, making the ternary structure similar in architecture to that of the BMP2-Alk3-ActRIIa structure (Fig. 4c) [313]. The Fn5 domain of NEO1, which takes on a typical Fn-fold, directly binds to BMP2 at the fingertips of the protein in a similar location as to ActRIIa. However, as outlined in this study, NEO1 and BMP2 do not directly interact, suggesting that binding of NEO1 to BMP2 within this structure is a direct result of the crystallization process and not physiologically represented [313]. In addition, RGMB and NEO1 do not interact utilizing the Fn5 domain of NEO1. Instead, a major interaction between RGMB and NEO1 occurs distal to the mature BMP2 ligand, where Fn6 of NEO1 and the CD of RGMB form intimate contacts between two predominantly β-strand-rich domains, similar to the RGM-NEO structure [312, 313]. These domains are closer to the cell surface and transmembrane regions of the co-receptors. As RGM-NEO1 signals, it has been hypothesized that BMP2 functions to propagate this event by allowing clustering of tandem RGM-NEO1 complexes, which has been experimentally supported through TIRF (total internal resonance fluorescence) dSTORM (direct stochastic optical reconstruction) microscopy experiments (Fig. 4a) [313]. With this in mind, cells may be able to

modulate the context of SMAD activation or RGM-NEO1 signaling based upon which receptors are present and the expression levels of BMP ligands. While the exact interplay of RGM-NEO1 with BMP signaling is not fully understood, future studies need to address how these pathways synergize and what this means for SMAD activation and downstream signaling.

9 Summary of BMP Regulation Through Different Mechanisms

With the emerging structures that have become available in the TGF-β/BMP field, we now have a deeper understanding of the mechanisms dictating activation and regulation of these highly important pathways. While there are numerous questions abound, these structures reveal a number of important findings: (1) BMP ligands interact with a host of receptors, antagonists, prodomains, and co-receptors, indicating a set of complex interactions where it is unlikely that ligands exist in a "free" or unbound state but are more likely in equilibrium with a host of binding proteins; (2) that BMP ligands utilize conserved surfaces to interact with numerous different families of structurally unrelated proteins that most certainly have evolved independently; (3) despite each BMP ligand sharing highly similar tertiary structures, individual ligands contain unique surfaces and amino acids that impart specificity toward the binding of different antagonists and receptors (such as noggin binding to BMP2 with very high affinity but not at all to BMP9); and (4) the basis for specific BMP-related diseases, thus expanding the number of therapies that can be developed for modulating BMP signaling to enhance patient outcomes.

As observed in the structures outlined above, most BMP-binding proteins utilize both the Type I and Type II receptor interfaces to inhibit and/or enhance BMP signaling. In general, it appears that the Type II interface functions as the main interaction domain for these proteins, where large, typically hydrophobic surfaces account for the major affinity of these interactions. In addition, most antagonists utilize, rather uniquely, varying mechanisms to bind the Type I receptor motif, which exhibits significant sequence variation across family members. While not providing the majority of the binding interaction, this epitope likely provides the specificity needed to give individual BMP regulatory proteins their preferences for unique subsets of BMP and TGF-β ligands.

In summary, our understanding of the mechanisms governing BMP and TGF-β signaling and modulation/antagonism has been greatly enhanced through structural resolution of protein-ligand complexes. Without this information, our understanding of the regions necessary for signaling and how these play into ligand specificity would be severely underdeveloped. As we build upon this knowledge, we enhance our ability to generate and engineer novel molecules capable of targeting desired subsets or specific ligands, which can ultimately be used as biological tools (e.g., in stem cell protocols) for discovery and, also, disease treatment.

References

1. Bragdon B et al (2011) Bone morphogenetic proteins: a critical review. Cell Signal 23:609–620
2. Rider CC, Mulloy BB (2010) Bone morphogenetic protein and growth differentiation factor cytokine families and their protein antagonists. Biochem J 429:1–12
3. Mueller TD, Nickel J (2012) Promiscuity and specificity in BMP receptor activation. FEBS Lett 586:1846–1859
4. Mueller TD (2015) Mechanisms of BMP-receptor interaction and activation. Vitam Horm 99:1–61
5. Hinck AP (2012) Structural studies of the TGF-βs and their receptors - insights into evolution of the TGF-β superfamily. FEBS Lett 586:1860–1870
6. Harrison CA, Al-Musawi SL, Walton KL (2011) Prodomains regulate the synthesis, extracellular localisation and activity of TGF-β superfamily ligands. Growth Factors 29:174–186
7. Constam DB (2014) Regulation of TGFβ and related signals by precursor processing. Semin Cell Dev Biol 32:85–97
8. Lee MJ et al (2003) Bone morphogenetic protein-7 inhibits constitutive and interleukin-1 beta-induced monocyte chemoattractant protein-1 expression in human mesangial cells: role for JNK/AP-1 pathway. J Immunol 170:2557–2563
9. Guicheux J et al (2003) Activation of p38 mitogen-activated protein kinase and c-Jun-NH2-terminal kinase by BMP-2 and their implication in the stimulation of osteoblastic cell differentiation. J Bone Miner Res 18:2060–2068
10. Biver E, Thouverey C, Magne D, Caverzasio J (2014) Crosstalk between tyrosine kinase receptors, GSK3 and BMP2 signaling during osteoblastic differentiation of human mesenchymal stem cells. Mol Cell Endocrinol 382:120–130
11. Voloshenyuk TG, Landesman ES, Khoutorova E, Hart AD, Gardner JD (2011) Induction of cardiac fibroblast lysyl oxidase by TGF-β1 requires PI3K/Akt, Smad3, and MAPK signaling. Cytokine 55:90–97
12. Li CJ, Madhu V, Balian G, Dighe AS, Cui Q (2015) Cross-talk between VEGF and BMP-6 pathways accelerates osteogenic differentiation of human adipose-derived stem cells. J Cell Physiol 230:2671–2682
13. Li B et al (2012) Adenovirus-mediated overexpression of BMP-9 inhibits human osteosarcoma cell growth and migration through downregulation of the PI3K/AKT pathway. Int J Oncol 41:1809–1819
14. Viñals F, López-Rovira T, Rosa JL, Ventura F (2002) Inhibition of PI3K/p70 S6 K and p38 MAPK cascades increases osteoblastic differentiation induced by BMP-2. FEBS Lett 510:99–104
15. Sugimori K et al (2005) BMP-2 prevents apoptosis of the N1511 chondrocytic cell line through PI3K/Akt-mediated NF-kappaB activation. J Bone Miner Metab 23:411–419
16. Koseki T et al (2002) Role of TGF-beta family in osteoclastogenesis induced by RANKL. Cell Signal 14:31–36
17. Usui M et al (2008) Murine and chicken chondrocytes regulate osteoclastogenesis by producing RANKL in response to BMP2. J Bone Miner Res 23:314–325
18. Gallea S et al (2001) Activation of mitogen-activated protein kinase cascades is involved in regulation of bone morphogenetic protein-2-induced osteoblast differentiation in pluripotent C2C12 cells. Bone 28:491–498
19. Hoppler S, Moon RT (1998) BMP-2/-4 and Wnt-8 cooperatively pattern the Xenopus mesoderm. Mech Dev 71:119–129
20. Letamendia A, Labbé E, Attisano L (2001) Transcriptional regulation by Smads: crosstalk between the TGF-beta and Wnt pathways. J Bone Joint Surg Am 83-A(Suppl 1):S31–S39
21. Seystahl K, Tritschler I, Szabo E, Tabatabai G, Weller M (2015) Differential regulation of TGF-β-induced, ALK-5-mediated VEGF release by SMAD2/3 versus SMAD1/5/8 signaling in glioblastoma. Neuro Oncol 17:254–265

22. Liu Z et al (2009) VEGF and inhibitors of TGFbeta type-I receptor kinase synergistically promote blood-vessel formation by inducing alpha5-integrin expression. J Cell Sci 122:3294–3302
23. Massagué J (2012) TGFβ signalling in context. Nat Rev Mol Cell Biol 13:616–630
24. Guo X, Wang XF (2009) Signaling cross-talk between TGF-beta/BMP and other pathways. Cell Res 19:71–88
25. Rahman MS, Akhtar N, Jamil HM, Banik RS, Asaduzzaman SM (2015) TGF-β/BMP signaling and other molecular events: regulation of osteoblastogenesis and bone formation. Bone Res 3:15005
26. van den Bosch MH et al (2015) [published online ahead of print] Wnts talking with the TGF-β superfamily: WISPers about modulation of osteoarthritis. Rheumatology (Oxford) 55:1536–1547. doi:10.1093/rheumatology/kev402
27. Cheruku HR, Mohamedali A, Cantor DI, Tan SH (2015) Transforming growth factor-β, MAPK and Wnt signaling interactions in colorectal cancer. EuPA Open 8:104–115
28. Daopin S, Piez KA, Ogawa Y, Davies DR (1992) Crystal structure of transforming growth factor-beta 2: an unusual fold for the superfamily. Science 257:369–373
29. Schlunegger MP, Grütter MG (1992) An unusual feature revealed by the crystal structure at 2.2 A resolution of human transforming growth factor-beta 2. Nature 358:430–434
30. Griffith DL, Keck PC, Sampath TK, Rueger DC, Carlson WD (1996) Three-dimensional structure of recombinant human osteogenic protein 1: structural paradigm for the transforming growth factor beta superfamily. Proc Natl Acad Sci U S A 93:878–883
31. Hinck AP et al (1996) Transforming growth factor beta 1: three-dimensional structure in solution and comparison with the X-ray structure of transforming growth factor beta 2. Biochemistry 35:8517–8534
32. Scheufler C, Sebald W, Hülsmeyer M (1999) Crystal structure of human bone morphogenetic protein-2 at 2.7 A resolution. J Mol Biol 287:103–115
33. Oefner C, D'Arcy A, Winkler FK, Eggimann B, Hosang M (1992) Crystal structure of human platelet-derived growth factor BB. EMBO J 11:3921–3926
34. Muller YA et al (1997) Vascular endothelial growth factor: crystal structure and functional mapping of the kinase domain receptor binding site. Proc Natl Acad Sci U S A 94:7192–7197
35. Fox KM, Dias JA, Van Roey P (2001) Three-dimensional structure of human follicle-stimulating hormone. Mol Endocrinol 15:378–389
36. Keller S, Nickel J, Zhang J-L, Sebald W, Mueller TD (2004) Molecular recognition of BMP-2 and BMP receptor IA. Nat Struct Mol Biol 11:481–488
37. Allendorph GP, Vale WW, Choe S (2006) Structure of the ternary signaling complex of a TGF-beta superfamily member. Proc Natl Acad Sci U S A 103:7643–7648
38. Weber D et al (2007) A silent H-bond can be mutationally activated for high-affinity interaction of BMP-2 and activin type IIB receptor. BMC Struct Biol 7:6
39. Kotzsch A, Nickel J, Seher A, Sebald W, Müller TD (2009) Crystal structure analysis reveals a spring-loaded latch as molecular mechanism for GDF-5-type I receptor specificity. EMBO J 28:937–947
40. Greenwald J et al (2003) The BMP7/ActRII extracellular domain complex provides new insights into the cooperative nature of receptor assembly. Mol Cell 11:605–617
41. Townson SA et al (2012) Specificity and structure of a high affinity activin receptor-like kinase 1 (ALK1) signaling complex. J Biol Chem 287:27313–27325
42. Groppe J et al (2008) Cooperative assembly of TGF-beta superfamily signaling complexes is mediated by two disparate mechanisms and distinct modes of receptor binding. Mol Cell 29:157–168
43. Radaev S et al (2010) Ternary complex of transforming growth factor-beta1 reveals isoform-specific ligand recognition and receptor recruitment in the superfamily. J Biol Chem 285:14806–14814

44. Hart PJ et al (2002) Crystal structure of the human TbetaR2 ectodomain--TGF-beta3 complex. Nat Struct Biol 9:203–208
45. Greenwald J et al (2004) A flexible activin explains the membrane-dependent cooperative assembly of TGF-beta family receptors. Mol Cell 15:485–489
46. Klammert U et al (2015) GDF-5 can act as a context-dependent BMP-2 antagonist. BMC Biol 13:77
47. Thompson TB (2003) Structures of an ActRIIB: activin A complex reveal a novel binding mode for TGF-beta ligand:receptor interactions. EMBO J 22:1555–1566
48. Yadin D, Knaus P, Mueller TD (2015) [published online ahead of print] Structural insights into BMP receptors: specificity, activation and inhibition. Cytokine Growth Factor Rev 27:13–34. doi:10.1016/j.cytogfr.2015.11.005
49. Heinecke K et al (2009) Receptor oligomerization and beyond: a case study in bone morphogenetic proteins. BMC Biol 7:59
50. Cash JN, Rejon CA, McPherron AC, Bernard DJ, Thompson TB (2009) The structure of myostatin:follistatin 288: insights into receptor utilization and heparin binding. EMBO J 28:2662–2676
51. Nickel J, Kotzsch A, Sebald W, Mueller TD (2005) A single residue of GDF-5 defines binding specificity to BMP receptor IB. J Mol Biol 349:933–947
52. Kotzsch A et al (2007) Structure analysis of bone morphogenetic protein-2 type I receptor complexes reveals a mechanism of receptor inactivation in juvenile polyposis syndrome. J Biol Chem 283:5876–5887
53. Yoon BH et al (2014) An activin A/BMP2 chimera, AB204, displays bone-healing properties superior to those of BMP2. J Bone Miner Res 29:1950–1959
54. Allendorph GP et al (2011) Designer TGFβ superfamily ligands with diversified functionality. PLoS One 6:e26402
55. Esquivies L et al (2014) Designer nodal/BMP2 chimeras mimic nodal signaling, promote chondrogenesis, and reveal a BMP2-like structure. J Biol Chem 289:1788–1797
56. Brazil DP, Church RH, Surae S, Godson C, Martin F (2015) BMP signalling: agony and antagony in the family. Trends Cell Biol 25:249–264
57. Smith WC, Harland RM (1992) Expression cloning of noggin, a new dorsalizing factor localized to the Spemann organizer in Xenopus embryos. Cell 70:829–840
58. Smith WC, Knecht AK, Wu M, Harland RM (1993) Secreted noggin protein mimics the Spemann organizer in dorsalizing Xenopus mesoderm. Nature 361:547–549
59. Lamb TM et al (1993) Neural induction by the secreted polypeptide noggin. Science 262:713–718
60. Re'em-Kalma Y, Lamb T, Frank D (1995) Competition between noggin and bone morphogenetic protein 4 activities may regulate dorsalization during Xenopus development. Proc Natl Acad Sci U S A 92:12141–12145
61. Knecht AK, Good PJ, Dawid IB, Harland RM (1995) Dorsal-ventral patterning and differentiation of noggin-induced neural tissue in the absence of mesoderm. Development 121:1927–1935
62. Drossopoulou G et al (2000) A model for anteroposterior patterning of the vertebrate limb based on sequential long- and short-range Shh signalling and Bmp signalling. Development 127:1337–1348
63. Capdevila J, Johnson RL (1998) Endogenous and ectopic expression of noggin suggests a conserved mechanism for regulation of BMP function during limb and somite patterning. Dev Biol 197:205–217
64. Merino R et al (1998) Morphogenesis of digits in the avian limb is controlled by FGFs, TGFbetas, and noggin through BMP signaling. Dev Biol 200:35–45
65. Nifuji A, Noda M (1999) Coordinated expression of noggin and bone morphogenetic proteins (BMPs) during early skeletogenesis and induction of noggin expression by BMP-7. J Bone Miner Res 14:2057–2066
66. Zehentner BK, Haussmann A, Burtscher H (2002) The bone morphogenetic protein antagonist Noggin is regulated by Sox9 during endochondral differentiation. Dev Growth Differ 44:1–9

67. Brunet LJ, McMahon JA, McMahon AP, Harland RM (1998) Noggin, cartilage morphogenesis, and joint formation in the mammalian skeleton. Science 280:1455–1457
68. Pathi S, Rutenberg JB, Johnson RL, Vortkamp A (1999) Interaction of Ihh and BMP/Noggin signaling during cartilage differentiation. Dev Biol 209:239–253
69. Tsumaki N et al (2002) Bone morphogenetic protein signals are required for cartilage formation and differently regulate joint development during skeletogenesis. J Bone Miner Res 17:898–906
70. Matsui M, Klingensmith J (2014) Multiple tissue-specific requirements for the BMP antagonist Noggin in development of the mammalian craniofacial skeleton. Dev Biol 392: 168–181
71. Wijgerde M, Karp S, McMahon J, McMahon AP (2005) Noggin antagonism of BMP4 signaling controls development of the axial skeleton in the mouse. Dev Biol 286:149–157
72. Nesterenko AM et al (2015) Affinity of the heparin binding motif of noggin1 to heparan sulfate and its visualization in the embryonic tissues. Biochem Biophys Res Commun 468:331–336
73. Masuda S et al (2014) A mutation in the heparin-binding site of noggin as a novel mechanism of proximal symphalangism and conductive hearing loss. Biochem Biophys Res Commun 447:496–502
74. Paine-Saunders S, Viviano BL, Economides AN, Saunders S (2002) Heparan sulfate proteoglycans retain Noggin at the cell surface: a potential mechanism for shaping bone morphogenetic protein gradients. J Biol Chem 277:2089–2096
75. Viviano BL, Paine-Saunders S, Gasiunas N, Gallagher J, Saunders S (2004) Domain-specific modification of heparan sulfate by Qsulf1 modulates the binding of the bone morphogenetic protein antagonist Noggin. J Biol Chem 279:5604–5611
76. Zimmerman LB, De Jesús-Escobar JM, Harland RM (1996) The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. Cell 86:599–606
77. Groppe J et al (2002) Structural basis of BMP signalling inhibition by the cystine knot protein Noggin. Nature 420:636–642
78. Albers CE et al (2012) L51P – A BMP2 variant with osteoinductive activity via inhibition of Noggin. Bone 51:401–406
79. Seemann P et al (2009) Mutations in GDF5 reveal a key residue mediating BMP inhibition by NOGGIN. PLoS Genet 5:e1000747
80. Song K et al (2010) Identification of a key residue mediating bone morphogenetic protein (BMP)-6 resistance to noggin inhibition allows for engineered BMPs with superior agonist activity. J Biol Chem 285:12169–12180
81. Wang Y et al (2013) Noggin resistance contributes to the potent osteogenic capability of BMP9 in mesenchymal stem cells. J Orthop Res 31:1796–1803
82. Wang J et al (2015) [published online ahead of print] A new subtype of multiple-synostoses syndrome is caused by a mutation in GDF6 that decreases its sensitivity to noggin and enhances its potency as a BMP signal. J Bone Miner Res 31:882–889. doi:10.1002/jbmr.2761
83. Ueno N et al (1987) Isolation and partial characterization of follistatin: a single-chain Mr 35,000 monomeric protein that inhibits the release of follicle-stimulating hormone. Proc Natl Acad Sci U S A 84:8282–8286
84. Ying SY, Becker A, Swanson G, Tan P, Ling N (1987) Follistatin specifically inhibits pituitary follicle stimulating hormone release in vitro. Biochem Biophys Res Commun 149:133–139
85. Nakamura T et al (1990) Activin-binding protein from rat ovary is follistatin. Science 247:836–838
86. Shimasaki S et al (1989) Follistatin gene expression in the ovary and extragonadal tissues. Mol Endocrinol 3:651–659
87. Carroll RS, Corrigan AZ, Gharib SD, Vale W, Chin WW (1989) Inhibin, activin, and follistatin: regulation of follicle-stimulating hormone messenger ribonucleic acid levels. Mol Endocrinol 3:1969–1976
88. Shimasaki S et al (1988) Primary structure of the human follistatin precursor and its genomic organization. Proc Natl Acad Sci U S A 85:4218–4222

89. Sidis Y et al (2006) Biological activity of follistatin isoforms and follistatin-like-3 is dependent on differential cell surface binding and specificity for activin, myostatin, and bone morphogenetic proteins. Endocrinology 147:3586–3597
90. Shimasaki S, Koga M, Esch F, Mercado M (1988) Porcine follistatin gene structure supports two forms of mature follistatin produced by alternative splicing. Biochem Biophys Res Commun 152:717–723
91. Sugino K et al (1993) Molecular heterogeneity of follistatin, an activin-binding protein. Higher affinity of the carboxyl-terminal truncated forms for heparan sulfate proteoglycans on the ovarian granulosa cell. J Biol Chem 268:15579–15587
92. Inouye S et al (1991) Recombinant expression of human follistatin with 315 and 288 amino acids: chemical and biological comparison with native porcine follistatin. Endocrinology 129:815–822
93. Shibanuma M, Mashimo J, Mita A (1993) Cloning from a mouse osteoblastic cell line of a set of transforming-growth-factor-β1-regulated genes, one of which seems to encode a follistatin-related polypeptide. Eur J Biochem 217:13–19
94. Hayette S et al (1998) FLRG (follistatin-related gene), a new target of chromosomal rearrangement in malignant blood disorders. Oncogene 16:2949–2954
95. Zwijsen A et al (1994) Characterization of a rat C6 glioma-secreted follistatin-related protein (FRP). Cloning and sequence of the human homologue. Eur J Biochem 225:937–946
96. Trexler M, Banyai L, Patthy L (2001) A human protein containing multiple types of protease-inhibitory modules. Proc Natl Acad Sci U S A 98:3705–3709
97. Trexler M, Bányai L, Patthy L (2002) Distinct expression pattern of two related human proteins containing multiple types of protease-inhibitory modules. Biol Chem 383:223–228
98. Tsuchida K et al (2000) Identification and characterization of a novel follistatin-like protein as a binding protein for the TGF-beta family. J Biol Chem 275:40788–40796
99. Tanaka M et al (2010) DIP2 disco-interacting protein 2 homolog A (Drosophila) is a candidate receptor for follistatin-related protein/follistatin-like 1--analysis of their binding with TGF-β superfamily proteins. FEBS J 277:4278–4289
100. Sidis Y et al (2002) Follistatin-related protein and follistatin differentially neutralize endogenous vs. exogenous activin. Endocrinology 143:1613–1624
101. Schneyer A et al (2004) Differential actions of follistatin and follistatin-like 3. Mol Cell Endocrinol 225:25–28
102. Thompson TB, Lerch TF, Cook RW, Woodruff TK (2005) The structure of the follistatin: activin complex reveals antagonism of both type I and type II receptor binding. Dev Cell 9:535–543
103. Stamler R et al (2008) The structure of FSTL3.activin A complex. Differential binding of N-terminal domains influences follistatin-type antagonist specificity. J Biol Chem 283:32831–32838
104. Cash JN et al (2012) Structure of myostatin·follistatin-like 3: N-terminal domains of follistatin-type molecules exhibit alternate modes of binding. J Biol Chem 287:1043–1053
105. Harrington AE et al (2006) Structural basis for the inhibition of activin signalling by follistatin. EMBO J 25:1035–1045
106. Innis CA, Hyvönen M (2003) Crystal structures of the heparan sulfate-binding domain of follistatin. Insights into ligand binding. J Biol Chem 278:39969–39977
107. Lerch TF, Shimasaki S, Woodruff TK, Jardetzky TS (2007) Structural and biophysical coupling of heparin and activin binding to follistatin isoform functions. J Biol Chem 282:15930–15939
108. Iemura S et al (1998) Direct binding of follistatin to a complex of bone-morphogenetic protein and its receptor inhibits ventral and epidermal cell fates in early Xenopus embryo. Proc Natl Acad Sci U S A 95:9337–9342
109. Cash JN, Angerman EB, Keutmann HT, Thompson TB (2012) Characterization of follistatin-type domains and their contribution to myostatin and activin A antagonism. Mol Endocrinol 26:1167–1178

110. Geng Y et al (2011) Follistatin-like 1 (Fstl1) is a bone morphogenetic protein (BMP) 4 signaling antagonist in controlling mouse lung development. Proc Natl Acad Sci 108: 7058–7063
111. Sylva M et al (2011) The BMP antagonist follistatin-like 1 is required for skeletal and lung organogenesis. PLoS One 6:e22616
112. Sasai Y et al (1994) Xenopus chordin: a novel dorsalizing factor activated by organizer-specific homeobox genes. Cell 79:779–790
113. Piccolo S, Sasai Y, Lu B, De Robertis EM (1996) Dorsoventral patterning in Xenopus: inhibition of ventral signals by direct binding of chordin to BMP-4. Cell 86:589–598
114. Holley SA et al (1995) A conserved system for dorsal-ventral patterning in insects and vertebrates involving sog and chordin. Nature 376:249–253
115. Sasai Y, Lu B, Steinbeisser H, De Robertis EM (1995) Regulation of neural induction by the Chd and Bmp-4 antagonistic patterning signals in Xenopus. Nature 376:333–336
116. Fisher S, Halpern ME (1999) Patterning the zebrafish axial skeleton requires early chordin function. Nat Genet 23:442–446
117. Délot EC, Shneyder N, Zhang H, Bachiller D (2007) Abnormal venous and arterial patterning in chordin mutants. Dev Dyn 236:2586–2593
118. Dale K et al (1999) Differential patterning of ventral midline cells by axial mesoderm is regulated by BMP7 and chordin. Development 126:397–408
119. Blader P, Rastegar S, Fischer N, Strähle U (1997) Cleavage of the BMP-4 antagonist chordin by zebrafish tolloid. Science 278:1937–1940
120. Piccolo S et al (1997) Cleavage of chordin by Xolloid metalloprotease suggests a role for proteolytic processing in the regulation of spemann organizer activity. Cell 91:407–416
121. Streit A, Stern CD (1999) Mesoderm patterning and somite formation during node regression: differential effects of chordin and noggin. Mech Dev 85:85–96
122. Sakuta H et al (2001) Ventroptin: a BMP-4 antagonist expressed in a double-gradient pattern in the retina. Science 293:111–115
123. Nakayama N et al (2004) A novel chordin-like BMP inhibitor, CHL2, expressed preferentially in chondrocytes of developing cartilage and osteoarthritic joint cartilage. Development 131:229–240
124. Lin J et al (2005) Kielin/chordin-like protein, a novel enhancer of BMP signaling, attenuates renal fibrotic disease. Nat Med 11:387–393
125. Moser M et al (2003) BMPER, a novel endothelial cell precursor-derived protein, antagonizes bone morphogenetic protein signaling and endothelial cell differentiation. Mol Cell Biol 23:5664–5679
126. Zhang JL, Huang Y, Qiu LY, Nickel J, Sebald W (2007) von Willebrand factor type C domain-containing proteins regulate bone morphogenetic protein signaling through different recognition mechanisms. J Biol Chem 282:20002–20014
127. Zhang JL et al (2008) Crystal structure analysis reveals how the chordin family member crossveinless 2 blocks BMP-2 receptor binding. J Mol Biol 14:739–750
128. Larraín J et al (2000) BMP-binding modules in chordin: a model for signalling regulation in the extracellular space. Development 127:821–830
129. Xie J, Fisher S (2005) Twisted gastrulation enhances BMP signaling through chordin dependent and independent mechanisms. Development 132:383–391
130. Coles E, Christiansen J, Economou A, Bronner-Fraser M, Wilkinson DG (2004) A vertebrate crossveinless 2 homologue modulates BMP activity and neural crest cell migration. Development 131:5309–5317
131. Troilo H et al (2014) Nanoscale structure of the BMP antagonist chordin supports cooperative BMP binding. Proc Natl Acad Sci 111:13063–13068
132. Ozaki T, Sakiyama S (1994) Tumor-suppressive activity of N03 gene product in v-src-transformed rat 3Y1 fibroblasts. Cancer Res 54:646–648
133. Pearce JJ, Penny G, Rossant J (1999) A mouse cerberus/Dan-related gene family. Dev Biol 209:98–110

134. Hsu DR, Economides AN, Wang X, Eimon PM, Harland RM (1998) The Xenopus dorsalizing factor Gremlin identifies a novel family of secreted proteins that antagonize BMP activities. Mol Cell 1:673–683
135. Minabe-Saegusa C, Saegusa H, Tsukahara M, Noguchi S (1998) Sequence and expression of a novel mouse gene PRDC (protein related to DAN and cerberus) identified by a gene trap approach. Dev Growth Differ 40:343–353
136. Marques S et al (2004) The activity of the Nodal antagonist Cerl-2 in the mouse node is required for correct L/R body axis. Genes Dev 18:2342–2347
137. Laurikkala J, Kassai Y, Pakkasjärvi L, Thesleff I, Itoh N (2003) Identification of a secreted BMP antagonist, ectodin, integrating BMP, FGF, and SHH signals from the tooth enamel knot. Dev Biol 264:91–105
138. Brunkow ME et al (2001) Bone dysplasia sclerosteosis results from loss of the SOST gene product, a novel cystine knot-containing protein. Am J Hum Genet 68:577–589
139. Bouwmeester T, Kim S, Sasai Y, Lu B, De Robertis EM (1996) Cerberus is a head-inducing secreted factor expressed in the anterior endoderm of Spemann's organizer. Nature 382:595–601
140. Nolan K, Thompson TB (2014) The DAN family: modulators of TGF-β signaling and beyond. Protein Sci 23:999–1012
141. Zúñiga A, Haramis AP, McMahon AP, Zeller R (1999) Signal relay by BMP antagonism controls the SHH/FGF4 feedback loop in vertebrate limb buds. Nature 401:598–602
142. Khokha MK, Hsu D, Brunet LJ, Dionne MS, Harland RM (2003) Gremlin is the BMP antagonist required for maintenance of Shh and Fgf signals during limb patterning. Nat Genet 34:303–307
143. Merino R et al (1999) The BMP antagonist Gremlin regulates outgrowth, chondrogenesis and programmed cell death in the developing limb. Development 126:5515–5522
144. Michos O et al (2004) Gremlin-mediated BMP antagonism induces the epithelial-mesenchymal feedback signaling controlling metanephric kidney and limb organogenesis. Development 131:3401–3410
145. Michos O et al (2007) Reduction of BMP4 activity by gremlin 1 enables ureteric bud outgrowth and GDNF/WNT11 feedback signalling during kidney branching morphogenesis. Development 134:2397–2405
146. Marchant V et al (2015) Tubular overexpression of gremlin in transgenic mice aggravates renal damage in diabetic nephropathy. Am J Physiol Renal Physiol 309:F559–F568
147. Roxburgh SA et al (2009) Allelic depletion of grem1 attenuates diabetic kidney disease. Diabetes 58:1641–1650
148. Yanagita M et al (2004) USAG-1: a bone morphogenetic protein antagonist abundantly expressed in the kidney. Biochem Biophys Res Commun 316:490–500
149. Yanagita M et al (2006) Uterine sensitization-associated gene-1 (USAG-1), a novel BMP antagonist expressed in the kidney, accelerates tubular injury. J Clin Invest 116:70–79
150. Tanaka M et al (2008) Expression of BMP-7 and USAG-1 (a BMP antagonist) in kidney development and injury. Kidney Int 73:181–191
151. Tanaka M et al (2010) Loss of the BMP antagonist USAG-1 ameliorates disease in a mouse model of the progressive hereditary kidney disease Alport syndrome. J Clin Invest 120:768–777
152. Cahill E et al (2012) Gremlin plays a key role in the pathogenesis of pulmonary hypertension. Circulation 125:920–930
153. Wellbrock J et al (2015) Intrinsic BMP antagonist Gremlin-1 as a novel circulating marker in pulmonary arterial hypertension. Lung 193:567–570
154. Ciuclan L et al (2013) Treatment with anti-gremlin 1 antibody ameliorates chronic hypoxia/SU5416-induced pulmonary arterial hypertension in mice. Am J Pathol 183:1461–1473
155. Karagiannis GS, Berk A, Dimitromanolakis A, Diamandis EP (2013) Enrichment map profiling of the cancer invasion front suggests regulation of colorectal cancer progression by the bone morphogenetic protein antagonist, gremlin-1. Mol Oncol 7:826–839
156. Karagiannis GS et al (2015) Bone morphogenetic protein antagonist gremlin-1 regulates colon cancer progression. Biol Chem 396:163–183

157. Tamminen JA, Parviainen V, Rönty M, Wohl AP (2013) Gremlin-1 associates with fibrillin microfibrils in vivo and regulates mesothelioma cell survival through transcription factor slug. Oncogenesis 2:e66
158. Li Y et al (2012) Gremlin-mediated decrease in bone morphogenetic protein signaling promotes aristolochic acid-induced epithelial-to-mesenchymal transition (EMT) in HK-2 cells. Toxicology 297:68–75
159. Kim M et al (2012) Gremlin-1 induces BMP-independent tumor cell proliferation, migration, and invasion. PLoS One 7:e35100
160. Namkoong H et al (2006) The bone morphogenetic protein antagonist gremlin 1 is overexpressed in human cancers and interacts with YWHAH protein. BMC Cancer 6:74
161. Müller II et al (2012) Functional modeling in zebrafish demonstrates that the atrial-fibrillation-associated gene GREM2 regulates cardiac laterality, cardiomyocyte differentiation and atrial rhythm. Dis Model Mech 6:332–341
162. Tanwar V et al (2014) Gremlin 2 promotes differentiation of embryonic stem cells to atrial fate by activation of the JNK signaling pathway. Stem Cells 32:1774–1788
163. van Bezooijen RL et al (2004) Sclerostin is an osteocyte-expressed negative regulator of bone formation, but not a classical BMP antagonist. J Exp Med 199:805–814
164. Kusu N et al (2003) Sclerostin is a novel secreted osteoclast-derived bone morphogenetic protein antagonist with unique ligand specificity. J Biol Chem 278:24113–24117
165. Winkler DG et al (2003) Osteocyte control of bone formation via sclerostin, a novel BMP antagonist. EMBO J 22:6267–6276
166. Suen PK et al (2015) Sclerostin antibody treatment increases bone formation, bone mass, and bone strength of Intact bones in adult male rats. Sci Rep 5:15632
167. Yao W et al (2016) Sclerostin-antibody treatment of glucocorticoid-induced osteoporosis maintained bone mass and strength. Osteoporos Int 27:283–294
168. Sinder BP et al (2016) Effect of anti-sclerostin therapy and osteogenesis imperfecta on tissue-level properties in growing and adult mice while controlling for tissue age. Bone 84:222–229
169. Grafe I et al (2016) Sclerostin antibody treatment improves the bone phenotype of Crtap(−/−) mice, a model of recessive osteogenesis imperfecta. J Bone Miner Res 31:1030–1040
170. Tinsley BA et al (2015) Systemic administration of sclerostin antibody enhances bone morphogenetic protein-induced femoral defect repair in a rat model. J Bone Joint Surg Am 97:1852–1859
171. Eddleston A et al (2009) A short treatment with an antibody to sclerostin can inhibit bone loss in an ongoing model of colitis. J Bone Miner Res 24:1662–1671
172. Li X et al (2009) Sclerostin antibody treatment increases bone formation, bone mass, and bone strength in a rat model of postmenopausal osteoporosis. J Bone Miner Res 24:578–588
173. Worthley DL et al (2015) Gremlin 1 identifies a skeletal stem cell with bone, cartilage, and reticular stromal potential. Cell 160:269–284
174. Sakaki-Yumoto M, Katsuno Y, Derynck R (2013) TGF-β family signaling in stem cells. Biochim Biophys Acta 1830:2280–2296
175. Veverka V et al (2009) Characterization of the structural features and interactions of sclerostin: molecular insight into a key regulator of Wnt-mediated bone formation. J Biol Chem 284:10890–10900
176. Weidauer SE et al (2009) NMR structure of the Wnt modulator protein Sclerostin. Biochem Biophys Res Commun 380:160–165
177. Nolan K et al (2013) Structure of protein related to dan and cerberus: Insights into the mechanism of bone morphogenetic protein antagonism. Structure 21:1417–1429
178. Nolan K et al (2015) Structure of neuroblastoma suppressor of tumorigenicity 1 (NBL1): insights for the functional variability across bone morphogenetic protein (BMP) antagonists. J Biol Chem 290:4759–4771
179. Kattamuri C et al (2012) Members of the DAN family are BMP antagonists that form highly stable noncovalent dimers. J Mol Biol 424:313–327

180. Lintern KB, Guidato S, Rowe A, Saldanha JW, Itasaki N (2009) Characterization of wise protein and its molecular mechanism to interact with both Wnt and BMP signals. J Biol Chem 284:23159–23168
181. Tatsinkam AJ, Mulloy B, Rider CC (2015) Mapping the heparin-binding site of the BMP antagonist gremlin by site-directed mutagenesis based on predictive modelling. Biochem J 470:53–64
182. Aykul S, Martinez-Hackert E (2016) [published online ahead of print] New ligand binding function of human Cerberus and role of proteolytic processing in regulating ligand-receptor interactions and antagonist activity. J Mol Biol 428:590–602. doi:10.1016/j.jmb.2016.01.011
183. Avsian-Kretchmer O, Hsueh AJW (2004) Comparative genomic analysis of the eight-membered ring cystine knot-containing bone morphogenetic protein antagonists. Mol Endocrinol 18:1–12
184. Hung WT, Wu FJ, Wang CJ, Luo CW (2012) DAN (NBL1) specifically antagonizes BMP2 and BMP4 and modulates the actions of GDF9, BMP2, and BMP4 in the rat ovary. Biol Reprod 86:1–9
185. Hernandez P, Whitty C, John Wardale R, Henson FMD (2014) New insights into the location and form of sclerostin. Biochem Biophys Res Commun 446:1108–1113
186. Sun J et al (2006) BMP4 activation and secretion are negatively regulated by an intracellular gremlin-BMP4 interaction. J Biol Chem 281:29349–29356
187. Aykul S, Ni W, Mutatu W, Martinez-Hackert E (2015) Human cerberus prevents nodal-receptor binding, inhibits nodal signaling, and suppresses nodal-mediated phenotypes. PLoS One 10:e0114954
188. Gray AM, Mason AJ (1990) Requirement for activin A and transforming growth factor--beta 1 pro-regions in homodimer assembly. Science 247:1328–1330
189. Walton KL et al (2010) Two distinct regions of latency-associated peptide coordinate stability of the latent transforming growth factor-beta1 complex. J Biol Chem 285:17029–17037
190. Buzzard JJ et al (2004) Changes in circulating and testicular levels of inhibin A and B and activin A during postnatal development in the rat. Endocrinology 145:3532–3541
191. Lau CP, Ledger WL, Groome NP, Barlow DH, Muttukrishna S (1999) Dimeric inhibins and activin A in human follicular fluid and oocyte-cumulus culture medium. Hum Reprod 14:2525–2530
192. McIntosh CJ et al (2008) The proregion of mouse BMP15 regulates the cooperative interactions of BMP15 and GDF9. Biol Reprod 79:889–896
193. Little SC, Mullins MC (2009) Bone morphogenetic protein heterodimers assemble heteromeric type I receptor complexes to pattern the dorsoventral axis. Nat Cell Biol 11:637–643
194. Nachtigal MW, Ingraham HA (1996) Bioactivation of Müllerian inhibiting substance during gonadal development by a kex2/subtilisin-like endoprotease. Proc Natl Acad Sci U S A 93:7711–7716
195. Miyazono K, Hellman U, Wernstedt C, Heldin CH (1988) Latent high molecular weight complex of transforming growth factor beta 1. Purification from human platelets and structural characterization. J Biol Chem 263:6407–6415
196. Wakefield LM, Smith DM, Flanders KC, Sporn MB (1988) Latent transforming growth factor-beta from human platelets. A high molecular weight complex containing precursor sequences. J Biol Chem 263:7646–7654
197. Pircher R, Lawrence DA, Jullien P (1984) Latent beta-transforming growth factor in non-transformed and Kirsten sarcoma virus-transformed normal rat kidney cells, clone 49F. Cancer Res 44:5538–5543
198. Lawrence DA, Pircher R, Jullien P (1985) Conversion of a high molecular weight latent beta-TGF from chicken embryo fibroblasts into a low molecular weight active beta-TGF under acidic conditions. Biochem Biophys Res Commun 133:1026–1034
199. Hill JJ et al (2002) The myostatin propeptide and the follistatin-related gene are inhibitory binding proteins of myostatin in normal serum. J Biol Chem 277:40735–40741

200. Zimmers TA et al (2002) Induction of cachexia in mice by systemically administered myostatin. Science 296:1486–1488
201. Lee SJ, McPherron AC (2001) Regulation of myostatin activity and muscle growth. Proc Natl Acad Sci U S A 98:9306–9311
202. Ge G, Hopkins DR, Ho WB, Greenspan DS (2005) GDF11 forms a bone morphogenetic protein 1-activated latent complex that can modulate nerve growth factor-induced differentiation of PC12 cells. Mol Cell Biol 25:5846–5858
203. Madisen L et al (1989) Expression and characterization of recombinant TGF-beta 2 proteins produced in mammalian cells. DNA 8:205–212
204. Thies RS et al (2001) GDF-8 propeptide binds to GDF-8 and antagonizes biological activity by inhibiting GDF-8 receptor binding. Growth Factors 18:251–259
205. Miller DM et al (1992) Characterization of the binding of transforming growth factor-beta 1, -beta 2, and -beta 3 to recombinant beta 1-latency-associated peptide. Mol Endocrinol 6:694–702
206. Lawrence DA, Pircher R, Krycève-Martinerie C, Jullien P (1984) Normal embryo fibroblasts release transforming growth factors in a latent form. J Cell Physiol 121:184–188
207. Krycève-Martinerie C, Lawrence DA, Crochet J, Jullien P, Vigier P (1982) Cells transformed by Rous sarcoma virus release transforming growth factors. J Cell Physiol 113:365–372
208. Saharinen J, Keski-Oja J (2000) Specific sequence motif of 8-Cys repeats of TGF-β binding proteins, LTBPs, creates a hydrophobic interaction surface for binding of small latent TGF-β. Mol Biol Cell 11:2691–2704
209. Anderson SB, Goldberg AL, Whitman M (2008) Identification of a novel pool of extracellular pro-myostatin in skeletal muscle. J Biol Chem 283:7027–7035
210. Nunes I, Gleizes PE, Metz CN, Rifkin DB (1997) Latent transforming growth factor-beta binding protein domains involved in activation and transglutaminase-dependent cross-linking of latent transforming growth factor-beta. J Cell Biol 136:1151–1163
211. Isogai Z et al (2003) Latent transforming growth factor beta-binding protein 1 interacts with fibrillin and is a microfibril-associated protein. J Biol Chem 278:2750–2757
212. Nistala H et al (2010) Fibrillin-1 and -2 differentially modulate endogenous TGF-β and BMP bioavailability during bone formation. J Cell Biol 190:1107–1121
213. Robertson I, Jensen S, Handford P (2011) TB domain proteins: evolutionary insights into the multifaceted roles of fibrillins and LTBPs. Biochem J 433:263–276
214. Munger JS, Harpel JG, Giancotti FG (1998) Interactions between growth factors and integrins: latent forms of transforming growth factor-β are ligands for the integrin αvβ1. Mol Biol Cell 9:2627–2638
215. Munger JS et al (1999) The integrin alpha v beta 6 binds and activates latent TGF beta 1: a mechanism for regulating pulmonary inflammation and fibrosis. Cell 96:319–328
216. Annes JP, Chen Y, Munger JS, Rifkin DB (2004) Integrin alphaVbeta6-mediated activation of latent TGF-beta requires the latent TGF-beta binding protein-1. J Cell Biol 165:723–734
217. Annes JP, Rifkin DB, Munger JS (2002) The integrin alphaVbeta6 binds and activates latent TGFbeta3. FEBS Lett 511:65–68
218. Yang Z et al (2007) Absence of integrin-mediated TGFbeta1 activation in vivo recapitulates the phenotype of TGFbeta1-null mice. J Cell Biol 176:787–793
219. Li S et al (2010) Activin A binds to perlecan through its pro-region that has heparin/heparan sulfate binding activity. J Biol Chem 285:36645–36655
220. Schultz-Cherry S, Ribeiro S, Gentry L, Murphy-Ullrich JE (1994) Thrombospondin binds and activates the small and large forms of latent transforming growth factor-beta in a chemically defined system. J Biol Chem 269:26775–26782
221. Young GD, Murphy-Ullrich JE (2004) Molecular interactions that confer latency to transforming growth factor-beta. J Biol Chem 279:38032–38039
222. Wolfman NM et al (2003) Activation of latent myostatin by the BMP-1/tolloid family of metalloproteinases. Proc Natl Acad Sci U S A 100:15842–15846
223. Jenkins G (2008) The role of proteases in transforming growth factor-beta activation. Int J Biochem Cell Biol 40:1068–1078

224. Ge G, Greenspan DS (2006) BMP1 controls TGFbeta1 activation via cleavage of latent TGFbeta-binding protein. J Cell Biol 175:111–120
225. Ho DM, Yeo C-Y, Whitman M (2010) The role and regulation of GDF11 in Smad2 activation during tailbud formation in the Xenopus embryo. Mech Dev 127:485–495
226. Gregory KE et al (2005) The prodomain of BMP-7 targets the BMP-7 complex to the extracellular matrix. J Biol Chem 280:27970–27980
227. di Clemente N et al (2010) Processing of anti-mullerian hormone regulates receptor activation by a mechanism distinct from TGF-beta. Mol Endocrinol 24:2193–2206
228. Sengle G, Ono RN, Lyons KM, Bächinger HP, Sakai LY (2008) A new model for growth factor activation: type II receptors compete with the prodomain for BMP-7. J Mol Biol 381:1025–1039
229. Sengle G et al (2008) Targeting of bone morphogenetic protein growth factor complexes to fibrillin. J Biol Chem 283:13874–13888
230. Sengle G, Ono RN, Sasaki T, Sakai LY (2011) Prodomains of transforming growth factor beta (TGFbeta) superfamily members specify different functions: extracellular matrix interactions and growth factor bioavailability. J Biol Chem 286:5087–5099
231. Akiyama T, Marqués G, Wharton KA (2012) A large bioactive BMP ligand with distinct signaling properties is produced by alternative proconvertase processing. Sci Signal 5:ra28
232. Künnapuu J et al (2014) Cleavage of the Drosophila screw prodomain is critical for a dynamic BMP morphogen gradient in embryogenesis. Dev Biol 389:149–159
233. Sopory S, Kwon S, Wehrli M, Christian JL (2010) Regulation of Dpp activity by tissue-specific cleavage of an upstream site within the prodomain. Dev Biol 346:102–112
234. Fritsch C et al (2012) Different requirements for proteolytic processing of bone morphogenetic protein 5/6/7/8 ligands in *Drosophila melanogaster*. J Biol Chem 287:5942–5953
235. Janssens K et al (2006) Camurati-Engelmann disease: review of the clinical, radiological, and molecular data of 24 families and implications for diagnosis and treatment. J Med Genet 43:1–11
236. Ye M et al (2010) Mutation of the bone morphogenetic protein GDF3 causes ocular and skeletal anomalies. Hum Mol Genet 19:287–298
237. Everman DB et al (2002) The mutational spectrum of brachydactyly type C. Am J Med Genet 112:291–296
238. Plöger F et al (2008) Brachydactyly type A2 associated with a defect in proGDF5 processing. Hum Mol Genet 17:1222–1233
239. Asai-Coakwell M et al (2009) Incomplete penetrance and phenotypic variability characterize Gdf6-attributable oculo-skeletal phenotypes. Hum Mol Genet 18:1110–1121
240. Suzuki S et al (2009) Mutations in BMP4 are associated with subepithelial, microform, and overt cleft lip. Am J Hum Genet 84:406–411
241. Wyatt AW, Osborne RJ, Stewart H, Ragge NK (2010) Bone morphogenetic protein 7 (BMP7) mutations are associated with variable ocular, brain, ear, palate, and skeletal anomalies. Hum Mutat 31:781–787
242. Dixit H et al (2006) Missense mutations in the BMP15 gene are associated with ovarian failure. Hum Genet 119:408–415
243. Di Pasquale E, Beck-Peccoz P, Persani L (2004) Hypergonadotropic ovarian failure associated with an inherited mutation of human bone morphogenetic protein-15 (BMP15) gene. Am J Hum Genet 75:106–111
244. Di Pasquale E et al (2006) Identification of new variants of human BMP15 gene in a large cohort of women with premature ovarian failure. J Clin Endocrinol Metab 91:1976–1979
245. Noor A et al (2009) Oligodontia is caused by mutation in LTBP3, the gene encoding latent TGF-beta binding protein 3. Am J Hum Genet 84:519–523
246. Urban Z et al (2009) Mutations in LTBP4 cause a syndrome of impaired pulmonary, gastrointestinal, genitourinary, musculoskeletal, and dermal development. Am J Hum Genet 85:593–605
247. Robinson PN, Godfrey M (2000) The molecular genetics of Marfan syndrome and related microfibrillopathies. J Med Genet 37:9–25

248. Chen Y et al (2005) Amino acid requirements for formation of the TGF-beta-latent TGF-beta binding protein complexes. J Mol Biol 345:175–186
249. Shi M et al (2011) Latent TGF-β structure and activation. Nature 474:343–349
250. Gleizes PE, Beavis RC, Mazzieri R, Shen B (1996) Identification and characterization of an eight-cysteine repeat of the latent transforming growth factor-beta binding protein-1 that mediates bonding to the latent transforming growth factor-beta1 *J*. Biol Chem 27:29891–29896
251. Rifkin DB (2005) Latent transforming growth factor-beta (TGF-beta) binding proteins: orchestrators of TGF-beta availability. J Biol Chem 280:7409–7412
252. Saharinen J, Taipale J, Keski-Oja J (1996) Association of the small latent transforming growth factor-beta with an eight cysteine repeat of its binding protein LTBP-1. EMBO J 15:245–253
253. Szláma G, Trexler M, Patthy L (2013) Latent myostatin has significant activity and this activity is controlled more efficiently by WFIKKN1 than by WFIKKN2. FEBS J 280:3822–3839
254. Mi LZ et al (2015) Structure of bone morphogenetic protein 9 procomplex. Proc Natl Acad Sci 112:3710–3715
255. Suzuki A et al (1994) A truncated bone morphogenetic protein receptor affects dorsal-ventral patterning in the early Xenopus embryo. Proc Natl Acad Sci U S A 91:10255–10259
256. Graff JM, Thies RS, Song JJ, Celeste AJ, Melton DA (1994) Studies with a Xenopus BMP receptor suggest that ventral mesoderm-inducing signals override dorsal signals in vivo. Cell 79:169–179
257. Onichtchouk D et al (1999) Silencing of TGF-beta signalling by the pseudoreceptor BAMBI. Nature 401:480–485
258. Sammar M et al (2004) Modulation of GDF5/BRI-b signalling through interaction with the tyrosine kinase receptor Ror2. Genes Cells 9:1227–1238
259. Sammar M, Sieber C, Knaus P (2009) Biochemical and functional characterization of the Ror2/BRIb receptor complex. Biochem Biophys Res Commun 381:1–6
260. Jin W, Yun C, Kim HS, Kim SJ (2007) TrkC binds to the bone morphogenetic protein type II receptor to suppress bone morphogenetic protein signaling. Cancer Res 67:9869–9877
261. Jin W, Yun C, Kwak MK, Kim TA, Kim SJ (2007) TrkC binds to the type II TGF-beta receptor to suppress TGF-beta signaling. Oncogene 26:7684–7691
262. Bernabeu C, Lopez-Novoa JM, Quintanilla M (2009) The emerging role of TGF-beta superfamily coreceptors in cancer. Biochim Biophys Acta 1792:954–973
263. Sweet K et al (2005) Molecular classification of patients with unexplained hamartomatous and hyperplastic polyposis. JAMA 294:2465–2473
264. Fujita K et al (2009) Endoglin (CD105) as a urinary and serum marker of prostate cancer. Int J Cancer 124:664–669
265. Wong VCL et al (2008) Identification of an invasion and tumor-suppressing gene, Endoglin (ENG), silenced by both epigenetic inactivation and allelic loss in esophageal squamous cell carcinoma. Int J Cancer 123:2816–2823
266. Bilandzic M et al (2009) Loss of betaglycan contributes to the malignant properties of human granulosa tumor cells. Mol Endocrinol 23:539–548
267. Turley RS et al (2007) The type III transforming growth factor-beta receptor as a novel tumor suppressor gene in prostate cancer. Cancer Res 67:1090–1098
268. Dong M et al (2007) The type III TGF-beta receptor suppresses breast cancer progression. J Clin Invest 117:206–217
269. Gougos A, Letarte M (1990) Primary structure of endoglin, an RGD-containing glycoprotein of human endothelial cells. J Biol Chem 265:8361–8364
270. Morén A, Ichijo H, Miyazono K (1992) Molecular cloning and characterization of the human and porcine transforming growth factor-β type III receptors. Biochem Biophys Res Commun 189:356–362
271. Wong SH, Hamel L, Chevalier S (2000) Endoglin expression on human microvascular endothelial cells association with betaglycan and formation of higher order complexes with TGF-ß signalling receptors. Eur J Biochem 267:5550–5560

272. Chen W, Kirkbride KC, How T, Nelson CD, Mo J (2003) ß-Arrestin 2 mediates endocytosis of type III TGF-ß receptor and down-regulation of its signaling. Science 301:1394–1397
273. Blobe GC, Liu X, Fang SJ, How T, Lodish HF (2001) A novel mechanism for regulating transforming growth factor beta (TGF-beta) signaling. Functional modulation of type III TGF-beta receptor expression through interaction with the PDZ domain protein, GIPC. J Biol Chem 276:39608–39617
274. Lewis KA et al (2000) Betaglycan binds inhibin and can mediate functional antagonism of activin signalling. Nature 404:411–414
275. Kirkbride KC, Townsend TA, Bruinsma MW, Barnett JV, Blobe GC (2008) Bone morphogenetic proteins signal through the transforming growth factor-beta type III receptor. J Biol Chem 283:7628–7637
276. Massagué J (1990) The transforming growth factor-beta family. Annu Rev Cell Biol 6:597–641
277. Wiater E, Harrison CA, Lewis KA, Gray PC, Vale WW (2006) Identification of distinct inhibin and transforming growth factor beta-binding sites on betaglycan: functional separation of betaglycan co-receptor actions. J Biol Chem 281:17011–17022
278. Castonguay R et al (2011) Soluble endoglin specifically binds bone morphogenetic proteins 9 and 10 via its orphan domain, inhibits blood vessel formation, and suppresses tumor growth. J Biol Chem 286:30034–30046
279. Alt A et al (2012) Structural and functional insights into endoglin ligand recognition and binding. PLoS One 7:e29948
280. Cheifetz S et al (1992) Endoglin is a component of the transforming growth factor-beta receptor system in human endothelial cells. J Biol Chem 267:19027–19030
281. López-Casillas F et al (1991) Structure and expression of the membrane proteoglycan betaglycan, a component of the TGF-beta receptor system. Cell 67:785–795
282. López-Casillas F, Wrana JL, Massagué J (1993) Betaglycan presents ligand to the TGFβ signaling receptor. Cell 73:1435–1444
283. Wang XF et al (1991) Expression cloning and characterization of the TGF-beta type III receptor. Cell 67:797–805
284. Eickelberg O, Centrella M, Reiss M, Kashgarian M, Wells RG (2002) Betaglycan inhibits TGF-beta signaling by preventing type I-type II receptor complex formation. Glycosaminoglycan modifications alter betaglycan function. J Biol Chem 277:823–829
285. Letamendia A et al (1998) Role of endoglin in cellular responses to transforming growth factor-beta. A comparative study with betaglycan. J Biol Chem 273:33011–33019
286. Barbara NP, Wrana JL, Letarte M (1999) Endoglin is an accessory protein that interacts with the signaling receptor complex of multiple members of the transforming growth factor-beta superfamily. J Biol Chem 274:584–594
287. Scharpfenecker M et al (2007) BMP-9 signals via ALK1 and inhibits bFGF-induced endothelial cell proliferation and VEGF-stimulated angiogenesis. J Cell Sci 120:964–972
288. Lastres P, Martín-Perez J, Langa C (1994) Phosphorylation of the human-transforming-growth-factor-β-binding protein endoglin. Biochem J 301:765–768
289. Guerrero-Esteo M, Sánchez-Elsner T, Letamendia A, Bernabeu C (2002) Extracellular and cytoplasmic domains of endoglin interact with the transforming growth factor-beta receptors I and II. J Biol Chem 277:29197–29209
290. Koleva RI et al (2006) Endoglin structure and function: determinants of endoglin phosphorylation by transforming growth factor-beta receptors. J Biol Chem 281:25110–25123
291. Blobe GC et al (2001) Functional roles for the cytoplasmic domain of the type III transforming growth factor beta receptor in regulating transforming growth factor beta signaling. J Biol Chem 276:24627–24637
292. Velasco-Loyden G, Arribas J, López-Casillas F (2004) The shedding of betaglycan is regulated by pervanadate and mediated by membrane type matrix metalloprotease-1. J Biol Chem 279:7721–7733
293. Hawinkels LJAC et al (2010) Matrix metalloproteinase-14 (MT1-MMP)-mediated endoglin shedding inhibits tumor angiogenesis. Cancer Res 70:4141–4150

294. Van Le B et al (2009) Structural and functional characterization of soluble endoglin receptor. Biochem Biophys Res Commun 383:386–391
295. Blanco FJ et al (2005) Interaction and functional interplay between endoglin and ALK-1, two components of the endothelial transforming growth factor-beta receptor complex. J Cell Physiol 204:574–584
296. Fonsatti E, Altomonte M, Nicotra MR, Natali PG, Maio M (2003) Endoglin (CD105): a powerful therapeutic target on tumor-associated angiogenetic blood vessels. Oncogene 22:6557–6563
297. Takahashi N, Kawanishi-Tabata R, Haba A (2001) Association of serum endoglin with metastasis in patients with colorectal, breast, and other solid tumors, and suppressive effect of chemotherapy on the serum endoglin *Clin*. Cancer Res 7:524–532
298. Li C et al (2000) Plasma levels of soluble CD105 correlate with metastasis in patients with breast cancer. Int J Cancer 89:122–126
299. Lin SJ, Hu Y, Zhu J, Woodruff TK, Jardetzky TS (2011) Structure of betaglycan zona pellucida (ZP)-C domain provides insights into ZP-mediated protein polymerization and TGF-beta binding. Proc Natl Acad Sci 108:5232–5236
300. Monnier PP et al (2002) RGM is a repulsive guidance molecule for retinal axons. Nature 419:392–395
301. Babitt JL et al (2005) Repulsive guidance molecule (RGMa), a DRAGON homologue, is a bone morphogenetic protein co-receptor. J Biol Chem 280:29820–29827
302. Babitt JL et al (2006) Bone morphogenetic protein signaling by hemojuvelin regulates hepcidin expression. Nat Genet 38:531–539
303. Samad TA et al (2005) DRAGON, a bone morphogenetic protein co-receptor. J Biol Chem 280:14122–14129
304. Rajagopalan S et al (2004) Neogenin mediates the action of repulsive guidance molecule. Nat Cell Biol 6:756–762
305. Matsunaga E, Chedotal A (2004) Repulsive guidance molecule/neogenin: a novel ligand-receptor system playing multiple roles in neural development. Dev Growth Differ 46:481–486
306. Muramatsu R et al (2011) RGMa modulates T cell responses and is involved in autoimmune encephalomyelitis. Nat Med 17:488–494
307. Li VSW et al (2009) Frequent inactivation of axon guidance molecule RGMA in human colon cancer through genetic and epigenetic mechanisms. Gastroenterology 137:176–187
308. Mirakaj V et al (2011) Repulsive guidance molecule-A (RGM-A) inhibits leukocyte migration and mitigates inflammation. Proc Natl Acad Sci 108:6555–6560
309. Yamashita T, Mueller BK, Hata K (2007) Neogenin and repulsive guidance molecule signaling in the central nervous system. Curr Opin Neurobiol 17:29–34
310. Papanikolaou G et al (2004) Mutations in HFE2 cause iron overload in chromosome 1q-linked juvenile hemochromatosis. Nat Genet 36:77–82
311. Itokazu T, Fujita Y, Takahashi R, Yamashita T (2012) Identification of the neogenin-binding site on the repulsive guidance molecule A. PLoS One 7:e32791
312. Bell CH et al (2013) Structure of the repulsive guidance molecule (RGM)-neogenin signaling hub. Science 341:77–80
313. Healey EG et al (2015) Repulsive guidance molecule is a structural bridge between neogenin and bone morphogenetic protein. Nat Struct Mol Biol 22:458–465

Novel In Vitro Assay Models to Study Osteogenesis and Chondrogenesis for Human Skeletal Disorders

Takenobu Katagiri

Abstract Bone morphogenetic proteins (BMPs)/growth and differentiation factors (GDFs) are involved not only in the physiological development of skeletal tissues but also in the pathological conditions in the tissues. Osteogenesis and chondrogenesis during skeletal formation can be studied in vitro using cell lines and primary cultured cells, which are able to differentiate into osteoblasts and chondrocytes in response to BMP/GDF signaling. These in vitro model systems have been applied to the examination of molecular mechanisms of skeletal disorders related to BMPs/GDFs. Moreover, these in vitro model systems are useful for the development of novel treatments for the disorders.

Keywords Osteoblast differentiation • Chondrocyte differentiation • Mesenchymal cells • In vitro model systems

Osteogenic members of the transforming growth factor -β (TGF-β) family, such as bone morphogenetic proteins (BMPs) and growth and differentiation factors (GDFs), regulate skeletal development during embryogenesis and tissue regeneration in various vertebrates [1, 2]. Optimal activity of BMPs and GDFs is required for normal skeletal tissue because inadequate activity or overactivity causes skeletal disorders in humans and other animals (please see other chapters for details). As Marshall R. Urist originally reported [3], new bone formation is induced by osteogenic BMPs and GDFs in vivo. It has been reported that the implantation of a pharmacological dosage of BMPs or GDFs induces chondrocytes and osteoblasts in the implants within a week [4–8], suggesting that those osteogenic ligands directly regulate differentiation of chondrocytes and osteoblasts from progenitor cells. In this chapter, I will describe in vitro assay models to study molecular mechanisms underlying skeletal disorders in humans.

T. Katagiri
Division of Pathophysiology, Research Center for Genomic Medicine, Saitama Medical University, 1397-1 Yamane, Hidaka-shi, Sa-tama 350-1241, Japan
e-mail: katagiri@saitama-med.ac.jp

S. Vukicevic, K.T. Sampath (eds.), *Bone Morphogenetic Proteins: Systems Biology Regulators*, Progress in Inflammation Research,
DOI 10.1007/978-3-319-47507-3_5

1 Phenotypic Markers of Skeletal Tissue-Forming Cells

1.1 Osteoblasts

Osteoblasts are unique and specialized cells that form bone tissue in vivo [9–11]. They are believed to develop from undifferentiated mesenchymal cells during embryonic development. The bone-forming osteoblasts secrete typical organic components of bone matrices, such as type I collagen, bone sialoprotein, osteonectin, and osteocalcin [9–11]. Moreover, osteoblasts regulate the accumulation of minerals, such as hydroxyapatite (calcium phosphate) crystals, in the organic bone matrix (osteoid) by removing pyrophosphate with high alkaline phosphatase (ALP) activity [9]. Osteoclast-dependent bone resorption is indirectly regulated by osteoblasts via expression of receptor activated nuclear factor-kB ligand (RANKL) and its decoy receptor osteoprotegerin (OPG) as well as macrophage colony-stimulating factor (M-CSF) [10, 12, 13]. These differentiations and functions of osteoblasts are regulated by various extracellular stimuli, including a calcium hormone, parathyroid hormone (PTH), various cytokines, and growth factors. Runx2 and Osterix are transcription factors abundantly expressed in osteoblasts. Thus, the expression levels of these phenotypic markers are examined to study osteoblast differentiation in vitro.

1.2 Chondrocytes

Although chondrocytes, similar to osteoblasts, are also derived from undifferentiated mesenchymal cells, they are specialized cells that form cartilage tissue, which is a template before bone formation in endochondral ossification [11, 14–16]. Commitment of chondrocyte differentiation in progenitor cells is regulated by critical transcription factors, such as SOX9, SOX5, and SOX6 [11, 14–16]. Type II collagen and aggrecan are abundantly secreted by proliferating chondrocytes in growth plates. Terminally differentiated hypertrophic chondrocytes express type X collagen, metalloproteinase-13 (MMP-13), and ALP and induce a transition from cartilage to bone tissues [11, 14–16].

2 C2C12 Myoblasts for BMP/GDF Research

2.1 C2C12 Myoblasts

The murine myoblast cell line C2C12 was originally established from regenerating thigh muscle for studying the molecular mechanisms of myogenesis [17]. Indeed, C2C12 myoblasts proliferate as mononuclear cells expressing MyoD, a skeletal muscle-specific transcription factor, and differentiate into myocytes expressing proteins for muscle contraction such as myosin heavy chain and troponin T (Fig. 1).

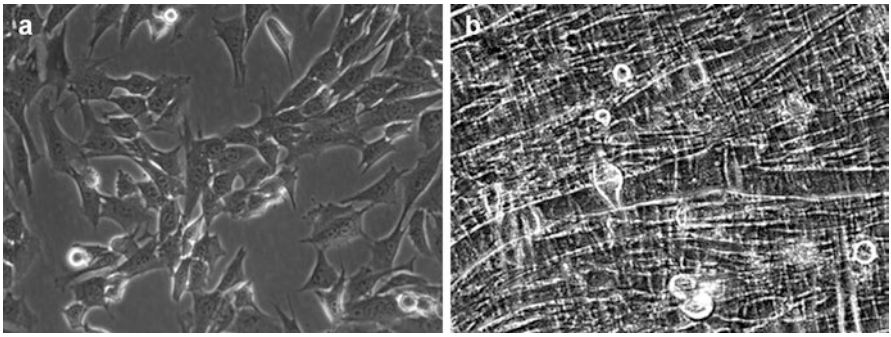


Fig. 1 Murine C2C12 myoblasts. C2C12 cells cultured for 1 (**a**) and 14 (**b**) days in vitro. They proliferate as mononuclear cells and form multinucleated myotubes after differentiation

The cells fuse together and form multinucleated myotubes in vitro (Fig. 1). However, C2C12 myoblasts are also widely used for studying BMP signaling and BMP-induced osteoblastic differentiation.

2.2 Osteoblastic Differentiation of C2C12 Myoblasts by BMPs

In the early 1990s, just after molecular cloning of several BMPs, in in vitro assay systems that reflect the bone-inducing activity of BMPs in vivo were developed [18–21]. These systems allowed the evaluation of biological activity of each recombinant BMP produced, which was needed to study intracellular signal transduction of BMPs. Among the cells examined, C2C12 myoblasts showed the high response to BMP-2, which was evaluated by the induction of ALP activity in vitro [20]. Moreover, the expression of other phenotypic markers related to osteoblast differentiation, such as osteocalcin secretion and PTH receptor, were also induced by the treatment with BMP-2 in C2C12 cells [20]. In contrast, the C2C12 myoblasts treated with BMP-2 were suppressed to express markers related to skeletal muscle differentiation, such as myogenin, myosin heavy chain, and troponin T, and they remained as mononuclear cells [20]. Although BMP-2 induced the expression of type II collagen mRNA and small droplets stained with oil red-O staining, it was still unclear whether chondrogenesis and/or adipogenesis occurred in the cells.

It has been reported that TGF-β1 does not induce heterotopic ossification in vivo [22]. In C2C12 cell cultures in vitro, TGF-β1 has been shown to inhibit myogenesis, but it does not induce any markers related to osteoblast differentiation in vitro [20]. Other inhibitors of myogenesis, such as fibroblast growth factors (FGFs), suppress myogenesis of C2C12 myoblasts but do not induce the markers of osteoblastic differentiation, suggesting that the inducing capacity of osteoblastic differentiation of C2C12 cells is limited for the osteogenic members of the TGF-β family. In addition, all-trans-retinoic acid has been shown to induce ALP activity in C2C12 and another

mesenchymal cell line, C3H101/2 clone 8, but it does not induce other markers related to osteoblast differentiation [20]. Together, these findings suggest that C2C12 cells can reflect the osteogenic and non-osteogenic activities of the members of TGF-β family in vitro.

The differentiation capacity of C2C12 cells into osteoblastic cells in vitro has been applied to evaluate osteogenic activity of 14 types of human BMP (BMP-2 through BMP-15), which were individually overexpressed in the cells using adenovirus vectors [23, 24]. In the assay, not only BMP-2 but also BMP-4, BMP-6, BMP-7, and BMP-9 have been found to induce ALP activity [23, 24]. After transplantation of these C2C12 cells expressing each BMP in mice, BMP-2, BMP-6, BMP-7, and BMP-9 have been found to induce heterotopic ossification in vivo [24]. It is possible that the failure of BMP-4 to induce heterotopic ossification in vivo was due to rapid diffusion of the ligand.

2.3 Applications of C2C12 Myoblasts for Signaling Molecules of BMPs/GDFs

The osteoblastic differentiation of C2C12 cells in response to osteogenic BMPs is clear and easily detectable because the basal levels of ALP or osteocalcin expression are quite low in the untreated C2C12 cells. Thus, this cell line has been used to elucidate intracellular signaling molecules of BMPs, and the findings have been expanded to studies of human skeletal disorders related to BMPs later.

2.3.1 Receptors

Osteogenic BMPs and GDFs bind to type II receptors (BMPR-II, ActR-IIA, and ActR-IIB) and type I receptors (ALK1, ALK2, ALK3, and ALK6) [25–31], and they are expressed in C2C12 cells even though ALK1 and ALK6 are quite low [32, 33]. Each BMP and GDF binds to various combinations of the type I and type II receptors that are expressed on target cell plasma membranes and activates intracellular signaling.

Both types of receptors are transmembrane serine/threonine kinase. Although type II receptors are active regardless of ligand binding, type I receptors are inactive and activated by type II receptors via phosphorylation at the glycine/serine rich domain (GS domain) in the intracellular region at the juxtamembrane [25–31]. Type I receptors, rather than type II receptors, determine intracellular signaling pathways. Overexpression of constitutively active forms of type I receptors for osteogenic BMPs and GDFs (ALK1, ALK2, ALK3, or ALK6) in C2C12 cells can inhibit myogenesis and induce osteoblastic differentiation without the addition of exogenous ligands [33–35]. In contrast, overexpression of dominant negative forms of type I receptors, which have the extracellular ligand-binding domain and transmembrane domain but lack a functional intracellular kinase domain, inhibits osteoblastic

differentiation of C2C12 cells even in the presence of ligands [32]. These findings support a hypothesis that type I receptors are downstream effectors of type II receptors.

2.3.2 Transcription Factors

The type I receptors bound to osteogenic ligands phosphorylate intracellular signaling molecules in the cytoplasm and subsequently transduce intracellular signaling. Smad1, Smad5, and Smad9 (also known as Smad8) are known as major substrates critical for downstream signaling [25–31]. The type I receptors phosphorylate two serine residues in the serine-valine-serine (SVS) motif at the carboxyl termini of these Smad proteins [25–31]. The phosphorylation of the carboxyl termini leads to a conformational change of Smad proteins and allows them to form complexes with other transcriptional regulators, such as coactivators and corepressors including Smad4 and RAN-binding domain-containing protein 2, respectively [36–38].

Indeed, co-expression of a constitutively active type I receptor kinase, with Smad1 or Smad5, which is a substrate of the receptor kinase, induces osteoblastic differentiation of C2C12 cells [39–41]. This induction is blocked by addition of a small molecule inhibitor of type I receptor kinases, such as LDN-193189 and LDN-212854, thus supporting that the kinase activity of type I receptors is essential for intracellular signal transduction through Smad proteins [39–41].

Stimulation of cells with a ligand of BMP/GDF members activates multiple intracellular signaling pathways, including Smad1, Smad5, Smad9, phosphatidylinositol-3 kinase, and p38 mitogen activated protein kinase. C2C12 cells have been used to examine the role of each Smad pathway in osteoblastic differentiation. To activate only one phosphorylated Smad without activating the others, the constitutively activated forms of Smad1, Smad5, and Smad9 have been generated by substituting the SVS motif with the DVD sequence in each Smad [36, 38]. The DVD forms of Smad1, Smad5, and Smad9 are recognized by an antibody against phosphorylated Smad1/Smad5/Smad9 [36, 38]. Moreover, they activate transcription of target genes and osteoblastic differentiation of C2C12 cells without addition of ligands or active receptors [36, 38]. However, Smad9 shows weaker transcriptional activity than Smad1 and Smad5 and fails to activate osteoblastic differentiation of the cells, owing to a deletion of a small part of the linker region [38]. Interestingly, expression of Smad9 mRNA has been found to be induced by BMP-4 stimulation in C2C12 cells, similarly to that of an inhibitory Smad, Smad6 [38]. Although Smad6 suppresses BMP receptors, Smad9 suppresses a constitutively active form of Smad1 as a dominant negative Smad [38].

Osterix (also known as SP7) is a transcription factor essential for bone formation during embryogenesis in mice. Osterix was identified as a novel transcription factor in C2C12 cells stimulated with BMP [42]. Osterix-deficient mice lack bone formation due to a loss of osteoblast differentiation similarly to Runx2-deficient mice. A premature natural mutation of Osterix/SP7 has been identified in a patient with osteogenesis imperfecta, type XII (MIM: 613849) [43]. Heterozygous

loss-of-function mutation of RUNX2 has been found in patients with cleidocranial dysplasia (MIM: 119600) [44–46].

2.3.3 Early Response Genes of BMP Signaling

Although BMP/GDF proteins are multifunctional growth factors, they activate transcription of common genes within an hour after binding in various types of cells. The inhibitor of differentiation/DNA binding (Id) genes, *Id1*, *Id2*, and *Id3*, have been identified as the early responsive genes of BMP signaling [47, 48]. The regulatory elements in their 5′ enhancer regions have a conserved GGCGCC sequence, which is recognized by a complex of Smad1/5 and Smad4 [47, 48]. The same sequence has been found in the 5′ region of BMP-inducible transcript-1, which is also induced by BMP signaling within an hour [48]. The regulatory regions of the BMP early responsive genes can be placed in front of the luciferase gene to examine quantitatively examine BMP-specific intracellular signaling [47, 48]. However, the direct targets of Smad proteins, which regulate osteoblastic differentiation upstream of Osterix and/or Runx2 in the BMP/GDF signaling pathway, still need to be clarified.

3 Models of Chondrogenesis

Because osteogenic BMPs and GDFs induce cartilage before the induction of bone tissue in vivo, the differentiation of chondrocytes in response to ligands are examined in vitro.

3.1 Skeletal Muscle Cells

The induction of cartilage in skeletal muscle by BMPs in vivo suggests that skeletal muscle contains progenitor cells of chondrocytes. Thus, the minced skeletal muscle has been cultured on the demineralized bone matrix to examine the chondrogenesis in vitro [49]. Histological analysis has identified chondrocytes in the cavities formed in the bone matrix, confirming that the possibility of the presence of chondrocyte progenitor cells in the skeletal muscle [49]. Chondrogenesis-inducing activity in the extracts of the demineralized bone matrix has been examined in vitro using skeletal muscle cells embedded in agarose by monitoring synthesis of cartilage-specific proteoglycans [50]. Recent studies of cell lineage tracing using fluorescent proteins have revealed that the progenitor cells in the skeletal muscle tissue, which differentiate into chondrocytes and/or osteoblasts in response to BMP signaling, are interstitial mesenchymal cells, not satellite cells or endothelial cells [51].

3.2 Embryonic Fibroblasts, Embryonic Stem Cells, and Induced Pluripotent Stem Cells

Embryonic fibroblasts and embryonic stem (ES) cells are used in vitro as sources of pluripotent cells. In these types of cells, chondrogenesis is inducible in pellet cultures in the presence of TGF-β and/or BMPs. Cells prepared from chicken or mouse embryonic limb buds also show the chondrogenic activity in high-density micromass cultures. Recently, induced pluripotent stem (iPS) cells established from patients with skeletal disorders have also been used in chondrogenesis in vitro.

4 Analysis of Skeletal Disorders Related to BMP Activity In Vitro

4.1 Fibrodysplasia Ossificans Progressiva

Fibrodysplasia ossificans progressiva (FOP) (MIM: 135100) is a rare disorder characterized by progressive heterotopic ossification in soft tissues, such as skeletal muscle, tendon, and ligaments, after birth [27, 28, 52]. Although the soft tissues are almost normal at birth, muscle trauma induces an acute heterotopic ossification. The incidence of FOP is estimated to be one in two million, regardless of race, gender, location, or age [52]. The involvement of BMP signaling has been suggested in heterotopic ossification in FOP [53]. Although there is no approved treatment for inhibiting heterotopic ossification in FOP, the in vitro models are useful for studying the molecular mechanisms of the disease and the development of novel treatments.

4.1.1 Functional Changes of ALK2/ACVR1 in FOP

In 2006, a recurrent mutation in both familial and sporadic cases of FOP was identified in one BMP type I receptor, ALK2/ACVR1 [54]. The mutation, c.617G>A, causes an Arg to His substitution mutation of ALK2 at position 206 (p.R206H) in the GS domain, which is a phosphorylation site of BMP type II receptors [54]. The mutation changes conformation of the GS domain and affects the interaction between the GS domain and kinase domain.

Functional changes of the mutant ALK2 has been examined in vitro using C2C12 cells. Transient overexpression of ALK2(R206H) in C2C12 cells induces phosphorylation of Smad1/Smad5/Smad9 and activates a BMP-specific luciferase reporter driven by an enhancer region of the early responsive gene of BMP signaling, such as Id1 [39–41]. ALP activity, a typical marker of osteoblastic differentiation of C2C12 cells, is also induced when ALK2(R206H) is co-expressed with Smad1 or Smad5 [39–41]. Moreover, myogenesis of is suppressed in the ALK(R206H)-

expressing C2C12 cells [39, 40]. These BMP activities induced by the mutant ALK2 are blocked by treatment with a small chemical inhibitor against BMP type I receptor kinases [39–41], suggesting that the kinase activity of the mutant ALK2 is increased and phosphorylated Smad1 and/or Smad5 mediate the biological activity of the receptor.

4.1.2 Molecular Mechanisms of the Activation of ALK2 in FOP

To date, more than 10 different mutations in the intracellular region, such as the GS domain and the kinase domain, of ALK2 have been identified from patients with typical or atypical FOP [28, 29]. All of the mutant ALK2 identified activate BMP signaling when they are overexpressed in C2C12 cells, although some mutants show quite weak activity [55]. FKBP12, a small binding protein for an immunosuppressor FK506, has been shown to bind to type I receptors for the TGF-β family and stabilize the inactive state of the kinase [56]. Crystal structures of the cytoplasmic domain of ALK2 and FKBP12 have revealed that the FOP mutations break critical interactions with FKBP12 [57].

In C2C12 cells, co-expression of BMP type II receptor, such as BMPR-II or ActR-IIB but not ActR-IIA, synergistically increases the kinase activity of the mutant forms of ALK2 associated with FOP but not the wild type or associated with heart diseases [55]. This stimulation depends on the kinase activity of the type II receptors. ALK2(Q207D), a constitutively active form created by genetic engineering, is activated even by the kinase activity-deficient type II receptors in C2C12 cells [55]. This suggests that the mutant forms of ALK2 associated with FOP are not constitutively active but require upstream effectors, such as type II receptors and possible ligands [55]. The threonine residue at position 203 in ALK2 is essential for the type II receptor-dependent activation of BMP signaling through regulating the phosphorylation levels of ALK2 by the type II receptors [55]. Moreover, the conserved Thr residues in other BMP type I receptors, such as ALK1, ALK3, and ALK6, are also required for the ligand-induced intracellular signaling [55].

Recently, Activin A, which is a non-osteogenic member of the TGF-β family, has been shown to activate BMP-like activity in cells expressing ALK2(R206H) [58]. Moreover, anti-Activin A antibody suppressed heterotopic ossification in conditional-on knock-in mice of human ALK2(R206H) [58]. These findings suggest that heterotopic ossification in patients with FOP is a ligand-dependent event, and Activin A is responsible for it.

4.1.3 Chondrogenesis Models in Vitro for FOP

Heterotopic skeletal tissues in FOP are formed through an endochondral ossification process, suggesting that mutant forms of ALK2 induce chondrocyte differentiation in progenitor cells in the soft tissues.

Murine ES cells that express human ALK2(R206H) under the control of the Tet-off system have been established, and their chondrogenic capacity in vitro has been analyzed [59]. Withdrawal of doxycycline from the culture medium induces the expression of ALK2(R206H), the phosphorylation of Smad1/5 and the expression of markers related to chondrocyte differentiation, such as type II collagen and aggrecan [59]. As expected, a small chemical inhibitor of the BMP type I receptor kinases inhibits these doxycycline-dependent events except for the expression of human ALK2 [59].

Knock-in mice of the ALK2(R206H) mutation have been examined. Although they show the malformation of great toe and the heterotopic ossification in soft tissues, similarly to patients with FOP, these mice die after birth [60]. Embryonic fibroblasts prepared from the knock-in mice show enhanced chondrogenic activity in vitro compared to that of wild-type mice [61]. iPS cells have been established from patients with typical FOP who carry the R206H mutation [62]. The iPS cells show accelerated chondrogenic ability in vitro compared to that of the gene-corrected and rescued iPS cells [63].

4.2 Brachydactyly, Symphalangism, and Multiple Synostosis Syndrome

Among BMPs/GDFs, GDF5 is a key regulator of skeletal development during embryogenesis, especially for digit and joint formation. Loss-of-function and gain-of-function mutations have been identified in a ligand (GDF5), receptor (BMPR-IB/ALK6), and antagonist (Noggin) in patients with skeletal disorders, such as brachydactyly, symphalangism, and multiple synostosis syndrome.

4.2.1 Gain-of-Function and Loss-of Function Mutations in GDF5

Human GDF5 has been shown to be mutated in skeletal malformation syndromes including brachydactyly type C (BDC) (MIM: 113100), which is characterized by the shortening of digits and hypersegmentation of phalanges and the recessive acromesomelic dysplasias of the Hunter-Thompson, Grebe, and DuPan types, which are characterized by short stature, severe limb shortening, and profound brachydactyly.

A mutation of p.L441P in GDF5 has been identified from patients showing short index fingers and variable clinodactyly, similarly to the patients with brachydactyly type A2 (BDA2) (MIM: 112600), which is caused by a mutation in BMPR-IB/ALK6 [64]. Another mutation in GDF5, p.R438L, has been identified in patients with proximal symphalangism (SYM1) (MIM: 185800) and is characterized by a bony fusion between the proximal and middle phalanges in the digits [64]. C2C12 cells express BMPR-IA/ALK3, but they do not express functional levels of BMPR-IB/ALK6. Thus, the cells respond to BMP-2, but they do not respond to GDF5 [64]. GDF5

stimulates chondrogenesis of chicken limb bud cells in micromass cultures [64]. The p.L441P mutant GDF5 seems to be a loss-of-function mutation because it does not show BMP/GDF-like activity in vitro [64]. In contrast, the p.R438L mutant is a gain-of-function mutation, because it has increased in binding affinity to BMPR-IA/ALK3 [64]. The p.R438L mutant GDF5, but not p.L441P, induces ALP activity in C2C12 cells and suppresses myogenesis similarly to BMP-2 [64].

Additional mutations in GDF5, p.N445T/K, and p.W414R have been identified from patients with SYM1 and combined clinical features of brachydactyly type A2 (BDA2) and multiple synostosis syndrome 2 (SYNS2) (MIM: 610017), respectively [65]. These mutant GDF5 are insensitive and resistant to Noggin, similarly to BMP-9 and BMP-10 [65]. Overexpression of the mutant GDF5 or BMP-9 in the micromass cultures of chicken limb bud cells shows high chondrogenic activity [65], suggesting that normal joint formation induced by GDF5 requires a negative feedback through an antagonist, i.e., Noggin.

5 Conclusion

The original bone-inducing activity of BMPs can be reflected, at least in part, in in vitro model systems using cell lines or primary cultured cells. These systems have been applied to the examination of molecular mechanisms of skeletal disorders related to BMPs/GDFs. Moreover, these in vitro model systems are useful for the development of novel treatments for the disorders.

Acknowledgments I would like to thank the members of the Division of Pathophysiology, Research Center for Genomic Medicine, Saitama Medical University for their helpful discussions. This work was supported, in part, by JSPS KAKENHI Numbers 15K15556 and 25293326 and a grant-in-aid from the Support Project for the Formation of a Strategic Center in a Private University from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT) (S1311002).

References

1. Jin L, Li X (2013) Growth differentiation factor 5 regulation in bone regeneration. Curr Pharm Des 19(19):3364–3373
2. Pignatti E, Zeller R, Zuniga A (2014) To BMP or not to BMP during vertebrate limb bud development. Semin Cell Dev Biol 32:119–127
3. Urist MR (1965) Bone: formation by autoinduction. Science 150:893–899
4. Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Whitters MJ, Kriz RW, Hewick RM, Wang EA (1988) Novel regulators of bone formation: molecular clones and activities. Science 242(4885):1528–1534
5. Celeste AJ, Iannazzi JA, Taylor RC, Hewick RM, Rosen V, Wang EA, Wozney JM (1990 Dec) Identification of transforming growth factor beta family members present in bone-inductive protein purified from bovine bone. Proc Natl Acad Sci U S A 87(24):9843–9847
6. Sampath TK, Coughlin JE, Whetstone RM, Banach D, Corbett C, Ridge RJ, Ozkaynak E, Oppermann H, Rueger DC (1990) Bovine osteogenic protein is composed of dimers of OP-1

and BMP-2A, two members of the transforming growth factor-beta superfamily. J Biol Chem 265(22):13198–13205
7. Sampath TK, Maliakal JC, Hauschka PV, Jones WK, Sasak H, Tucker RF, White KH, Coughlin JE, Tucker MM, Pang RH et al (1992) Recombinant human osteogenic protein-1 (hOP-1) induces new bone formation in vivo with a specific activity comparable with natural bovine osteogenic protein and stimulates osteoblast proliferation and differentiation in vitro. J Biol Chem 267(28):20352–20362
8. Chang SC, Hoang B, Thomas JT, Vukicevic S, Luyten FP, Ryba NJ, Kozak CA, Reddi AH, Moos M Jr (1994) Cartilage-derived morphogenetic proteins. New members of the transforming growth factor-beta superfamily predominantly expressed in long bones during human embryonic development. J Biol Chem 269(45):28227–28234
9. Yamaguchi A, Komori T, Suda T (2000) Regulation of osteoblast differentiation mediated by bone morphogenetic proteins, hedgehogs, and Cbfa1. Endocr Rev 21(4):393–411
10. Katagiri T, Takahashi N (2002) Regulatory mechanisms of osteoblast and osteoclast differentiation. Oral Dis 8(3):147–159
11. Gómez-Picos P, Eames BF (2015) On the evolutionary relationship between chondrocytes and osteoblasts. Front Genet 6:297
12. Nakamura I, Takahashi N, Jimi E, Udagawa N, Suda T (2012 Apr) Regulation of osteoclast function. Mod Rheumatol 22(2):167–177
13. Kobayashi Y, Uehara S, Koide M, Takahashi N (2015 Jul 1) The regulation of osteoclast differentiation by Wnt signals. Bonekey Rep 4:713
14. Somoza RA, Welter JF, Correa D, Caplan AI (2014) Chondrogenic differentiation of mesenchymal stem cells: challenges and unfulfilled expectations. Tissue Eng Part B Rev 20(6):596–608
15. Jiang Y, Tuan RS (2015) Origin and function of cartilage stem/progenitor cells in osteoarthritis. Nat Rev Rheumatol 11(4):206–212
16. Abdallah BM, Jafari A, Zaher W, Qiu W, Kassem M (2015) Skeletal (stromal) stem cells: an update on intracellular signaling pathways controlling osteoblast differentiation. Bone 70:28–36
17. Blau HM, Chiu CP, Webster C (1983) Cytoplasmic activation of human nuclear genes in stable heterocaryons. Cell 32:1171–1180
18. Katagiri T, Yamaguchi A, Ikeda T, Yoshiki S, Wozney JM, Rosen V, Wang EA, Tanaka H, Omura S, Suda T (1990) The non-osteogenic mouse pluripotent cell line, C3H10T1/2, is induced to differentiate into osteoblastic cells by recombinant human bone morphogenetic protein-2. Biochem Biophys Res Commun 172(1):295–299
19. Yamaguchi A, Katagiri T, Ikeda T, Wozney JM, Rosen V, Wang EA, Kahn AJ, Suda T, Yoshiki S (1991) Recombinant human bone morphogenetic protein-2 stimulates osteoblastic maturation and inhibits myogenic differentiation in vitro. J Cell Biol 113(3):681–687
20. Katagiri T, Yamaguchi A, Komaki M, Abe E, Takahashi N, Ikeda T, Rosen V, Wozney JM, Fujisawa-Sehara A, Suda T (1994) Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. J Cell Biol 127(6 Pt 1): 1755–1766
21. Rosen V, Nove J, Song JJ, Thies RS, Cox K, Wozney JM (1994) Responsiveness of clonal limb bud cell lines to bone morphogenetic protein 2 reveals a sequential relationship between cartilage and bone cell phenotypes. J Bone Miner Res 9(11):1759–1768
22. Sampath TK, Muthukumaran N, Reddi AH (1987) Isolation of osteogenin, an extracellular matrix-associated, bone-inductive protein, by heparin affinity chromatography. Proc Natl Acad Sci U S A 84(20):7109–7113
23. Cheng H, Jiang W, Phillips FM, Haydon RC, Peng Y, Zhou L, Luu HH, An N, Breyer B, Vanichakarn P, Szatkowski JP, Park JY, He TC (2003) Osteogenic activity of the 14 types of human bone morphogenetic proteins (BMPs). J Bone Joint Surg Am 85-A(8):1544–1552 Erratum in: J Bone Joint Surg Am. 2004 Jan;86-A(1):141
24. Kang Q, Sun MH, Cheng H, Peng Y, Montag AG, Deyrup AT, Jiang W, Luu HH, Luo J, Szatkowski JP, Vanichakarn P, Park JY, Li Y, Haydon RC, He TC (2004) Characterization of

the distinct orthotopic bone-forming activity of 14 BMPs using recombinant adenovirus-mediated gene delivery. Gene Ther 11(17):1312–1320
25. Katagiri T, Suda T, Miyazozo K (2008) The bone morphogenetic proteins. In: Miyazono K, Derynck R (eds) The TGF-βFamily. Cold Spring Harbor Press, New York, pp. 121–149
26. Miyazono K, Kamiya Y, Morikawa M (2010) Bone morphogenetic protein receptors and signal transduction. J Biochem 147:35–51
27. Katagiri T (2010) Heterotopic bone formation induced by bone morphogenetic protein signaling: fibrodysplasia ossificans progressiva. J Oral Biosci 52:33–41
28. Katagiri T (2012) Recent topics in fibrodysplasia ossificans progressiva. J Oral Biosci 54:119–123
29. Katagiri T, Tsukamoto S (2013) The unique activity of bone morphogenetic proteins in bone: a critical role of the Smad signaling pathway. Biol Chem 394:703–714
30. Katagiri T, Tsukamoto S, Osawa K, and Kokabu S. Ligand-receptor interactions and their implications in delivering certain signaling for bone regeneration. In A tissue regeneration approach to bone and cartilage repair, Mechanical Engineering Series,Zreiqat H Rosen V and Dunstan C, editors. Springer, London, pp 1–15, 2014.
31. Katagiri T, Osawa K, Tsukamoto S, Fujimoto M, Miyamoto A, Mizuta T (2015) Bone morphogenetic protein-induced heterotopic bone formation: what have we learned from the history of a half century? Jpn Dent Sci Rev 51:42–50
32. Namiki M, Akiyama S, Katagiri T, Suzuki A, Ueno N, Yamaji N, Rosen V, Wozney JM, Suda T (1997) A kinase domain-truncated type I receptor blocks bone morphogenetic protein-2-induced signal transduction in C2C12 myoblasts. J Biol Chem 272(35):22046–22052
33. Akiyama S, Katagiri T, Namiki M, Yamaji N, Yamamoto N, Miyama K, Shibuya H, Ueno N, Wozney JM, Suda T (1997) Constitutively active BMP type I receptors transduce BMP-2 signals without the ligand in C2C12 myoblasts. Exp Cell Res 235(2):362–369
34. Fujii M, Takeda K, Imamura T, Aoki H, Sampath TK, Enomoto S, Kawabata M, Kato M, Ichijo H, Miyazono K (1999) Roles of bone morphogenetic protein type I receptors and Smad proteins in osteoblast and chondroblast differentiation. Mol Biol Cell 10(11):3801–3813
35. Aoki H, Fujii M, Imamura T, Yagi K, Takehara K, Kato M, Miyazono K (2001) Synergistic effects of different bone morphogenetic protein type I receptors on alkaline phosphatase induction. J Cell Sci 114(Pt 8):1483–1489
36. Nojima J, Kanomata K, Takada Y, Fukuda T, Kokabu S, Ohte S, Takada T, Tsukui T, Yamamoto TS, Sasanuma H, Yoneyama K, Ueno N, Okazaki Y, Kamijo R, Yoda T, Katagiri T (2010) Dual roles of Smad proteins in the conversion from myoblasts to osteoblastic cells by bone morphogenetic proteins. J Biol Chem 285(20):15577–15586
37. Ohte S, Kokabu S, Iemura S, Sasanuma H, Yoneyama K, Shin M, Suzuki S, Fukuda T, Nakamura Y, Jimi E, Natsume T, Katagiri T (2012) Identification and functional analysis of Zranb2 as a novel Smad-binding protein that suppresses BMP signaling. J Cell Biochem 113(3):808–814
38. Tsukamoto S, Mizuta T, Fujimoto M, Ohte S, Osawa K, Miyamoto A, Yoneyama K, Murata E, Machiya A, Jimi E, Kokabu S, Katagiri T (2014) Smad9 is a new type of transcriptional regulator in bone morphogenetic protein signaling. Sci Rep 4:7596. doi:10.1038/srep07596
39. Fukuda T, Kanomata K, Nojima J, Kokabu S, Akita M, Ikebuchi K, Jimi E, Komori T, Maruki Y, Matsuoka M, Miyazono K, Nakayama K, Nanba A, Tomoda H, Okazaki Y, Ohtake A, Oda H, Owan I, Yoda T, Haga N, Furuya H, Katagiri T (2008) A unique mutation of ALK2, G356D, found in a patient with fibrodysplasia ossificans progressiva is a moderately activated BMP type I receptor. Biochem Biophys Res Commun 377(3):905–909
40. Fukuda T, Kohda M, Kanomata K, Nojima J, Nakamura A, Kamizono J, Noguchi Y, Iwakiri K, Kondo T, Kurose J, Endo K, Awakura T, Fukushi J, Nakashima Y, Chiyonobu T, Kawara A, Nishida Y, Wada I, Akita M, Komori T, Nakayama K, Nanba A, Maruki Y, Yoda T, Tomoda H, PB Y, Shore EM, Kaplan FS, Miyazono K, Matsuoka M, Ikebuchi K, Ohtake A, Oda H, Jimi E, Owan I, Okazaki Y, Katagiri T (2009) Constitutively activated ALK2 and increased SMAD1/5 cooperatively induce bone morphogenetic protein signaling in fibrodysplasia ossificans progressiva. J Biol Chem 284(11):7149–7156

41. Ohte S, Shin M, Sasanuma H, Yoneyama K, Akita M, Ikebuchi K, Jimi E, Maruki Y, Matsuoka M, Namba A, Tomoda H, Okazaki Y, Ohtake A, Oda H, Owan I, Yoda T, Furuya H, Kamizono J, Kitoh H, Nakashima Y, Susami T, Haga N, Komori T, Katagiri T (2011) A novel mutation of ALK2, L196P, found in the most benign case of fibrodysplasia ossificans progressiva activates BMP-specific intracellular signaling equivalent to a typical mutation, R206H. Biochem Biophys Res Commun 407(1):213–218
42. Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, de Crombrugghe B (2002) The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell 108(1):17–29
43. Lapunzina P, Aglan M, Temtamy S, Caparrós-Martín JA, Valencia M, Letón R, Martínez-Glez V, Elhossini R, Amr K, Vilaboa N, Ruiz-Perez VL (2010) Identification of a frameshift mutation in Osterix in a patient with recessive osteogenesis imperfecta. Am J Hum Genet 87(1):110–114
44. Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao YH, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S, Kishimoto T (1997) Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 89(5):755–764
45. Otto F, Thornell AP, Crompton T, Denzel A, Gilmour KC, Rosewell IR, Stamp GW, Beddington RS, Mundlos S, Olsen BR, Selby PB, Owen MJ (1997) Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell 89(5):765–771
46. Mundlos S, Otto F, Mundlos C, Mulliken JB, Aylsworth AS, Albright S, Lindhout D, Cole WG, Henn W, Knoll JH, Owen MJ, Mertelsmann R, Zabel BU, Olsen BR (1997) Mutations involving the transcription factor CBFA1 cause cleidocranial dysplasia. Cell 89(5):773–779
47. Katagiri T, Imada M, Yanai T, Suda T, Takahashi N, Kamijo R (2002) Identification of a BMP-responsive element in Id1, the gene for inhibition of myogenesis. Genes Cells 7:949–960
48. Shin M, Ohte S, Fukuda T, Sasanuma H, Yoneyama K, Kokabu S, Miyamoto A, Tsukamoto S, Hohjoh H, Jimi E, Katagiri T (2013) Identification of a novel bone morphogenetic protein (BMP)-inducible transcript, BMP-inducible transcript-1, by utilizing the conserved BMP-responsive elements in the Id genes. J Bone Miner Metab 31(1):34–43
49. Nogami H, Urist MR (1974) Substrata prepared from bone matrix for chondrogenesis in tissue culture. J Cell Biol 62:510–519
50. Seyedin SM, Thompson AY, Bentz H, Rosen DM, McPherson JM, Conti A et al (1986) Cartilage-inducing factor-A. Apparent identity to transforming growth factor-beta. J Biol Chem 261:5693–5695
51. Wosczyna MN, Biswas AA, Cogswell CA, Goldhamer DJ (2012) Multipotent progenitors resident in the skeletal muscle interstitium exhibit robust BMP-dependent osteogenic activity and mediate heterotopic ossification. J Bone Miner Res 27:1004–1017
52. Kitterman JA, Kantanie S, Rocke DM, Kaplan FS (2005) Iatrogenic harm caused by diagnostic errors in fibrodysplasia ossificans progressiva. Pediatrics 116(5):e654–e661
53. Kaplan FS, Tabas JA, Zasloff MA (1990) Fibrodysplasia ossificans progressiva: a clue from the fly? Calcif Tissue Int 47:117–125
54. Shore EM, Xu M, Feldman GJ, Fenstermacher DA, Cho TJ, Choi IH, Connor JM, Delai P, Glaser DL, LeMerrer M, Morhart R, Rogers JG, Smith R, Triffitt JT, Urtizberea JA, Zasloff M, Brown MA, Kaplan FS (2006) A recurrent mutation in the BMP type I receptor ACVR1 causes inherited and sporadic fibrodysplasia ossificans progressiva. Nat Genet 38(5):525–527
55. Fujimoto M, Ohte S, Osawa K, Miyamoto A, Tsukamoto S, Mizuta T, Kokabu S, Suda N, Katagiri T (2015) Mutant activin-like kinase 2 in fibrodysplasia ossificans progressiva are activated via T203 by BMP type II receptors. Mol Endocrinol 29(1):140–152
56. Wang T, Li BY, Danielson PD, Shah PC, Rockwell S, Lechleider RJ, Martin J, Manganaro T, Donahoe PK (1996) The immunophilin FKBP12 functions as a common inhibitor of the TGF beta family type I receptors. Cell 86(3):435–444
57. Chaikuad A, Alfano I, Kerr G, Sanvitale CE, Boergermann JH, Triffitt JT, von Delft F, Knapp S, Knaus P, Bullock AN (2012) Structure of the bone morphogenetic protein receptor

ALK2 and implications for fibrodysplasia ossificans progressiva. J Biol Chem 287(44): 36990–36998
58. Hatsell SJ, Idone V, Wolken DM, Huang L, Kim HJ, Wang L, Wen X, Nannuru KC, Jimenez J, Xie L, Das N, Makhoul G, Chernomorsky R, D'Ambrosio D, Corpina RA, Schoenherr CJ, Feeley K, Yu PB, Yancopoulos GD, Murphy AJ, Economides AN (2015) ACVR1R206H receptor mutation causes fibrodysplasia ossificans progressiva by imparting responsiveness to activin A. Sci Transl Med 7(303):303ra137
59. Fujimoto M, Ohte S, Shin M, Yoneyama K, Osawa K, Miyamoto A, Tsukamoto S, Mizuta T, Kokabu S, Machiya A, Okuda A, Suda N, Katagiri T (2014) Establishment of a novel model of chondrogenesis using murine embryonic stem cells carrying fibrodysplasia ossificans progressiva-associated mutant ALK2. Biochem Biophys Res Commun 455(3–4):347–352
60. Chakkalakal SA, Zhang D, Culbert AL, Convente MR, Caron RJ, Wright AC, Maidment AD, Kaplan FS, Shore EM (2012) An Acvr1 R206H knock-in mouse has fibrodysplasia ossificans progressiva. J Bone Miner Res 27(8):1746–1756
61. Culbert AL, Chakkalakal SA, Theosmy EG, Brennan TA, Kaplan FS, Shore EM (2014) Alk2 regulates early chondrogenic fate in fibrodysplasia ossificans progressiva heterotopic endochondral ossification. Stem Cells 32:1289–1300
62. Matsumoto Y, Hayashi Y, Schlieve CR, Ikeya M, Kim H, Nguyen TD, Sami S, Baba S, Barruet E, Nasu A, Asaka I, Otsuka T, Yamanaka S, Conklin BR, Toguchida J, Hsiao EC (2013) Induced pluripotent stem cells from patients with human fibrodysplasia ossificans progressiva show increased mineralization and cartilage formation. Orphanet J Rare Dis 8:190
63. Matsumoto Y, Ikeya M, Hino K, Horigome K, Fukuta M, Watanabe M, Nagata S, Yamamoto T, Otsuka T, Toguchida J (2015) New protocol to optimize iPS cells for genome analysis of Fibrodysplasia Ossificans Progressiva. Stem Cells 33:1730–1742
64. Seemann P, Schwappacher R, Kjaer KW, Krakow D, Lehmann K, Dawson K, Stricker S, Pohl J, Plöger F, Staub E, Nickel J, Sebald W, Knaus P, Mundlos S (2005) Activating and deactivating mutations in the receptor interaction site of GDF5 cause symphalangism or brachydactyly type A2. J Clin Invest 115:2373–2381
65. Degenkolbe E, König J, Zimmer J, Walther M, Reißner C, Nickel J, Plöger F, Raspopovic J, Sharpe J, Dathe K, Hecht JT, Mundlos S, Doelken SC, Seemann P. A GDF5 point mutation strikes twice–causing BDA1 and SYNS2. PLoS Genet 2013. 9:e1003846.

Toward Advanced Therapy Medicinal Products (ATMPs) Combining Bone Morphogenetic Proteins (BMP) and Cells for Bone Regeneration

Wei Ji, Johanna Bolander, Yoke Chin Chai, Hiroki Katagiri, Marina Marechal, and Frank P. Luyten

Abstract Cell-based implants with or without osteoinductive biomolecules on optimal carrier materials as an advanced therapeutic medicinal product (ATMP) are a promising strategy for poorly healing long-bone defects. This chapter will focus on ATMPs combining bone morphogenetic proteins (BMPs) and progenitor cells for the clinical treatment of large bone defects in compromised environments. We describe BMP signaling involved in the process of bone fracture healing with specific emphasis on clinically relevant BMP ligands, followed by characterization and BMP responsiveness of progenitor cells obtained from different sources. Then we explore different biomaterials and their contribution to achieve optimal BMP release and osteoinduction. Finally, we provide a perspective on the applicability of ATMPs in bone repair by reviewing the preclinical studies carried out so far in various animal models. We believe the era of regenerative medicine has just started. First-generation BMP and stem cell technologies have demonstrated that in the postnatal environment, one can successfully enhance the healing of damaged tissues by recapitulating the principles of developmental tissue formation. A second generation of products is needed that leads to successful bone healing in compromised environments.

Keywords Bone tissue regeneration • Bone morphogenetic proteins • Bone marrow stromal cells • Periosteal-derived cells • Pluripotent stem cells • Scaffold • Advanced therapy medicinal product • Animal model

W. Ji • J. Bolander • Y.C. Chai • H. Katagiri • M. Marechal • F.P. Luyten (✉)
Prometheus, Division of Skeletal Tissue Engineering, KU Leuven, Leuven, Belgium

Skeletal Biology and Engineering Research Center, Department of Development and Regeneration, KU Leuven, Leuven, Belgium
e-mail: frank.luyten@uzleuven.be

S. Vukicevic, K.T. Sampath (eds.), *Bone Morphogenetic Proteins: Systems Biology Regulators*, Progress in Inflammation Research,
DOI 10.1007/978-3-319-47507-3_6

1 Introduction

Long-bone fractures are most frequently the result of trauma but can also be associated with a variety of conditions including osteoporosis, infection, tumors, and congenital diseases. Moreover, over 10 % of tibia fractures lead to delayed healing or nonunion, which greatly affects quality of life for the individual. This patient population ultimately demands an effective restoration strategy to fulfill functional requirements. Current state of the art for the reconstruction of skeletal defects involves transplantation of autologous or allogenic bone grafts, which can be harvested from sites such as the iliac crest, fibula, scapula, or radius [189]. However, the inherent drawbacks of this approach, including insufficient autologous resources, pain, and donor-site morbidity, strongly urge clinicians and researchers to explore alternative therapeutic strategies.

Several alternative strategies are emerging to treat nonhealing fractures: (1) a "smart" biomaterial device with or without growth factors, which is frequently used in non-compromised conditions, and (2) an advanced therapeutic medicinal product (ATMP) composed of cell-based implants with or without osteoinductive biomolecules on optimal carrier materials, which is typically targeted for use in compromised conditions. The combined factors in such ATMP should function synergistically with a potent regenerative effect. Hypothetically, when implanted in vivo, they act as a robust engine steering bone formation and integration, subsequently leading to successful healing of the defect [104]. Indeed, it is envisioned that cell-based ATMPs can overcome the limited and defective regenerative capacity of the patient. Moreover, in contrast to the single use of growth factors which seems to require high doses, the combined cell growth factor ATMP is expected to eliminate the necessity of supraphysiological doses of growth factors which could potentially induce adverse clinical complications [25]. It is anticipated that the soluble growth factors will stimulate proliferation and differentiation of progenitor cells both in carriers and defect site to form new bone tissue. Meanwhile, the implanted progenitor cells cross talk with the surrounding tissue via the secretion of signaling molecules to accelerate tissue formation, integration, and remodeling.

This chapter will focus on ATMPs combining bone morphogenetic proteins (BMPs) and stem cells for the clinical treatment of large bone defects in compromised environments. We will describe the BMP signaling that is involved in the process of bone fracture healing with specific emphasis on clinically relevant BMP ligands, followed by characterization and BMP responsiveness of stem cells obtained from different sources. Then we will explore different biomaterials and their contribution to achieve optimal BMP release and osteoinduction. Finally, we will provide a perspective on the applicability of ATMPs in bone repair by reviewing the preclinical studies carried out so far in various animal models.

2 Lessons from Biology: BMP Signaling Involved in Bone Healing

2.1 Biological Fundamentals of Bone and Fracture Healing

Bone formation during embryonic development involves three distinct structures that generate the skeleton. The somites give rise to the axial skeleton, the lateral plate mesoderm generates the limb skeleton and the cranial neural crest gives rise to the craniofacial cartilage and bones. Depending on the bone to be formed, two major modes of bone formation occur where both involve the transformation of a pre-existing mesenchymal tissue into bone tissue. Intramembranous ossification is a slow process that involves direct conversion of mesenchymal tissue into bone, primarily giving rise to the flat bones of the skull. The second bone-forming process, endochondral bone formation, gives rise to the long bones through a process where progenitor cells differentiate into cartilage, which subsequently is degraded, remodeled, and replaced by bone.

Throughout the life span of an individual, bones continuously undergo remodeling, leading to changes in bone size, shape, and density during growth and load-induced damage, adapting the bone to an individual's development. This remodeling process is tightly coordinated between bone-forming osteoblasts and bone-resorbing osteoclasts, the latter ones originating from hematopoietic stem cells. The interplay between these cells is regulated on both the systemic and local level by hormones, cytokines, mechanical signals, and metabolites. Imbalance, upon aging or immobilization, between bone formation and resorption, often leads to reduced bone density, osteoporosis, and fractures [68].

In healthy individuals, the skeleton acts as a scaffold by providing support and protection for the soft tissues that together make up the body. Subsequently, the bone has a complex structure and can stand high-impact and mechanical load. Fracture occurs upon severe trauma or on minor trauma in diseased bones such as osteoporosis. The majority of the fractures can heal spontaneously, due to the high regenerative potential of our skeletal system. The healing process, initiated by trauma causing the fracture, can be divided in four stages: (1) initial inflammatory response and hematoma formation, (2) callus formation, (3) remodeling of callus to immature bone, and subsequently (4) remodeling to mature lamellar bone [127]. During the initial inflammatory response, cytokines and growth factors are secreted by cells at the fracture site to recruit skeletal progenitor cells from mostly the periosteum to aid in the succeeding stages [7]. The nature of secreted stimulatory signals is partially driven by the type of fracture, hence also which healing process that will be initiated.

Fracture healing can occur through two different routes, depending on the mechanical stabilization of the fracture: intramembranous (stable fractures) or

endochondral (unstable fractures) fracture healing. In the former, osteoblasts directly produce and deposit woven bone. This process often takes place in impact or compression fractures, where the mechanical stability is high. In more mechanically unstable fractures, bone is formed through an intermediate cartilaginous tissue that can function under hypoxic conditions. The cartilage intermediate contributes to stabilization of the fracture, and upon matrix calcification, angiogenesis occurs and with new bone formation and remodeling through resorption by osteoclasts delivered through the invading blood vessels.

In clinics, over 10 % of annual tibial fractures lead to delayed or nonunions, due to the critical size of the defect, severely damaged or infected surrounding tissue, and/or genetic disorders [47]. Typically, nonunions can be characterized as hypertrophic or atrophic nonunions or a combination of both (Fig. 1) [131] . Hypertrophic nonunions are caused by excessive motion at the fracture site, causing abnormal vascularity and abundant callus formation, and these can often be successfully treated by a stabilizing fixation. Atrophic nonunions are the result of inadequate biological conditions, causing fibrous tissue to fill the fracture.

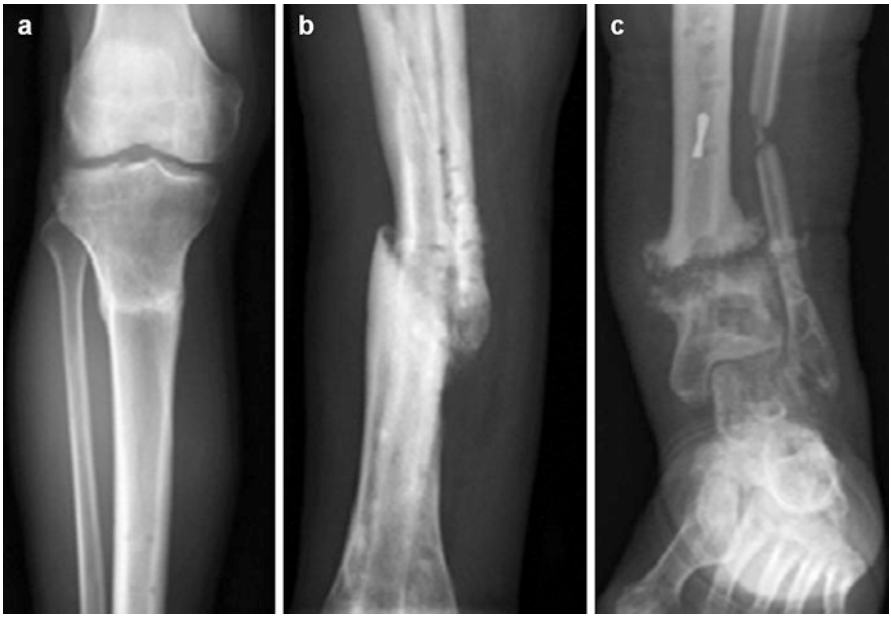


Fig. 1 Long-bone fractures. A fracture of long bones such as tibiae heals spontaneously under normal conditions (**a**). Under specific circumstances, the fracture can develop into an atrophic (**b**) or hypertrophic (**c**) nonunion (Radiographic images received from Professor, J. Lammens, UZ Leuven, Belgium)

2.2 BMPs Involved in Bone Development and Fracture Healing

Among the different ligands of the BMP family, BMP-2, BMP-4, BMP-5, BMP-6, BMP-7, BMP-9, and GDF5 play important roles during bone development and fracture healing. During the early stages of non-compromised endochondral fracture healing, BMP-2, BMP-4, BMP-5, BMP-9, and GDF5 can be detected in activated periosteal cells and inflammatory cells in the granulation tissue [28]. As the fracture healing progresses, the expression level of these signals decreases/fluctuates. The proliferating chondrocytes express BMP-2, BMP-6, BMP-7, and BMP-9, while pre-hypertrophic chondrocytes express BMP-2, BMP-6, and BMP-7. Once cells have differentiated to hypertrophic chondrocytes, they are strongly positive for BMP-2, BMP-4, BMP-5, BMP-6, BMP-7, and BMP-9 [28, 222].

While many of the BMP ligands can exert a similar function during fracture healing as in bone development, some of them seem to play more crucial roles than others. For instance, global loss of BMP-2 leads to embryonic lethality [224]. In a limb-specific knockout of BMP-2, embryogenesis was not affected but spontaneous postnatal fractures occurred that did not heal. These data confirm that other ligands cannot compensate for the absence of BMP-2, hence ratifying its crucial role in postnatal bone development and fracture repair [196]. In similarity to BMP-2, BMP-4 and BMP-7 are present during all stages of bone development and regeneration. However, both have been reported dispensable in these processes in mice [138, 197, 198].

Nonsense mutations of the BMP-5 gene give rise to a short-ear phenotype in mice and lead to reduced plate growth and height as well as body mass [87, 133]. Upon fracture, these mice display a delayed formation and maturation of the cartilaginous fracture callus, only half the volume of healthy fracture callus [60].

BMP-6 is highly expressed in the growth plate as well as during the different stages of fracture repair. However, the BMP-6 ligand is not crucial for skeletal development, maintenance, or fracture healing [59, 100]. Nevertheless, BMP-6 mutant mice displayed a reduced size of long bones, impaired growth plate function, and a delayed ossification of the developing sternum [149, 182]. GDF5, another member of the BMP family, is found throughout the growth plate of the developing long bones, and mutations in this gene have been shown to cause impaired joint morphogenesis and brachypodism in mice and man [185, 194]. During fracture repair, deletion of GDF5 does not compromise long-term fracture healing, but a delay in callus formation and remodeling suggests a role in the early phase of bone repair [30]. BMP-9 is mainly known for its regulatory role in angiogenesis, evidenced by arteriovenous malformations in BMP-9-deficient mice [155, 218]. Interestingly, recent research efforts suggest BMP-9 to be one of the most osteogenic ligands, and a first report on skeletal malformations in BMP-9-deficient mice

is currently being processed [155]. Moreover, additional support for BMP-9 as an interesting osteoinductive factor was evidenced by its role during trauma-induced heterotopic ossification [58].

The BMP signaling pathway is strictly regulated; hence, BMP antagonists are also present in fluctuating levels during fracture healing. In cartilage- and bone-forming cells as well as in granulation tissue, BMP-3, noggin, chordin, gremlin, SMAD6, and SMAD7 have been detected [222]. Moreover, BMP ligands and receptors, phosphorylated SMAD1/5/8, and BMP inhibitors are also express in nonunions in similarity to non-compromised fractures [92]. Interestingly, an imbalance between the level of ligands and inhibitors was reported with the most striking differences in the early cartilaginous tissue intermediates. Potentially, the disrupted balance in BMP signaling may be a mechanistic cause of the nonunion (Table 1).

2.3 Current Status of BMPs in Clinical Application

Since the discovery by Marshall Urist of BMPs and their potent bone-inducing capacity in 1965, comprehensive research efforts have led to the characterization of several ligands from the family. When it comes to bone regenerative medicine and the treatment of nonunions, BMP-7 and BMP-2 have gained most attention

Table 1 The functions of different BMPs during bone development and fracture healing

BMP-	Knockout phenotype	Fracture healing	References
BMP-2	Embryonically lethal; limb, spontaneous fractures and impaired fracture repair; chondrocyte, severe chondrodysplasia	Expressed during inflammatory, chondrogenic, and osteogenic stages	[28, 90, 91, 181, 196, 224]
BMP-4	Embryonically lethal, limb: defective patterning	Expressed during chondrogenic and osteogenic stages	[28, 90, 91, 174, 210]
BMP-5	Short-ear mice, reduced growth plate height, growth rate, and body mass	Expressed during mesenchymal condensation, delayed fracture callus formation and maturation	[60, 87, 133]
BMP-6	Delay in sternum ossification, smaller long bones	Expressed during inflammatory, chondrogenic, and osteogenic stages	[28, 149, 182]
BMP-7	Die after birth, defect in skeletal patterning, in limb: no effect	Expressed during chondrogenic and osteogenic stages	[28, 38, 90, 91, 119, 171, 198]
BMP-9	Skeletal malformations, phenotype not yet published	Decreased mean levels in nonunions	[200]
GDF5	Brachypodism, joint phenotype delayed callus formation, and remodeling	Expressed during chondrogenic stage	[28, 30]

for a number of reasons including biotech-driven focus. In 2001 and 2002, FDA approved the clinical products OP-1® (BMP-7) and Infuse® (BMP-2) for the treatment of long-bone nonunion and anterior lumbar interbody fusions, respectively [2, 44]. In the following years, these approvals were extended to posterolateral fusion, posterolateral lumbar pseudarthrosis, and nonhealing tibia shaft fractures [3–5, 140].

Currently, 11 clinical trials are registered under bone morphogenetic proteins for critical bone fractures, one for BMP-7 and the remaining for BMP-2 [71] (Table 2). In the majority of these studies, the BMP ligand is delivered through the use of an adsorbable collagen sponge (ACS), a calcium phosphate matrix (CPM), or as a liquid solution in buffer. The investigated concentrations of BMP-2 are reported between 1–12 mg/ml, and the product efficacy in fracture healing was compared to autograft or allograft transplants.

Reports from these studies display that approved BMP devices function as an alternative treatment, providing similar efficacy as autologous transplants, but does not result in an superior outcome [33, 46, 81, 121]. Even though promising, a debated therapeutic outcome has been reported due to safety issues and side effects possibly related to the usage of supraphysiological doses [49, 187, 211].

2.4 BMP Signaling Pathway

2.4.1 Ligand-Receptor Binding and Oligomerization

When inducing physiological cellular responses, BMP ligands activate intracellular signaling by binding to their respective transmembrane receptors. The active receptor complex involves typically one of the type 1 receptors, activin receptor-like kinase-1 (ALK)1., ALK2, ALK3, or ALK6, and one of the type 2 receptors, BMP-receptor type 2 (BMPR2) or activin type 2 receptor (ACVR2 or ACVR2b) [178]. It has been reported that BMP-2 and BMP-4 preferentially and predominantly bind to ALK3 or ALK6, whereas BMP-6 and BMP-7 primarily bind to ALK2 [41, 193]. Moreover, BMP ligands bind to type 1 and type 2 receptors with different affinities, likely due to their structural conformation [96]. For instance, while BMP-2 and BMP-4 bind with high affinity to their type 1 receptor, BMP-7 binds with high affinity to the type 2 receptors ACVR2a or ACVR2b and less to the type 1 receptors [57, 94].

Ligand-receptor oligomerization occurs through two different mechanisms, formation of a pre-formed receptor complex (PRC) or a BMP-induced receptor complex (BRC), causing distinct downstream signaling mechanisms [139]. PRC induces signaling through the SMAD-dependent signaling pathway, while BRC-induced signaling activates the (mitogen-activated protein kinases) MAPK pathway (Fig. 2). The difference in downstream signaling, induced by the oligomerization mechanism, has been explained by two different endocytosis routes, clathrin dependent or independent [54, 69, 139].

Table 2 Clinical trials involving BMPs

Treatment	Intervention	Control	BMP-concentration	Phase	Endpoint study	Status	References
Nonunion diaphysary tibial fractures	BMP-7 in adjunct to fresh frozen allograft	Allograft together with DBM	OP-1®	4	Efficacy	Suspended	NCT00551941
Nonunion diaphyseal Fractures	BMP-2 on ACS	Iliac crest autograft	InFuse®	4	Safety/efficacy	Withdrawn	NCT00856479
Nonunion fracture	Mesenchymal stem cells, BMP-2, and collagen scaffold	ND	ND	2	Safety/efficacy study	Recruiting	NCT01958502
Critical size tibial defects	Recombinant bone morphogenetic protein 2	Autogenous iliac crest bone graft	InFuse® (1.5–12 mg)	4	Safety/efficacy	Recruiting	NCT00853489
Radius fractures	BMP-2/CPM	ND	ND	1	Safety	Completed	NCT00161629
Tibia fracture	BMP-2/CPM	Buffer/CPM	1-2 mg/ml	2/3	Safety	Terminated	NCT00387686
Closed fractures of the humerus	BMP-2/CPM	Buffer/CPM	1-2 mg/ml	2	Safety/efficacy	Completed	NCT00384852
Critical-sized fracture	Mesenchymal stem cell, HA-CaSO4,BMP-2	ND	ND	1	Efficacy	Unknown	NCT01725698
Osteoporosis	BMP-2/CPM injection and bisphosphonates, calcium, and vitamin D	Bisphosphonates, calcium, and vitamin D	1–2 mg/ml unilateral intraosseous injection of 6 mL	2	Safety/efficacy	Completed	NCT00752557
Pseudarthrosis	BMP-2 on ACS	Autologous	InFuse®	ND	Efficacy	Not yet recruiting	NCT01756144

2.4.2 SMAD-Dependent Signaling During Bone Formation

The SMAD-dependent signaling cascade is initiated, as the constitutively active type 2 receptor phosphorylates the (glycine-serine rich) GS domain of the type 1 receptor which subsequently phosphorylates and activates the receptor-regulated SMAD1/5/8 complex (Fig. 2) [165]. These SMADs commonly consist of a DNA-binding domain at the N-terminus and a protein-protein interaction domain at the C-terminus domain, connected through a linker domain [83]. Upon phosphorylation of the C-terminus domain by the common mediator SMAD4, the R-SMAD complex is formed and translocates to the nucleus where it regulates the expression of BMP-responsive genes [97, 118, 165, 180].

The downstream signaling cascade of the R-SMADs can be modulated by phosphorylation of the linker region by other cellular kinases such as MAPKs and glycogen synthase kinase 3-beta (GSK-β). These compete with the receptor-mediated phosphorylation for deactivation through proteasomal-mediated degradation [50, 162]. Further fine-tuning of the signaling cascade is regulated by intracellular mediators such as small C-terminal domain phosphatases (SCP)-1 and SCP-2 and transcriptional cofactor BMP type 2 receptor-associated protein cGMP-dependent

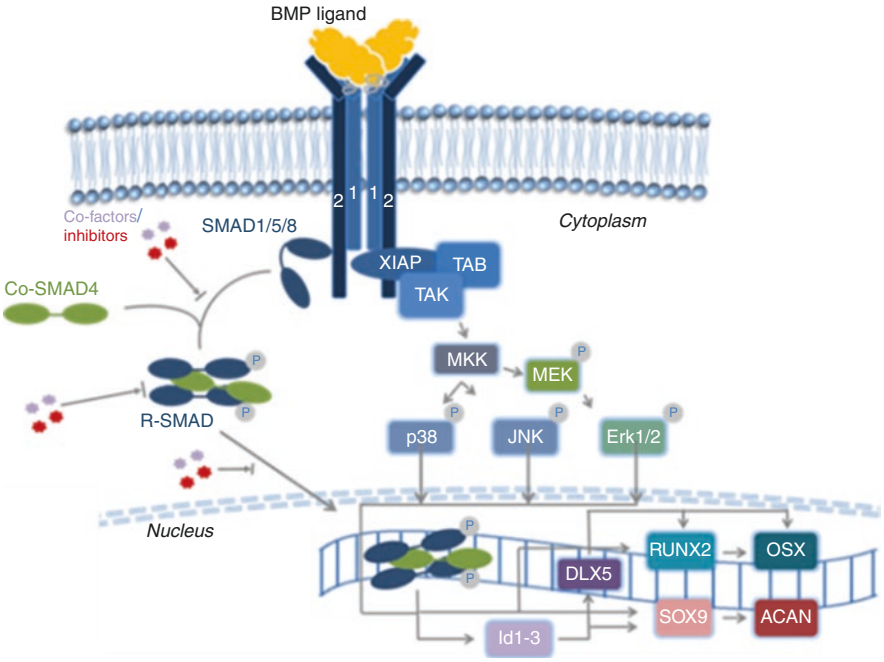


Fig. 2 Schematic view of BMP signal transduction. BMP ligands activate intracellular signaling by binding to their related transmembrane type 1 and type 2 receptors. Ligand-receptor oligomerization occurs through two different mechanisms where formation of a preformed receptor complex (PRC) mainly induces signaling through the SMAD-dependent signaling route, while BMP-induced receptor complex (BRC) preferentially activates the MAPK pathway

protein kinase 1 (cGK1) [163, 167]. Ubiquitination is another mode of regulating SMAD activity, which can lead to either proteasomal-mediated degradation causing repressed signal transduction or protein aggregate formation and regulate cellular processes as a potential protective mechanism [168]. SMAD6 and SMAD7 are also called inhibitory SMADs (I-SMADs), due to their antagonizing of the activation of R- and Co-SMADs. SMAD6 mitigates BMP signaling through competing with SMAD4 for complex formation [70]; SMAD7, on the other hand, is recruited to the receptor and induces degradation of the type 1 receptor kinase together with SMURF1 [40].

2.4.3 SMAD-Independent Signaling During Bone Formation

While the SMAD-dependent BMP signaling pathway is well investigated, less is known regarding the SMAD-independent pathways. Upon ligand binding to a preformed complex of the types 1 and 2 receptors, activation on gene transcription level occurs through the activation of the MAPK pathway (Fig. 2). MAPKs are evolutionary conserved enzymes that convert various extracellular stimuli into different cellular responses during biological processes such as fracture healing. The key effector enzymes p38, extracellular signal-regulated kinase (ERK), and c-Jun N-terminal kinase 1-3 (JNK) are part of a multistep cascade which is tightly regulated by phosphorylation and dephosphorylation processes [61, 80, 141]. JNK signaling is mainly known for its regulatory role in inflammation, apoptosis, and cell migration [136, 195]. The ERK-1 and ERK-2 kinases modulate cell survival, proliferation, and differentiation as well as protein synthesis in multiple cell lineages [144, 216]. Altered ERK-1/ERK-2 signaling is found in several genetic diseases with skeletal phenotypes such as neurofibromatosis type 1, suggesting a role in the regulation of skeletal development [9]. BMP-induced ERK signaling occurs through MEK1 activation, subsequently increasing Runx2 stability and transcriptional activity [82].

Among the various MAPK subfamilies, p38 kinase has attracted elevated attention in the last years and has proven essential for skeletogenesis and bone homeostasis due to its role in cell proliferation, differentiation, apoptosis, senescence, as well as cytokine production and function [56, 79, 106]. Upon BMP receptor phosphorylation, it associates with TAK1, TAB1, and XIAP, leading to activation of p38 which translocates to the nucleus [56]. Then, p38 activates transcriptional factors ATF2, c-Jun, or c-Fos to regulate BMP target genes such as RUNX2, OSX, OPN, ACAN, and ALP [103]. Each of the pathways has been proven of importance, since an effect can be seen upon inhibition, and the system is tightly controlled through fine-tuning between the activated MAPK pathways [98]. Moreover, cross talk between MAPK and SMAD signaling occurs, since it has been shown that TAK1 can modulate the duration and intensity of SMAD signaling [15, 72, 167, 177].

3 Candidate Cell Types for BMP/Cell-Based ATMPs

As aforementioned, the cells can be a driving force for tissue regeneration in cell-based ATMPs. Moreover, the necessity of (stem) cells to be included in the development of ATMPs becomes particularly important for fractures in compromised conditions, such as severely damaged surrounding tissues, elderly patients with suboptimal conditions (e.g., diabetes and osteoporosis), or in young children with congenital disease (e.g., neurofibromatosis type 1), which all may lead to poor healing of the fracture. In such compromised conditions, the surrounding tissue may not be able to efficiently respond to the BMP stimuli. In view of this, it is a potential advantage to pre-seed the scaffold with (stem, progenitor) cells combined with a physiological dose of BMP. From a clinical point of view, it is preferable for cell-based ATMPs to have a source of human stem cells that can be derived from a small biopsy via a noninvasive initial harvest and can proliferate in large numbers and be BMP responsive including proliferate and/or differentiate into the osteochondrogenic lineage upon BMP exposure [126].

3.1 Bone Marrow Stromal Cells (BMSCs)

Bone marrow, which is composed of the hematopoietic compartment and the stroma, is the conventional source to obtain human somatic stromal cells for use in regenerative medicine. In the hematopoietic compartment, hematopoietic stem cells and committed progenitors of different specific hematopoietic lineages reside. In the stroma, stromal cells, accessory cells, extracellular matrix components, and soluble factors have been described [77]. Taking the heterogeneous population of cells into account, it is of relevance to choose a well-defined and robust methodology to isolate, characterize, and study the functionality of the expanded stromal cell [45].

3.1.1 Isolation and Expansion

BMSCs are usually isolated by cultivation of cells adherent to plastic and obtained from untreated whole bone marrow in the form of bone marrow explant or bone marrow filter washout [148]. However, this method may lead to low yield of isolation because a large proportion of erythrocytes reside in the untreated bone marrow and their presence may interfere with the initial attachment of BMSCs [6]. An alternative method to isolate BMSCs is through an initial isolation of mononuclear cells by a Ficoll-Hypaque gradient before further cultivation [45]. By removing the unwanted high-density blood cells, this method is helpful to increase the number of colony-forming unit (CFU) in the primary BMSC culture [6].

The isolated BMSCs are usually cultured for expansion in basal medium supplemented with irradiated fetal bovine serum (FBS) [105]. FBS batches may differ from one to another, which could deeply affect the proliferation rate, reproducibility, and consistency of the production process [23]. Furthermore, FBS raises a general concern regarding immunological issues due to potential transfer of xenogeneic proteins as well as communicable disease such as prion-transmitted bovine spongiform encephalopathy, hence, posing potentially a long-term health risk [122]. In consequence, the regulatory authorities encourage replacing the FBS with a non-xenogeneic alternative, albeit GMP-compliant FBS batches are available and used in clinical-grade manufacturing [23].

As an alternative, human platelet lysate (hPL), a blood-derived product prepared as a clinical-grade reagent, has drawn attention for BMSC expansion, since it is a rich source of growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and basic fibroblast growth factor (bFGF)[19]. Previous studies revealed that hPL-expanded MSCs have comparable characteristics with those cultured in the presence of FBS [16]. Furthermore, hPL increases proliferation capacity of BMSCs, hence providing more efficient expansion [22]. However, also hPL shows important variability in its growth factor content, and a clinical-grade preparation poses still concern.

3.1.2 Characterization and BMP Responsiveness

In vitro, BMSCs represent a phenotypically heterogeneous population of cells. Fernandez Vallone et al. comprehensively reviewed the current progress on the phenotypic characterization of BMSCs using the fluorescence activated-cell sorter (FACS) and magnetic separation techniques [45]. Also our results demonstrated that primary cultures of human BMSCs are positive for the following markers: Strol-1, CD73, CD49, CD105, CD90, CD146, CD147, and lack of expression of CD14, CD20, CD34, and CD45. However, the aforementioned marker expression decreases during in vitro passaging, in association with the disappearance of multipotency of BMSCs [137]. When subjected to appropriate culture conditions, BMSCs readily differentiate into the osteoblastic and chondrogenic lineages, which is particularly of interest for bone regeneration. Research showed that BMSCs even possess greater osteogenic potential than either chondrogenic or adipogenic potential [137]. Moreover, their osteogenic potential appeared to be one of the last lineage commitment phenotypes to be lost [137, 188].

The age and skeletal site of harvest of BMSCs can affect their responses to BMP exposure. Osyczka et al. [142] assessed BMP-2 responsiveness (100 ng/ml supplemented in serum-containing and serum-free medium) of BMSCs harvested from adult maxilla, mandible, and iliac crest BMSCs from the same individuals and pediatric iliac crest. Their results showed that adult orofacial BMSCs were more BMP-2 responsive than iliac crest BMSCs based on higher gene transcripts of alkaline phosphatase, osteopontin, and osteogenic transcription factors MSX-2 and Osterix in serum-free insulin-containing medium. Pediatric iliac crest BMSCs were more

responsive to recombinant human BMP-2 (rhBMP-2) than adult iliac crest BMSCs based on higher expression of alkaline phosphatase and osteopontin in serum-containing medium [142].

Nevertheless, it is noted that BMPs are relatively inefficient in inducing human BMSC to undergo osteogenesis, albeit they are strong inducers for rat and mouse BMSCs [143]. It is shown that mouse-derived BMSCs respond to BMP-2, BMP-4, BMP-6, BMP-7, and GDF5 and further undergo chondrogenic differentiation [24, 43, 172, 173]. However, human BMSCs respond in a different way to distinct BMPs. Continuous stimulation of BMP-2, -4, or -7 upregulated the osteochondrogenic gene expression (e.g., NOGGIN, BMP-2, osteopontin) in human BMSCs [36]. However, they failed to enhance alkaline phosphatase activity, an indicator of osteogenic differentiation [36, 37]. In addition, continuous stimulation of BMP-2 with relatively high dosage (100 ng/ml) significantly increased human BMSC proliferation [36]. In contrast, short-term BMP-2 stimulation at lower doses (10–20 ng/ml) is more effective to induce in vitro osteogenic differentiation, evidenced by significantly increased gene expression of RUNX2, COLI, ALP, and OCN, as well as protein levels of COLI and ALP [36]. It was hypothesized that the impaired BMP response of human BMSCs is correlated with the absence of ALK6 expression [143]. However, the overexpression of ALK6 in human BMSCs had no effect on alkaline phosphatase mRNA transcripts, suggesting that the precise relationship between BMP receptor ALK6 and osteoblast-related genes remains to be defined [143]. There is limited research focusing on systematic in vivo evaluation of cell-based implants combining BMSCs and BMP. Wang et al. [207] reported a moderate increase of bone formation when loading BMSCs and BMP on calcium phosphate cements subcutaneously implanted in nude rats after 8 weeks, and such improved bone formation can be further enhanced by additional low dosage of bFGF (50 ng/ml).

3.2 Periosteum-Derived Cells (PDCs)

Anatomically, the periosteum is a thin vascular membrane that covers the external surface of the bone except for the articular joint surfaces of the long bones. It serves as an attachment site for tendons, ligaments, and muscles and is a rich source of blood vessels that deliver 70–80 % of the blood supply to the bone cortex [26]. Microscopically, the periosteum is composed of an outer fibrous layer and an inner cambium layer. The fibrous layer contains fibroblasts, collagen, and elastin along with a nerve and microvascular network [8], while the inner cambium layer consists of progenitor cells with the capacity to differentiate into osteoblasts and chondrocytes [64, 183].

The osteogenic potential of the periosteum was revealed early in the eighteenth century, when the integrity of the periosteum was found crucial to achieve successful fracture healing [39, 102]. Upon fracture, progenitor cells in the periosteum adjacent to the fracture undergo extensive expansion and differentiation to form a

cartilaginous fracture callus [31]. The cartilaginous callus progressively bridges the fractured bone fragments, followed by replacement by the bone, resulting in the formation of a hard callus which eventually is remodeled to the original cortical and trabecular bone configuration by osteoclasts.

3.2.1 Isolation and Expansion

To isolate periosteal tissue from the patient, a periosteum elevator, shaped like a curved chisel, is typically used to cut off the Sharpey's fibers that anchor the periosteum to the bone, hence maintaining the integrity of the periosteum [27]. Periosteum-derived cells (PDCs) are then harvested by enzymatic digestion of the tissue or by spontaneous cell egression from the biopsy onto plastic cell culture flasks [156].

In culture, PDCs exhibit a fibroblast-like morphology, which is stably maintained over several passages [156]. During in vitro expansion, PDCs do not express osteogenic and chondrogenic properties; however, they can be induced to differentiate into the osteogenic, chondrogenic, and adipogenic lineage by exposing them to specific differentiation medium [34, 156, 202], confirming their multi-lineage potential at the single-cell level.

3.2.2 Characterization and BMP Responsiveness

During expansion, over 90 % of human PDCs express CD73, CD90, and CD105 [156, 202], while lacking the presence of hematopoietic markers such as CD14, CD20, CD34, and CD45 (Ji et al. submitted). In addition, it has been reported that PDCs express perivascular cell markers, including αSMA [130], CD146 [156], and PDGF receptor beta [202], most likely due to their perivascular location [132, 159]. This concept is further underscored by our recent report that PDC enhanced vasculogenesis by adapting a pericyte-like phenotype when they were implanted in vivo [202].

Our data show that continuous in vitro stimulation of BMP-2, BMP-4, BMP-6, and BMP-9 (100 ng/ml) significantly enhanced the osteochondrogenic differentiation of human PDCs, evidenced by the upregulation of *SOX9*, *ACAN*, *RUNX2*, *OSX*, *DLX5*, *and ID1*. Through mRNA transcript analysis, the BMP-induced differentiation could be correlated to the expression of BMP type 1 and type 2 receptors Bolander et al., Eur Cell Mater. 2016 Jan 5;31:11-25. PMID: 26728496.

Upon coating onto calcium phosphate (CaP) carriers followed by hPDC seeding and 5-week in vivo implantation in nude mice, only BMP-2- and BMP-6-containing constructs gave rise to ossicle formation, including cartilage intermediates, trabeculae-like structures embedded in bone marrow with a surrounding cortex-like bone structure. In these ossicles, the implanted human PDCs contributed to 20 % of de novo bone (Bolander et al. submitted). Such enhanced in vivo bone formation might be correlated with the activation of SMAD-dependent pathway and MAPK pathway within hPDCs induced by BMP and Ca^{2+} exposure (Bolander et al. submitted).

3.3 Induced Pluripotent Stem Cells (iPSCs)

Induced pluripotent stem cells (iPSCs) are adult cells that have been genetically reprogrammed to an embryonic stem cell-like state by being forced to express genes and factors important for maintaining the defined properties of embryonic stem cells [124]. Since iPSCs can be derived directly from adult tissues, they not only bypass the need for embryos, but can be made in a patient-matched manner, which means that each individual could have their own pluripotent stem cell line, revealing a potential in personalized medicine.

3.3.1 Generation of iPSCs

In 2006, Yamanaka et al. first reported the generation of mouse iPSCs using retroviral transduction with 24 transcription factors highly expressed in embryonic stem (ES) cells [89]. This cluster of genes was gradually reduced to four key genes that encode the transcription factors OCT4, SOX2, KLF4, and c-Myc [191]. Shortly after the initial reprogramming success in the mouse, Yamanaka et al. [190] reported the generation of iPS cells from adult human dermal fibroblasts using a retroviral system with the same four factors: *OCT3/4*, *SOX2*, *KLF4*, and *c-Myc*. Concurrently, Yu et al. [219] reported the generation of human iPSCs from human somatic cells with lentivirus using a slightly different combination of genes including OCT4, SOX2, NANOG, and LIN28. Notably, the conversion from human somatic fibroblast to iPSCs is very low, with reported transduction rate from 0.001 to 1 %, depending on different vectors and gene combinations [89]. In 2012, Zhou et al. [229] reported a detailed protocol for generating human iPSCs from exfoliated renal epithelial cells present in urine, which allow a less-invasive and cost-effective sample harvest procedure and up to 4 % retroviral transduction efficiency.

3.3.2 Characterizations and BMP Responsiveness

Human iPS cells are similar to human ES cells in morphology, proliferation, surface antigens, gene expression, epigenetic status of pluripotent cell-specific genes, and telomerase activity, with capacity to further differentiate into cell types of the three germ layers including teratoma formation. Based on the guideline from the International Stem Cell Banking Initiative (ISCBI), Marti et al. [128] published a detailed characterization of iPSCs. In summary, human iPSCs demonstrate the following characteristics: (i) pluripotency – human iPSCs positively express human ES cell markers, such as pluripotent markers placental alkaline phosphatase (hPLAP); nuclear transcription factors OCT4, NANOG, and SOX2; the keratin sulfate antigens Tra-1-60 and Tra-1-81; and the glycolipid antigens SSEA3 and SSEA4. (ii) Differentiation – In vitro, human iPSCs colonies can form large aggregates called embryoid bodies (EBs), which should differentiate

spontaneously to different cell types derived from the three germ layers (spontaneous differentiation) or can be cultured in different substrates with different media to favoring differentiation toward a specific lineage (guided differentiation). Furthermore, the iPSCs will proliferate and differentiate in vivo in the tissue where they are injected and ultimately form a teratoma that contains multiple tissues from the three primordial germinal layers characterized by specific markers [11] (Table 3).

Recently, we reported in collaboration with Tsumaki labs the reprogramming of human dermal fibroblast into induced chondrogenic cells (iChon cells) using lentivirus system for Klf4, c-Myc, and Sox9 [192]. The iChon cells demonstrated a highly hypertrophic differentiation capacity in vitro and direct or indirect contribution to cartilage and bone formation in vivo [192], which highlights the promise of cellular reprogramming for the creation of functional skeletal cells that can be used for novel bone healing strategies.

The generation of iPSCs is regulated by multiple types of signaling cascades, including those mediated by BMPs. A recent study demonstrated that BMP signaling during the early stage of iPSC induction can induce a set of miRNAs associated with the mesenchymal-to-epithelial transition (MET), which can accelerate the generation of iPSCs [161]. Such enhancement might be mediated by a receptor complex consisting of ALK3 and BMPR2, since suppression of ALK3 and BMPR2 inhibited the generation of iPSCs [161]. Hamasaki et al. [66] recently showed that constitutive activation of ALK2 affected both the upregulation of pluripotent markers and the downregulation of fibroblastic markers during the early phase of iPSC generation, thus resulting in incomplete reprogramming. The role of ALK3 and ALK6 in the generation of iPSCs in cellular reprogramming still remains unknown.

Similar to ES cells, pluripotency and differentiation of iPSCs are also regulated by BMPs. However, many studies have highlighted differences between mouse and human ES cells regarding the response to extrinsic signals. For instance, Ying et al. [217] reported that BMP-4 sustains self-renewal of mouse ES cells by inducing the expression of ID genes. In contrast, in human ES cells, BMP-4 has been shown to

Table 3 Characteristic markers expressed in human iPSCs

Pluripotency		hPLAP, Oct4, Nanog, Sox2 Tra-1-60, Tra-1-81; SSEA3, SSEA4, SSEA1 (mouse)
Differentiation	Endoderm	α-1-Fetoprotein FoxA2
	Mesoderm	Brachyury (nuclear) (Muscle-like tissue) α-smooth muscle actin and α-sarcomeric actin (Cartilage-like tissue) Sox9, fibronectin, chondroitin sulfate
	Ectoderm	Pax6, Sox1, Tuj1

induce specification into the trophoblastic lineage [212], as well as germ cell lineage differentiation [209]. Consistently, Hamasaki et al. [66] showed that the BMP-4 or BMP-7 reduced the colony-forming capacity of iPSCs and directed iPSCs into both mesodermal and endodermal lineage. Therefore, we should be very careful to interpret the data obtained from mouse iPSCs and to extrapolate the results for studies using human cells.

4 Scaffolding Material for BMP Cell-Based ATMPs for Bone Regeneration

4.1 Clinical Perspectives of Desired Scaffold Properties

Effective clinical repair of bone defects is highly dependent on mechanical stability in the defect site and requires osteogenic cells and osteoinductive growth factors in combination with a proper delivery system, conceptualized as the "diamond concept" that provides the optimum mechano-biological conditions for bone regeneration [53]. The standard clinical practice for fracture immobilization is by using internal or external fixators to prevent micro-motion that will lead to scar tissue or cartilage formation. This technique is necessary especially when non-load-bearing biomaterial is used as the BMP delivery system/scaffold within an ATMP. Alternatively, metallic scaffolds with high mechanical strength could play a role in alleviating the adverse effects arising from mechanical instability. Although metallic scaffolds provide temporary supports to patients to regain immediate mobility, the non-biodegradability of the metals has limited its clinical applications. Nevertheless, significant research efforts on developing biodegradable metallic scaffolds with high mechanical strength are being carried out in order to overcome this limitation [223].

In addition to the mechanical stability aspect, the biodegradation kinetic of a biomaterial needs to match the bone formation process, to precisely control the release of BMPs, to guide cell differentiation and bone tissue formation, and to timely provide free space for blood vessel ingrowth and bone tissue formation. It is being suggested that an ideal biomaterial for bone defect repair should be partially degraded by 7 weeks and fully degraded around 14 weeks post-implantation, slightly depending on the defect nature including defect site, size, and patient profile. Moreover, the degradation by-products should not or minimally interfere with the activation of BMP signaling, if possible rather enhance the molecular and cellular cascade of bone healing. Therefore, the pharmacokinetic profile of a BMP-based ATMP should preferably be sustained over an appropriate period of time that matches the bone healing process in accordance to the cell proliferation and differentiation and mineralization effects elicited by BMP, instead of long but low concentration of BMP release or initial burst release. In fact, a delicate balance in concentration of BMP loading onto scaffolds is required. Furthermore, the pharmacokinetic profile should be specific to BMP ligands (due to different amino acid sequences of the BMP subtypes), the type of fracture or the application, host species

(different optimal release profiles are required), and implantation site. These factors would determine the form of the BMP delivery system conformations (from injection, micro-/nanoparticles to 3D porous scaffold), formulation (single or composite materials), and the type and amount of BMP in use.

Host environment is another crucial factor that needs to be considered thoroughly for designing an effective BMP-based ATMP therapy for bone defects, including the suitable BMP dosing and the concentration of BMP at the graft site. However, findings from animal studies are not easy to be translated into a clinical protocol as the BMP concentration used in animal studies appears to be lower than the dose required in patients. Moreover, the host environment is rich in a variety of organic and inorganic molecules that potentially influence the interaction between the biomaterials and BMP as well as the BMP bioactivity, such as in vivo temperature and bodily fluid pH and osmolarity. Other clinical implications of BMP treatment that require careful considerations include the route of administration and BMP antibody formation (i.e., 38 % of treated patients in some trials).

Ideally, a BMP carrier should (1) be biodegradable or present adequate porosity to allow the formation of an interface with the surrounding biological tissues for cell infiltration, vascularization, and new bone formation, (2) possess full biodegradability for complete integration of healed bone tissues, (3) provoke some mild inflammatory responses to activate the healing process, and (4) protect BMPs from deactivation while releasing the protein in a time- and space-controlled way to promote bone regeneration. On top of the requirements from the biomaterial's point of view, other stringent criteria for clinical usage include adaptability to the wound site, surgical malleability, as well as patient specificity or customization in respect to the treatment duration, anatomical geometry of the defect, and vascularity [65]. Lastly, the ATMPs need to be sterile without either loss of material integrity or deactivation of BMPs. Therefore, the respective manufacturing pipelines require special production and handling processes that would give rise to conveniently sterilizable, surgeon-friendly implants, stable over time with well-defined storage procedures (long shelf life). By combining manufacturing technologies, minimal manual intervention in the production pipeline is highly preferable for efficient commercial scale-up manufacturing of the respective BMP-based ATMPs, an additional criterion that would facilitate approval by regulatory agencies.

4.2 Injectable Materials for BMP- and Cell-Based ATMPs

Due to its water solubility, albeit rather poorly soluble, BMPs can be dissolved in water-based buffer solutions (e.g., physiological saline) and delivered in vivo simply via injection. New generation of BMPs is being developed that improve the solubility. Local injection is a potential minimally invasive delivery technique for treatment of delayed and nonunions, spinal fusion, and acceleration of healing of closed fractures. However, injection of BMPs in solution results in burst release of

BMP molecules, hence, a rapid clearance from the defect into surrounding tissues which reduces the differentiation effect and potentially causes toxicity and heterotopic bone formation.

To overcome these potential adverse effects, BMPs are often added into a protein carrier for precise injection into the defect to ensure sustainable BMP release to enhance long duration of local-acting differentiation effects. Besides maintaining the local BMP concentration, the carriers also provide protection to BMPs from deactivation by harmful conditions such as endogenous enzyme digestion and protein denaturation due to pH shifts. For this, collagen is often used as BMP delivery vehicle because it is easy to prepare in an injectable hydrogel form and can be obtained as purified recombinant human collagens that are free of animal components from reliable and chemically defined sources. Moreover, the binding affinity of BMPs to collagen can be modulated by changing the pH or isoelectric point of the two proteins in order to obtain specific BMP-release profiles to enhance bone formation [52]. Alternatively, gelatin is a cost-effective collagen-derived protein carrier that could provide controlled BMP release, by changing the electrical nature of gelatin via acidic or alkaline preparation process of collagen. In fact, delivery of BMP using collagen or gelatin as carriers showed increased retention ranging from 15 to 55 % as compared to less than 5 % of BMP dose remaining at the application site when no carrier was used [73].

Furthermore, the BMP release and bioactivity can be modulated by varying the extent [213] or employing site-specific enzymatic cross-linking of BMP onto gelatin [101]. Hyaluronic acid, a natural extracellular matrix (ECM), has been used as an effective injectable control release system to augment bone formation due to its specific chemical structures that allow chemical modification to ease cross-linking and for covalent binding of BMPs [129]. Self-assembly silk fibroin is another interesting injectable BMP hydrogel due to its processing flexibility, biodegradability, biocompatibility, and high mechanical toughness. The BMP release can be tailored by adjusting the enzymatic degradability of silk fibroin via the control of the crystalline state, molecular weight, and secondary structure [150, 157, 226]. Other natural-origin biopolymers that are used as injectable BMP hydrogel include alginate [18, 76], fibrin [145, 214], chitosan/chitin [184], and heparin [107]. Several studies showed that the composites of the above mentioned biopolymers either simply by mixing two biopolymers [147] or by conjugation [e.g., heparin-conjugated fibrin [215] provided more sustainable BMP release and improved in vivo bone regeneration as compared to using collagen alone as a carrier.

Synthetic polymers offer an advantage over the natural-origin biopolymers of being free from the risk of disease transmission. These polymer carriers are biodegradable, and thus allow for a controlled release of BMPs by fine-tuning the material degradation kinetics to match in vivo bone healing processes. Poly(lactic acid) (PLA) was an initial carrier to be used for BMP delivery, but it was ineffective due to the release of acidic degradation by-products that deactivate BMP. Subsequently, poly(lactic-co-glycolic acid) (PLGA) received particular attention because it combines the absorptive stability of PLA with mechanical strength of polyglycolic acid (PGA) and offers tunable biodegradability by varying the proportion of the two

components. Poly(ethylene glycol) (PEG) is a bio-inert hydrophilic polymer that is versatile for hydrogel formation or for conjugating with biomolecules including growth factors, cell adhesion peptides, and enzymes for controlling matrix degradation (e.g., matrix metalloproteinase)[179, 230]. Because of its unique chemical structure (i.e., two hydroxyl end groups), PEG can be converted into other functional groups to obtain a tunable physical state. This tunable state renders the PEG injectable and in situ cross-linkable either via a temperature-dependent liquid-semisolid transition [called thermosensitive polymers, such as polypropylene fumarate-co-ethylene glycol [14], poly-D,L-lactic acid-polyethylene glycol (PLA-PEG) block copolymers [85, 160]] or via in situ polymerization through chemical [e.g., poly(ethylene glycol) fumarate[179]] or photo-cross-linking mechanisms [e.g., poly(ethylene glycol) diacrylate [35, 228]]. Furthermore, synthetic polymers also provide higher mechanical properties (such as torsional strength) than the biopolymers which are crucial for healing large bone defects. However, additional materials for intervention may hinder BMP release from the bulk or alter BMP molecular integrity and thus compromise its bioactivity. Nonetheless, these materials are often bio-inert and lacking bone-inducing effects. This has led to the development of injectable, in situ setting ceramic cements as BMP delivery carriers.

Ceramic cements, such as calcium phosphate or hydroxyapatite, have been shown to have high binding affinity for BMP molecules [108, 206], thus making them suitable carriers for effective delivery of BMP in addition to their well-known osteoconductive and osteoinductive effects. The osteoinductive effect of calcium phosphate is beneficial as BMP devices as currently formulated must be used at very high concentrations to be effective [55]. In fact, ceramic pastes incorporated with rhBMP-2 showed to accelerate healing of critical-sized bone defects in preclinical large animals, such as canine [42] and nonhuman primate [170]. Bioactive glass is another promising bone-inducing biomaterial and delivery vehicle for BMPs due to its unique ability to bond to living bone and promote bone regeneration [220]. It has been reported that BMPs can be covalently immobilized onto bioactive glass effectively via surface functionalization techniques such as silanization [205] or physical absorption onto apatite coating formed on bioactive glass [123]. The benefits of injectable synthetic polymer and ceramic carriers for BMP delivery gave rise to the development of injectable composite carriers that were found to enhance bone formation and were linearly dependent on the amount of additional calcium phosphate powder in respect to the rhBMP-2/calcium phosphate ratio [84]. Nevertheless, lack of open-pore structures or low porosity of the hardened paste appears to be the major drawback of this delivery method, which may interrupt BMP release kinetics and prevent ingrowth of surrounding tissues and the formation of neo-tissues, thereby compromising or delaying bone formation. For this, injectable micro- or nanocarriers that are encapsulated or chemically immobilized with BMPs are developed to circumvent these drawbacks by providing a higher specific area for BMP release and interparticle open spaces for tissue growth. For instances, these injectable micro- or nanocarriers have been reported to be successfully made from PLGA [152], chitosan [125], silk fibroin [17], polycaprolactone [12], and calcium phosphate [208].

Recently, carbon nanotube (CNT) was reported to be a promising biomaterial for bone tissue engineering [1]. In addition to the high mechanical strength, surface functionalizing the nanotube surface with BMPs was shown to be feasible and gave rise to controlled release of BMPs and accelerated chondrogenic and osteogenic differentiation of progenitor cells and in vivo bone formation [112]. Interestingly, an inhibitory effect of CNT was found on carboxylated CNT that showed to inhibit proliferation and differentiation of precursor cells which may be modulated via a SMAD-dependent BMP signaling pathway [113]. This indicates that further investigation is necessary to gain more insights into the biomedical applicability of CNT as BMP delivery system, in addition to the potential cytotoxicity effects due to intracellular accumulation of CNTs [62] or generation of reactive oxygen species [151].

4.3 Solid Porous Scaffolds for BMP- and Cell-Based ATMPs

Three-dimensional (3D) scaffolds play an important role in tissue regeneration by providing attachment sites, void spaces, as well as bioactive signals for cells to grow and differentiate into specific lineages. Specifically, it aims to provide a precise microenvironment for optimal expansion and control of differentiation of precursor cells that subsequently lead to 3D functional organ formation. Conventional techniques are employed to produce 3D porous scaffolds in solid (e.g., salt leaching, porogen sacrifice, and gas foaming), fibrous (e.g., electrospinning), and microspheres (e.g., water-to-oil emulsion and droplet generation). These scaffolds could act as efficient drug delivery systems, delivering BMP homogeneously in a three-dimensional manner which is an important criterion to elicit bone formation in all or a targeted direction.

It is known that the clinical efficacy of recombinant human BMPs (rhBMPs) will depend on the carrier system used to ensure an effective delivery of adequate protein concentrations to the defect site [134]. Various modes of BMPs incorporation into the scaffolds have been developed and showed promising bone formation outcomes [20]. The most convenient method is by physical absorption onto porous scaffold, whereby BMPs are randomly impregnated within the delivery matrix without chemical bonding. However, physical absorption will lead to an initial burst release of BMPs. BMPs can also be incorporated into the porous scaffolds by entrapment within a hydrophobic polymeric matrix during scaffold production in order to obtain an extended period of BMP release. The risk of BMP protein denaturation and loss of bioactivity could arise due to temperature changes during the production process or pH shift due to material degradation. Hydrogel scaffolds made from extracellular matrix (e.g., hyaluronic acid, heparin sulfate, heparin proteoglycans) or charged polymers (e.g., chitosan, alginate, or synthetic polyelectrolytes) are interesting biomaterials for BMP delivery, attributed to the strong affinity of BMPs or via ion complexation binding of BMPs with the biomaterials. Modification of surface chemistries of the porous scaffolds for immobilization of BMPs via covalent binding showed to be more promising than any nonspecific immobilization methods. This immobilization can be achieved

by either modifying the chemical backbone structures of the biomaterials or grafting functional groups that are specific for BMP molecules onto the surface of scaffolds. Alternatively, BMP protein with a domain of specific binding to the scaffolds can be produced due to the great versatility of the recombinant technology nowadays. Therefore, chemical immobilization of BMPs has provided feasibility to develop "smart" BMP-releasing scaffolds which guaranteed precise dosing and control over BMP release such as via cell-mediated activity [114], light [93], temperature [115], and pH changes [51]. Incorporation of other essential biological cues to enhance cell adhesion and growth on the porous scaffolds is an attractive approach to enhance the biological functions of the porous scaffolds. For instance, hyaluronic acid scaffold was reported to be superior over collagen gel as carrier for a gradual and sustainable release of functional rhBMP-2 [86], and covalent grafting of fibronectin fragments within the hyaluronic acid structures enhanced cell attachment and spreading, as well as improved quality of ectopic bone formation [88].

Besides biochemical signals between cells, physical parameters of the scaffolds are shown to exhibit significant effects on tissue formation starting at the single-cell level. Indeed, the behavior of stem cells or osteochondro-progenitors is strongly influenced by the geometrical features of scaffold pores. It is reported that small pore sizes (<500 μm in diameter) gave rise to lower scaffold permeability (than the bigger pore size; >500 μm in diameter), thus, resulting in significantly higher in vitro cell seeding efficiency but faster occlusion of the pores that blocked further cell growth [201]. In vivo, subcutaneous implantation of porous hydroxyapatite scaffolds (in combination with BMP-2) with pore sizes of 300–400 μm resulted in highest bone formation, whereas pore size of 90–120 μm gave rise to cartilage tissues, a phenomenon that was dependent on the vascular invasion [99, 199]. Additionally, the pore curvature imposed active mechanical forces that influenced the speed of cell growth, which resulted in a curvature-driven cell growth pattern that was associated with distinct patterns of actin organization and alignment [63, 95]. Interestingly, a study using sheep critical-sized bone defects showed that the scaffold architecture directed bone tissue organization through structural guidance and load transfer, while BMP stimulation accelerated bone formation without altering the bone tissue microstructure at different length scales [29]. These findings indicated important implications toward the understanding of natural processes of bone defect healing and bone remodeling, as well as important clues for designing optimum 3D porous scaffolds [158].

Advances in 3D additive manufacturing (e.g., selective laser melting/sintering, fused deposition modeling, and solid free-form fabrication) have opened up the feasibility to fabricate synthetic 3D microenvironments that mimic the regulatory characteristics of natural extracellular matrices (ECMs) and ECM-bound growth factors in addition to the indispensable biological and physical criteria required on the scaffolds to warrant success during in vitro 3D culture and in vivo tissue formation [10]. Since BMPs are delicate proteins that are vulnerable to temperature and pH, it is of utmost importance that the employed 3D printing technology must not compromise the bioactivity of the incorporated BMPs; otherwise incorporation of BMPs is to be carried out on the surface of scaffolds after the production process via the aforementioned methods. Examples of 3D-printed porous scaffolds for BMP delivery include

polymer-based scaffolds [e.g., polycaprolactone [225]], hydrogels [e.g., PEG [164, 175]], ceramics [e.g., biphasic calcium phosphate [186]], and metallic [e.g., titanium alloys [227]]. This technology has potential to fulfill the needs for engineering an efficient upscale production of ATMPs with quality attributes of high controllability and reproducibility. Table 4 shows a summary of different types of biomaterials and their advantages and disadvantages as potential BMP-related ATMPs.

Table 4 Types of biomaterials as potential BMP-related ATMPs: advantages and disadvantages

Types of biomaterials	Advantages	Disadvantages
A. Non-ceramic based		
1. *Natural-origin* (e.g., collagen, gelatin, fibrin, chitin/chitosan, alginate, hyaluronic acid, and agarose)	Biocompatible and biodegradable Allows clinical malleability according to defect geometry and application (injectable, moldable putty like, sponges, hydrogels, 3D-printed porous scaffolds) Simple incorporation of BMP into the biomaterials	Risk of disease transmission and immunogenic Limited sources and impurities contamination Low mechanical properties
2. *Synthetic polymers* (e.g., polylactic acid, polylactic-glycolic acid, polycaprolactone, polyethylene oxide, polyethylene glycol, polypropylene, polyvinyl alcohol)	Biocompatible, biodegradable, and free of risk of disease transmission Available by mass production via chemical synthesis Tunable chemical and material properties for specific BMP release and material degradation profiles to match bone healing Allows cells encapsulation and chemical immobilization of biomolecules to enhance biological activity No or low intervention with BMP bioactivity Presence in hydrogel form or 3D-printed porous scaffolds to allow patient-customary implant design	Potential deactivation of BMP and immunogenic due to acidic degradation products Insufficient mechanical strength for load-bearing applications No bone-inducing property thus required high BMP dosage to achieve the desired therapeutic effect
B. Ceramic-based biomaterials		
1. *An organic animals' bone granules*	Confers superior osteoinductivity due to high similarity of chemical composition and structure to native bone Biocompatible, biodegradable, and non-immunogeni Ideal delivery vehicle for BMPs due to high binding affinity and material degradation Possess physiological calcium and phosphate ions release kinetics for stimulating bone formation	Risk of zoonosis transmission and limited sources Limited sources and impurities contamination and toxicity Inconsistent bone formation outcomes due to the variation in animals and production factors

(continued)

Table 4 (continued)

Types of biomaterials	Advantages	Disadvantages
2. *Calcium carbonate* (e.g., corals, egg shells)	Alternative resources and cost effectiv Can be synthesized into calcium phosphate-based apatites	Risk of zoonosis transmission and impurities contamination
3. *Calcium phosphate and bioactive glasses* (e.g., hydroxyapatite, tricalcium phosphate, biphasic CaP, octacalcium phosphate, calcium pyrophosphate, dicalcium phosphate)	Widely used as synthetic bone substitutes due to its excellent osteoconductivity and osteoinductivity Possess higher biomechanical strength than polymer- or hydrogel-based biomaterials Allows fine-tuning of the material degradation and BMP release kinetics Synthetic and thus free of risk of disease transmission and impurities contamination High affinity for BMPs binding, and unlimited availability Can be formed into paste-like or 3D-printed porous scaffolds	Rigid, brittle, and requires fixators in load-bearing application May induce adverse inflammatory responses and osteoclastic resorption May interfere BMP signaling activation
C. Metals (e.g., titanium-based, cobalt-chromium, zirconium, stainless steel, tantalum, magnesium alloys)	Biocompatible and offers excellent mechanical strength Can be produced into implants with desired defect geometry and 3D-printed porous scaffolds Allows surface immobilization of BMPs for controlled delivery applications Bio-inert and thus not interfering with BMP effects Provide immediate mechanical support and mobility to patients	Nonbiodegradable and requires surgical intervention due to implant wear off Risk of metal toxicity or chronic inflammatory responses
D. Composites made from the above biomaterials (e.g. Composites of CaP with collagen, hydrogels or polymers; Hydrogel or CaP-coated of collagen, polymer or metallic sponges or scaffolds)	Improved mechanical strength, osteoconductivity and osteoinductivity Higher versatility and flexibility in fine-tuning the material properties and bioactivities Offers higher technological flexibility for different clinical implications and demands Improved BMPs delivery as compared to single material delivery	Lack of technological tools as well as knowledge on the BMP-material-host interactions for developing an ideal biomaterial that optimally elicits bone regeneration based on BMP technology

5 Toward ATMP Combining BMP and Cells

Since powerful "raw materials" are now available in clinical grade such as BMPs, CE-approved smart biomaterials, and GMP-manufactured cell suspensions such as BMSCs or periosteal-derived cell populations, we have set out to produce combinations of these that exceed the biological potency of the single products such as BMPs or biomaterials only. These combination products are envisioned to be of use for large bone defects in compromised environments, with sick tissues, lack of stem or progenitor cells close by, and where the implant needs to drive semiautonomously the process of tissue formation and integration despite an unfavorable environment. This may be in genetic diseases such as NF1, where the periosteum compartment is simply ineffective or an aging patient with diabetes and osteoporosis or osteomalacia.

The search for these optimal combination products is quite challenging and should be based on the principles of developmental engineering as a concept of "in vitro biomimetics of in vivo tissue development" [109, 110]. In short, the design of cell-based products should integrate the concepts of developmental biology, so that the behavior of networks of genes, proteins, or cells that govern the unfolding of developmental processes could be related to the design parameters. In addition, it is necessary to involve new methodologies such as design of experiment (DoE) approach to determine the optimal setup for each design parameters. We recently conducted a full-factor DoE analysis of bone formation capacity induced by ATMPs with different calcium phosphate scaffolds, BMP loading dosage, and cell seeding dosage (Ji and Kerkhofs et al. in preparation). Our data indicates that indeed the proper dosage combinations of BMPs and cells seeded on specific scaffolds can generate skeletal tissue intermediates with higher bone-forming potency, improved bone quality, and more active contribution from donor cells, exceeding these of smart biomaterials only with growth factors or cells.

To turn this into robust manufacturing processes, new enabling technologies such as perfusion bioreactors in combination with biosensors are required. Such setup provides several advantages for a manufacturing pipeline, including (1) direct cell-cell and cell-extracellular matrix interaction, (2) direct control over shear stress development, and (3) accurate sensor readouts at the outlet of the bioreactors. It also helps to develop structurally defined and functionally effective complex 3D-engineered constructs at the patient scale using scale-out strategies [146]. In addition, noninvasive imaging will be necessary to further tailor the quality characteristics of specific stem cell culture as well as for more complex 3D TE construct culture [146]. Furthermore, regulatory requirements are evolving for these novel 3D products and their manufacturing processes. Therefore, the effective bioreactor systems with incorporation of multiple sensors would provide information-rich processes for the manufacturing of TE products that could meet regulatory demands [146].

6 Preclinical Evaluation of ATMPs Combining BMP and Cells

BMP, stem cells, and biomaterials can be considered as "raw materials" in the development of ATMPs. Although recent progress has been achieved in BMP production, (stem) cell culture and expansion as well as new biomaterials fabrication, respectively, the translation of knowledge from in vitro model systems to in vivo and upscaling to the clinical setting is still challenging. Therefore, it is necessary to use sequential animal model systems to fully understand the biological performance of these devices in a living organism before translation into the clinics can be made. The following section will focus on animal models suitable for preclinical evaluation of BMP-/cell-based ATMPs.

6.1 Ectopic Model

Particularly for bone regeneration, the ectopic model provides a relatively controlled and clean system to evaluate the in vivo de novo bone formation capacity of human cell-based ATMPs. Therefore, this is suitable as a first-line screening model to identify the biocompatibility, toxicity, and bioactivity of ATMPs. The three most commonly used ectopic models are subcutaneous, intramuscular thigh, and under-the-kidney capsule implantation [169]. Despite the advantages of the ectopic model, the differences in the inflammatory, immunological, biochemical, and mechanical environment between ectopic and orthotopic locations are distinct, which greatly affects the bone-forming process induced by the ATMP. For instance, Levi et al. [111] showed that adipose-derived stem cells successfully ossified a critical size defect. However, the same implants did not result in significant bone formation in the ectopic model.

Another concern comes from the different tissue responses between immune-deficient and immune-competent animals upon ATMP implantation. Liu et al. [116] showed that hydroxyapatite tricalcium phosphate (HA-TCP) scaffolds combined with mouse BMSCs were much less osteoinductive in syngeneic immune-competent mice than immune-deficient mice when implanted ectopically. Furthermore, recipient T lymphocytes were found to inhibit bone formation in immune-competent mice via inflammatory factors such as IFN gamma and TNF alpha. In a different study, gene expression profiles of the implants showed that T lymphocyte differentiation and activation gene markers were upregulated in immune-competent mice in comparison to immune-deficient mice [21]. Our recent data confirmed that BMP-6-mouse PDCs combined implants induced bone formation in the ectopic model in immune-deficient mice, but failed to do so when tested in immune-competent mice (Fig. 3).

On the other hand, it is well known that a proper inflammatory response is an essential part of the natural bone healing process [135]. Consequently, modulation of inflammation in ATMP implantation is of utmost importance. More recent

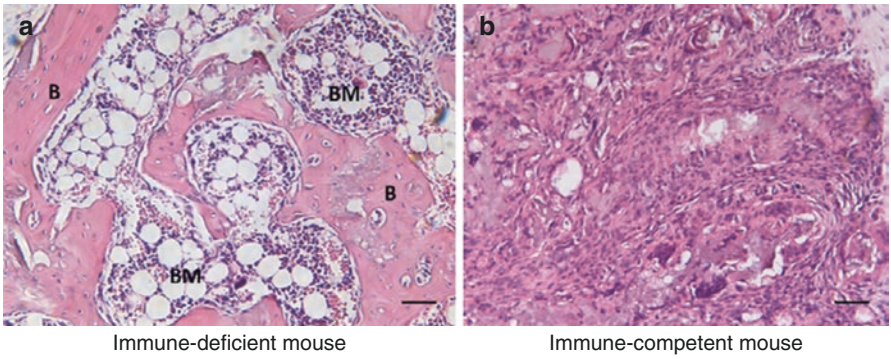


Fig. 3 Mouse PDC-mediated bone formation in immune-deficient and immune-competent mouse. HE staining of tissue explants from immune-deficient mouse (**a**) and immune-competent mouse (**b**) 6 weeks after subcutaneous implantation of BMP-6-coated scaffolds with syngeneic mouse PDCs (scale bar = 100 μm, *B* bone, *BM* bone marrow)

emphasis has been given to the modulation of the inflammatory reaction toward improved bone regeneration. New strategies based on surface modifications of biomaterials, coupling of anti-inflammatory drugs to biomaterials, delivery of growth factors, and infusion of MSCs have been explored [48, 117, 153, 176]. For instance, it was reported that Nel-like molecule-1 (NELL-1), a protein first identified for its osteoinductive properties in craniosynostosis patients, could suppress the BMP-2-induced inflammatory reaction in vivo [176]. Furthermore, systemic infusion of MSCs had a positive effect on reducing IFN gamma and TNF alpha and promoted bone formation when scaffolds combined with MSCs were implanted ectopically in immune-competent mice [117]. Nevertheless, there are no methods that generate the same amount of bone in immune-competent mice compared to immune-deficient mice without concerns regarding its clinical safety. Therefore, further studies are required to fully understand the interaction between the immune system and bone tissue formation, providing new insights for successful application of bone tissue engineering strategies.

6.2 Orthotopic Model

Orthotopic models refer to studies in which the bone is formed in its correct and relevant anatomical location. These can be applied in different species to fulfill specific research questions, which can be categorized as (1) understanding of mechanism of action and (2) clinical upscaling, feasibility, safety, and efficacy prediction. For instance, to understand the mechanism underlying bone formation, small rodents such as mice and rats are preferred due to availability of immune-deficient animals for xenograft-based experiment [169]. For clinical translation, the defect should be upscaled in a clinically relevant setting with critical size, which is “above

Table 5 Orthotopic models in different animals with critical size

Animal	Advantages	Disadvantages	Calvarial defect	Segmental long-bone defect	References
Rodents (mice and rat)	Easy to perform surgery Availability of Immunodeficient animals Availability of specific disease-target gene knockout animal	Relatively thicker and fewer trabeculae than humans Mice and rat do not have Haversian system Periosteum in rats and is well vascularized, hence improving bone healing	Mice: 5 mm diameter Rat: 8 mm diameter	Mice: 0.4 cm in the radius, 0.5 cm in the femur Rat: 1 cm in the radius, 0.4–0.5 cm in the tibia, 0.5-1 cm in the femur	[13, 32, 203, 204]
Dog	Tractable nature Similar bone mineral density to humans	Higher rate of solid bony fusion when compared to humans Low nonunion rates Ethical issues and negative public perception Significant inter-animal variations due to breed diversity	N/A	0.3–2.5 cm in the radius 2–2.5 cm in the ulna 2.1–7 cm in the femur	[74, 154]
Sheep	Docile animals with easy outdoor housing Similar body weight to humans Hind limb anatomy similar to humans Dimension of long bones suitable for human implants	Higher trabecular bone density than humans Late skeletal maturity,, with Haversian remodeling at 7–9 years of age	N/A	3–3.5 cm in the tibia 2.5 cm in the femur	[74, 203, 221]
Pig	Better social acceptance Bone mineral density and healing similar to humans	Hind limb anatomy is different to humans Rapid growth rate Difficult handling	N/A	2.5–3 cm in the radius	[74, 203, 221]

the threshold size intraosseous defect dimensions that will not heal spontaneously during the lifetime of the animal" [166]. Therefore, large animals are more appropriate. Table 5 summarizes the advantages and disadvantages when applying orthotopic models in different animal species.

From a surgical point of view, orthotopic models can be categorized as (1) calvarial defect and (2) segmental long-bone defect, which has different critical sizes

depending on location, age, and animal species (Table 5). The calvarial defect model provides a good non-loading-bearing bone-healing environment with relative biological inertness due to poor blood supply and limited access of bone marrow, which is thought to resemble the atrophic mandibular bone in humans. Furthermore, it provides a good simultaneous environment to study the intramembranous ossification and allows the establishment of a uniform, reproducible, and standardized defect. The standard rodent calvarial bone defect is typically created by using a trephine drill that makes a circular defect in the cranial skeleton on the midline [189]. It is suggested that the sagittal suture and the dura mater underlying the defect have to be carefully protected during the surgery which is important for the cranial skeleton healing. Furthermore, the filling materials should be strong and sufficiently resistant to avoid the dilation of brain tissue beneath the defect [78]. The rodent models are the first-choice models for in vivo testing of regenerative and/or therapeutic approaches but are not suited to the establishment of long-term studies and immediately translation to a clinical setting.

Segmental long-bone defects allow researchers to test and understand the tissue formation destined for long-bone healing with mechanical loading and in upscaled treatment modalities for clinical application. The creation of segmental long-bone defects is usually done in an osteotomy approach, which utilizes a drill or saw to surgically remove the required length of the bone from a predetermined site, producing a consistent defect in all animal species. After filling the defect, it can be internally fixed with either bone plates or intramedullary rods [74] or by external fixation such as the Ilizarov fixation technique. In addition, we recently developed a sheep segmental tibial defect bone model, which provides additional insights on the handling, safety, feasibility, and upscaling possibilities of different regenerative treatments. However, also in this large-animal bone defect model, discussions still remain in defining a critical size defect being the one that does not achieve spontaneous healing during the lifetime of the animal. Therefore, the design of a large-animal model has to be stringent, where factors such as the age of the animal, the defect size, and the fixation material used will have a significant impact. Moreover, this phenomenon of spontaneous bone regeneration, which can occur in a large-animal bone model and thus can interfere with a regenerative treatment applied in the defect, can be seen as "background noise" and can therefore lead to over-enthusiastic conclusions about the actual effect of a regenerative treatment (Fig. 4).

7 Cell-Based Combination Products: Challenges and Perspectives

Bone fracture healing is essential for the quality of life and even survival. Therefore, a natural tightly regulated cascade of cellular and molecular events has evolved in evolution leading to a successful healing process allowing the individual to survive and resume normal function within 6–10 weeks. However, the bone-healing process gets delayed and leads to a nonunion or nonhealing fracture when the defects are too

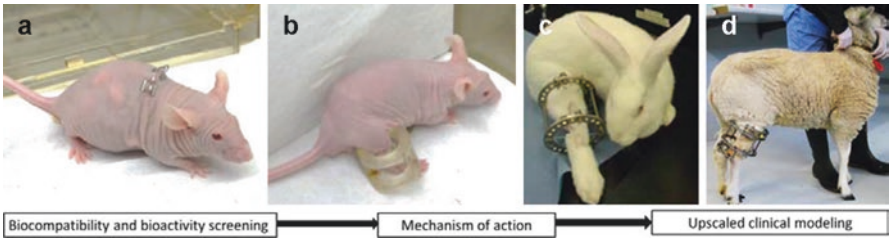


Fig. 4 Animal models for preclinical evaluation of regenerative treatment possibilities for bone regeneration. (**a**) Ectopic model in rodents is mainly used for biocompatibility and bioactivity screening. Orthotopic defect with Ilizarov fixation technique in mouse (**b**) and rabbits (**c**) is usually used to study the mechanism of action underlying the tissue formation. The upscaled orthotopic defect in sheep (**d**) is useful for clinical translation

large or comprising conditions such as infection and diseased bones arise. In the animal world, a nonunion or nonhealing fracture results inevitably to death. In humans we have the challenge to try to obtain healing by other means in an attempt to restore function and thus independence of the patient.

Novel solutions have been developed in the past decades, including the discovery of antibiotics to fight infection, new surgical techniques and instrumentation to obtain full immobilization, and bone distraction osteogenesis as developed by Ilizarov [67, 75]. In addition, impressive progress in our knowledge on the cell and developmental biology of bone as well as fracture healing has triggered the discovery of new growth and differentiation factors such as BMPs and the development of smart biomaterials. This in turn has led to an unprecedented number of opportunities and strategies to enhance bone healing.

Despite all these stellar developments, there are still quite some clinical challenges, and growing in number, also due to the aging population. These include large bone defects in compromised environments in the patient with comorbidities such as cardiovascular disease, diabetes, osteoporosis, and osteomalacia. In addition, large bone defects as a result of revisions of joint prostheses are becoming a real challenge in daily clinical practice.

In view of this, we need to turn to more sophisticated strategies, combining and improving all the powerful tools and insights that nature has provided us. Opportunities include the use of (stem) cell technologies, the development of more sophisticated growth factor formulations, and the optimization of biologically relevant scaffolds that are enhancing the biological processes and not just sitting there as an inert material. Ultimately, the dream is to combine all these to create living tissue intermediates or provisional tissues that upon implantation steer the healing process in the right direction, also called developmental engineering [109, 110]. Growing knowledge and insights on both materials engineering and cell biology is crucial to implement the essential natural temporal and spatial complexity within the synthetic microenvironment that recapitulates developmental and healing processes of cell proliferation, differentiation, and tissue morphogenesis [120].

To produce these living tissues "of the shelf," we have serious manufacturing challenges. In combination with robust in vitro culture technology that mimics closely the in vivo "biological chamber," upscaled tissue engineering constructs or ATMPs could be engineered into sufficiently pre-differentiated tissue intermediates that are directly recognized by the microenvironment and readily initiate the cascade of bone regeneration. In this perspective, bioreactors with sophisticated online monitoring systems tracking all relevant cellular metabolic profiles and culture environment readouts become critical assets. Novel enabling technologies such as biosensors will be instrumental for industrial manufacturing modular processes for cell-based combination products.

In conclusion, we believe the era of regenerative medicine has just started. First-generation BMP and stem cell technologies have demonstrated that in the postnatal environment, one can successfully enhance the healing of damaged tissues by recapitulating the principles of developmental tissue formation. The stage is set; it is up to us to take on the challenge for the second-generation products that lead to the creation of living replacement body parts.

References

1. Abarrategi A, Gutierrez MC, Moreno-Vicente C, Hortiguela MJ, Ramos V, Lopez-Lacomba JL, Ferrer ML, Del Monte F (2008) Multiwall carbon nanotube scaffolds for tissue engineering purposes. Biomaterials 29:94–102
2. Administration, U. F. A. D (2002) Infuse™ Bone Graft/Lt-Cage™ Lumbar Tapered Fusion device – P000058 [Online]. Available: http://www.Fda.Gov/Medicaldevices/Productsandmedicalprocedures/Deviceapprovalsandclearances/Recently-Approveddevices/Ucm083423.Htm. Accessed Aug 28 2015
3. Administration, U. F. A. D (2004a) Infuse® Bone Graft – P000054 [Online]. Available: http://www.Fda.Gov/Medicaldevices/Productsandmedicalprocedures/Deviceapprovalsandclearances/Recently-Approveddevices/Ucm081154.Htm. Accessed 28 Aug 2015
4. Administration, U. F. A. D (2004b) Op-1 Putty – H020008 [Online]. Available: http://www.Fda.Gov/Medicaldevices/Productsandmedicalprocedures/Deviceapprovalsandclearances/Recently-Approveddevices/Ucm081181.Htm. Accessed 28 Aug 2015
5. Administration, U. F. A. D (2007) Infuse® Bone Graft – P050053 [Online]. Available: http://www.Fda.Gov/Medicaldevices/Productsandmedicalprocedures/Deviceapprovalsandclearances/Recently-Approveddevices/Ucm077024.Htm. Accessed 28 Aug 2015
6. Hideki Agata (2013). Isolation of Bone Marrow Stromal Cells: Cellular Composition is Technique-Dependent, Regenerative Medicine and Tissue Engineering, Prof. Jose A. Andrades (Ed.), InTech, DOI: 10.5772/55543. Available from: http://www.intechopen.com/books/regenerative-medicine-and-tissueengineering/isolation-of-bone-marrow-stromal-cells-cellular-composition-is-technique-dependent
7. Ai-Aql ZS, Alagl AS, Graves DT, Gerstenfeld LC, Einhorn TA (2008) Molecular mechanisms controlling bone formation during fracture healing and distraction osteogenesis. J Dent Res 87:107–118
8. Allen MR, Hock JM, Burr DB (2004) Periosteum: biology, regulation, and response to osteoporosis therapies. Bone 35:1003–1012
9. Aoki Y, Niihori T, Narumi Y, Kure S, Matsubara Y (2008) The RAS/MAPK syndromes: novel roles of the RAS pathway in human genetic disorders. Hum Mutat 29:992–1006
10. Arafat MT, Gibson I, Li X (2014) State of the art and future direction of additive manufactured scaffolds-based bone tissue engineering. Rapid Prototyping J 20:13–26

11. Asprer JS, Lakshmipathy U (2015) Current methods and challenges in the comprehensive characterization of human pluripotent stem cells. Stem Cell Rev 11:357–372
12. Balmayor ER, Feichtinger GA, Azevedo HS, Van Griensven M, Reis RL (2009) Starch-poly-epsilon-caprolactone microparticles reduce the needed amount of BMP-2. Clin Orthop Relat Res 467:3138–3148
13. Barak MM, Lieberman DE, Hublin JJ (2013) Of mice, rats and men: trabecular bone architecture in mammals scales to body mass with negative allometry. J Struct Biol 183:123–131
14. Behravesh E, Jo S, Zygourakis K, Mikos AG (2002) Synthesis of in situ cross-linkable macroporous biodegradable poly(propylene fumarate-co-ethylene glycol) hydrogels. Biomacromolecules 3:374–381
15. Bengtsson L, Schwappacher R, Roth M, Boergermann JH, Hassel S, Knaus P (2009) PP2A regulates Bmp signalling by interacting with Bmp receptor complexes and by dephosphorylating both the C-terminus and the linker region of Smad1. J Cell Sci 122:1248–1257
16. Bernardo ME, Avanzini MA, Perotti C, Cometa AM, Moretta A, Lenta E, Del Fante C, Novara F, De Silvestri A, Amendola G, Zuffardi O, Maccario R, Locatelli F (2007) Optimization of in vitro expansion of human multipotent mesenchymal stromal cells for cell-therapy approaches: further insights in the search for a fetal calf serum substitute. J Cell Physiol 211:121–130
17. Bessa PC, Balmayor ER, Azevedo HS, Nurnberger S, Casal M, Van Griensven M, Reis RL, Redl H (2010) Silk fibroin microparticles as carriers for delivery of human recombinant Bmps. Physical characterization and drug release. J Tissue Eng Regen Med 4:349–355
18. Bidarra SJ, Barrias CC, Granja PL (2014) Injectable alginate hydrogels for cell delivery in tissue engineering. Acta Biomater 10:1646–1662
19. Bieback K, Hecker A, Kocaomer A, Lannert H, Schallmoser K, Strunk D, Kluter H (2009) Human alternatives to fetal bovine serum for the expansion of mesenchymal stromal cells from bone marrow. Stem Cells 27:2331–2341
20. Blackwood KA, Bock N, Dargaville TR, Woodruff MA (2012) Scaffolds for growth factor delivery as applied to bone tissue engineering. Int J Polymer Sci 2012:174942
21. Bouvet-Gerbettaz S, Boukhechba F, Balaguer T, Schmid-Antomarchi H, Michiels JF, Scimeca JC, Rochet N (2014) Adaptive immune response inhibits ectopic mature bone formation induced by BMSCs/BCP/plasma composite in immune-competent mice. Tissue Eng Part A 20:2950–2962
22. Capelli C, Domenghini M, Borleri G, Bellavita P, Poma R, Carobbio A, Mico C, Rambaldi A, Golay J, Introna M (2007) Human platelet lysate allows expansion and clinical grade production of mesenchymal stromal cells from mall samples of bone marrow asopirates or marrow filter washouts. Bone Marrow Transplant 40:785–791
23. Capelli C, Pedrini O, Valgardsdottir R, Da Roit F, Golay J, Introna M (2015) Clinical grade expansion of MSCs. Immunol Lett 168(2):222–227
24. Caron MM, Emans PJ, Cremers A, Surtel DA, Coolsen MM, Van Rhijn LW, Welting TJ (2013) Hypertrophic differentiation during chondrogenic differentiation of progenitor cells is stimulated by BMP-2 but suppressed by BMP-7. Osteoarthritis Cartilage 21:604–613
25. Carragee EJ, Hurwitz EL, Weiner BK (2011) A critical review of recombinant human bone morphogenetic protein-2 trials in spinal surgery: emerging safety concerns and lessons learned. Spine J 11:471–491
26. Chanavaz M (1995) Anatomy and histophysiology of the periosteum: quantification of the periosteal blood supply to the adjacent bone with 85Sr and gamma spectrometry. J Oral Implantol 21:214–219
27. Chang H, Knothe Tate ML (2012) Concise review: the periosteum: tapping into a reservoir of clinically useful progenitor cells. Stem Cells Transl Med 1:480–491
28. Cho TJ, Gerstenfeld LC, Einhorn TA (2002) Differential temporal expression of members of the transforming growth factor beta superfamily during murine fracture healing. J Bone Miner Res 17:513–520
29. Cipitria A, Wagermaier W, Zaslansky P, Schell H, Reichert JC, Fratzl P, Hutmacher DW, Duda GN (2015) BMP delivery complements the guiding effect of scaffold architecture without

altering bone microstructure in critical-sized long bone defects: A multiscale analysis. Acta Biomater 23:282–294
30. Coleman CM, Scheremeta BH, Boyce AT, Mauck RL, Tuan RS (2011) Delayed fracture healing in growth differentiation factor 5-deficient mice: a pilot study. Clin Orthop Relat Res 469:2915–2924
31. Colnot C (2009) Skeletal cell fate decisions within periosteum and bone marrow during bone regeneration. J Bone Miner Res 24:274–282
32. Cooper GM, Mooney MP, Gosain AK, Campbell PG, Losee JE, Huard J (2010) Testing the critical size in calvarial bone defects: revisiting the concept of a critical-size defect. Plast Reconstr Surg 125:1685–1692
33. Das SP, Ganesh S, Pradhan S, Singh D, Mohanty RN (2014) Effectiveness of recombinant human bone morphogenetic protein-7 in the management of congenital pseudoarthrosis of the tibia: a randomised controlled trial. Int Orthop 38:1987–1992
34. De Bari C, Dell'accio F, Luyten FP (2001) Human periosteum-derived cells maintain phenotypic stability and chondrogenic potential throughout expansion regardless of donor age. Arthritis Rheum 44:85–95
35. Dickerson M, Winquist N, Bae Y (2014) Photo-inducible crosslinked nanoassemblies for pH-controlled drug release. Pharm Res 31:1254–1263
36. Diefenderfer DL, Osyczka AM, Garino JP, Leboy PS (2003a) Regulation of BMP-induced transcription in cultured human bone marrow stromal cells. J Bone Joint Surg Am 85-A(Suppl 3):19–28
37. Diefenderfer DL, Osyczka AM, Reilly GC, Leboy PS (2003b) BMP responsiveness in human mesenchymal stem cells. Connect Tissue Res 44(Suppl 1):305–311
38. Dudley AT, Lyons KM, Robertson EJ (1995) A requirement for bone morphogenetic protein-7 during development of the mammalian kidney and eye. Genes Dev 9:2795–2807
39. Duhamel H (1742) Sur le devéloppement et la crue des os des animaux. Mem Acad R Sci Paris 55:354–370
40. Ebisawa T, Fukuchi M, Murakami G, Chiba T, Tanaka K, Imamura T, Miyazono K (2001) Smurf1 interacts with transforming growth factor-beta type I receptor through Smad7 and induces receptor degradation. J Biol Chem 276:12477–12480
41. Ebisawa T, Tada K, Kitajima I, Tojo K, Sampath TK, Kawabata M, Miyazono K, Imamura T (1999) Characterization of bone morphogenetic protein-6 signaling pathways in osteoblast differentiation. J Cell Sci 112(Pt 20):3519–3527
42. Edwards RB 3rd, Seeherman HJ, Bogdanske JJ, Devitt J, Vanderby R Jr, Markel MD (2004) Percutaneous injection of recombinant human bone morphogenetic protein-2 in a calcium phosphate paste accelerates healing of a canine tibial osteotomy. J Bone Joint Surg Am 86-A:1425–1438
43. Estes BT, Wu AW, Guilak F (2006) Potent induction of chondrocytic differentiation of human adipose-derived adult stem cells by bone morphogenetic protein 6. Arthritis Rheum 54: 1222–1232
44. FDA (2001) *Op-1™ – H010002* [Online]. Available: http://www.Fda.Gov/Medicaldevices/Productsandmedicalprocedures/Deviceapprovalsandclearances/Recently-Approveddevices/Ucm085026.Htm
45. Fernandez Vallone VB, Romaniuk MA, Choi H, Labovsky V, Otaegui J, Chasseing NA (2013) Mesenchymal stem cells and their use in therapy: what has been achieved? Differentiation 85:1–10
46. Flierl MA, Smith WR, Mauffrey C, Irgit K, Williams AE, Ross E, Peacher G, Hak DJ, Stahel PF (2013) Outcomes and complication rates of different bone grafting modalities in long bone fracture nonunions: a retrospective cohort study in 182 patients. J Orthop Surg Res 8:33
47. Fong K, Truong V, Foote CJ, Petrisor B, Williams D, Ristevski B, Sprague S, Bhandari M (2013) Predictors of nonunion and reoperation in patients with fractures of the tibia: an observational study. BMC Musculoskelet Disord 14:103

48. Franz S, Rammelt S, Scharnweber D, Simon JC (2011) Immune responses to implants - a review of the implications for the design of immunomodulatory biomaterials. Biomaterials 32:6692–6709
49. Friedlaender GE, Perry CR, Cole JD, Cook SD, Cierny G, Muschler GF, Zych GA, Calhoun JH, Laforte AJ, Yin S (2001) Osteogenic protein-1 (bone morphogenetic protein-7) in the treatment of tibial nonunions. J Bone Joint Surg Am 83-A(Suppl 1):S151–S158
50. Fuentealba LC, Eivers E, Ikeda A, Hurtado C, Kuroda H, Pera EM, De Robertis EM (2007) Integrating patterning signals: Wnt/GSK3 regulates the duration of the BMP/Smad1 signal. Cell 131:980–993
51. Gan Q, Zhu JY, Yuan Y, Liu HL, Qian JC, Lib YS, Liu CS (2015) A dual-delivery system of pH-responsive chitosan-functionalized mesoporous silica nanoparticles bearing BMP-2 and dexamethasone for enhanced bone regeneration. J Mater Chem B 3:2056–2066
52. Geiger M, Li RH, Friess W (2003) Collagen sponges for bone regeneration with rhBMP-2. Adv Drug Deliv Rev 55:1613–1629
53. Giannoudis PV, Panteli M, Calori GM (2014) Bone healing: the diamond concept. Eur Instructional Lect 14(14):3–16
54. Gilboa L, Nohe A, Geissendorfer T, Sebald W, Henis YI, Knaus P (2000) Bone morphogenetic protein receptor complexes on the surface of live cells: a new oligomerization mode for serine/threonine kinase receptors. Mol Biol Cell 11:1023–1035
55. Govender S, Csimma C, Genant HK, Valentin-Opran A, Amit Y, Arbel R, Aro H, Atar D, Bishay M, Borner MG, Chiron P, Choong P, Cinats J, Courtenay B, Feibel R, Geulette B, Gravel C, Haas N, Raschke M, Hammacher E, Van Der Velde D, Hardy P, Holt M, Josten C, Ketterl RL, Lindeque B, Lob G, Mathevon H, Mccoy G, Marsh D, Miller R, Munting E, Oevre S, Nordsletten L, Patel A, Pohl A, Rennie W, Reynders P, Rommens PM, Rondia J, Rossouw WC, Daneel PJ, Ruff S, Ruter A, Santavirta S, Schildhauer TA, Gekle C, Schnettler R, Segal D, Seiler H, Snowdowne RB, Stapert J, Taglang G, Verdonk R, Vogels L, Weckbach A, Wentzensen A, Wisniewski T, Group, B. M. P. E. I. S. F. T. T. S (2002) Recombinant human bone morphogenetic protein-2 for treatment of open tibial fractures: a prospective, controlled, randomized study of four hundred and fifty patients. J Bone Joint Surg Am 84-A:2123–2134
56. Greenblatt MB, Shim JH, Zou W, Sitara D, Schweitzer M, Hu D, Lotinun S, Sano Y, Baron R, Park JM, Arthur S, Xie M, Schneider MD, Zhai B, Gygi S, Davis R, Glimcher LH (2010) The p38 MAPK pathway is essential for skeletogenesis and bone homeostasis in mice. J Clin Invest 120:2457–2473
57. Greenwald J, Groppe J, Gray P, Wiater E, Kwiatkowski W, Vale W, Choe S (2003) The BMP7/ActRII extracellular domain complex provides new insights into the cooperative nature of receptor assembly. Mol Cell 11:605–617
58. Grenier G, Leblanc E, Faucheux N, Lauzier D, Kloen P, Hamdy RC (2013) BMP-9 expression in human traumatic heterotopic ossification: a case report. Skelet Muscle 3:29
59. Grimsrud CD, Romano PR, D'souza M, Puzas JE, Reynolds PR, Rosier RN, O'keefe RJ (1999) BMP-6 is an autocrine stimulator of chondrocyte differentiation. J Bone Miner Res 14: 475–482
60. Guenther CA, Wang Z, Li E, Tran MC, Logan CY, Nusse R, Pantalena-Filho L, Yang GP, Kingsley DM (2015) A distinct regulatory region of the Bmp5 locus activates gene expression following adult bone fracture or soft tissue injury. Bone 77:31–41
61. Guicheux J, Lemonnier J, Ghayor C, Suzuki A, Palmer G, Caverzasio J (2003) Activation of p38 mitogen-activated protein kinase and c-Jun-NH2-terminal kinase by BMP-2 and their implication in the stimulation of osteoblastic cell differentiation. J Bone Miner Res 18:2060–2068
62. Guo YY, Zhang J, Zheng YF, Yang J, Zhu XQ (2011) Cytotoxic and genotoxic effects of multi-wall carbon nanotubes on human umbilical vein endothelial cells in vitro. Mutat Res 721:184–191
63. Guyot Y, Papantoniou I, Chai YC, Van Bael S, Schrooten J, Geris L (2014) A computational model for cell/ECM growth on 3D surfaces using the level set method: a bone tissue engineering case study. Biomech Model Mechanobiol 13:1361–1371

64. Gysel C (1983) Henri-Louis Duhamel du Monceau (1700-1782-1982), growth and osteogenic function of the periosteum. Orthod Fr 54:605–621
65. Haidar ZS, Hamdy RC, Tabrizian M (2009) Delivery of recombinant bone morphogenetic proteins for bone regeneration and repair. Part A: current challenges in BMP delivery. Biotechnol Lett 31:1817–1824
66. Hamasaki M, Hashizume Y, Yamada Y, Katayama T, Hohjoh H, Fusaki N, Nakashima Y, Furuya H, Haga N, Takami Y, Era T (2012) Pathogenic mutation of ALK2 inhibits induced pluripotent stem cell reprogramming and maintenance: mechanisms of reprogramming and strategy for drug identification. Stem Cells 30:2437–2449
67. Reggie C. Hamdy, Juan S. Rendon and Maryam Tabrizian (2012). Distraction Osteogenesis and Its Challenges in Bone Regeneration, Bone Regeneration, Prof. Haim Tal (Ed.), InTech, DOI: 10.5772/32229. Available from: http://www.intechopen.com/books/bone-regeneration/distraction-osteogenesis-and-itschallenges-in-bone-regeneration
68. Harada S, Rodan GA (2003) Control of osteoblast function and regulation of bone mass. Nature 423:349–355
69. Hartung A, Bitton-Worms K, Rechtman MM, Wenzel V, Boergermann JH, Hassel S, Henis YI, Knaus P (2006) Different routes of bone morphogenic protein (BMP) receptor endocytosis influence BMP signaling. Mol Cell Biol 26:7791–7805
70. Hata A, Lagna G, Massague J, Hemmati-Brivanlou A (1998) Smad6 inhibits BMP/Smad1 signaling by specifically competing with the Smad4 tumor suppressor. Genes Dev 12:186–197
71. Health, U. S. N. I. O. 2015. *Clinicaltrials.Gov* [Online]. Available: https://Clinicaltrials.Gov/Ct2/Results?Term=Bmp%2c+Fracture&Search=Search. Accessed 28 Aug 2015
72. Hoffmann A, Preobrazhenska O, Wodarczyk C, Medler Y, Winkel A, Shahab S, Huylebroeck D, Gross G, Verschueren K (2005) Transforming growth factor-beta-activated kinase-1 (TAK1), a MAP3K, interacts with Smad proteins and interferes with osteogenesis in murine mesenchymal progenitors. J Biol Chem 280:27271–27283
73. Hollinger JO, Schmitt JM, Buck DC, Shannon R, Joh SP, Zegzula HD, Wozney J (1998) Recombinant human bone morphogenetic protein-2 and collagen for bone regeneration. J Biomed Mater Res 43:356–364
74. Horner EA, Kirkham J, Wood D, Curran S, Smith M, Thomson B, Yang XB (2010) Long bone defect models for tissue engineering applications: criteria for choice. Tissue Eng Part B Rev 16:263–271
75. Ilizarov S (2006). The ilizarov method: history and scope. In: Rozbruch R, Ilizarov S (eds) Limb lengthening and reconstruction surgery. CPC Press. Florida, USA
76. Jain D, Bar-Shalom D (2014) Alginate drug delivery systems: application in context of pharmaceutical and biomedical research. Drug Dev Ind Pharm 40:1576–1584
77. Janowska-Wieczorek A, Majka M, Ratajczak J, Ratajczak MZ (2001) Autocrine/paracrine mechanisms in human hematopoiesis. Stem Cells 19:99–107
78. Ji W, Wang H, Van Den Beucken JJ, Yang F, Walboomers XF, Leeuwenburgh S, Jansen JA (2012) Local delivery of small and large biomolecules in craniomaxillofacial bone. Adv Drug Deliv Rev 64:1152–1164
79. Jiang Y, Chen C, Li Z, Guo W, Gegner JA, Lin S, Han J (1996) Characterization of the structure and function of a new mitogen-activated protein kinase (p38beta). J Biol Chem 271:17920–17926
80. Johnson GL, Lapadat R (2002) Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science 298:1911–1912
81. Jones AL, Bucholz RW, Bosse MJ, Mirza SK, Lyon TR, Webb LX, Pollak AN, Golden JD, Valentin-Opran A, Group, B. M. P. E. I. S. F. T. T.-A. S (2006) Recombinant human BMP-2 and allograft compared with autogenous bone graft for reconstruction of diaphyseal tibial fractures with cortical defects. A randomized, controlled trial. J Bone Joint Surg Am 88:1431–1441
82. Jun JH, Yoon WJ, Seo SB, Woo KM, Kim GS, Ryoo HM, Baek JH (2010) BMP2-activated Erk/MAP kinase stabilizes Runx2 by increasing p300 levels and histone acetyltransferase activity. J Biol Chem 285:36410–36419

83. Katagiri T, Tsukamoto S (2013) The unique activity of bone morphogenetic proteins in bone: a critical role of the Smad signaling pathway. Biol Chem 394:703–714
84. Kato M, Namikawa T, Terai H, Hoshino M, Miyamoto S, Takaoka K (2006a) Ectopic bone formation in mice associated with a lactic acid/dioxanone/ethylene glycol copolymer-tricalcium phosphate composite with added recombinant human bone morphogenetic protein-2. Biomaterials 27:3927–3933
85. Kato M, Toyoda H, Namikawa T, Hoshino M, Terai H, Miyamoto S, Takaoka K (2006b) Optimized use of a biodegradable polymer as a carrier material for the local delivery of recombinant human bone morphogenetic protein-2 (rhBMP-2). Biomaterials 27: 2035–2041
86. Kim HD, Valentini RF (2002) Retention and activity of BMP-2 in hyaluronic acid-based scaffolds in vitro. J Biomed Mater Res 59:573–584
87. King JA, Marker PC, Seung KJ, Kingsley DM (1994) BMP5 and the molecular, skeletal, and soft-tissue alterations in short ear mice. Dev Biol 166:112–122
88. Kisiel M, Martino MM, Ventura M, Hubbell JA, Hilborn J, Ossipov DA (2013) Improving the osteogenic potential of BMP-2 with hyaluronic acid hydrogel modified with integrin-specific fibronectin fragment. Biomaterials 34:704–712
89. Kiskinis E, Eggan K (2010) Progress toward the clinical application of patient-specific pluripotent stem cells. J Clin Invest 120:51–59
90. Kloen P, Di Paola M, Borens O, Richmond J, Perino G, Helfet DL, Goumans MJ (2003) BMP signaling components are expressed in human fracture callus. Bone 33:362–371
91. Kloen P, Doty SB, Gordon E, Rubel IF, Goumans MJ, Helfet DL (2002) Expression and activation of the BMP-signaling components in human fracture nonunions. J Bone Joint Surg Am 84-A:1909–1918
92. Kloen P, Lauzier D, Hamdy RC (2012) Co-expression of BMPs and BMP-inhibitors in human fractures and non-unions. Bone 51:59–68
93. Kloxin AM, Kasko AM, Salinas CN, Anseth KS (2009) Photodegradable hydrogels for dynamic tuning of physical and chemical properties. Science 324:59–63
94. Knaus P, Sebald W (2001) Cooperativity of binding epitopes and receptor chains in the BMP/TGFbeta superfamily. Biol Chem 382:1189–1195
95. Knychala J, Bouropoulos N, Catt CJ, Katsamenis OL, Please CP, Sengers BG (2013) Pore geometry regulates early stage human bone marrow cell tissue formation and organisation. Ann Biomed Eng 41:917–930
96. Koenig BB, Cook JS, Wolsing DH, Ting J, Tiesman JP, Correa PE, Olson CA, Pecquet AL, Ventura F, Grant RA et al (1994) Characterization and cloning of a receptor for BMP-2 and BMP-4 from NIH 3 T3 cells. Mol Cell Biol 14:5961–5974
97. Korchynskyi O, Ten Dijke P (2002) Identification and functional characterization of distinct critically important bone morphogenetic protein-specific response elements in the Id1 promoter. J Biol Chem 277:4883–4891
98. Kozawa O, Hatakeyama D, Uematsu T (2002) Divergent regulation by p44/p42 MAP kinase and p38 MAP kinase of bone morphogenetic protein-4-stimulated osteocalcin synthesis in osteoblasts. J Cell Biochem 84:583–589
99. Kuboki Y, Jin Q, Takita H (2001) Geometry of carriers controlling phenotypic expression in BMP-induced osteogenesis and chondrogenesis. J Bone Joint Surg Am 83-A(Suppl 1):S105–S115
100. Kugimiya F, Kawaguchi H, Kamekura S, Chikuda H, Ohba S, Yano F, Ogata N, Katagiri T, Harada Y, Azuma Y, Nakamura K, Chung UI (2005) Involvement of endogenous bone morphogenetic protein (BMP) 2 and BMP6 in bone formation. J Biol Chem 280:35704–35712
101. Kuwahara K, Fang JY, Yang Z, Han B (2011) Enzymatic crosslinking and degradation of gelatin as a switch for bone morphogenetic protein-2 activity. Tissue Eng Part A 17:2955–2964
102. L O (1867) Traite Experimental Et Clinique De La Regeration Des Os Et De La Production Artificiele Du Tissu Osseux. In: Masson V (ed) Paris

103. Lai CF, Cheng SL (2002) Signal transductions induced by bone morphogenetic protein-2 and transforming growth factor-beta in normal human osteoblastic cells. J Biol Chem 277:15514–15522
104. Langer R, Vacanti JP (1993) Tissue engineering. Science 260:920–926
105. Le Blanc K, Frassoni F, Ball L, Locatelli F, Roelofs H, Lewis I, Lanino E, Sundberg B, Bernardo ME, Remberger M, Dini G, Egeler RM, Bacigalupo A, Fibbe W, Ringden O (2008) Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. Lancet 371:1579–1586
106. Lechner C, Zahalka MA, Giot JF, Moller NP, Ullrich A (1996) ERK6, a mitogen-activated protein kinase involved in C2C12 myoblast differentiation. Proc Natl Acad Sci U S A 93:4355–4359
107. Lee J, Choi WI, Tae G, Kim YH, Kang SS, Kim SE, Kim SH, Jung Y, Kim SH (2011) Enhanced regeneration of the ligament-bone interface using a poly(L-lactide-co-epsilon-caprolactone) scaffold with local delivery of cells/BMP-2 using a heparin-based hydrogel. Acta Biomater 7:244–257
108. Lee WH, Loo CY, Rohanizadeh R (2014) A review of chemical surface modification of bioceramics: effects on protein adsorption and cellular response. Colloids Surf B Biointerfaces 122:823–834
109. Lenas P, Moos M, Luyten FP (2009a) Developmental engineering: a new paradigm for the design and manufacturing of cell-based products. Part I: from three-dimensional cell growth to biomimetics of in vivo development. Tissue Eng Part B Rev 15:381–394
110. Lenas P, Moos M, Luyten FP (2009b) Developmental engineering: a new paradigm for the design and manufacturing of cell-based products. Part II: from genes to networks: tissue engineering from the viewpoint of systems biology and network science. Tissue Eng Part B Rev 15:395–422
111. Levi B, James AW, Nelson ER, Peng M, Wan DC, Commons GW, Lee M, Wu B, Longaker MT (2011) Acute skeletal injury is necessary for human adipose-derived stromal cell-mediated calvarial regeneration. Plast Reconstr Surg 127:1118–1129
112. Li X, Liu H, Niu X, Yu B, Fan Y, Feng Q, Cui FZ, Watari F (2012) The use of carbon nanotubes to induce osteogenic differentiation of human adipose-derived MSCs in vitro and ectopic bone formation in vivo. Biomaterials 33:4818–4827
113. Liu D, Yi C, Zhang D, Zhang J, Yang M (2010) Inhibition of proliferation and differentiation of mesenchymal stem cells by carboxylated carbon nanotubes. ACS Nano 4:2185–2195
114. Liu T, Wu G, Zheng Y, Wismeijer D, Everts V, Liu Y (2014a) Cell-mediated BMP-2 release from a novel dual-drug delivery system promotes bone formation. Clin Oral Implants Res 25:1412–1421
115. Liu X, Zhao K, Gong T, Song J, Bao CY, Luo E, Weng J, Zhou SB (2014b) Delivery of growth factors using a smart porous nanocomposite scaffold to repair a mandibular bone defect. Biomacromolecules 15:1019–1030
116. Liu Y, Wang L, Kikuiri T, Akiyama K, Chen C, Xu X, Yang R, Chen W, Wang S, Shi S (2011) Mesenchymal stem cell-based tissue regeneration is governed by recipient T lymphocytes via IFN-gamma and TNF-alpha. Nat Med 17:1594–1601
117. Liu Y, Yang R, Shi S (2015) Systemic infusion of mesenchymal stem cells improves cell-based bone regeneration via upregulation of regulatory T cells. Tissue Eng Part A 21:498–509
118. Lopez-Rovira T, Chalaux E, Massague J, Rosa JL, Ventura F (2002) Direct binding of Smad1 and Smad4 to two distinct motifs mediates bone morphogenetic protein-specific transcriptional activation of Id1 gene. J Biol Chem 277:3176–3185
119. Luo G, Hofmann C, Bronckers AL, Sohocki M, Bradley A, Karsenty G (1995) BMP-7 is an inducer of nephrogenesis, and is also required for eye development and skeletal patterning. Genes Dev 9:2808–2820
120. Lutolf MP, Hubbell JA (2005) Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. Nat Biotechnol 23:47–55

121. Lyon T, Scheele W, Bhandari M, Koval KJ, Sanchez EG, Christensen J, Valentin A, Huard F (2013) Efficacy and safety of recombinant human bone morphogenetic protein-2/calcium phosphate matrix for closed tibial diaphyseal fracture: a double-blind, randomized, controlled phase-II/III trial. J Bone Joint Surg Am 95:2088–2096
122. Ma J, Both SK, Yang F, Cui FZ, Pan J, Meijer GJ, Jansen JA, Van Den Beucken JJ (2014) Concise review: cell-based strategies in bone tissue engineering and regenerative medicine. Stem Cells Transl Med 3:98–107
123. Magyari K, Baia L, Vulpoi A, Simon S, Popescu O, Simon V (2015) Bioactivity evolution of the surface functionalized bioactive glasses. J Biomed Mater Res B Appl Biomater 103:261–272
124. Maherali N, Hochedlinger K (2008) Guidelines and techniques for the generation of induced pluripotent stem cells. Cell Stem Cell 3:595–605
125. Mantripragada VP, Jayasuriya AC (2014) Injectable chitosan microparticles incorporating bone morphogenetic protein-7 for bone tissue regeneration. J Biomed Mater Res A 102:4276–4289
126. Marolt D, Knezevic M, Novakovic GV (2010) Bone tissue engineering with human stem cells. Stem Cell Res Ther 1:10
127. Marsell R, Einhorn TA (2011) The biology of fracture healing. Injury 42:551–555
128. Marti M, Mulero L, Pardo C, Morera C, Carrio M, Laricchia-Robbio L, Esteban CR, Izpisua Belmonte JC (2013) Characterization of pluripotent stem cells. Nat Protoc 8:223–253
129. Martinez-Sanz E, Ossipov DA, Hilborn J, Larsson S, Jonsson KB, Varghese OP (2011) Bone reservoir: injectable hyaluronic acid hydrogel for minimal invasive bone augmentation. J Control Release 152:232–240
130. Matthews BG, Grcevic D, Wang L, Hagiwara Y, Roguljic H, Joshi P, Shin DG, Adams DJ, Kalajzic I (2014) Analysis of alphaSMA-labeled progenitor cell commitment identifies notch signaling as an important pathway in fracture healing. J Bone Miner Res 29:1283–1294
131. Megas P (2005) Classification of non-union. Injury 36(Suppl 4):S30–S37
132. Mendez-Ferrer S, Michurina TV, Ferraro F, Mazloom AR, Macarthur BD, Lira SA, Scadden DT, Ma'ayan A, Enikolopov GN, Frenette PS (2010) Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. Nature 466:829–834
133. Mikic B, Van Der Meulen MC, Kingsley DM, Carter DR (1996) Mechanical and geometric changes in the growing femora of BMP-5 deficient mice. Bone 18:601–607
134. Mont MA, Ragland PS, Biggins B, Friedlaender G, Patel T, Cook S, Etienne G, Shimmin A, Kildey R, Rueger DC, Einhorn TA (2004) Use of bone morphogenetic proteins for musculoskeletal applications – an overview. J Bone Joint Surg Am 86a:41–55
135. Mountziaris PM, Mikos AG (2008) Modulation of the inflammatory response for enhanced bone tissue regeneration. Tissue Eng Part B Rev 14:179–186
136. Mukai T, Otsuka F, Otani H, Yamashita M, Takasugi K, Inagaki K, Yamamura M, Makino H (2007) TNF-alpha inhibits BMP-induced osteoblast differentiation through activating SAPK/JNK signaling. Biochem Biophys Res Commun 356:1004–1010
137. Muraglia A, Cancedda R, Quarto R (2000) Clonal mesenchymal progenitors from human bone marrow differentiate in vitro according to a hierarchical model. J Cell Sci 113(Pt 7):1161–1166
138. Nilsson O, Parker EA, Hegde A, Chau M, Barnes KM, Baron J (2007) Gradients in bone morphogenetic protein-related gene expression across the growth plate. J Endocrinol 193:75–84
139. Nohe A, Hassel S, Ehrlich M, Neubauer F, Sebald W, Henis YI, Knaus P (2002) The mode of bone morphogenetic protein (BMP) receptor oligomerization determines different BMP-2 signaling pathways. J Biol Chem 277:5330–5338
140. Ong KL, Villarraga ML, Lau E, Carreon LY, Kurtz SM, Glassman SD (2010) Off-label use of bone morphogenetic proteins in the United States using administrative data. Spine (Phila Pa 1976) 35:1794–1800
141. Ortuno MJ, Ruiz-Gaspa S, Rodriguez-Carballo E, Susperregui AR, Bartrons R, Rosa JL, Ventura F (2010) p38 regulates expression of osteoblast-specific genes by phosphorylation of osterix. J Biol Chem 285:31985–31994

142. Osyczka AM, Damek-Poprawa M, Wojtowicz A, Akintoye SO (2009) Age and skeletal sites affect BMP-2 responsiveness of human bone marrow stromal cells. Connect Tissue Res 50:270–277
143. Osyczka AM, Diefenderfer DL, Bhargave G, Leboy PS (2004) Different effects of BMP-2 on marrow stromal cells from human and rat bone. Cells Tissues Organs 176:109–119
144. Pages G, Guerin S, Grall D, Bonino F, Smith A, Anjuere F, Auberger P, Pouyssegur J (1999) Defective thymocyte maturation in p44 MAP kinase (Erk 1) knockout mice. Science 286:1374–1377
145. Pan W, Wei Y, Zhou L, Li D (2011) Comparative in vivo study of injectable biomaterials combined with BMP for enhancing tendon graft osteointegration for anterior cruciate ligament reconstruction. J Orthop Res 29:1015–1021
146. Papantoniou I, Mantalaris A, Sonnaert M, Lambrechts T, Aerts J-M, Geris L, Schrooten J (2014) Product and process design: toward industrial TE manufacturing. In: Van Blitterswijk CA, De Boer J (eds) Tissue engineering, 2nd ed. Ed.: Elsevier.
147. Park DJ, Choi BH, Zhu SJ, Huh JY, Kim BY, Lee SH (2005) Injectable bone using chitosan-alginate gel/mesenchymal stem cells/BMP-2 composites. J Craniomaxillofac Surg 33:50–54
148. Pereira RF, O'hara MD, Laptev AV, Halford KW, Pollard MD, Class R, Simon D, Livezey K, Prockop DJ (1998) Marrow stromal cells as a source of progenitor cells for nonhematopoietic tissues in transgenic mice with a phenotype of osteogenesis imperfecta. Proc Natl Acad Sci U S A 95:1142–1147
149. Perry MJ, Mcdougall KE, Hou SC, Tobias JH (2008) Impaired growth plate function in bmp-6 null mice. Bone 42:216–225
150. Pritchard EM, Kaplan DL (2011) Silk fibroin biomaterials for controlled release drug delivery. Expert Opin Drug Deliv 8:797–811
151. Pulskamp K, Diabate S, Krug HF (2007) Carbon nanotubes show no sign of acute toxicity but induce intracellular reactive oxygen species in dependence on contaminants. Toxicol Lett 168:58–74
152. Qutachi O, Vetsch JR, Gill D, Cox H, Scurr DJ, Hofmann S, Muller R, Quirk RA, Shakesheff KM, Rahman CV (2014) Injectable and porous PLGA microspheres that form highly porous scaffolds at body temperature. Acta Biomater 10:5090–5098
153. Ratanavaraporn J, Furuya H, Tabata Y (2012) Local suppression of pro-inflammatory cytokines and the effects in BMP-2-induced bone regeneration. Biomaterials 33:304–316
154. Reichert JC, Saifzadeh S, Wullschleger ME, Epari DR, Schutz MA, Duda GN, Schell H, Van Griensven M, Redl H, Hutmacher DW (2009) The challenge of establishing preclinical models for segmental bone defect research. Biomaterials 30:2149–2163
155. Ricard N, Ciais D, Levet S, Subileau M, Mallet C, Zimmers TA, Lee SJ, Bidart M, Feige JJ, Bailly S (2012) BMP9 and BMP10 are critical for postnatal retinal vascular remodeling. Blood 119:6162–6171
156. Roberts SJ, Van Gastel N, Carmeliet G, Luyten FP (2015) Uncovering the periosteum for skeletal regeneration: the stem cell that lies beneath. Bone 70:10–18
157. Rockwood DN, Preda RC, Yucel T, Wang X, Lovett ML, Kaplan DL (2011) Materials fabrication from *Bombyx mori* silk fibroin. Nat Protoc 6:1612–1631
158. Rumpler M, Woesz A, Dunlop JW, Van Dongen JT, Fratzl P (2008) The effect of geometry on three-dimensional tissue growth. J R Soc Interface 5:1173–1180
159. Sacchetti B, Funari A, Michienzi S, Di Cesare S, Piersanti S, Saggio I, Tagliafico E, Ferrari S, Robey PG, Riminucci M, Bianco P (2007) Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. Cell 131:324–336
160. Saito N, Okada T, Horiuchi H, Ota H, Takahashi J, Murakami N, Nawata M, Kojima S, Nozaki K, Takaoka K (2003) Local bone formation by injection of recombinant human bone morphogenetic protein-2 contained in polymer carriers. Bone 32:381–386
161. Samavarchi-Tehrani P, Golipour A, David L, Sung HK, Beyer TA, Datti A, Woltjen K, Nagy A, Wrana JL (2010) Functional genomics reveals a BMP-driven mesenchymal-to-epithelial transition in the initiation of somatic cell reprogramming. Cell Stem Cell 7:64–77

162. Sapkota G, Alarcon C, Spagnoli FM, Brivanlou AH, Massague J (2007) Balancing BMP signaling through integrated inputs into the Smad1 linker. Mol Cell 25:441–454
163. Sapkota G, Knockaert M, Alarcon C, Montalvo E, Brivanlou AH, Massague J (2006) Dephosphorylation of the linker regions of Smad1 and Smad2/3 by small C-terminal domain phosphatases has distinct outcomes for bone morphogenetic protein and transforming growth factor-beta pathways. J Biol Chem 281:40412–40419
164. Sawkins MJ, Mistry P, Brown BN, Shakesheff KM, Bonassar LJ, Yang J (2015) Cell and protein compatible 3D bioprinting of mechanically strong constructs for bone repair. Biofabrication 7:035004
165. Schmierer B, Hill CS (2007) TGFbeta-SMAD signal transduction: molecular specificity and functional flexibility. Nat Rev Mol Cell Biol 8:970–982
166. Schmitz JP, Hollinger JO (1986) The critical size defect as an experimental model for craniomandibulofacial nonunions. Clin Orthop Relat Res (205):299–308
167. Schwappacher R, Weiske J, Heining E, Ezerski V, Marom B, Henis YI, Huber O, Knaus P (2009) Novel crosstalk to BMP signalling: cGMP-dependent kinase I modulates BMP receptor and Smad activity. EMBO J 28:1537–1550
168. Schwartz AL, Ciechanover A (2009) Targeting proteins for destruction by the ubiquitin system: implications for human pathobiology. Annu Rev Pharmacol Toxicol 49:73–96
169. Scott MA, Levi B, Askarinam A, Nguyen A, Rackohn T, Ting K, Soo C, James AW (2012) Brief review of models of ectopic bone formation. Stem Cells Dev 21:655–667
170. Seeherman H, Li R, Bouxsein M, Kim H, Li XJ, Smith-Adaline EA, Aiolova M, Wozney JM (2006) rhBMP-2/calcium phosphate matrix accelerates osteotomy-site healing in a nonhuman primate model at multiple treatment times and concentrations. J Bone Joint Surg Am 88:144–160
171. Segklia A, Seuntjens E, Elkouris M, Tsalavos S, Stappers E, Mitsiadis TA, Huylebroeck D, Remboutsika E, Graf D (2012) Bmp7 regulates the survival, proliferation, and neurogenic properties of neural progenitor cells during corticogenesis in the mouse. PLoS One 7: E34088
172. Sekiya I, Colter DC, Prockop DJ (2001) BMP-6 enhances chondrogenesis in a subpopulation of human marrow stromal cells. Biochem Biophys Res Commun 284:411–418
173. Sekiya I, Vuoristo JT, Larson BL, Prockop DJ (2002) In vitro cartilage formation by human adult stem cells from bone marrow stroma defines the sequence of cellular and molecular events during chondrogenesis. Proc Natl Acad Sci U S A 99:4397–4402
174. Selever J, Liu W, Lu MF, Behringer RR, Martin JF (2004) Bmp4 in limb bud mesoderm regulates digit pattern by controlling AER development. Dev Biol 276:268–279
175. Serra T, Ortiz-Hernandez M, Engel E, Planell JA, Navarro M (2014) Relevance of PEG in PLA-based blends for tissue engineering 3D-printed scaffolds. Mater Sci Eng C Mater Biol Appl 38:55–62
176. Shen J, James AW, Zara JN, Asatrian G, Khadarian K, Zhang JB, Ho S, Kim HJ, Ting K, Soo C (2013) BMP2-induced inflammation can be suppressed by the osteoinductive growth factor NELL-1. Tissue Eng Part A 19:2390–2401
177. Shim JH, Greenblatt MB, Xie M, Schneider MD, Zou W, Zhai B, Gygi S, Glimcher LH (2009) TAK1 is an essential regulator of BMP signalling in cartilage. EMBO J 28: 2028–2041
178. Shimasaki S, Moore RK, Otsuka F, Erickson GF (2004) The bone morphogenetic protein system in mammalian reproduction. Endocr Rev 25:72–101
179. Shin H, Quinten Ruhe P, Mikos AG, Jansen JA (2003) In vivo bone and soft tissue response to injectable, biodegradable oligo(poly(ethylene glycol) fumarate) hydrogels. Biomaterials 24:3201–3211
180. Shin M, Ohte S, Fukuda T, Sasanuma H, Yoneyama K, Kokabu S, Miyamoto A, Tsukamoto S, Hohjoh H, Jimi E, Katagiri T (2013) Identification of a novel bone morphogenetic protein (BMP)-inducible transcript, BMP-inducible transcript-1, by utilizing the conserved BMP-responsive elements in the Id genes. J Bone Miner Metab 31:34–43

181. Shu B, Zhang M, Xie R, Wang M, Jin H, Hou W, Tang D, Harris SE, Mishina Y, O'keefe RJ, Hilton MJ, Wang Y, Chen D (2011) BMP2, but not BMP4, is crucial for chondrocyte proliferation and maturation during endochondral bone development. J Cell Sci 124:3428–3440
182. Solloway MJ, Dudley AT, Bikoff EK, Lyons KM, Hogan BL, Robertson EJ (1998) Mice lacking Bmp6 function. Dev Genet 22:321–339
183. Squier CA, Ghoneim S, Kremenak CR (1990) Ultrastructure of the periosteum from membrane bone. J Anat 171:233–239
184. Stephan SJ, Tholpady SS, Gross B, Petrie-Aronin CE, Botchway EA, Nair LS, Ogle RC, Park SS (2010) Injectable tissue-engineered bone repair of a rat calvarial defect. Laryngoscope 120:895–901
185. Storm EE, Huynh TV, Copeland NG, Jenkins NA, Kingsley DM, Lee SJ (1994) Limb alterations in brachypodism mice due to mutations in a new member of the TGF beta-superfamily. Nature 368:639–643
186. Strobel LA, Rath SN, Maier AK, Beier JP, Arkudas A, Greil P, Horch RE, Kneser U (2014) Induction of bone formation in biphasic calcium phosphate scaffolds by bone morphogenetic protein-2 and primary osteoblasts. J Tissue Eng Regen Med 8:176–185
187. Swiontkowski MF, Aro HT, Donell S, Esterhai JL, Goulet J, Jones A, Kregor PJ, Nordsletten L, Paiement G, Patel A (2006) Recombinant human bone morphogenetic protein-2 in open tibial fractures. A subgroup analysis of data combined from two prospective randomized studies. J Bone Joint Surg Am 88:1258–1265
188. Szpalski C, Barbaro M, Sagebin F, Warren SM (2012) Bone tissue engineering: current strategies and techniques--part II: Cell types. Tissue Eng Part B Rev 18:258–269
189. Szpalski C, Barr J, Wetterau M, Saadeh PB, Warren SM (2010) Cranial bone defects: current and future strategies. Neurosurg Focus 29:E8
190. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131:861–872
191. Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676
192. Tam WL, O DF, Hiramatsu K, Tsumaki N, Luyten FP, Roberts SJ (2014) Sox9 reprogrammed dermal fibroblasts undergo hypertrophic differentiation in vitro and trigger endochondral ossification in vivo. Cell Reprogram 16:29–39
193. Ten Dijke P, Yamashita H, Sampath TK, Reddi AH, Estevez M, Riddle DL, Ichijo H, Heldin CH, Miyazono K (1994) Identification of type I receptors for osteogenic protein-1 and bone morphogenetic protein-4. J Biol Chem 269:16985–16988
194. Thomas JT, Lin K, Nandedkar M, Camargo M, Cervenka J, Luyten FP (1996) A human chondrodysplasia due to a mutation in a TGF-beta superfamily member. Nat Genet 12:315–317
195. Tibbles LA, Woodgett JR (1999) The stress-activated protein kinase pathways. Cell Mol Life Sci 55:1230–1254
196. Tsuji K, Bandyopadhyay A, Harfe BD, Cox K, Kakar S, Gerstenfeld L, Einhorn T, Tabin CJ, Rosen V (2006) BMP2 activity, although dispensable for bone formation, is required for the initiation of fracture healing. Nat Genet 38:1424–1429
197. Tsuji K, Cox K, Bandyopadhyay A, Harfe BD, Tabin CJ, Rosen V (2008) BMP4 is dispensable for skeletogenesis and fracture-healing in the limb. J Bone Joint Surg Am 90(Suppl 1): 14–18
198. Tsuji K, Cox K, Gamer L, Graf D, Economides A, Rosen V (2010) Conditional deletion of BMP7 from the limb skeleton does not affect bone formation or fracture repair. J Orthop Res 28:384–389
199. Tsuruga E, Takita H, Itoh H, Wakisaka Y, Kuboki Y (1997) Pore size of porous hydroxyapatite as the cell-substratum controls BMP-induced osteogenesis. J Biochem 121: 317–324
200. Van Baardewijk LJ, Van Der Ende J, Lissenberg-Thunnissen S, Romijn LM, Hawinkels LJ, Sier CF, Schipper IB (2013) Circulating bone morphogenetic protein levels and delayed fracture healing. Int Orthop 37:523–527

201. Van Bael S, Chai YC, Truscello S, Moesen M, Kerckhofs G, Van Oosterwyck H, Kruth JP, Schrooten J (2012) The effect of pore geometry on the in vitro biological behavior of human periosteum-derived cells seeded on selective laser-melted Ti6AL4V bone scaffolds. Acta Biomater 8:2824–2834
202. Van Gastel N, Torrekens S, Roberts SJ, Moermans K, Schrooten J, Carmeliet P, Luttun A, Luyten FP, Carmeliet G (2012) Engineering vascularized bone: osteogenic and proangiogenic potential of murine periosteal cells. Stem Cells 30:2460–2471
203. Van Griensven M (2015) Preclinical testing of drug delivery systems to bone. Adv Drug Deliv Rev 94:151–164
204. Vashishth D (2008) Small animal bone biomechanics. Bone 43:794–797
205. Verne E, Vitale-Brovarone C, Bui E, Bianchi CL, Boccaccini AR (2009) Surface functionalization of bioactive glasses. J Biomed Mater Res A 90:981–992
206. Wang K, Zhou C, Hong Y, Zhang X (2012) A review of protein adsorption on bioceramics. Interface Focus 2:259–277
207. Wang L, Huang Y, Pan K, Jiang X, Liu C (2010) Osteogenic responses to different concentrations/ratios of BMP-2 and bFGF in bone formation. Ann Biomed Eng 38:77–87
208. Wang Z, Wang K, Lu X, Li M, Liu H, Xie C, Meng F, Jiang O, Li C, Zhi W (2015) Bmp-2 encapsulated polysaccharide nanoparticle modified biphasic calcium phosphate scaffolds for bone tissue regeneration. J Biomed Mater Res A 103:1520–1532
209. West FD, Roche-Rios MI, Abraham S, Rao RR, Natrajan MS, Bacanamwo M, Stice SL (2010) Kit ligand and bone morphogenetic protein signaling enhances human embryonic stem cell to germ-like cell differentiation. Hum Reprod 25:168–178
210. Winnier G, Blessing M, Labosky PA, Hogan BL (1995) Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse. Genes Dev 9:2105–2116
211. Woo EJ (2013) Adverse events after recombinant human BMP2 in nonspinal orthopaedic procedures. Clin Orthop Relat Res 471:1707–1711
212. Xu RH, Chen X, Li DS, Li R, Addicks GC, Glennon C, Zwaka TP, Thomson JA (2002) BMP4 initiates human embryonic stem cell differentiation to trophoblast. Nat Biotechnol 20:1261–1264
213. Yamamoto M, Ikada Y, Tabata Y (2001) Controlled release of growth factors based on biodegradation of gelatin hydrogel. J Biomater Sci Polym Ed 12:77–88
214. Yang HS, La WG, Bhang SH, Jeon JY, Lee JH, Kim BS (2010) Heparin-conjugated fibrin as an injectable system for sustained delivery of bone morphogenetic protein-2. Tissue Eng Part A 16:1225–1233
215. Yang HS, La WG, Cho YM, Shin W, Yeo GD, Kim BS (2012) Comparison between heparin-conjugated fibrin and collagen sponge as bone morphogenetic protein-2 carriers for bone regeneration. Exp Mol Med 44:350–355
216. Yao Y, Li W, Wu J, Germann UA, Su MS, Kuida K, Boucher DM (2003) Extracellular signal-regulated kinase 2 is necessary for mesoderm differentiation. Proc Natl Acad Sci U S A 100:12759–12764
217. Ying QL, Nichols J, Chambers I, Smith A (2003) Bmp induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. Cell 115:281–292
218. Yoshimatsu Y, Lee YG, Akatsu Y, Taguchi L, Suzuki HI, Cunha SI, Maruyama K, Suzuki Y, Yamazaki T, Katsura A, Oh SP, Zimmers TA, Lee SJ, Pietras K, Koh GY, Miyazono K, Watabe T (2013) Bone morphogenetic protein-9 inhibits lymphatic vessel formation via activin receptor-like kinase 1 during development and cancer progression. Proc Natl Acad Sci U S A 110:18940–18945
219. Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin I, Thomson JA (2007) Induced pluripotent stem cell lines derived from human somatic cells. Science 318:1917–1920
220. Yu L, Li Y, Zhao K, Tang Y, Cheng Z, Chen J, Zang Y, Wu J, Kong L, Liu S, Lei W, Wu Z (2013a) A novel injectable calcium phosphate cement-bioactive glass composite for bone regeneration. PLoS One 8:E62570

221. Yu N, Plachokova A, Yang F, Walboomers X, Jansen J (2013b) Engineering of dental tissues: scaffolds and preclinical models. In: Stem cells in craniofacial development, regeneration and repair. Wiley-Blackwell Press, Hoboken
222. Yu YY, Lieu S, Lu C, Miclau T, Marcucio RS, Colnot C (2010) Immunolocalization of BMPS, BMP antagonists, receptors, and effectors during fracture repair. Bone 46:841–851
223. Yusop AH, Bakir AA, Shaharom NA, Abdul Kadir MR, Hermawan H (2012) Porous biodegradable metals for hard tissue scaffolds: a review. Int J Biochem 2012:641430
224. Zhang H, Bradley A (1996) Mice deficient for BMP2 are nonviable and have defects in amnion/chorion and cardiac development. Development 122:2977–2986
225. Zhang H, Migneco F, Lin CY, Hollister SJ (2010) Chemically-conjugated bone morphogenetic protein-2 on three-dimensional polycaprolactone scaffolds stimulates osteogenic activity in bone marrow stromal cells. Tissue Eng Part A 16:3441–3448
226. Zhang WJ, Wang XL, Wang SY, Zhao J, Xu LY, Zhu C, Zeng DL, Chen J, Zhang ZY, Kaplan DL, Jiang XQ (2011) The use of injectable sonication-induced silk hydrogel for VEGF(165) and BMP-2 delivery for elevation of the maxillary sinus floor. Biomaterials 32:9415–9424
227. Zhang XS, Zheng GQ, Wang JQ, Zhang YG, Zhang GQ, Li ZL, Wang Y (2013) Porous Ti6al4v scaffold directly fabricated by sintering: preparation and in vivo experiment. J Nanomater 2013:205076
228. Zhong C, Wu J, Reinhart-King CA, Chu CC (2010) Synthesis, characterization and cytotoxicity of photo-crosslinked maleic chitosan-polyethylene glycol diacrylate hybrid hydrogels. Acta Biomater 6:3908–3918
229. Zhou T, Benda C, Dunzinger S, Huang Y, Ho JC, Yang J, Wang Y, Zhang Y, Zhuang Q, Li Y, Bao X, Tse HF, Grillari J, Grillari-Voglauer R, Pei D, Esteban MA (2012) Generation of human induced pluripotent stem cells from urine samples. Nat Protoc 7:2080–2089
230. Zhu J (2010) Bioactive modification of poly(Ethylene Glycol) hydrogels for tissue engineering. Biomaterials 31:4639–4656

BMP Signaling in Articular Cartilage Repair and Regeneration: Potential Therapeutic Opportunity for Osteoarthritis

Susan Chubinskaya and David C. Rueger

Abstract Eight years ago we reviewed the role of bone morphogenetic proteins (BMPs) in articular cartilage repair in the last edition of this series on BMPs. Since that time our understanding of the function of BMPs and especially BMP-7, also called osteogenic protein-1 (OP-1), in cartilage homeostasis and repair has significantly increased. The primary focus of this chapter is the potential therapeutic opportunity for BMPs in the treatment of osteoarthritis. The intervening data confirm that among BMPs, BMP-7 exhibits the most robust evidence supporting its use for cartilage repair and regeneration. In the current review, we continue to unravel more of the underlying mechanisms of the anabolic and anti-catabolic activities of BMPs to provide a better understanding of the interactions between BMPs and signaling pathways and highlight the increased role BMP-7 and other BMPs play in human cartilage homeostasis. In regard to in vivo activities, exciting new data have been published demonstrating that BMP-7, in multiple models of osteoarthritis, can delay or inhibit degradation of the articular cartilage. Most interesting is that for the first time, a clinical trial has been reported, and Phase I data evaluation of the effect of a single injection of BMP-7 into osteoarthritic knees demonstrated enough of a positive response to warrant a Phase II study. Together, recent studies continue to indicate a significant opportunity for BMPs and particularly BMP-7 as therapeutics for osteoarthritis.

Keywords BMP-7 • Articular Cartilage Homeostasis • Osteoarthritis • Cartilage repair

1 Introduction

Cartilage repair and regeneration is a major obstacle in orthopedic medicine [1, 2]. Mature human articular cartilage has a limited innate ability to regenerate. The consequence is enormous since osteoarthritis (OA) is a major cause of disability among

S. Chubinskaya (✉)
Department of Pediatrics, Rush University Medical Center, Chicago, IL 60612, USA
e-mail: susanna_chubinskaya@rush.edu

D.C. Rueger
Rueger Consulting, Southborough, MA 01772, USA
e-mail: dwkrueger@msn.com

S. Vukicevic, K.T. Sampath (eds.), *Bone Morphogenetic Proteins: Systems Biology Regulators*, Progress in Inflammation Research,
DOI 10.1007/978-3-319-47507-3_7

the adult population in the United States. Knee OA affects between 19 and 28 % of Americans over age 45 [3–4] and, of this number, a significant number had an identifiable acute trauma to the joint. OA is considered a disease of the whole joint. In regard to cartilage, it is viewed as a process of attempted, but gradually failing, repair of damaged extracellular matrix, as the balance between synthesis and breakdown of matrix components is disturbed and shifted toward catabolism. There is a potential window for intervention with biologic agents to prevent progression of OA or even to reverse accumulated damage. In recent times members of the bone morphogenetic protein (BMP) family of proteins have demonstrated a great potential as anabolic factors for treatment of focal osteochondral defects and posttraumatic OA because of their ability to induce matrix synthesis and promote repair in cartilage defect models [5].

Since the first BMP genes were identified in the late 1980s, the corresponding recombinant proteins have been produced, and two of these early BMPs, BMP-7 and BMP-2, have been extensively characterized both biochemically and biologically. Initial in vivo characterization involved a variety of animal models to evaluate the therapeutic potential in bone repair applications. These studies led to the demonstration of bone repair in humans and eventually in BMP-7 and BMP-2 receiving regulatory approval as the first commercial BMPs. The purpose of our previous chapter was to review the knowledge to 2008 on BMPs in cartilage biology from the standpoint of both in vitro studies and a variety of animal repair studies. The data clearly showed that BMPs have an important role in cartilage, both in normal homeostasis and in repair, and predicted a bright future for the use of certain BMPs in the engineering of cartilage.

In vitro studies demonstrated that many BMPs are endogenously expressed in cartilage, and some act as anabolic factors for chondrocytes in culture. BMP-2, BMP-3, BMP-4, BMP-5, BMP-6, and BMP-7 and GDF-5 and GDF-6 have been localized to cartilage, and BMP-7 has also been localized to synovial fluid, synovium, ligament, tendon, and meniscus. In regard to the anabolic activity, the role that exogenously applied BMPs play in stimulating chondrocyte differentiation, extracellular matrix production, and maintenance of the adult chondrocytic phenotype has been well documented. However, few direct comparisons of the different BMPs had been reported, and the only extensively studied BMP has been BMP-7. Application of recombinant BMP-7 was shown to stimulate the synthesis of all the major cartilage extracellular matrix proteins and to counteract the degenerative effect of numerous catabolic mediators. Thus the data from in vitro studies clearly demonstrated that at least one BMP, BMP-7, is very important in articular cartilage homeostasis. It was concluded that a more detailed analysis of the importance of other BMPs needed to be done.

Data from numerous studies in animals showed that at least three BMPs, BMP-7, BMP-2, and GDF-5, have therapeutic potential for cartilage repair, both in articular cartilage models and models of damaged intervertebral discs. Although most studies reported the use of recombinant proteins, a few described the potential use of a BMP in gene therapy. In several large osteochondral defect studies, both BMP-7 and BMP-2 were observed to induce a significant improvement in repair of both the cartilage and bone compartment over that observed in untreated control defects; the

BMP-treated sites exhibited less fibrocartilage and more hyaline-like cartilage. In a large chondral defect study in sheep, BMP-7 was shown to induce significant repair in a model where no repair takes place in the controls; the repair was hyaline like and well bonded with the surrounding cartilage. However, the goal of perfectly repaired cartilage was not achieved. In preliminary results of studies evaluating models of osteoarthritis (OA), BMP-7 was shown to possibly prevent the development of damage and in some models reverse the damage. It was concluded that the potential of BMPs and especially BMP-7 as therapeutics for OA should be the focus of future animal studies. There needed to be extensive evaluations of a variety of formulations, scaffolds, methods of administration, and possibly combinations with other factors. In addition a wider range of BMPs needed to be evaluated since in the limited number of BMPs tested thus far, there appeared to be differences between BMPs, and particular BMPs may be better suited for different OA stages.

The conclusion from the data presented in our 2008 chapter stated that a BMP-based therapy for damaged cartilage would appear to have significant clinical potential. The clinical demand is immense for new cartilage repair procedures particularly to address OA. Animal studies have clearly demonstrated that one BMP, BMP-7, is efficacious and can safely be delivered to the joint. It was concluded that pilot clinical studies with at least BMP-7 should be initiated. In addition, the use of an injectable BMP, most probably in a slow-release formulation, would seem to be the ideal route of administration. In the present chapter, we present data from studies that use these strategies and, in fact, demonstrate significant progress toward realizing the clinical potential of BMPs for OA. These studies have, as in our previous chapter, focused on BMP-7.

2 In Vitro Studies

Recent in vitro studies covering BMPs in cartilage repair are reviewed emphasizing data that extends the characterization of the role of BMPs in cartilage homeostasis and OA. In the first part of this section, studies are reviewed evaluating the effects of exogenously applied samples of BMPs on chondrocytes either embedded in native cartilage matrix and cultured as explants or isolated from the extracellular matrix and cultured under a variety of conditions. In the second part, studies are reviewed evaluating the expression and roles of endogenous BMPs in chondrocytes in culture or in cartilage tissue. The focus of this section will be on recent data evaluating human cartilage samples.

2.1 Exogenous Activity

In recent years, the number of BMPs evaluated under in vitro conditions has diminished with few studies reported for BMPs other than BMP-7 and BMP-2. However, one study reported a side-by-side comparison of the anabolic and anti-catabolic

activities of BMP-2, BMP-4, BMP-6, BMP-7, and CDMP-1 (GDF-5) and CDMP-2 in cultures of normal human adult articular chondrocytes [6]. Proteoglycan synthesis was stimulated to a greater extent by BMP-2, BMP-4, and BMP-7 with BMP-7 treatment resulting in maximal proteoglycan synthesis. However, only BMP-7 showed consistent anti-catabolic activity as demonstrated by restoration of proteoglycan synthesis after IL-1 treatment. Other studies have demonstrated that some BMPs, such as BMP-2, can stimulate cartilage degradation by stimulating MMP-13 production [7]. In regard to the effects on mesenchymal cell preparations, BMP-2, BMP-6, and BMP-7 can induce chondrogenesis, but the results depend on culture conditions [8]. These results confirmed the importance of BMPs and stimulated additional studies evaluating the effects of particularly BMP-7 in cartilage homeostasis.

In regard to anabolic activity, treatment of both human and animal chondrocytes with BMP-7 has demonstrated increased production of a large number of cartilage-specific extracellular proteins, such as collagens type II and VI, aggrecan, decorin, fibronectin, and hyaluronan, via upregulation of enzymes such as hyaluronan synthase [9]. When applied to other cell types in the knee, BMP-7 has been shown to increase extracellular matrix (ECM) synthesis in synovial- and bone marrow-derived MSCs, both alone and in combination with TGF-β [10–11]. This profound anabolic response stems from BMP-7 regulatory properties as a modulator of other growth factors, such as insulin-like growth factor-1 and fibroblast growth factor, as well as their receptors, kinases involved in signaling, inhibitory binding proteins, and downstream transcription factors [12]. Furthermore, BMP-7 has been shown to restore tissue responsiveness to IGF-1 [13]. The evaluation of anti-catabolic activities of BMP-7 has also been extended. BMP-7 has been shown to downregulate multiple catabolic mediators (IL-1, IL-6, IL-8, IL-11, and tumor necrosis factor [TNF-α]) and inhibit both baseline- and cytokine-induced expression of MMP-1 and MMP-13 [12]. Lastly, BMP-7 modulates expression of receptors for certain matrix components, such as CD44 [14], and the synthesis of chondrocyte cytoskeleton proteins, such as talin, paxillin, and focal adhesion kinase [15], bolstering the cartilage scaffold and strengthening newly formed tissue. While several growth factors have shown decreased efficacy with aged or diseased chondrocytes, BMP-7 induces an anabolic response across a variety of age groups and different stages of cartilage degeneration and OA [9, 16]. In summary, the recent in vitro data continue to point to BMP-7 as the BMP with the most short-term promise for cartilage repair clinical trials.

2.2 Endogenous Expression

Although clinical application of recombinant BMPs is the primary focus, the understanding of the regulation and function of endogenously expressed BMPs in adult articular cartilage offers significant supporting data. Knowledge of the mechanisms that control their synthesis, activation, induction, signaling, and interaction with

other pathways in articular cartilage provides critical information which is necessary to develop and correct strategies for the application of recombinant BMPs for cartilage restoration and repair in OA. Of importance is confirming which BMPs offer the most therapeutic opportunity.

In recent reports, there has been a renewed effort to evaluate BMP expression in cartilage and synovia from OA patients in order to determine if a correlation could be found between expression levels and the disease state. Early studies had demonstrated that BMP-2, BMP-3, BMP-4, BMP-6, BMP-7, and CDMP-1 (GDF-5) and CDMP-2 are expressed in human normal and OA cartilage [17–18]. However, in subsequent studies, different groups have reported differences in expression levels. For example, the intra-articular expression and localization of BMP-2 and BMP-7 and their receptors BMPR-1A, BMPR-1B, and BMPR-2 were evaluated in clinical samples taken from patients undergoing autologous chondrocyte implantation [19]. BMP-2, BMP-7, and BMPR-1A were found expressed in the cartilage and synovia from these knees. BMP-7 was highly expressed in all samples with BMP-2 found in about half of the samples. In addition, increased levels of BMPR-1A, but not of BMPR-1B and BMPR-2, were found in all synovia and about half of the cartilage samples. Although duration of symptoms and localization of lesions of the patients were evaluated, there was no correlation with protein expression. This data conflicted with an earlier study that showed BMP-2 to be more consistently expressed in the knees with local chondromalacia compared to BMP-7 [20]. In this study BMP-2 but not BMP-7 levels were associated with a better clinical outcome. However, BMP-7 expression has been extensively investigated in a variety of cartilage samples from both normal donors and OA patients. It was found that BMP-7 gene and protein expressions were dramatically reduced with cartilage aging and degeneration [16]. It was suggested that one of the mechanisms responsible for this decrease in BMP-7 production with aging is the methylation of the BMP-7 promoter [21]. This might provide an explanation for the apparent conflict in the results on BMP expression reported by different groups.

Early studies had demonstrated endogenously expressed and synthesized BMP-7 in adult articular cartilage of a variety of species, suggesting that this BMP had a functional role in the maintenance of normal cartilage homeostasis. This was confirmed by inhibition studies with a BMP-7 antisense probe [22], where transfection of human adult articular chondrocytes with the BMP-7 probe led to about 70 % inhibition in the BMP-7 gene expression. The downregulation of BMP-7 mRNA induced a significant inhibition of aggrecan expression, aggrecan core protein synthesis, and PG synthesis. Histological evaluation of cartilage explants cultured in the presence of the BMP-7 antisense oligonucleotides revealed a remarkable depletion of prostaglandins, paucity of chondrocytes, initial fibrillation of the cartilage surface, and a decrease in Safranin O staining in the upper and middle cartilage zones. Similar results were obtained with the inhibition of BMP-7 gene expression using the siRNA approach. Thus these data, together with previous results, provide strong evidence for endogenous BMP-7 being a critical factor that controls cartilage matrix integrity and is involved in the maintenance of normal cartilage homeostasis.

In addition, the data strongly suggest that the lack of endogenous BMP-7 could predispose cartilage to degenerate processes and make the tissue more susceptible to the influence of catabolic agents.

To confirm the role of BMP-7 in cartilage, the Affymetrix GeneChip technology was used to monitor BMP-7 regulation of 22,000 genes from the human genome with specific emphasis on genes that are relevant to adult articular cartilage [12]. These included matrix proteins, anabolic and catabolic gene products, as well as their intracellular regulators and receptors. In this analysis, the role of BMP-7 was evaluated under conditions where the BMP-7 antisense probe inhibited BMP-7 gene expression, or BMP-7 signaling was activated or enhanced by recombinant BMP-7 using high-density human chondrocyte monolayers. The results confirmed and considerably extended the knowledge about the role of BMP-7. In summary, the data showed that BMP-7 controls cartilage homeostasis on multiple levels including regulation of genes responsible for the chondrocyte cytoskeleton (cyclin D, Talin1, and cyclin M1), matrix production, and other anabolic pathways (TGF-β/BMP, IGF, VEGF, genes responsible for bone formation) as well as regulation of cytokines, neuromediators, and various catabolic pathways responsible for matrix degradation and cell death. In many of these cases, BMP-7 modulated the expression of not only the ligands but also their receptors, mediators of downstream signaling, kinases responsible for an activation of the pathways, binding proteins responsible for the inhibition of the pathways, and transcription factors that induce transcriptional responses. Of most importance, the data led to the conclusion that BMP-7 is clearly a unique growth factor in its capacity to display simultaneously pro-anabolic and anti-catabolic activities.

Finally, gene knockout studies have recently been conducted to further explore the role of endogenous locally produced BMP-7 in the joint. Since early studies showed that the complete knockout of the BMP-7 gene led to perinatal lethality of the mice [23–24], limb mesenchyme-specific BMP-7 conditional knockout mice were established to define the roles of BMP-7 in potential bone and joint homeostasis [25]. Initial studies examined bone homeostasis and demonstrated that the conditional deletion of endogenous BMP-7 from the limb skeleton did not affect bone formation or fracture repair in these animals. Subsequent studies evaluated the role of endogenous BMP-7 in the cartilage of these animals [26]. Mice were sacrificed at 4, 8, and 24 weeks for evaluation. The results demonstrated that the absence of BMP-7 led to a significant reduction at 24 weeks in the amount of proteoglycan and aggrecan present in the articular cartilage and an increase in the MMP-13. In addition, extensive synovial hyperplasia and macrophage infiltration were observed, as well as enhanced expression of activin A, a proinflammatory cytokine. In the early time points observed, there was no effect on the formation of articular cartilage, but with age the cartilage degeneration became evident. In regard to type II collagen expression, there were no changes observed at any time point, and histological analysis showed that cartilage morphology and thickness were normal, as was the morphology of the meniscus and underlying bone. The data led the authors to suggest that other factors, along with BMP-7, are necessary for the progression of OA. In regard to other BMPs, earlier data from these authors [27] demonstrated

reduced type II collagen expression during cartilage formation in their study using BMP-2 conditional knockout mice, but there was no extensive cartilage evaluation done as with the current BMP-7 study. The BMP-7 analysis also demonstrated that endogenous BMP-7 was not directly involved in the proliferation, hypertrophic differentiation, and survival of articular chondrocytes. These results are in contrast to data reported using exogenous BMP-7 [28], but the authors suggested the difference may be due to the high doses used in those studies. In summarizing their data, the authors believe that loss of locally produced BMP-7 clearly leads to degenerative changes in articular cartilage, and these changes correlate with the development of age-related OA. Thus the data is consistent with a critical role of endogenous BMP-7 activity in synovial joint homeostasis and suggests along with the data from the in vitro studies that supplementing endogenous BMP-7 with recombinant BMP-7 may be beneficial to prevent or slow the development of OA.

3 Animal Studies

The pivotal role of BMPs in the development and regeneration process of the skeleton had originally suggested a role in articular cartilage repair. Furthermore, the accumulation of data from in vitro studies has clearly demonstrated that certain BMPs have an important role in chondrocyte differentiation and extracellular matrix production as well as the maintenance of adult chondrocyte phenotype. For the most part, two BMPs, BMP-7 and BMP-2, were the subjects of most of the initial animal models of cartilage repair, although another BMP, GDF-5 (also called CDMP-1 or MP-52), was also investigated [5]. Studies were reported in a wide range of animal species and involved both articular cartilage models, as well as non-articular cartilage tissue, particularly the intervertebral disc. Numerous studies were reported using deep osteochondral defects in articular cartilage with the BMPs delivered locally into the defect site on a collagen scaffold that was press fitted into the defect site. However, a few studies were also reported using the more difficult chondral (partial thickness) defect models where the defect did not penetrate the calcified cartilage layer. For these studies the BMP was delivered by a variety of methods to the defects, including via a mini-pump, into the synovial fluid. To summarize, these animal studies demonstrated that certain BMPs can improve both cartilage and bone repair in osteochondral defects. However, the repaired tissues are not perfect, and some studies show the repaired cartilage may not be stable over extended periods. Few studies have been done with chondral defects, but the data showed that the repaired cartilage is more hyaline than has been obtained using osteochondral defects. In regard to OA, treatments are seldom administered at the time of injury, and most patients with impending or early osteoarthritis will have areas of hypocellular cartilage matrix, superficial zone delamination, and fibrillation. Thus, the animal study data relating to repair of focal chondral and osteochondral defects also supports the use of BMPs in early or posttraumatic OA. Since we last reviewed animal studies, the research focus has been on extending the investigations

evaluating BMPs for the treatment of OA. Studies have been reported with BMP-7 using several established animal models of OA, but it is important to recognize that the majority of these models represent posttraumatic or early OA, in which a well-defined insult to the joint has been introduced (Table 7.1). These studies have involved rats, rabbits, and sheep, and OA was induced using anterior cruciate ligament (ACL) transection, impaction, or mechanical stress models. For the most part, BMP-7 was injected into the knee, but one study delivered BMP-7 via an implanted osmotic pump. In this section, we review these results.

A series of studies were done evaluating the potential of liquid BMP-7 to inhibit articular cartilage damage using the rabbit ACL transection model of induced OA [29–30]. The BMP-7 was delivered via Alzet osmotic pump implanted in the thigh with an intra-articular catheter. An initial study was done implanting the pump at the same time as the ACL transection. The BMP-7 was delivered for approximately 6 weeks, and the animals sacrificed at 9 weeks. Analysis showed a clear effect in reducing the development of OA by gross observation, histological staining, and semiquantitative polymerase chain reaction (PCR). A subsequent study to evaluate BMP-7 as a treatment when OA had already developed was done using the same model, but the pump was implanted 4 weeks after the ACL transection, and the animals were sacrificed 5 weeks later. This data showed that BMP-7 again had a positive effect in reducing the amount of cartilage loss, and this delayed treatment showed only a slightly smaller protective effect when comparing gross histology and histomorphometry. Interestingly, both studies also showed enhanced expression of the anabolic genes aggrecan and collagen type II, and decreases in the catabolic mediators aggrecanase, MMP-3 and MMP-13 in the BMP-7-treated joints.

A second series of studies were done evaluating the potential of liquid BMP-7 to inhibit articular cartilage damage [31–32], but those were conducted in rats using a strenuous running model to induce OA, and BMP-7 was injected rather than continuously delivered via osmotic pump. Five days after cessation of running,

Table 7.1 OA animal studies: BMP-7 as therapeutic

Citation	Species	Formulation	Model	Treatment	OA progression
Badlani et al. (2008) [29]	Rabbit	Liquid	ACL transection	At 0 weeks/osmotic pump for 6 weeks	Prevention
Badlani et al. (2009) [30]	Rabbit	Liquid	ACL transection	At 4 weeks/osmotic pump for 6 weeks	Inhibition
Sekiya et al. (2009) [31]	Rat	Liquid	Strenuous running	At 5 days/6 weekly injections	Inhibition
Hayashi et al. (2010) [32]	Rabbit	Liquid	ACL transection	At 0 weeks At 4 weeks/8 weekly injections	Prevention Inhibition
Hurtig et al. (2009) [28]	Sheep	Putty	Impaction	At 0 weeks At 3 weeks At 12 weeks/two injections, 1 week apart	Prevention Inhibition No effect

BMP-7 was injected into the test knee and repeated five more times at weekly intervals, and the animals sacrificed 1 week later. The analysis showed that although BMP-7 did not block progression of OA completely, it significantly delayed progression of cartilage degeneration in this model. As a result of the positive data, studies were extended to the ACL transection model in rabbits. BMP-7 was injected intra-articularly at weekly intervals starting immediately after transection and at 4 weeks after transection for a total of eight injections. After sacrifice at 12 weeks, the knees were evaluated by gross morphology, histology, immunohistochemistry, and micro-CT. The results showed when BMP-7 was injected immediately after transection there was inhibition of progression of cartilage degeneration, and thus BMP-7 demonstrated an ability to prevent the development of OA in this model. When BMP-7 was injected 4 weeks after transection and mild degeneration had already taken place in the joint, the data demonstrated that BMP-7 inhibited the progression of cartilage degeneration. In other words, BMP-7 did not induce regeneration of the damaged cartilage but delayed further damage; all BMP-7-treated knees showed less OA damage than the control knees by all analytical tools. In addition, there were no adverse effects observed such as osteophytes or ectopic bone or fibrosis.

Finally, the most advanced series of studies were done using a sheep impaction model for OA and an injectable formulation of BMP-7. This formulation, called BMP-7 Putty, was bound to type I collagen and mixed with carboxymethyl cellulose and phosphate-buffered saline [28]. In advance of these studies, bioavailability of BMP-7 was evaluated by injecting BMP-7 Putty into the knee joint of adult sheep and the BMP-7 levels in the synovial fluid measured periodically for 5 days. Peak synovial fluid BMP-7 concentrations occurred 24 hours after intra-articular injection (1.9 μg +/−0.17 μg/ml), and detectable levels were still present at 48 and 72 hours (80 ng +/−1.0 ng/ml and 4.7 ng +/−1.9 ng/ml, respectively). BMP-7 was administered bound to the collagen vehicle because the half-life of liquid BMP-7 was believed to be on the order of a few hours. In this model of direct injury to the knee joint [33], focal cartilage lesions developed in the medial femorotibial joint compartment of horses, dogs, and sheep by 3 months and progressed to severe medial femorotibial compartment arthritis by 6 months. The ability of BMP-7 to preempt lesion development and progression was tested in six sheep by intra-articular injection of 300 μg of BMP-7 in the putty formulation at the time of injury and 1 week later. The contralateral limb received carrier alone. BMP-7 injections resulted in significant improvements in histological scores, cell viability, and proteoglycan content of the injured cartilage 12 weeks after injury. Lesions at the impact site were absent or very subtle in three of six animals and consisted of minor superficial zone delamination in the remaining three; however, all six contralateral joints had severe cartilage degeneration in the medial condyle. A similar experiment using a single injection 1 week post-injury also suppressed proteoglycan loss and progression of histological degeneration in the medial femoral condyle. In subsequent experiments the ability of BMP-7 to reverse an established injury was studied by delayed administration of 300 μg BMP-7 in the putty formulation 3 and 4 weeks after injury. Assessments were made 12 weeks after the last injection. Macroscopic and histological damage to the femoral condyle was reduced, as was the C3/C4 short

collagen epitope immunostaining, the latter indicating that there was protection against metalloproteinase-mediated collagen breakdown. Another experimental group received the same dose of BMP-7 12 and 13 weeks after injury, but when these animals were sacrificed 12 weeks after the last intra-articular injection, there was only minor improvement in histological scores and no other indications of efficacy. This was not surprising given that well-established lesions were present in control animals 12 weeks post-injury, and any improvement would have required extensive regeneration and repair. However, these studies demonstrated that BMP-7 afforded protection against the development of posttraumatic cartilage degeneration when administered immediately after injury and again 1 week later. Delayed treatment 1 month after injury still prevented progression of degeneration, but the original injury remained. Delayed treatment 12 weeks after injury was not protective, and degeneration progressed beyond the original injury site. In addition, it was noted that in all the sheep experiments, there were no intra-articular bone formation or osteophytes in joints that received intra-articular BMP-7 Putty.

In summary, this body of preclinical evidence supports a role for the administration of BMP-7 in prevention and treatment of early posttraumatic injuries and osteoarthritis. Although BMP-7 seems to restore intrinsic cartilage repair, data from the sheep studies suggested that BMP-7 did not induce chondrocyte proliferation during repair but allowed survival and retention of the native chondrocytes that replenished and remodeled the damaged matrix. Despite its anabolic capacity, BMP-7 has not been shown to induce chondrocyte hypertrophy or other changes in chondrocytic phenotype, nor have BMP-7-treated animal knees displayed any histological evidence of uncontrolled fibroblast proliferation or radiographically detectable osteophyte formation. In regard to clinical studies, the duration of exposure and concentration of BMP-7 needed to create the repair response seen in the OA animal studies in patients is unknown. Furthermore, the window of opportunity to address developing lesions may differ depending upon the energy absorbed and size of the impact zone of the injury, but the sheep data suggest that in many cases, treatment within the first 4–6 weeks should be beneficial.

4 Clinical Studies

Human clinical trials evaluating BMP-7 to treat OA have begun as a result of the promising data accumulated from animal and in vitro studies. Data from in vitro studies suggesting a potential role for BMP-7 in reducing pain had also added to its importance for human testing [12]. The first clinical study reported was a Phase I study of BMP-7 used to treat symptomatic knee OA [34]. The study was a double-blind, randomized, multicenter, placebo-controlled, single-dose escalation safety study evaluating four doses of a liquid formulation of BMP-7. The primary study objective was to determine the safety and tolerability of BMP-7, and secondary objectives were to determine improvement in WOMAC pain and function and changes in OARSI responder criteria. The 33 participants enrolled had symptomatic

knee OA, were over 40 years of age, and were evaluated at 4, 8, 12, and 24 weeks. Doses of BMP-7, 0 (placebo), 0.03 mg, 0.1 mg, 0.3 mg, and 1.0 mg in 5 % lactose, were injected intra-articularly**.** The results showed more injection site pain at the highest BMP-7 dose, but otherwise there were no overall differences in toxicity or adverse event rates between BMP-7 and the placebo group, and no patient developed anti-BMP-7-binding antibodies during the study. In regard to the lack of antibodies, BMP-7 levels were evaluated 1 hour post-injection and were observed to be extremely low, suggesting a rapid clearance rate. Patients receiving the BMP-7 injections at the midrange doses (0.1 mg and 0.3 mg) reported some symptomatic improvement and anti-pain effects. These effects were not seen in the high- and low-dose cohorts. However, it was concluded that the trend to a positive response, together with the lack of toxicity provided support for the continued development of BMP-7 for the treatment of OA. As a result, a Phase II clinical study was conducted to further evaluate the BMP-7 formulations that showed the greatest promise. Although this second trial has not appeared to be successful, the data has not yet been published (personal communication). Hopefully, the reasons for such an outcome can be determined and more trials initiated.

5 Conclusion

The purpose of this chapter is to review the current knowledge of BMPs in cartilage biology with a focus on the potential as a therapeutic for OA. Since our last chapter, the data have clearly expanded what is known about the role BMPs play in normal homeostasis and in repair. A large body of additional in vitro evidence has accumulated for BMP-7 that suggests a very important role as an anabolic agent to increase matrix components and as an anti-catabolic agent to decrease components active in degeneration. These activities have translated to the effects seen in multiple animal studies using different models of OA and have demonstrated, in certain models, an inhibition or a delay of degeneration in BMP-7-treated joints. In the initial clinical study with a BMP used to treat cartilage repair, certain doses of BMP-7 injected into the knees of OA patients demonstrated some symptomatic improvement and anti-pain effects. In addition, there was no toxicity or adverse events observed in these patients.

Although a BMP-7-based therapy appears to have significant clinical potential in treating cartilage degeneration, many unanswered questions remain. Future short-term goals should include obtaining a better understanding of the pathophysiology of cartilage degeneration so that growth factor therapy can be tailored to various stages of the healing process [35–36]. Optimal doses and formulations must be determined in order to maximize clinical response and minimize side effects. In this regard, joint clearance studies must be done to evaluate whether slow delivery formulations should be developed. In addition, BMP-7 and other BMPs must be studied further in hostile, inflammatory environments to better understand their efficacy in disease states. This will likely underscore a difference in potential therapy for

posttraumatic chondral defects versus therapy for chronic degenerative joint disease, and future clinical trials must be conducted with carefully selected patient cohorts. Reliable delivery of therapeutic proteins to the synovial environment is difficult, but the stability of the BMP-7 protein confers an advantage over more labile agents, and a series of timed injections, with or without slow-release carriers, may be able to maintain therapeutic levels. This may be quite useful in the context of sports injuries where the injury time, such as a tear of the ACL, is known and surgical reconstruction anticipated. However, a significant amount of effort will need to be undertaken to address issues regarding formulation and what disease state and study end points would be most appropriate for new clinical trials.

Historically, most growth factors have been evaluated on an independent basis rather than in combination, to assess their effects on cartilage homeostasis in vitro or in vivo. Given the array and interactions of growth factors that are involved in cartilage development and homeostasis, it is possible that any single growth factor will not lead to acceptable cartilage repair, but rather a combination of factors might be required [37]. This has been shown by the fact that BMP-7 produces better cartilage repair when applied in combination with TGF-β or IGF-1[13]. BMP-7 and IGF-1 would seem to be an ideal first combination. However, other growth factors, such as other BMPs, TGF-β, PDGF, and FGF family members, have also shown some promise in cartilage repair, and recently an initial clinical study using FGF-18 has been reported [38].

Because of the difficulty in translating the potential of large proteins like BMPs to therapeutics in OA, alternative strategies that boost signaling such as small molecule inducers or antagonist inhibitors may provide an alternative route for investigators [39]. For instance, increases in the level of several BMP antagonists including noggin, chordin, and follistatin have been implicated in OA [40]. Thus inhibitors, possibly antibodies against these antagonists, could be tested for therapeutic potential, and in fact an anti-gremlin antibody has been demonstrated to be useful in ameliorating pulmonary disease in a mouse model [41]. Furthermore, small molecule agonists or antagonists could be developed and tested. In this regard, a small molecule called tilerone has been shown to increase the expression of BMP-7 [42]. Also a small peptide mimetic of BMP-7 called THR123 has been described which activates the BMP ALK3 receptor [43], and a small molecule called dorsomorphin has been described as an inhibitor of BMP signaling [44]. In addition, a new class of ALK2 inhibitor, the lead compound of which is called KO2288, has been shown to inhibit BMP-stimulated Smad1/5/8 phosphorylation [45]. In summary, these are some of the molecules of the future that demonstrate a different and exciting new path in realizing the therapeutic potential of BMPs in OA, and the well-developed in vitro models for evaluating their activities create obvious opportunities for screening these compounds.

In summary, the future of BMP-7 as an initial BMP therapeutic for OA seems bright. Aside from which formulations or delivery procedures produce optimal regeneration, future studies will need to be determined whether clinicians and researchers should strive to use exogenous recombinant BMP or perhaps to boost endogenous BMP production. Other BMPs should be evaluated, and combinations

of growth factors should be tested in animal models. Finally, in addition to protein therapy, small molecules should become a part of the OA investigations where mimetics and antagonists are compared directly with BMP-7. Thus, the potential to use BMPs as therapeutics in OA has become more complex but has also brought a significant number of new opportunities and made the future of this field very exciting. Although the clinical path will not be simple, the reward for millions of OA sufferers will be tremendous.

References

1. Moran CJ, Pascual-Garrido C, Chubinskaya S, Potter HG, Warren RF, Cole BJ, Rodeo SA (2014) Restoration of articular cartilage. J Bone Joint Surg Am 96:336–344
2. Pulsatelli L, Addimanda O, Brusi V, Pavloska B, Meliconi R (2013) New findings in osteoarthritis pathogenesis: therapeutic implications. Ther Adv Chronic Dis 4:23–43
3. Felson DT, Naimark A, Anderson J, Kazis L, Castelli W, Meenan RF (1987) The prevalence of knee osteoarthritis in the elderly. the framingham osteoarthritis study. Arthritis Rheum 30(8):914–918
4. Jordan JM, Helmick CG, Renner JB, Luta G, Dragomir AD, Woodard J, Fang F, Schwartz TA, Abbate LM, Callahan LF, Kalsbeek WD, Hochberg MC (2007) Prevalence of knee symptoms and radiographic and symptomatic knee osteoarthritis in African Americans and Caucasians: the Johnston County Osteoarthritis Project. J Rheumatol 34(1):172–180
5. Chubinskaya S, Hurtig M, Rueger DC (2008) Bone Morphogen proteins in cartilage biology. In: Vukicevic S, Sampath KT (eds) Bone morphogenetic proteins: from local to systemic therapeutics. Birkhauser, Basel, pp. 277–315
6. Chubinskaya S, Segalite D, Pikovsky D, Hakimiyan AA, Rueger DC (2007) Effects induced by BMPS in cultures of human articular chondrocytes: comparative studies. Growth Factors 26:275–283
7. van der Kraan PM, Blaney Davidson EN, van den Berg WB (2010) Bone morphogenetic proteins and articular cartilage: to serve and protect or a wolf in sheep clothing's? Osteoarthritis Cartilage 18(6):735–741
8. Kemmis CM, Vahdati A, Weiss HE, Wagner DR (2010) Bone morphogenetic protein 6 drives both osteogenesis and chondrogenesis in murine adipose-derived mesenchymal cells depending on culture conditions. Biochem Biophys Res Commun 401(1):20–25
9. Chubinskaya S, Hurtig M, Rueger DC (2007) OP-1/BMP-7 in cartilage repair. Int Orthop 31(6):773–781
10. Miyamoto C, Matsumoto T, Sakimura K, Shindo H (2007) Osteogenic protein-1 with transforming growth factor-beta1: potent inducer of chondrogenesis of synovial mesenchymal stem cells in vitro. J Orthop Sci 12(6):555–561
11. Shen B, Wei A, Whittaker S, Williams LA, Tao H, Ma DD, Diwan AD (2010) The role of BMP-7 in chondrogenic and osteogenic differentiation of human bone marrow multipotent mesenchymal stromal cells in vitro. J Cell Biochem 109(2):406–416
12. Chubinskaya S, Otten L, Soeder S, Borgia JA, Aigner T, Rueger DC, Loeser RF (2011) Regulation of chondrocyte gene expression by osteogenic protein-1. Arthritis Res Ther 13(2):R55
13. Chubinskaya S, Hakimiyan A, Pacione C, Yanke A, Rappoport L, Aigner T, Rueger DC, Loeser RF (2007) Synergistic effect of IGF-1 and OP-1 on matrix formation by normal and OA chondrocytes cultured in alginate beads. Osteoarthritis Cartilage 15(4):421–430
14. Nishida Y, Knudson CB, Eger W, Kuettner KE, Knudson W (2000) Osteogenic protein 7 stimulates cells-associated matrix assembly by normal human articular chondrocytes: up-regulation of hyaluronan synthase, CD44, and aggrecan. Arthritis Rheum 43(1):206–214

15. Vinall RL, Lo SH, Reddi AH (2002) Regulation of articular chondrocyte phenotype by bone morphogenetic protein 7, interleukin 1, and cellular context is dependent on the cytoskeleton. Exp Cell Res 272:32–44
16. Loeser RF, Gandhi U, Long DL, Yin W, Chubinskaya S (2014) Aging and oxidative stress reduce the response of human articular chondrocytes to insulin-like growth factor 1 and osteogenic protein 1. Arthritis Rheumatol 66(8):2201–2209
17. Bobinac D, Spanjol J, Marinović M, Zoricić Cvek S, Marić I, Cicvarić T, Fuckar D, Markić D, Vojniković B (2008) Expression of bone morphogenetic proteins, cartilage-derived morphogenetic proteins and related receptors in normal and osteoarthritic human articular cartilage. Coll Antropol 32(Suppl 2):83–87
18. Bobacz K, Gruber R, Soleiman A, Erlacher L, Smolen JS, Graninger WB (2003) Expression of bone morphogenetic protein 6 in healthy and osteoarthritic human articular chondrocytes and stimulation of matrix synthesis in vitro. Arthritis Rheum 48(9):2501–2508
19. Schmal H, Mehlhorn AT, Pilz IH, Dovi-Akue D, Kirchhoff C, Südkamp NP, Gerlach U, Lohrmann C, Niemeyer P (2012) Immunohistological localization of BMP-2, BMP-7, and their receptors in knee joints with focal cartilage lesions. Scientific World Journal 2012:467892
20. Schmal H, Niemeyer P, Zwingmann J, Stoffel F, Südkamp NP, Mehlhorn AT (2010) Association between expression of the bone morphogenetic. proteins 2 and 7 in the repair of circumscribed cartilage lesions with clinical outcome. BMC Musculoskelet Disord 11:170
21. Loeser RF, Im HJ, Richardson B, Lu Q, Chubinskaya S (2009) Methylation of the OP-1 promoter: potential role in the age-related decline in OP-1 expression in cartilage. Osteoarthritis Cartilage 17(4):513–517
22. Soeder S, Hakimiyan A, Rueger D, Kuettner KE, Aigner T, Chubinskaya S (2005) Antisense inhibition of osteogenic protein-1 disturbs human articular cartilage integrity. Arthritis Rheum 52(2):468–478
23. Dudley A, Lyons K, Robertson E (1995) A requirement for bone morphogenetic protein-7 during development of the mammalian kidney and eye. Genes Dev 9:2795–2807
24. Luo G, Hofmann C, Bronckers A, Sohock M, Bradley A et al (1995) BMP-7 is an inducer of nephrogenesis, and is also required for eye development and skeletal patterning. Genes Dev 9:2808–2820
25. Tsuji K, Cox K, Gamer L, Graf D, Economides A, Rosen V (2010) Conditional deletion of BMP7 from the limb skeleton does not affect bone formation or fracture repair. J Orthop Res 28:384–389
26. Abula K, Muneta T, Miyatake K, Yamada J, Matsukura Y, Inoue M, Sekiya I, Graf D, Economides AN, Rosen V, Tsuji K (2015) Elimination of BMP7 from the developing limb mesenchyme leads to articular cartilage degeneration and synovial inflammation with increased age. FEBS Lett 589(11):1240–1248
27. Tsuji K, Bandyopadhyay A, Harfe BD, Cox K, Kakar S, Gerstenfeld L, Einhorn T, Tabin CJ, Rosen V (2006) BMP2 activity, although dispensable for bone formation, is required for the initiation of fracture healing. Nat Genet 38(12):1424–1429
28. Hurtig M, Chubinskaya S, Dickey J, Rueger D (2009) BMP-7 protects against progression of cartilage degeneration after impact injury. J Orthop Res 27(5):602–611
29. Badlani N, Inoue A, Healey R, Coutts R, Amiel D (2008) The protective effect of OP-1 on articular cartilage in the development of osteoarthritis. Osteoarthritis Cartilage 16(5): 600–606
30. Badlani N, Oshima Y, Healey R, Coutts R, Amiel D (2009) Use of bone morphogenic protein-7 as a treatment for osteoarthritis. Clin Orthop Relat Res 467(12):3221–3229
31. Sekiya I, Tang T, Hayashi M, Morito T, Ju YJ, Mochizuki T, Muneta T (2009) Periodic knee injections of BMP-7 delay cartilage degeneration induced by excessive running in rats. J Orthop Res 27(8):1088–1092
32. Hayashi M, Muneta T, Takahashi T, Ju YJ, Tsuji K, Sekiya I (2010) Intra-articular injections of bone morphogenetic protein-7 retard progression of existing cartilage degeneration. J Orthop Res 28(11):1502–1506

33. Bolam C, Hurtig M, Cruz A, McEwen B (2006) Characterization of a model of post-traumatic osteoarthritis in the equine femorotibial joint. Am J Vet Res 67(3):433–447
34. Hunter DJ, Pike MC, Jonas BL, Kissin E, Krop J, McAlindon T (2010) Phase 1 safety and tolerability study of BMP-7 in symptomatic knee osteoarthritis. BMC Musculoskelet Disord 11:232
35. Anderson DD, Chubinskaya S, Guilak F, Martin JA, Oegema TR, Olson SA, Buckwalter JA (2011) Post-traumatic osteoarthritis: improved understanding and opportunities for early intervention. J Orthop Res 29:802–809
36. Sakata R, Iwakura T, Reddi AH (2015) Regeneration of articular cartilage surface: morphogens, cells, and extracellular matrix scaffolds. Tissue Eng Part B 21:461–473
37. Fortier LA, Barker JU, Strauss EJ, McCarrel TM, Cole BJ (2011) The role of growth factors in cartilage repair. Clin Orthop Relat Res 469:2706–2715
38. Lohmander LS, Hellot S, Dreher D, Krantz EF, Kruger DS, Guermazi A, Eckstein F (2014) Intraarticular spifermin (recombinant human fibroblast growth factor 18) in knee osteoarthritis: a randomized, double-blind, placebo-controlled trial. Arthritis Rheumatol 66:1820–1831
39. Ali IH, Brazil DP (2014) Bone morphogenetic proteins and their antagonists: current and emerging clinical uses. Br J Pharmacol 171(15):3620–3632
40. Tardif G, Pelletier JP, Boileau C, Martel-Pelletier J (2009) The BMP antagonists follistatin and gremlin in normal and early osteoarthritic cartilage: an immunohistochemical study. Osteoarthritis Cartilage 17:263–270
41. Ciuclan L, Sheppard K, Dong L, Sutton D, Duggan N, Hussey M et al (2013) Treatment with anti-gremlin 1 antibody ameliorates chronic hypoxia/SU5416-induced pulmonary arterial hypertension in mice. Am J Pathol 183:1461–1473
42. Lepparanta O, Tikkanen JM, Bespalov MM, Kali K, Myllarniemi M (2013) Bone morphogenetic protein-inducer tilorone identified by high-throughput screening is antifibrotic in vivo. Am J Respir Cell Mol Biol 48:448–455
43. Sugimoto H, LeBleu VS, Bosukonda D, Keck P, Taduri G, Bechtel W et al (2012) Activin-like kinase 3 is important for kidney regeneration and reversal of fibrosis. Nat Med 18:396–404
44. Yu PB, Hong CC, Sachidanandan C, Babitt JL, Deng DY, Hoyng SA et al (2008b) Dorsomorphin inhibits BMP signals required for embryogenesis and iron metabolism. Nat Chem Biol 4:33–41
45. Sanvitale CE, Kerr G, Chaikuad A, Ramel MC, Mohedas AH, Reichert S et al (2013) A new class of small molecule inhibitor of BMP signaling. PLoS One 8:e62721

BMPs in Orthopaedic Medicine: Promises and Challenges

Peter V. Giannoudis and Nikolaos K. Kanakaris

Abstract Over the last 50 years the concept of inducing bone formation, using biologically active signalling molecules, has evolved significantly. The most potent of these osteoinductive molecules remain the Bone Morphogenetic Proteins, with established role on the chemotaxis, proliferation and differentiation of mesenchymal progenitor cells to form cartilage and bone.

The initial enthusiasm following the synthetic production of BMP2 and BMP7 using recombinant gene technology, was followed by an expansion of their use "in-" and "off-label" in clinical practice, on parallel to a large number of basic science and translational medicine studies attempting to define further their effect.

The key role of BMPs in bone repair stimulated their widespread use in the orthopaedic discipline including the management of delayed union and non-union of fractures, bone defects, open fractures, fusion of joints, spinal fusions, as well as treatment of osteoarthritis and intervertebral disc cartilage degeneration. It is quite evident that rhBMPs in humans have a different dose–response relationship in comparison to animal species, as well as that the final outcome of their use is also relevant to the specifics of their carrier and delivery system, their containment, the timing of their application, as well as the state of the recipient host local environment. The different effect of different BMPs, and their variable interaction with inhibiting molecules and negative feedback mechanisms, are nowadays better understood, widening further the horizon of contemporary research of bone, as well as of cartilage regeneration.

Conflict of Interest No funds were received in support of this study. No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article

P.V. Giannoudis, MB, BSc, MD, FACS, FRCS (Eng) (✉)
Academic Department of Trauma & Orthopaedics, School of Medicine, University of Leeds, Leeds, UK

NIHR Leeds Biomedical Research Unit, Chapel Allerton Hospital, LS7 4SA Leeds, West Yorkshire, Leeds, UK
e-mail: pgiannoudi@aol.com

N.K. Kanakaris
Academic Department of Trauma & Orthopaedics, School of Medicine, University of Leeds, Leeds, UK

S. Vukicevic, K.T. Sampath (eds.), *Bone Morphogenetic Proteins: Systems Biology Regulators*, Progress in Inflammation Research,
DOI 10.1007/978-3-319-47507-3_8

The reputation of BMPs has been negatively affected lately due to the recent redraw from the market of their recombinant synthetic forms, which is however attributed mostly to strategic commercial planning rather than their performance. New osteoinductive molecules emerge attempting to fill in the gap, whilst the challenge of evidence based clinical practice remains.

This article presents the contemporary understanding, as well as a summary of selected published evidence on the roles of the BMPs in bone and cartilage regeneration.

Keywords BMP • Bone morphogenetic protein • Cartilage degeneration • Bone repair • Intervertebral disc degeneration

Bone morphogenetic proteins (BMPs) are biologically active signalling molecules first described by Dr Marshall Urist in 1965 [1]. They are members of the transformation growth factor-β (TGF-β) superfamily possessing a unique osteoinductive property. At least 40 different subtypes have been described to date, and these have been divided into groups according to their primary amino acid sequence [2]. It is of interest that they have been isolated from various species and have been given alternative names, for instance, BMP-7 is OP-1, BMP-8 is OP-2, BMP-12 is growth and differentiation factor 7 (GDF-7) and BMP-13 is both GDF-6 and cartilage-derived morphogenetic protein-2. A number of studies in vivo have established the role of BMPs in inducing chemotaxis, proliferation and differentiation of mesenchymal progenitor cells to form cartilage and bone [3–5]. Consequently, it has soon become evident that the properties of BMPs could have clinical benefits, and this has generated great interest for commercial exploitation. Due to the fact that human cadaver bone contains only small quantities of human BMP (hBMP), commercial production of the purified composite protein was found to be impractical. Accordingly, industry has turned to recombinant gene technology and focused on the production of those isotypes with the greatest potential for bone induction, i.e. recombinant human (rh) BMP-2 and BMP-7 (OP-1). Noteworthy, these two molecules received different levels of approval from the US Food and Drug Administration (FDA) for use in trauma surgery (rhBMP-2 has full premarket approval for the treatment of fresh (open) tibial fractures, whereas rhBMP-7 (rhOP-1) has limited approval as part of a Humanitarian Device Exemption (HDE) for use as an alternative to bone autograft for the management of recalcitrant tibial non-unions). HDE is granted by the FDA when it is believed that a small group of patients may benefit from a treatment whose effectiveness has not yet been fully proven. Under such a waiver program, a device can be used in up to 4,000 patients a year.

The osteoinductivity of a single BMP vial appears to have a dose–response relationship unaffected by the individual characteristics of the recipient. Nevertheless, BMPs must be administered to humans in higher doses compared to other species in order to attain osteoinductive activity, but the subsequent bone formation is not consistent. It is thought that the dose must overcome a certain level before successful

induction of bone formation can happen. The dose–response curve becomes steeper with the progression from rodent to nonhuman primate models. The latter species, most closely related to humans, was used to derive the human therapeutic dosages of 3.5 mg rhOP-1/4 ml sterile saline solution or 0.88 mg rhOP-1/1 ml sterile saline solution and 12 mg rhBMP-2/8 ml sterile water or 1.5 mg rhBMP-2/1 ml sterile water. Currently, in the clinical setting, rhBMPs are used at concentrations that are 10–1000 times higher than those of endogenous BMPs [6]. These high doses are implanted in an effort to fabricate a clinical effect comparable with that shown to be osteoinductive in animal studies. Higher doses of BMPs have also been dictated by the multifaceted signalling mechanisms and quick local and systemic clearance of BMPs in higher species. It has been presumed that higher species have less responding cells than do lower species, which has generated important questions regarding combination therapies of BMPs with stem or progenitor cells and the development of more efficient and more cost-effective delivery systems [6].

When rhBMPs are implanted to the site of anatomical interest with a compromised bone repair response, they exhibit quite short biological half-lives. Moreover, they are difficult to retain at sites of local application. The large bolus doses implanted are coupled with a non-uniform release. For instance, from the bolus, there is rapid flux causing saturation of the surrounding tissues with very high concentrations, thus leading to systemic exposure. Subsequent release, albeit slower, results in much inferior, suboptimal concentrations [7]. Thus, if the dose of BMP is too low, there may be inadequate bone formation, and if it is too high, there may be more bone formation and more rapid osteoinduction than anticipated [8]. The increased bone formation eventually leads to intramembranous ossification, bypassing the intermediate phase of endochondral ossification occurring when lower doses are used. Still, with high doses of BMPs, initial localised resorption of bone can be caused by an increase in osteoclastic activity, as BMPs also stimulate osteoclastogenesis [9]. It has not been proven as yet under what specific conditions or with what predisposing risk factors resorption of bone may be elicited. Local overdoses of BMPs could be expected to lead to heterotopic ossification, but this phenomenon has not been consistently observed under physiological conditions [10]. A variety of carrier and delivery systems for BMPs have been explored including synthetic polymers, natural-origin polymers, inorganic materials and composites. Carriers range from nanoparticles to complex three-dimensional scaffolds, membranes for tissue-guided regeneration, biomimetic surfaces and smart thermosensitive hydrogels [11]. Carrier systems are usually absorbed over time, helping to sustain the concentration of the rhBMP at the treatment site, provide temporary scaffolding for osteogenesis and prevent ectopic bone formation. The rhBMP and carrier may be implanted in the recipient area using a delivery system that could also provide mechanical support. Interbody fusion cages being used for interbody spinal fusion represent an illustration of this concept [12]. As carrier and delivery systems are variables with great importance and different clinical applications require different dosages of rhBMP with different carriers and delivery systems, the results of one clinical application cannot be generalised to others [13].

Parameters such as the optimal therapeutic dosages, delivery systems and local conditions for bone repair are still under exploration. Moreover, it should be emphasised that fundamental surgical management to offer suitable environmental

circumstances of the recipient site (soft tissue coverage, host tissue bed vitality and biomechanical stability) continues to be considered essential. Overall, a more comprehensive understanding of the mechanisms that control BMP expression and signalling is required to design the most effective carrier systems and perhaps the concept of combination therapies with other BMPs or inductive molecules. For example, it has been shown that heterotopic ossification in fibrodysplasia ossificans progressiva may not be secondary to the genetic overexpression of BMP-4 but rather to the underexpression of noggin (extracellular antagonist of BMPs) [14]. In the animal model used, excessive ossification was preventable by the local delivery of noggin, thus demonstrating a highly regulated negative feedback mechanism for BMPs that could theoretically be used to prevent abnormal or heterotopic bone formation occurring with the use of high therapeutic doses of a BMP. Thus, the action of BMPs is controlled by corresponding BMP inhibitors, involving negative feedback loops and crosstalk of various pathways in order to decrease cellular exposure to the signalling molecules and temper their cellular activities [14].

These inhibitory effects can occur at different levels of the cascade: the extracellular compartment, the receptor level itself, the intracellular compartment and the nucleus. The different levels of inhibition reveal the complexity of signal regulation during various physiological processes. The balance between all the signalling molecules involved in bone formation with their inhibitors, and most importantly between BMPs and their antagonists, is a critical determinant of osteogenesis and therefore of skeletal development, fracture repair and bone remodelling. Furthermore, the potential to suppress BMP inhibitors is emerging as a biological therapeutic target in bone tissue engineering, to achieve unopposed synergy between the various growth factors that are involved in osteogenesis, in their physiological milieu [15].

The key role of BMPs in bone repair stimulated their widespread use in the orthopaedic discipline even in an ‘off-licence mode’. The basic objective related to their use is to speed up fracture healing and consolidation in situations where this might not naturally or reliably occur. Not surprisingly therefore, they have been used for the treatment of delayed union and non-union of fractures, bone defects, open fractures, fusion of joints, spinal fusions and even cartilage regeneration. Several studies have reported on their effectiveness particularly for fracture non-unions (Table 1) [16–25], (Fig. 1), open fractures (Table 2) [26–29], fusion of joints (Table 3) [30–32] and spinal fusions (Table 4) [33–37].

The use of BMPs for cartilage regeneration is based on the fact that an intermediate phase of the process of bone formation following the activation of multipotent mesenchymal cells is to differentiate into cartilage tissue consisting of proliferative, prehypertrophic and hypertrophic chondrocytes, which secondarily are replaced by bony tissue [38, 39]. Noteworthy, the interaction of the BMPs with the different chondrocytes and their matrix has been investigated in the specific setting of articular cartilage and osteoarthritis (OA), as well as of the cartilage of the intervertebral disc and its degeneration (DDD), where the cartilage represents the main affected tissue and the chondrocytes the targeted cell population, aiming to the development of new therapeutic and/or preventive strategies. Throughout the morphogenesis of the articular cartilage during its initial development, as well as its maintenance subsequently via

Table 1 Interaction of BMPs with fracture non-unions

Molecule – action	Type of evidence	Study	Combination of use	Union rates (%)
Efficacy of BMP-7 in the treatment of femoral non-unions	Prospective observational, 30 patients	Kanakaris et al. 2009	BMP-7 with revision of fixation (23 patients) and autograft (12 patients)	86.7
Efficacy of rhBMP-7 in treatment of symptomatic malunion of the distal radius after corrective osteotomy	Randomised controlled trial, 30 patients	Ekrol et al. 2008	Comparison of rhBMP-7 to autogenous bone graft	
Efficacy of BMPs in the treatment of tibial non-unions	Retrospective cohort study, 62 patients	Desmyter et al. 2008	Evaluation of the effectiveness of BMP-7 in non-union healing procedure	84.9
Evaluate the efficacy of BMP-7 in non-reactive posttraumatic long bone non-union or critical-size bone defect	Randomised controlled trial, 29 patients	Calori et al. 2006	Compare the efficacy of BMP-7 to treat non-unions to PRP efficacy	94
Effectiveness of BMP-7 in the treatment of femoral, tibial, clavicle, ankle, radius, scaphoid, humerus, olecranon non-union	Retrospective cohort study, 395 patients	Giannoudis et al. 2005		82
Tibial, femoral, humeral, forearm non-unions treated with BMP-7	Prospective observational, 25 patients	Dimitriou et al. 2005	Bone-stimulating agent in the treatment of persistent non-unions	92.3
Effectiveness of BMP-7 in non-union tibial shaft fracture acquired secondary to trauma	Model-based cost-effectiveness analysis (source of clinical data not stated)	Van Engen et al. 2003	Intramedullary rod or Ilizarov fixation with the aid of BMP-7 or autogenous iliac crest bone graft	
Establish both the safety and efficacy of this BMP in the treatment of tibial non-unions	Controlled, prospective, randomised, partially blinded, multicentre clinical trial, 122 patients	Friedlander et al., *Journal of Bone and Joint Surgery American*, 2001	Intramedullary rod with either rhOP-1 in a type I collagen carrier or by fresh bone autograft	75–81
Efficacy of BMP in the treatment of tibial non-union	Randomised controlled trial, 80 patients	Chen et al. 2000	Comparison of BMP to autograft bone	100
Effectiveness of BMP-7 in treatment of tibial non-unions	Randomised controlled trial, 30 patients	Cook et al. 1999	Comparison of BMP-7 to autogenous iliac bone graft	86

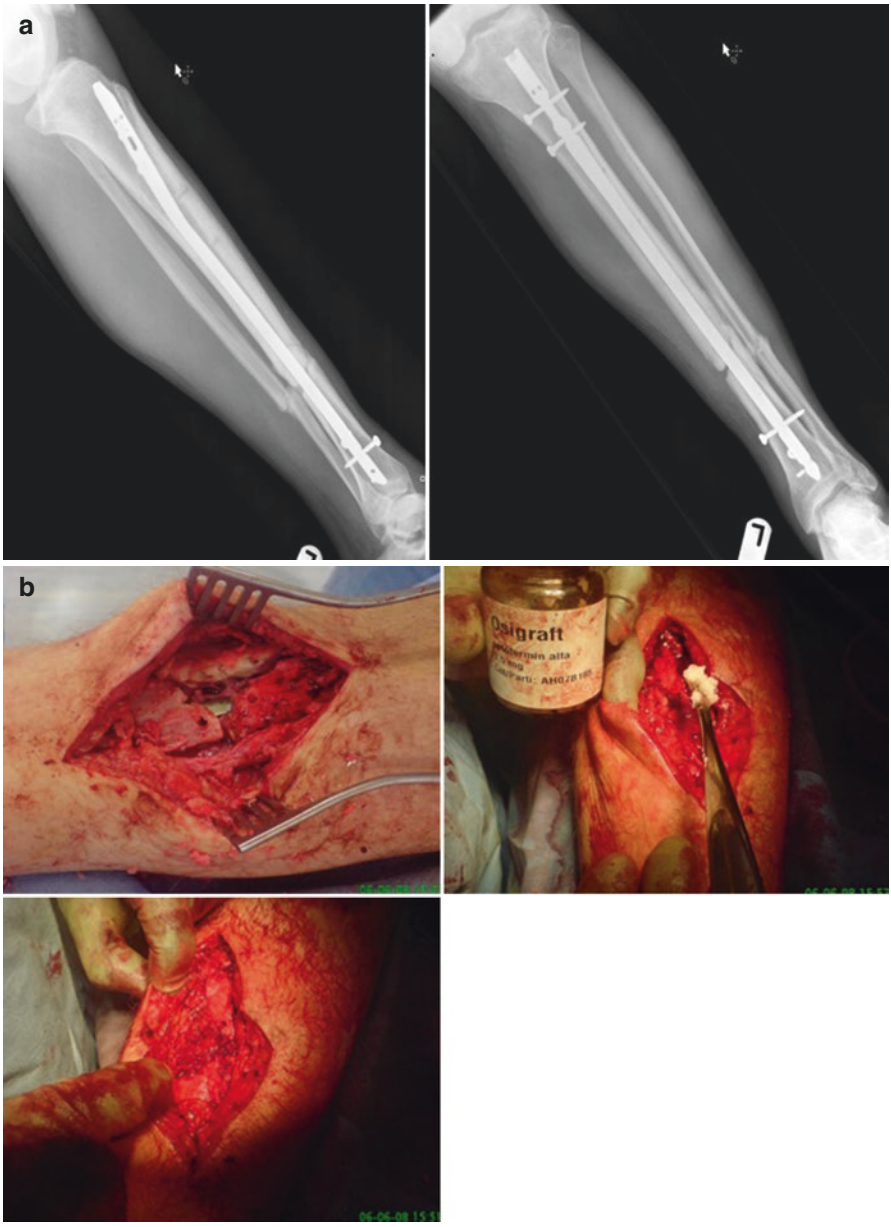


Fig. 1 (**a**) Radiographs AP and lateral of left tibial fracture 9 months after fixation with intramedullary nail with no evidence of bone healing. (**b**) Intraoperative images demonstrating the fracture non-union and the implantation of BMP-7. (**c**) Radiographs AP and lateral demonstrating fracture union 4 months later

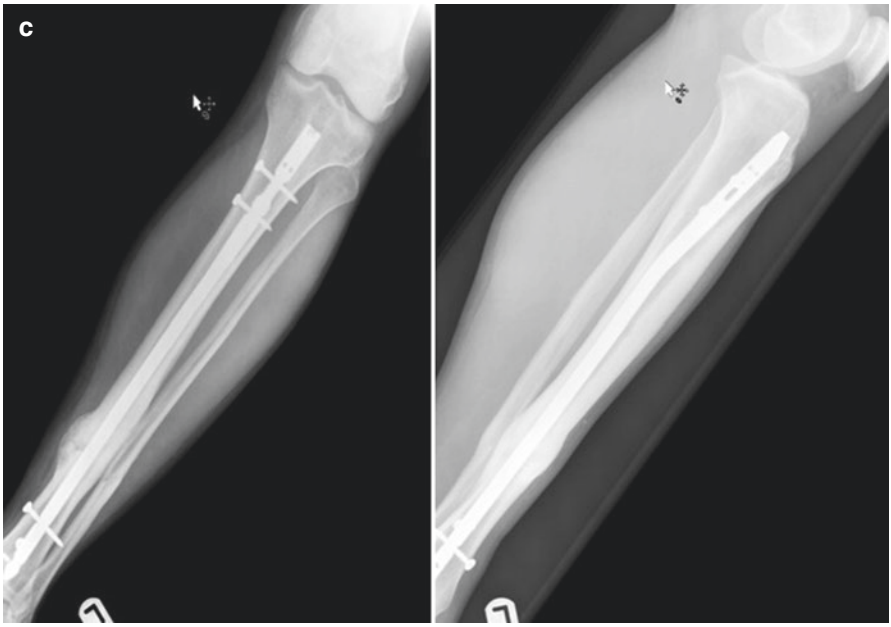


Fig. 1 (continued)

Table 2 Interaction of BMPs with open fractures

Molecule – action	Type of evidence	Study	Combination of use
Investigate the benefit and safety of the osteoinductive protein recombinant human bone morphogenetic protein-2 (rhBMP-2) when implanted on an absorbable collagen sponge in combination with freeze-dried cancellous allograft	Randomised, controlled trial, 30 patients	Jones et al., *Journal of Bone and Joint Surgery American*, 2006	Allograft (cancellous bone chips) in combination with rhBMP-2 on an absorbable collagen sponge
Evaluate the effectiveness of BMP in acute open tibial shaft fractures with main diaphyseal component	Cost analysis based on a single empirical study (Govender 2002), 291 patients	Alt et al., *Z Orthop Ihre Grenzgeb*, 2006	BMP used alone
Use of OP-1 in the treatment of open tibial shaft fractures was evaluated by the Canadian Orthopaedic Trauma Society	Prospective, randomised, multicentre controlled, 124 patients with open tibial fractures	McKee et al., Proceedings of the 18th Annual Meeting of the Orthopaedic Trauma Association, 2002	BMP used alone

(continued)

Table 2 (continued)

Molecule – action	Type of evidence	Study	Combination of use
Evaluation of the safety and efficacy of the use of recombinant human bone morphogenetic protein-2 (rhBMP-2; dibotermin alfa) to accelerate healing of open tibial shaft fractures and to reduce the need for secondary intervention	Prospective, randomised, controlled, single-blind study, 450 patients with an open tibial fracture	Govender et al., *Journal of Bone and Joint American*, 2002	BMP used alone

Table 3 Interaction of BMPs with joint fusion

Molecule – action	Type of evidence	Study	Combination of use	Union rates (%)
Evaluation of the efficacy of rhBMP-2 treatment in complex ankle arthrodesis	Retrospective chart study, 82 patients	Fourman et al. 2014	Application of Ilizarov frame and rhBMP-2 vs. control	93
Evaluation of the use of bone morphogenetic protein-2 (BMP-2) in revision tibiotalocalcaneal arthrodesis	Retrospective chart and radiographic review study, 23 patients	DeVries et al. 2012	Retrograde intramedullary nailing with the use of BMP-2 vs. control	71.4
Evaluation of the efficacy of BMP-7, bone morphogenetic protein-7/ OP-1 in joint fusion	Case series, 19 patients, ankle, subtalar, talonavicular, pubic and sacroiliac	Kanakaris et al. 2009	BMP-7 used alone or in combination with autograft or allograft	89

Table 4 Interaction of BMPs with spinal fusion

Molecule – action	Type of evidence	Study	Combination of use	Union rates (%)
rhBMP-2/ ACS vs. ICBG	Prospective randomised controlled trial, 40 patients	Michielsen et al. 2013	Single-level PLIF with pedicle screw fixation	100
rhBMP-2 vs. control	Retrospective study, 509 patients	Crandall et al. 2013	TLIF	98.40
rhBMP-2 vs. control	Prospective randomised study, 197 patients	Hurlbert et al. 2013	Posterior lumbar with pedicle screw fixation	96 (6 months) 94 (48 months)
rhBMP-2 vs. autograft	Prospective randomised controlled trial, 410 patients	Dimar et al. 2009	Single-level PLIF with pedicle screw fixation	96
rhBMP-2 vs. ICBG	Retrospective study, 148 patients	Glassman et al. 2007	Single-level PLIF with pedicle screw fixation	100 (non-smokers) 95.2 (smokers)

the homeostatic pathways, is governed by specific signalling molecules (Table 5) [40]. As far as our contemporary understanding, most molecular and biochemical research of the cartilage morphogenesis is influenced by work on the bone morphogenetic proteins (BMPs); therefore, all BMPs could be considered as cartilage morphogens.

At the early stages of posttraumatic osteoarthritis, a common condition affecting healthy adults, the mechanical disruption of the interaction between chondrocytes and matrix leads to irregular chondrocyte behaviour and transient increase of their proliferation and their metabolic activity. The latter is reflected to the appearance of cell clusters and changes of the quantity/composition of the matrix proteins with a decrease of the proteoglycans and cleavage of type II collagen [41–43]. During the evolution of osteoarthritis, part of the joint chondrocytes lose their stable phenotype and revert to changes resembling terminally differentiating cells, with basic characteristic the increased synthesis of the enzyme metalloproteinase-13 (MMP-13) [44, 45]. BMPs have been identified to be involved to all phases of chondrogenesis (mesenchymal condensation, chondrocyte proliferation, extracellular matrix deposition and terminal differentiation), regulating the expression of several chondrocyte-specific genes (Fig. 2) [46–48]. As shown in numerous in vivo and in vitro studies, BMPs (specifically the BMP-2, BMP-4, BMP-7) enhance the chondrocyte proliferation and the expression of type II collagen mRNA [49–51], regulate the activity of the essential transcription factor sox-9 [52] and stimulate the synthesis of aggrecans and of matrix [53, 54] (Table 6) [54–91]. The terminal differentiation of the chondrocytes during the process of endochondral bone formation, as well as the transformation of differentiated cartilage cells, is regulated by the BMP membrane receptors, Smad1 or Smad5, as well as the transcription factor Runx2 [92–95].

Table 5 Cartilage morphogenetic proteins

Cartilage-derived morphogenetic proteins CDMPs		
CDMP-1	GDF-5	Mesenchymal condensation, chondrogenesis
CDMP-2	GDF-6	Cartilage development and hypertrophy
CDMP-3	GDF-7	Ligament and tendon development
Bone morphogenetic proteins BMPs		
BMP-2	BMP-2A	Cartilage and bone morphogenesis
BMP-4	BMP-2B	Cartilage and bone morphogenesis
BMP-3	Osteogenesis	Bone formation
BMP-3B	GDF-10	Membrane bones
BMP-5	n/a	Bone morphogenesis
BMP-6	n/a	Hypertrophy of cartilage
BMP-7	Osteogenic protein	Bone differentiation
BMP-8	Osteogenic protein	Bone formation
BMP-9	n/a	
BMP-10	n/a	
BMP-11	GDF-11	

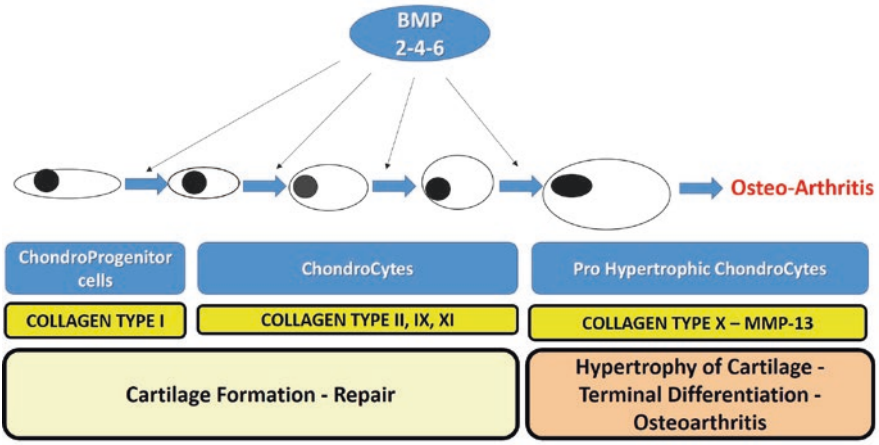


Fig. 2 Involvement of BMPs to all phases of chondrogenesis

BMP-2 and BMP-4 have been recognised of having a critical role to the morphogenesis and growth of the articular cartilage. The signalling by BMPs is modulated by extracellular BMP antagonists such as noggin and chondrin. As shown in vivo, the absence of noggin expression in a knockout mouse caused the complete absence of joints [96–98]. Furthermore, an additional standpoint of the role of the BMPs in healthy and diseased articular cartilage has been highlighted following research relevant to the endogenous production of these proteins. The local BMP environment has been implicated as far as either the maintenance of the cartilage homeostasis or the acceleration of its auto-destruction during the phases of OA. The existing evidence (Table 6) is limited and also contradicting as far as the measurable differences between the types and concentrations of different BMP molecules (BMP-2, BMP-4, BMP-6, BMP-11 and GDF-5) in normal and diseased articular cartilage [89–92]. It appears that locally produced BMPs are contributing to the regeneration of the articular cartilage after traumatic or inflammatory damage. An elevated local ratio of the BMPs to their inhibitors increases the BMP activity and is understood to enhance the chondrocyte differentiation/regeneration. A reverse ratio, with dominance of the inhibitors, has been identified to decrease the synthesis of matrix molecules. All in all, how these ratios differ in osteoarthritic cartilage and how these ratios affect the disease process have not been yet clarified. It appears that blocking intrinsic BMP activity either by overexpression of BMP inhibitors (noggin) [89] or by intraarticular IL-1 challenge using gremlin leads to decrease of aggrecan synthesis and depletion of the necessary proteoglycans [90].

The upregulation of the expression of BMP inhibitors, as the gremlin, follistatin and less that of noggin, has been reported by several authors in cartilage with OA [88, 91, 99]. Although the short-term effects of BMP activity are not clear-cut, following evidence from a number of publications, the long-term effects appear to lead to chondrocyte phenotype modulation and terminal differentiation with upregula-

Table 6 Selected evidence on the interaction of bone morphogenetic proteins with articular cartilage

Molecule – action	Type of evidence	Study
BMP-2		
Full-thickness trochlear articular cartilage defects showed improvement in the histological appearance and composition of the extracellular matrix at 1 year postoperatively, compared with controls	In vivo – rabbit model – articular cartilage defect and rhBMP-2/collagen sponge	Sellers RS, et al. 2000, *J Bone Joint Surg Am* [60]
Upregulates gene expression of SOX9	In vivo murine bone fracture model	Uusitalo H, et al. 2001, *J Bone Miner Res* [61]
Stimulation of the repair of articular cartilage defects of the mandibular condyle head in high-dose groups	In vivo – rabbit model – articular cartilage defect treated with BMPi2 lyophilised with collagen as the carrier	Suzuki T, et al. 2002, *Br J Oral Maxillofac Surg* [62]
Hardly present in normal human articular cartilage Clearly detected in both clustering and individual chondrocytes in osteoarthritic cartilage	In vitro human cartilage and in situ hybridisation and immune histochemistry for the expression of BMP-2	Nakase T, et al. 2003, *Osteoarthritis Cartilage* [26]
Increased expression following release of IL-1beta and TNF-α in the presence of an injury to human cartilage	In vitro – human chondrocytes – mRNA expression levels for BMP-2, BMP-4, BMP-6, cartilage-derived morphogenetic protein-1 (CDMP-1), connective tissue growth factor (CTGF) and activin	Fukui N, et al. 2003, *J Bone Joint Surg Am* [27]
mRNA expression in normal and osteoarthritic adult human cartilage	In vivo mouse models of osteoarthritis	Chen AL et al. 2004, *J Orthop Res* [31]
Strong upregulation in areas of cartilage lesions		
Increased expression following release of IL-1beta and TNF-α in the presence of an injury to human cartilage	In vitro human cartilage explant cultures	Dell' Accio F, et al. 2006, *Arthritis Res Ther* [28]
Highly inducing molecule from bone marrow MSCs into in vitro cartilage formation	In vitro bone marrow taken from normal adult donors	Sekiya I, et al. 2005, *Cell Tissue Res* [63]
A new biotechnology for articular cartilage repair: subchondral implantation of a composite of interconnected porous hydroxyapatite, synthetic polymer (PLA-PEG) and bone morphogenetic protein-2 (rhBMP-2)	In vivo – rabbit model – cartilage defect treated with triple composite (interconnected porous hydroxyapatite (IP-CHA), recombinant human bone morphogenetic protein-2 (rhBMP-2) and a synthetic biodegradable polymer [poly-d,l-lactic acid/ polyethylene glycol (PLA-PEG)] as a carrier)	Tamai N, et al. 2005, *Osteoarthritis Cartilage* [64]

(continued)

Table 6 (continued)

Molecule – action	Type of evidence	Study
rhBMP-2 was found to reduce the severity of cartilage lesions of lumbar facet joint compared with controls. However, higher-dose rhBMP-2 resulted in joint space obliteration caused by cartilage overgrowth, and there were significant synovium reactions	In vivo – rat model – osteoarthritis model and intraarticular injection of rhBMP-2	Yeh TT, et al. 2007, *Osteoarthritis Cartilage* [65]
Stimulates aggrecan synthesis	In vitro cultured human normal articular ankle chondrocytes	Chubinskaya S, et al. 2008, *Growth Factors* [17]
Stimulation with BMP-2 followed by IL-1 exposure led to increased expression of MMP-13	In vitro immortalised mouse chondrocytes	Majumdar MK, et al. 2008, J Cell Physiol. [66]
Long-term culture with BMP-2 upregulates the expression of MMP-13 Stimulated chondrocyte culture tissue was frailer than tissue cultured without it	In vitro culture of chondrocytes	Krawczak DA, et al. 2009, *Tissue Eng Part A* [35]
Repair of experimentally induced large osteochondral defects in rabbit knee with various concentrations of *Escherichia coli*-derived recombinant human bone morphogenetic protein-2	In vivo – rabbit model – large osteochondral defects	Tokuhara Y, et al. 2010, *Int Orthop* [67]
Spatiotemporal control of proliferation and differentiation of bone marrow-derived mesenchymal stem cells recruited using collagen hydrogel for repair of articular cartilage defects	In vivo – rabbit model – full-thickness chondral defects	Mimura T, et al. 2011, *J Biomed Mater Res B* [68]
Hyaline cartilage regeneration by combined therapy of microfracture and long-term bone morphogenetic protein-2 delivery	In vivo – rabbit model – long-term delivery of BMP-2 to cartilage defects subjected to microfracture	Yang HS, et al. 2011, *Tissue Eng Part A* [69]
Cartilage repair of experimentally induced osteochondral defects post mechanical drilling of the medial femoral condyle	In vivo – rabbit model – full-thickness osteochondral defect and intraarticular delivery of BMP-2	Aulin C, et al. 2013, *Lab Anim* [70]

BMP-4		
Stimulates aggrecan synthesis	In vitro cultured human normal articular ankle chondrocytes	Chubinskaya S, et al. 2008, *Growth Factors* [17]
Strong upregulation in areas of cartilage lesions	In vivo mouse models of osteoarthritis	
BMP-6		
Detected in both osteoarthritic and normal adult human articular cartilage	In vitro – human chondrocytes – mRNA expression levels for BMP-2, BMP-4, BMP-6, cartilage-derived morphogenetic protein-1 (CDMP-1), connective tissue growth factor (CTGF) and activing	Fukui N, et al., 2003, *J Bone Joint Surg Am* [27]
BMP-7		
Upregulates chondrocyte metabolism and protein synthesis without creating uncontrolled cell proliferation and formation of osteophytes	In vitro human chondrocytes	Flechtenmacher J, et al. 1996, *Arthritis Rheum* [71] Nishida Y, et al. 2000, *Arthritis Rheum* [72] Loeser RF, et al. 2003, *Arthritis Rheum* [73] Fan Z, et al. 2004, *Clin Exp Rheumatol* [74]
Generates normal, functional proteoglycans (PGs), with a hydrodynamic size unaltered	In vitro explants of porcine articular cartilage	Lietman S, et al. 1997, *J Bone J Surg Am* [75]
Using recombinant OP-1/BMP-7 delivered on bone-derived type I collagen particles press-fitted into large focal defects improved the repair of both bone and cartilage tissue	In vivo osteochondral defect rabbit model	Grgic M, et al. 1997, *Acta Med Croatica* [76]
OP-1/BMP-7 delivered on bone-derived type I collagen particles press-fitted into defects improved healing in both cartilage and bone	In vivo osteochondral defect goat and dog models	Louwerse RT, et al. 2000, *J Biomed Mater Res* [77] Cook SD, et al. 2003, *J Bone Joint Surg* [78]

(continued)

Table 6 (continued)

Molecule – action	Type of evidence	Study
Infused liquid OP-1/BMP-7 in acetate buffer delivered into the knee joint for 2 weeks led to the progressive filling of the defect by newly formed cartilage stained positive for collagen type II	In vivo large chondral sheep defect model	Jelic M, et al. 2001, *Growth Fact* [79]
BMP-7 expressing chondrocytes suppressed ingrowth of destructive fibrous connective tissue (pannus), so this protein may also be useful in inflammatory arthritis	In vivo mice model tissue engineering – transgenic chondrocytes were assembled in alginate or in bioresorbable copolymer fleeces	Kaps C, et al. 2002, *Arthritis Rheum* [80]
Induces similar anabolic responses in normal and OA chondrocytes from both young and old donors Does not cause chondrocyte hypertrophy or changes in chondrocytic phenotype	In vitro human arthritic cartilage	Loeser RF, et al. 2003, *Arthritis Rheum* [73] Merrihew C, et al. 2003, *J Ortho Res* [81] Chubinskaya S, et al. 2007, *Osteoarthritis Cartilage* [82]
Modulates the expression of various growth factors (insulin-like growth factor-1 [IGF-1], TGF-β/BMPs) and catabolic mediators (IL-6 family of pro-inflammatory cytokines)	In vitro – human chondrocytes	Im HJ, et al. 2003, *J Biol Chem* [83] Chubinskaya S, et al. 2008, *Growth Factors* [17]
Adding OP-1/BMP-7 to collagen material to augment a mosaicplasty (using osteochondral autograft) improved the histological outcome	In vivo osteochondral defect sheep models	Shimmin A, et al. 2003, *Trans ICRS* [84]
The OP-1/BMP-7 gene on an adenoviral vector was delivered to the defect site via transfected allogenic chondrocytes embedded in a fibrin clot. In comparison to the controls, accelerated healing and creation of hyaline-like morphology was noted, which was however neutralised by 8 weeks	In vivo large chondral horse defect model	Hidaka C, et al. 2003, *J Ortho Res* [85]
BMP-7 (100 ng/ml) dramatically improved cell-associated proteoglycan deposition and prevented matrix degradation caused by hyaluronan hexasaccharide depletion of the CD44 receptor cartilage explants	In vitro human chondrocytes	Nishida Y, et al. 2004, *Osteoarthritis Cart* [86]
Does not result in significant changes in MMP-13 expression	In vitro normal and osteoarthritic human chondrocytes	Fan Z, et al. 2004, *Clin Exp Rheumatol* [74]

Stimulates only cartilage-specific extracellular proteins: collagens type II and VI, aggrecan, decorin, fibronectin and hyaluronan	In vitro human and primate chondrocytes	Loeser R, et al. 2005, *Arthritis Rheum* [87] Chubinskaya S, et al. 2007, *Osteoarthritis Cartilage* [82]
Using OP-1/BMP-7 can augment the stimulating effect of the microfracture procedure	In vivo osteochondral defect rabbit model	Kuo AC, et al. 2006, *Osteoarthritis Cart* [88]
BMP-7 had a protective effect on cartilage degeneration. Significant improvements in histological and morphometric scores and expression of type II collagen were found in addition to suppression of aggrecanase activity	In vivo ACL transection model in rabbits	Badlani N, et al. 2008, *Osteoarthritis Cartilage* [89]
Stimulates aggrecan synthesis	In vitro cultured human normal articular ankle chondrocytes	Chubinskaya S, et al. 2008, *Growth Factors* [17]
Increased levels of BMP-7 were found in synovial fluid and tissues after joint injury, an arthrotomy incision or induction of osteoarthritis BMP-7 prevented posttraumatic osteoarthritis; macroscopic and histological damage to the articular surface was reduced, as was the C3/4 short collagen epitope immunostaining, indicating that there was protection against metalloproteinase-mediated collagen degradation Similar results were seen with two doses of BMP-7 injected three and four weeks after injury, but not when therapy was delayed for 12 weeks	In vivo – sheep model – two intraarticular injections of BMP-7 were given at the time of injury and one week later	Hurtig MB, 2009, *J Orthop Res* [90]
Enhances gene expression of the anabolic molecule tissue inhibitor of metalloproteinase (TIMP) in normal and OA chondrocytes	In vitro human chondrocytes transfected with OP-1 antisense oligonucleotide or treated with recombinant OP-1 for 48 h followed by RNA isolation – followed by selected gene array results, real-time PCR, in vitro measures of proteoglycan synthesis and signal transduction	Chubinskaya S, et al. 2011, *Arthritis Res Ther* [91]

tion of the MMP-13 synthesis [87, 100]. Besides the effect of BMPs on the chondrocyte differentiation, they also increase the synthesis of matrix molecules, which also represents a characteristic of osteoarthritis [101–103]. In arthritic cartilage, elevated levels of BMPs improve the synthesis of the matrix, contributing to the local reparative processes, but on the other hand may stimulate further cartilage degeneration by altering the characteristics of the chondrocyte population, stimulating the expression of MMP-13. As indicated in a number of publications, different BMPs will have different effects in the morphogenesis and regeneration of the articular cartilage. As evident from the effect of the BMPs to the bone tissue, where most of them stimulate bone formation while the BMP-3 acts as a negative regulator [104], it is expected that there are similar differences to their biological function on the chondrocyte lineage [105]. Additionally, osteoarthritis subtypes and patient ageing alter the expression of the BMP receptors, which subsequently changes the biological effects of the BMPs on their target cells [106, 107]. Overall, BMPs can have a protective role but also can be harmful to the articular cartilage.

Lately, the BMP-7 molecule has received significant attention mostly because of its availability in a synthetic form and experience gained from its wide use in bone regeneration. An array of in vivo and in vitro studies focusing on cartilaginous tissue have explored the unique function of this molecule that acts both as a strong pro-anabolic and a potent anti-catabolic agent (Table 6). It is a molecule endogenously expressed in cartilage, synovial fluid synovium, meniscus, ligaments and tendons. The ability of BMP-7 to enhance the repair of articular cartilage in several models of focal osteochondral and also of pure chondral defects, as well as of early osteoarthritis, has been pivotal for its inclusion to the future therapeutic strategies of articular cartilage pathologies. In a number of in vivo studies, BMP-7 has demonstrated significant chondroprotective effect in several histological and morphometric scores and expression of type II collagen in addition to suppression of aggrecanase activity. Especially the evidence from recent animal studies, where BMP-7 augmented significantly the efficiency of joint repair procedures (mosaicplasty, microfracture) after it was delivered locally to the chondral defect on an appropriate scaffold/carrier or even infused to the joint with a minipump, creates the basis of future clinical testing. The arthroscopic delivery of such molecules either alone or in conjunction to cell-based therapies to treat cartilage defects or prevent the progress of osteoarthritis represents some of the currently tested hypothesis.

However, there are two major concerns in regard to the use of the BMPs locally into the joints, which have been explored in all small and larger animal in vivo studies. The first concern is relevant to the formation of heterotopic or intraarticular bone, a condition which is clearly evident at the clinical setting of bone healing enhancement as a result of poor containment and limitations of the BMP carrier. Fortunately, at most of the existing studies (Table 6), it has not been observed to the degree of justifying these concerns. The second concern relates to the observed anabolic effect of the BMPs to the cartilage being attributed to the modulation of the remaining chondrocyte population to the area of implantation/concern and not to any extrinsic cartilage repair pathways. This finding is suggestive that their use should be at the early stages of arthritis when this population is higher and more potent.

It is of interest that the isolation and cloning of the BMP family from the bone has led to further research on the identification and characterisation of the cartilage-derived morphogenetic proteins (CDMPs) from the articular cartilage. More specifically, the key signalling molecule is the cartilage-derived morphogenetic protein-1 (CDMP-1) also known as growth/differentiation factor-5 (GDF-5). The homeostasis of the articular cartilage has been described as a balance between the anabolic agents as the BMPs and/or CDMPs and catabolic such as interleukin IL-1, IL-17 and/or the tumour necrosis factor-α (TNF-α). In combination, BMPs and CDMPs induce cartilage morphogenesis and maintenance. Furthermore, the morphogenesis of the cartilage is well associated to the supramolecular synthesis of the extracellular matrix. The cartilage matrix consists of collagens, glycoproteins and proteoglycans. Over 90 % of this collagen is type II with minor concentrations of collagens IX and XI [108]. As evident in a number of studies, genetic mutations in collagen II result in chondrodysplasias and cartilage degeneration [109, 110].

Overall, regenerative medicine of cartilage is currently based on the triad of signals, stem cells and scaffolds. Since the articular cartilage is damaged in joint arthritis, the growing interest over the last 20 years on the identification and manipulation of the signalling molecules is expected to lead to tissue engineering techniques recapitulating the embryonic cartilage regeneration, restoring local anatomy and function. Furthermore, BMPs/CDMPs modulators may be used to alleviate the pain of osteoarthritic patients via deceleration of the progress of arthritis.

The effect of BMPs at the intervertebral disc (IVD) cartilage has been tested in several in vivo and in vitro studies (Table 7) [111–121]. The aim of the researchers has been either to address the problem of degenerative disc disease, as well as that of discogenic back pain. For instance, both the BMP-2 and BMP-7 have been shown to boost the synthesis of extracellular matrix in several in vitro studies on rat, bovine and human intravertebral disc cells [110–115]. The delivery of these molecules has evolved through the years and ranges from the direct local injection of BMPs to the disc space to viral transfection methods leading to the modification of the target cells to stimulate the secretion of the specific growth factors [116]. Mainly small animal in vivo models have been used to study the effect of local injection of BMP-7 in normal as well as in models of degenerative/injured intervertebral discs. [122] Similarly, the role of BMP-2 in disc cartilage repair has been postulated, in a number of animal and in vitro studies, to involve promotion of both cartilage formation and subsequent cartilage degradation through hypertrophy and endochondral ossification [117, 123]. Direct administration of BMP-2 to IVD chondrocytes has been observed to stimulate the production of the extracellular matrix. Furthermore, the upregulation of the BMP pathway, via molecules as statins and LIM mineralisation protein-1, has led to similar observations [118].

Nonetheless, a major drawback of the existing evidence is based on in vivo studies of small animal models, in rodents and rabbits. These species retain their notochordal cells in adult life, whereas larger animals and humans loose them during their adolescence [124]. The notochordal cells are precursor cells of the nucleus pulposus and participate actively to the homeostasis of the extracellular matrix of the intravertebral disc. Thus, in such species, it may be that the regenerative effect of molecules as the BMPs may be exaggerated and inherently different from what could be observed in

Table 7 Published evidence in regards to the effect of BMP's at the intervertebral disc (IVD) cartilage (selected in vivo and in vitro studies)

Molecule – action	Type of evidence	Study
BMP-7		
Upregulation of the metabolism of extracellular matrix of rabbit annulus fibrosus and nucleus pulposus cells cultured in alginate beads	In vitro – rabbit – intravertebral disc cultures and OP-1/BMP-7	Masuda K, et al. 2003, *J Orthop Res.* [49]
Stimulation of cells of nucleus pulposus and annulus fibrosus to repair their matrix after chondroitinase ABC-induced in vitro chemonucleolysis	In vitro – rabbit – intravertebral disc cultures and OP-1/BMP-7	Takegami K, et al. 2005, *Spine J.* [48]
Stimulatory effect of OP-1/BMP-7 which increased the mean disc height of normal discs, relevant to a significant increase in the proteoglycan content of the nucleus pulposus	In vivo – rabbit model – intradiscal injection of OP-1/BMP-7	An HS, et al. 2005, *Spine*. [92]
Full restoration of the disc height in a posttraumatic degenerative rabbit disc model, after 6 weeks from the injection of liquid OP-1/BMP-7 Histological analysis showed less degeneration for the OP-1-treated discs, and biomechanical testing showed a restoration of the viscoelastic properties of the disc to the level of normal control discs	In vivo – rabbit model – intradiscal injection of liquid OP-1/BMP-7	Masuda K, et al. 2006, *Spine*. [52]
Increased cell proliferation and proteoglycan synthesis after stimulation of human nucleus pulposus and annulus fibrosis cells	In vitro – rabbit – intravertebral disc cultures and OP-1/BMP-7	Imai Y, et al. 2007, *Spine*. [50]
Restoration of the disc composition after injection of BMP-7 using chondroitinase ABC to induce degeneration of the disc	In vivo – rabbit model – intradiscal injection of liquid OP-1/BMP-7	Imai Y, et al. 2007, *Spine*. [93]
Reversion of the degenerative changes induced by chronic compression of vertebrae Immunohistochemically, the anti-catabolic effect of the BMP was expressed with reduction of the aggrecanase, MMP-13, TNF-α, IL-1β and substance P This was the first demonstration of an inhibitory effect on pain by OP-1/BMP-7	In vivo – rat model – intradiscal injection of OP-1/BMP-7	Kawakami M, et al. 2005, *Spine*. [54] Chubinskaya S, et al. 2007, *J Ortho Res*. [94]

BMP-2		
Stimulation of the repair in comparison to controls, after annular tear of the intervertebral disc	In vivo – rabbit model – intradiscal injections of rhBMP-2	Huang KY, et al. 2007, *Spine*. [56]
Delaying the course of intervertebral disc degeneration in an in vivo rabbit model	In vivo – rabbit model – intradiscal injection of adeno-associated virus serotype 2 (AAV2) vector carrying genes for either bone morphogenetic protein-2 (BMP-2) or tissue inhibitor of metalloproteinase 1 (TIMP1)	Leckie SK, et al. 2012, *Spine J*. [51]
No regenerative effect was observed in the studied groups	In vivo goat model mild intravertebral disc – slow delivery system for BMP-2 and BMP-2/BMP-7	Peeters M, et al. 2015, *Biores Open Acc*. [47]

humans. Further studies focusing on such strategies at the clinical setting are expected to supplement the amplitude of the existing in vitro and in vivo animal studies, translating these attractive experimental concepts to the bedside practice.

Despite all the intense research and clinical activity on the effectiveness of BMPs on musculoskeletal conditions, their widespread use has been hampered by several issues and concerns. For instance, the results obtained are not consistent and do vary from study to study. In addition, the excellent results seen in studies carried out in small animal models have never been replicated in humans. This discrepancy is reflected at the cellular level [125]. Of interest, the skeletal maturity of the rodents used in research, whose growth plates never close could be another reason of the differences seen between experimental and human studies. Moreover, regardless of the intrinsic biological differences between small laboratory animals and humans, the issue of scaling cannot also be ignored. In addition, many studies that have been done on cranial defects in animal models cannot be applied to human long bone healing. Besides, their use is associated with an increased cost compared to other graft materials. Finally, the supraphysiological dose delivered locally has been associated with intense inflammatory reactions causing wound breakdown and leakage. While initially this finding appears to be of aseptic origin, over time the wound can become septic compromising the final outcome in terms of bony union and functional outcome of the affected extremity. In such cases, early administration of antibiotics has been recommended until the wound discharge settles down.

In general terms, several parameters have been identified to influence their overall efficacy including their formulation, carrier characteristics, containment, timing of their implantation, the state of the soft tissues and the ideal dose of administration [126, 127]. Overall, despite their superiority with regard to their inductive potential, they are considered nowadays as good as the autologous iliac crest bone graft (AICBG), the gold standard of bone grafting materials. One however may question whether this comparison is valid. The AICBG possesses all the three important properties for bone regeneration: osteogenicity, osteoinductivity and osteoconductivity. In contrast, BMPs pose only one property, osteoinductivity. Consequently, one may argue that AICBG is actually more powerful in terms of biological bone inducing properties and as such any criticisms made of the BMPs is unfair. It is of note that most of the failures seen over the years following implantation of BMPs involved recalcitrant non-unions when patients had already undergone more than two to three procedures and after treatment of open fractures. In these difficult clinical circumstances where the soft tissue envelope is quite compromised, one has to consider whether the local environment contains sufficient osteoprogenitor cells to accept the stimulus from the BMPs allowing them to exert their positive bone repair effect. Accordingly, one may argue whether, under the above circumstances, BMPs should be routinely implanted in association with mesenchymal stem cells. There has also been a lot of concern with regard to the carcinogenic potential of BMPs. However, the available experimental data and clinical evidence are rather inadequate to allow any safe scientific conclusions. Clinical studies provide incomplete evidence to support the hypothesis that BMPs are carcinogenic. The available literature has several limitations including incomplete documentation, unreported data and inhered bias as a large number of trials have been funded by the industry [128].

Whereas, therefore, the initial clinical introduction of BMPs was associated with great enthusiasm and expectations, and while it has been widely accepted that BMPs constitute an important component of the conceptual frame of the so-called diamond concept for bone repair, almost 20 years later, their use and effectiveness has been questioned.

This can be attributed to the following reasons:

(i) Presented by the industry as the 'magic bullet' to clinical situations with a compromised bone repair response, even being superior to the autologous bone graft.
(ii) Most of the scientific evidence was accumulated in experimental studies of rodents which do not resemble the human physiology.
(iii) Inadequate knowledge of the pathways and negative feedback mechanisms regulating bone healing.
(iv) The optimum dose and timing of administration remains obscure.
(v) Selection of the right carrier and formulation is yet to be determined.
(vi) Poor containment of the BMP at the site of implantation.
(vii) Increased risk of carcinogenesis.
(viii) Increased cost to use the active molecule.

In addition, one may argue that the future of BMPs has entered some uncertainty following the withdrawal of BMP-7 by Olympus Biotech from the market. While the decision appears to be purely of strategic nature, one cannot hide that the reputation of BMPs has been greatly negatively affected. Lately, the use of peptides has emerged as an alternative option for the delivery of an inductive stimulus to the compromised bone environment. The term peptide refers to short amino acid oligomers most commonly lacking a stable three-dimensional structure. In general, peptides exert their effect through binding to specific high-affinity receptors on the respective target cell receptors [129]. The discovery that small protein segments (peptides) have the capacity to exert a similar effect like the big protein molecule could overcome some of the previously mentioned problems related to selection and properties of carriers, instability of the active growth factor substance in vivo, impact of sterilisation on the active substance and the theoretical involvement in carcinogenesis. Peptides not only have low immunogenicity but also can be easily synthesised and handled. The challenge remains, however, whether this alternative path for bone repair would be proven effective in the clinical setting as appropriate level I trials are currently lacking.

References

1. Urist M (1965) Bone formation by autoinduction. Science 12:893–899
2. Giannoudis PV, Kanakaris NK, Einhorn TA (2007) Interaction of bone morphogenetic proteins with cells of the osteoclast lineage: review of the existing evidence. Osteoporos Int 18:1565–1581
3. Kanakaris NK, Calori GM, Verdonk R et al (2008) Application of BMP-7 to tibial nonunions: a 3-year multicenter experience. Injury 39(Suppl 2):S83–S90

4. Kanakaris NK, Paliobeis C, Manidakis N, Giannoudis PV (2007) Biological enhancement of tibial diaphyseal aseptic non-unions: the efficacy of autologous bone grafting, BMPs and reaming by-products. Injury 38(Suppl 2):S65–S75
5. Papathanasopoulos A, Giannoudis PV (2008) Biological considerations of mesenchymal stem cells and endothelial progenitor cells. Injury 39(Suppl 2):S21–S32
6. Termaat MF, Den Boer FC, Bakker FC, Patka P, Haarman HJ (2005) Bone morphogenetic proteins: development and clinical efficacy in the treatment of fractures and bone defects. J Bone Joint Surg Am 87:1367–1378
7. Uludag H, Gao T, Porter TJ, Friess W, Wozney JM (2001) Delivery systems for BMPs: factors contributing to protein retention at an application site. J Bone Joint Surg Am 83-A(Suppl 1, Pt 2):S128–S135
8. Valentin-Opran A, Wozney J, Csimma C, Lilly L, Riedel GE (2002) Clinical evaluation of recombinant human bone morphogenetic protein-2. Clin Orthop 395:110–120
9. Canalis E, Economides AN, Gazzerro E (2003) Bone morphogenetic proteins, their antagonists, and the skeleton. Endocr Rev 24:218–235
10. Axelrad TW, Steen B, Lowenberg DW, Creevy WR, Einhorn TA (2008) Heterotopic ossification after the use of commercially available recombinant human bone morphogenetic proteins in four patients. J Bone Joint Surg Br 90(12):1617–1622
11. Stylios G, Wan T, Giannoudis P (2007) Present status and future potential of enhancing bone healing using nanotechnology. Injury 38(Suppl 1):S63–S74
12. Mont MA, Ragland PS, Biggins B et al (2004) Use of bone morphogenetic proteins for musculoskeletal applications. An overview. J Bone Joint Surg Am 86(Suppl 2):41–55
13. Schmidmaier G, Schwabe P, Strobel C, Wildemann B (2008) Carrier systems and application of growth factors in orthopaedics. Injury 39(Suppl 2):S37–S43
14. Glaser DL, Economides AN, Wang L et al (2003) In vivo somatic cell gene transfer of an engineered noggin mutein prevents BMP4-induced heterotopic ossification. J Bone Joint Surg Am 85:2332–2342
15. Giannoudis PV, Einhorn TA (2009) Bone morphogenetic proteins in musculoskeletal medicine. Int J Care Injured 40:S3, S1–S3
16. Friedlaender GE, Perry CR, Cole JD, Cook SD, Cierny G, Muschler GF, Zych GA, Calhoun JH, LaForte AJ, Yin S (2001) Osteogenic protein-1 (bone morphogenetic protein-7) in the treatment of tibial nonunions. J Bone Joint Surg Am 83-A(Suppl 1(Pt 2)):S151–S158
17. Calori GM, D'Avino M, Tagliabue L, Albisetti W, d'Imporzano M, Peretti G (2006 Sep) An ongoing research for evaluation of treatment with BMPs or AGFs in long bone non-union: protocol description and preliminary results. Injury 37(Suppl 3):S43–S50
18. Ekrol I, Hajducka C, Court-Brown C, McQueen MM (2008 Sep) A comparison of RhBMP-7 (OP-1) and autogenous graft for metaphyseal defects after osteotomy of the distal radius. Injury 39(Suppl 2):S73–S82. doi:10.1016/S0020-1383(08)70018-4
19. Cook SD (1999 Jul) Preclinical and clinical evaluation of osteogenic protein-1 (BMP-7) in bony sites. Orthopedics 22(7):669–671
20. Chen G, Yang J, Xu H, Wang M (2000) The application of NNB/BMP complex in the treatment of ununited-tibia fracture. Orthop J China (Zhongguo Jiaoxing Waike Zazhi) 7:758–761. doi:10.3969/j.issn.1005-8478.2000.08.009
21. van Engen AK, Vinken A, Andrew G. The cost-effectiveness of Osigraft (Osteogenic Protein 1) in the treatment of tibial non-unions in the UK and Germany [poster]. 6th European Federation of National Associations of Orthopaedics and Traumatology (EFORT) Congress; 2003 June 4–10; Helsinki (FI).
22. Dimitriou R, Dahabreh Z, Katsoulis E, Matthews SJ, Branfoot T, Giannoudis PV (2005) Application of recombinant BMP-7 on persistent upper and lower limb non-unions. Injury 36(Suppl 4):S51–S59
23. Kanakaris NK, Lasanianos N, Calori GM, Verdonk R, Blokhuis TJ, Cherubino P, De Biase P, Giannoudis PV (2009) Application of bone morphogenetic proteins to femoral non-unions: a 4-year multicentre experience. Injury 40(Suppl 3):S54–S61

24. Giannoudis PV, Tzioupis C (2005) Clinical applications of BMP-7: the UK perspective. Injury 36(Suppl 3):S47–S50
25. Desmyter S, Goubau Y, Benahmed N, de Wever A, Verdonk R (2008) The role of bone morphogenetic protein-7 (Osteogenic Protein-1) in the treatment of tibial fracture non-unions. An overview of the use in Belgium. Acta Orthop Belg 74(4):534–537
26. Govender S, Csimma C, Genant HK et al (2002) (BESTT) Study Group. Recombinant human bone morphogenetic protein-2 for treatment of open tibial fractures: a prospective, controlled, randomized study of four hundred and fifty patients; BMP-2 evaluation in surgery for tibial trauma. J Bone Joint Surg Am 84-A(12):2123–2134
27. McKee MD, Schemitsch EH, Waddell JP et al (2002). The effect of human recombinant bone morphogenic protein (RHBMP-7) on the healing of open tibial shaft fractures: results of a multi-center, prospective, randomized clinical trial. In: Proceedings of the 18th annual meeting of the Orthopaedic Trauma Association; Oct 11–13; Toronto Ontario, Canada, pp. 157–8
28. Jones AL, Bucholz RW, Bosse MJ, Mirza SK, Lyon TR, Webb LX, Pollak AN, Golden JD, Valentin-Opran A (2006) BMP-2 Evaluation in Surgery for Tibial Trauma-Allgraft (BESTT-ALL) Study Group, Recombinant human BMP-2 and allograft compared with autogenous bone graft for reconstruction of diaphyseal tibial fractures with cortical defects. A randomized, controlled trial. J Bone Joint Surg Am 88(7):1431–1441
29. Alt V, Heissel A (2006) Economic considerations for the use of recombinant human bone morphogenetic protein-2 in open tibial fractures in Europe: the German model. Curr Med Res Opin 22(Suppl 1):S19–S22
30. Kanakaris NK, Mallina R, Calori GM, Kontakis G, Giannoudis PV (2009) Use of bone morphogenetic proteins in arthrodesis: clinical results. Injury 40(Suppl 3):S62–S66
31. Fourman MS, Borst EW, Bogner E, Rozbruch SR, Fragomen AT (2014) Recombinant human BMP-2 increases the incidence and rate of healing in complex ankle arthrodesis. Clin Orthop Relat Res 472(2):732–739
32. DeVries JG, Nguyen M, Berlet GC, Hyer CF (2012) The effect of recombinant bone morphogenetic protein-2 in revision tibiotalocalcaneal arthrodesis: utilization of the Retrograde Arthrodesis Intramedullary Nail database. J Foot Ankle Surg 51:426–432
33. Michielsen J, Sys J, Rigaux A, Bertrand C (2013) The effect of recombinant human bone morphogenetic protein-2 in single-level posterior lumbar interbody arthrodesis. J Bone Joint Surg Am 95(10):873–880
34. Dimar JR, Glassman SD, Burkus JK, Pryor PW, Hardacker JW et al (2009) Clinical and radiographic analysis of an optimized rhBMP-2 formulation as an autograft replacement in posterolateral lumbar spine arthrodesis. J Bone Joint Surg Am 91:1377–1386
35. Glassman SD, Dimar JR, Burkus K, Hardacker JW, Pryor PW, Boden SD, Carreon LY (2007) The efficacy of rhBMP-2 for posterolateral lumbar fusion in smokers. Spine (Phila Pa 1976). 32(15):1693–1698
36. Crandall DG, Revella J, Patterson J, Huish E, Chang M, McLemore R (2013) Transforaminal lumbar interbody fusion with rhBMP-2 in spinal deformity, spondylolisthesis, and degenerative disease – part 2: BMP dosage-related complications and long-term outcomes in 509 patients. Spine (Phila Pa 1976) 38(13):1137–1145
37. RJ H, Alexander D, Bailey S, Mahood J, Abraham E, McBroom R, Jodoin A, Fisher C (2013) rhBMP-2 for posterolateral instrumented lumbar fusion: a multicenter prospective randomized controlled trial. Spine (Phila Pa 1976) 38(25):2139–2148
38. Kronenberg HM (2003) Developmental regulation of the growth plate. Nature 423(6937):332–336
39. de Crombrugghe B, Lefebvre V, Behringer RR, Bi W, Murakami S, Huang W (2000) Transcriptional mechanisms of chondrocyte differentiation. Matrix Biol 19(5):389–394
40. Reddi AH (2003) Cartilage morphogenetic proteins: role in joint development, homoeostasis, and regeneration. Ann Rheum Dis 62(Suppl 2):ii73–ii78
41. Pritzker KP, Gay S, Jimenez SA, Ostergaard K, Pelletier JP, Revell PA, Salter D, van den Berg WB (2006) Osteoarthritis cartilage histopathology: grading and staging. Osteoarthritis Cartilage 14(1):13–29

42. Roach HI, Aigner T, Soder S, Haag J, Welkerling H (2007) Pathobiology of osteoarthritis: pathomechanisms and potential therapeutic targets. Curr Drug Targets 8(2):271–282
43. Sandell LJ (2007) Anabolic factors in degenerative joint disease. Curr Drug Targets 8(2):359–365
44. Wu W, Billinghurst RC, Pidoux I, Antoniou J, Zukor D, Tanzer M, Poole AR (2002) Sites of collagenase cleavage and denaturation of type II collagen in aging and osteoarthritic articular cartilage and their relationship to the distribution of matrix metalloproteinase 1 and matrix metalloproteinase 13. Arthritis Rheum 46(8):2087–2094
45. Tchetina EV, Squires G, Poole AR (2005) Increased type II collagen degradation and very early focal cartilage degeneration is associated with upregulation of chondrocyte differentiation related genes in early human articular cartilage lesions. J Rheumatol 32(5):876–886
46. Chimal-Monroy J, Rodriguez-Leon J, Montero JA, Ganan Y, Macias D, Merino R, Hurle JM (2003) Analysis of the molecular cascade responsible for mesodermal limb chondrogenesis: Sox genes and BMP signaling. Dev Biol 257(2):292–301
47. Pan Q, Yu Y, Chen Q, Li C, Wu H, Wan Y, Ma J, Sun F (2008) Sox9, a key transcription factor of bone morphogenetic protein-2-induced chondrogenesis, is activated through BMP pathway and a CCAAT box in the proximal promoter. J Cell Physiol 217(1):228–241
48. Pan Q, Wu Y, Lin T, Yao H, Yang Z, Gao G, Song E, Shen H (2009) Bone morphogenetic protein-2 induces chromatin remodeling and modification at the proximal promoter of Sox9 gene. Biochem Biophys Res Commun 379(2):356–361
49. Goldring MB, Tsuchimochi K, Ijiri K (2006) The control of chondrogenesis. J Cell Biochem 97(1):33–44
50. Chen D, Zhao M, Mundy GR (2004) Bone morphogenetic proteins. Growth Factors 22(4):233–241
51. Minina E, Wenzel HM, Kreschel C, Karp S, Gaffield W, McMahon AP, Vortkamp A (2001) BMP and Ihh/PTHrP signaling interact to coordinate chondrocyte proliferation and differentiation. Development 128(22):4523–4534
52. Zhao L, Li G, Zhou GQ (2009) SOX9 directly binds CREB as a novel synergism with the PKA pathway in BMP-2-induced osteochondrogenic differentiation. J Bone Miner Res 24(5):826–836
53. Aaijman A, D'Souza RN, Bronckers AL, Goei SW, Burger EH (1997) OP-1 (BMP-7) affects mRNA expression of type I, II, X collagen, and matrix Gla protein in ossifying long bones in vitro. J Bone Miner Res 12(11):1815–1823
54. Chubinskaya S, Segalite D, Pikovsky D, Hakimiyan AA, Rueger DC (2008) Effects induced by BMPS in cultures of human articular chondrocytes: comparative studies. Growth Factors 26(5):275–283
55. Sellers RS, Zhang R, Glasson SS, Kim HD, Peluso D, D'Augusta DA, Beckwith K, Morris EA (2000) Repair of articular cartilage defects one year after treatment with recombinant human bone morphogenetic protein-2 (rhBMP-2). J Bone Joint Surg Am 82(2):151–160
56. Uusitalo H, Hiltunen A, Ahonen M, Gao TJ, Lefebvre V, Harley V, Kahari VM, Vuorio E (2001) Accelerated up-regulation of L-Sox5, Sox6, and Sox9 by BMP-2 gene transfer during murine fracture healing. J Bone Miner Res 16(10):1837–1845
57. Suzuki T, Bessho K, Fujimura K, Okubo Y, Segami N, Iizuka T (2002) Regeneration of defects in the articular cartilage in rabbit temporomandibular joints by bone morphogenetic protein-2. Br J Oral Maxillofac Surg 40(3):201–206
58. Sekiya I, Larson BL, Vuoristo JT, Reger RL, Prockop DJ (2005) Comparison of effect of BMP-2, −4, and −6 on in vitro cartilage formation of human adult stem cells from bone marrow stroma. Cell Tissue Res 320(2):269–276
59. Tamai N, Myoui A, Hirao M, Kaito T, Ochi T, Tanaka J, Takaoka K, Yoshikawa H (2005) A new biotechnology for articular cartilage repair: subchondral implantation of a composite of interconnected porous hydroxyapatite, synthetic polymer (PLA-PEG), and bone morphogenetic protein-2 (rhBMP-2). Osteoarthritis Cartilage 13(5):405–417
60. Yeh TT, Wu SS, Lee CH, Wen ZH, Lee HS, Yang Z, Nimni ME, Han B (2007) The short-term therapeutic effect of recombinant human bone morphogenetic protein-2 on

collagenase-induced lumbar facet joint osteoarthritis in rats. Osteoarthritis Cartilage 15(12):1357–1366
61. Majumdar MK, Chockalingam PS, Bhat RA, Sheldon R, Keohan C, Blanchet T, Glasson S, Morris EA (2008) Immortalized mouse articular cartilage cell lines retain chondrocyte phenotype and respond to both anabolic factor BMP-2 and pro-inflammatory factor IL-1. J Cell Physiol 215(1):68–76
62. Tokuhara Y, Wakitani S, Imai Y, Kawaguchi A, Fukunaga K, Kim M, Kadoya Y, Takaoka K (2010) Repair of experimentally induced large osteochondral defects in rabbit knee with various concentrations of Escherichia coli-derived recombinant human bone morphogenetic protein-2. Int Orthop 34(5):761–767
63. Mimura T, Imai S, Okumura N, Li L, Nishizawa K, Araki S, Ueba H, Kubo M, Mori K, Matsusue Y (2011) Spatiotemporal control of proliferation and differentiation of bone marrow-derived mesenchymal stem cells recruited using collagen hydrogel for repair of articular cartilage defects. J Biomed Mater Res B Appl Biomater 98(2):360–368
64. Yang HS, La WG, Bhang SH, Kim HJ, Im GI, Lee H, Park JH, Kim BS (2011) Hyaline cartilage regeneration by combined therapy of microfracture and long-term bone morphogenetic protein-2 delivery. Tissue Eng Part A 17(13–14):1809–1818
65. Aulin C, Jensen-Waern M, Ekman S, Hagglund M, Engstrand T, Hilborn J, Hedenqvist P (2013) Cartilage repair of experimentally 11 induced osteochondral defects in New Zealand White rabbits. Lab Anim 47(1):58–65
66. Flechtenmacher J, Huch K, Thonar EJ, Mollenhauer JA, Davies SR, Schmid TM, Puhl W, Sampath TK, Aydelotte MB, Kuettner KE (1996) Recombinant human osteogenic protein 1 is a potent stimulator of the synthesis of cartilage proteoglycans and collagens by human articular chondrocytes. Arthritis Rheum 39(11):1896–1904
67. Nishida Y, D'Souza AL, Thonar EJ, Knudson W (2000) Stimulation of hyaluronan metabolism by interleukin-1alpha in human articular cartilage. Arthritis Rheum 43(6):1315–1326
68. Loeser RF, Pacione CA, Chubinskaya S (2003) The combination of insulin-like growth factor 1 and osteogenic protein 1 promotes increased survival of and matrix synthesis by normal and osteoarthritic human articular chondrocytes. Arthritis Rheum 48(8):2188–2196
69. Fan Z, Chubinskaya S, Rueger DC, Bau B, Haag J, Aigner T (2004) Regulation of anabolic and catabolic gene expression in normal and osteoarthritic adult human articular chondrocytes by osteogenic protein-1. Clin Exp Rheumatol 22(1):103–106
70. Lietman SA, Yanagishita M, Sampath TK, Reddi AH (1997) Stimulation of proteoglycan synthesis in explants of porcine articular cartilage by recombinant osteogenic protein-1 (bone morphogenetic protein-7). J Bone Joint Surg Am 79(8):1132–1137
71. Grgic M, Jelic M, Basic V, Basic N, Pecina M, Vukicevic S (1997) Regeneration of articular cartilage defects in rabbits by osteogenic protein-1 (bone morphogenetic protein-7). Acta Med Croatica 51(1):23–27
72. Louwerse RT, Heyligers IC, Klein-Nulend J, Sugihara S, van Kampen GP, Semeins CM, Goei SW, de Koning MH, Wuisman PI, Burger EH (2000) Use of recombinant human osteogenic protein-1 for the repair of subchondral defects in articular cartilage in goats. J Biomed Mater Res 49(4):506–516
73. Cook SD, Patron LP, Salkeld SL, Rueger DC (2003) Repair of articular cartilage defects with osteogenic protein-1 (BMP-7) in dogs. J Bone Joint Surg Am 85-A(Suppl 3):116–123
74. Jelic M, Pecina M, Haspl M, Kos J, Taylor K, Maticic D, McCartney J, Yin S, Rueger D, Vukicevic S (2001) Regeneration of articular cartilage chondral defects by osteogenic protein-1 (bone morphogenetic protein-7) in sheep. Growth Factors 19(2):101–113
75. Kaps C, Bramlage C, Smolian H, Haisch A, Ungethum U, Burmester GR, Sittinger M, Gross G, Haupl T (2002) Bone morphogenetic proteins promote cartilage differentiation and protect engineered artificial cartilage from fibroblast invasion and destruction. Arthritis Rheum 46(1):149–162
76. Merrihew C, Kumar B, Heretis K, Rueger DC, Kuettner KE, Chubinskaya S (2003) Alterations in endogenous osteogenic protein-1 with degeneration of human articular cartilage. J Orthop Res 21(5):899–907

77. Chubinskaya S, Hakimiyan A, Pacione C, Yanke A, Rappoport L, Aigner T, Rueger DC, Loeser RF (2007) Synergistic effect of IGF-1 and OP-1 on matrix formation by normal and OA chondrocytes cultured in alginate beads. Osteoarthritis Cartilage 15(4):421–430
78. Im HJ, Pacione C, Chubinskaya S, Van Wijnen AJ, Sun Y, Loeser RF (2003) Inhibitory effects of insulin-like growth factor-1 and osteogenic protein-1 on fibronectin fragment- and interleukin-1beta-stimulated matrix metalloproteinase-13 expression in human chondrocytes. J Biol Chem 278(28):25386–25394
79. Shimmin A, Young D, O'Leary S, Shih MS, Rueger DC, Walsh WR: Growth factor augmentation of an ovine mosaicplasty model. Trans ICRS. 2003; 2003.
80. Hidaka C, Goodrich LR, Chen CT, Warren RF, Crystal RG, Nixon AJ (2003) Acceleration of cartilage repair by genetically modified chondrocytes over expressing bone morphogenetic protein-7. J Orthop Res 21(4):573–583
81. Nishida Y, Knudson CB, Knudson W (2004) Osteogenic Protein-1 inhibits matrix depletion in a hyaluronan hexasaccharide-induced model of osteoarthritis. Osteoarthritis Cartilage 12(5):374–382
82. Loeser RF, Yammani RR, Carlson CS, Chen H, Cole A, Im HJ, Bursch LS, Yan SD (2005) Articular chondrocytes express the receptor for advanced glycation end products: Potential role in osteoarthritis. Arthritis Rheum 52(8):2376–2385
83. Kuo AC, Rodrigo JJ, Reddi AH, Curtiss S, Grotkopp E, Chiu M (2006) Microfracture and bone morphogenetic protein 7 (BMP-7) synergistically stimulate articular cartilage repair. Osteoarthritis Cartilage 14(11):1126–1135
84. Badlani N, Inoue A, Healey R, Coutts R, Amiel D (2008) The protective effect of OP-1 on articular cartilage in the development of osteoarthritis. Osteoarthritis Cartilage 16(5): 600–606
85. Hurtig M, Chubinskaya S, Dickey J, Rueger D (2009) BMP-7 protects against progression of cartilage degeneration after impact injury. J Orthop Res 27(5):602–611
86. Chubinskaya S, Otten L, Soeder S, Borgia JA, Aigner T, Rueger DC, Loeser RF (2011) Regulation of chondrocyte gene expression by osteogenic protein-1. Arthritis Res Ther 13(2):R55
87. Krawczak DA, Westendorf JJ, Carlson CS, Lewis JL (2009) Influence of bone morphogenetic protein-2 on the extracellular matrix, material properties, and gene expression of long-term articular chondrocyte cultures: loss of chondrocyte stability. Tissue Eng Part A 15(6):1247–1255
88. Chen AL, Fang C, Liu C, Leslie MP, Chang E, Di Cesare PE (2004) Expression of bone morphogenetic proteins, receptors, and tissue inhibitors in human fetal, adult, and osteoarthritic articular cartilage. J Orthop Res 22(6):1188–1192
89. Nakase T, Miyaji T, Tomita T, Kaneko M, Kuriyama K, Myoui A, Sugamoto K, Ochi T, Yoshikawa H (2003) Localization of bone morphogenetic protein-2 in human osteoarthritic cartilage and osteophyte. Osteoarthritis Cartilage 11(4):278–284
90. Fukui N, Zhu Y, Maloney WJ, Clohisy J, Sandell LJ (2003) Stimulation of BMP-2 expression by pro-inflammatory cytokines IL-1 and TNF-alpha in normal and osteoarthritic chondrocytes. J Bone Joint Surg Am 85-A(Suppl 3):59–66
91. Dell'Accio F, De Bari C, El Tawil NM, Barone F, Mitsiadis TA, O'Dowd J, Pitzalis C (2006) Activation of WNT and BMP signaling in adult human articular cartilage following mechanical injury. Arthritis Res Ther 8(5):R139
92. Bobacz K, Gruber R, Soleiman A, Erlacher L, Smolen JS, Graninger WB (2003) Expression of bone morphogenetic protein 6 in healthy and osteoarthritic human articular chondrocytes and stimulation of matrix synthesis in vitro. Arthritis Rheum 48(9):2501–2508
93. Javed A, Bae JS, Afzal F, Gutierrez S, Pratap J, Zaidi SK, Lou Y, van Wijnen AJ, Stein JL, Stein GS et al (2008) Structural coupling of Smad and Runx2 for execution of the BMP2 osteogenic signal. J Biol Chem 283(13):8412–8422
94. Javed A, Afzal F, Bae JS, Gutierrez S, Zaidi K, Pratap J, van Wijnen AJ, Stein JL, Stein GS, Lian JB (2009) Specific residues of RUNX2 are obligatory for formation of BMP2-induced RUNX2-SMAD complex to promote osteoblast differentiation. Cells Tissues Organs 189(1–4):133–137

95. Leboy P, Grasso-Knight G, D'Angelo M, Volk SW, Lian JV, Drissi H, Stein GS, SL A (2001) Smad-Runx interactions during chondrocyte maturation. J Bone Joint Surg Am 83-A Suppl 1(Pt 1):S15–S22
96. Reddi AH (1998) Role of morphogenetic proteins in skeletal tissue engineering and regeneration. Nat Biotechnol 16(3):247–252
97. Brunet LJ, McMahon JA, McMahon AP, Harland RM (1998) Noggin, cartilage morphogenesis, and joint formation in the mammalian skeleton. Science 280(5368):1455–1457
98. Pizette S, Niswander L (2000) BMPs are required at two steps of limb chondrogenesis: formation of prechondrogenic condensations and their differentiation into chondrocytes. Dev Biol 219(2):237–249
99. Lories RJ, Daans M, Derese I, Matthys P, Kasran A, Tylzanowski P, Ceuppens JL, Luyten FP (2006) Noggin haploinsufficiency differentially affects tissue responses in destructive and remodeling arthritis. Arthritis Rheum 54(6):1736–1746
100. Nakashima A, Tamura M (2006) Regulation of matrix metalloproteinase-13 and tissue inhibitor of matrix metalloproteinase-1 gene expression by WNT3A and bone morphogenetic protein-2 in osteoblastic differentiation. Front Biosci 11:1667–1678
101. Aigner T, Fundel K, Saas J, Gebhard PM, Haag J, Weiss T, Zien A, Obermayr F, Zimmer R, Bartnik E (2006) Large-scale gene expression profiling reveals major pathogenetic pathways of cartilage degeneration in osteoarthritis. Arthritis Rheum 54(11):3533–3544
102. Aigner T, Zien A, Gehrsitz A, Gebhard PM, McKenna L (2001) Anabolic and catabolic gene expression pattern analysis in normal versus osteoarthritic cartilage using complementary DNA-array technology. Arthritis Rheum 44(12):2777–2789
103. Hermansson M, Sawaji Y, Bolton M, Alexander S, Wallace A, Begum S, Wait R, Saklatvala J (2004) Proteomic analysis of articular cartilage shows increased type II collagen synthesis in osteoarthritis and expression of inhibin betaA (activin A), a regulatory molecule for chondrocytes. J Biol Chem 279(42):43514–43521
104. Gamer LW, Ho V, Cox K, Rosen V (2008) Expression and function of BMP3 during chick limb development. Dev Dyn 237(6):1691–1698
105. Allendorph GP, Isaacs MJ, Kawakami Y, Izpisua Belmonte JC, Choe S (2007) BMP-3 and BMP-6 structures illuminate the nature of binding specificity with receptors. Biochemistry 46(43):12238–12247
106. Blaney Davidson EN, Vitters EL, van der Kraan PM, van den Berg WB (2006) Expression of transforming growth factor-beta (TGFbeta) and the TGFbeta signalling molecule SMAD-2P in spontaneous and instability-induced osteoarthritis: role in cartilage degradation, chondrogenesis and osteophyte formation. Ann Rheum Dis 65(11):1414–1421
107. Blaney Davidson EN, Remst DF, Vitters EL, van Beuningen HM, Blom AB, Goumans MJ, van den Berg WB, van der Kraan PM (2009) Increase in ALK1/ALK5 ratio as a cause for elevated MMP-13 expression in osteoarthritis in humans and mice. J Immunol 182(12):7937–7945
108. Bruckner P, van der Rest M (1994) Structure and function of cartilage collagens. Microsc Res Tech 28(5):378–384
109. Vikkula M, Metsaranta M, Ala-Kokko L (1994) Type II collagen mutations in rare and common cartilage diseases. Ann Med 26(2):107–114
110. Freisinger P, Ala-Kokko L, LeGuellec D, Franc S, Bouvier R, Ritvaniemi P, Prockop DJ, Bonaventure J (1994) Mutation in the COL2A1 gene in a patient with hypochondrogenesis. Expression of mutated COL2A1 gene is accompanied by expression of genes for type I procollagen in chondrocytes. J Biol Chem 269(18):13663–13669
111. Peeters M, Detiger SE, Karfeld-Sulzer LS, Smit TH, Yayon A, Weber FE, Helder MN (2015) BMP-2 and BMP-2/7 heterodimers conjugated to a fibrin/hyaluronic acid hydrogel in a large animal model of mild intervertebral disc degeneration. Biores Open Access 4(1):398–406
112. Takegami K, An HS, Kumano F, Chiba K, Thonar EJ, Singh K, Masuda K (2005) Osteogenicprotein-1 is most effective in stimulating nucleus pulposus and annulus fibrosus cells to repair their matrix after chondroitinase ABC-induced in vitro chemonucleolysis. Spine J 5(3):231–238

113. Masuda K, Takegami K, An H, Kumano F, Chiba K, Andersson GB, Schmid T, Thonar E (2003) Recombinant osteogenic protein-1 upregulates extracellular matrix metabolism by rabbit annulus fibrosus and nucleus pulposus cells cultured in alginate beads. J Orthop Res 21(5):922–930
114. Imai Y, Miyamoto K, An HS, Thonar EJ, Andersson GB, Masuda K (2007) Recombinant human osteogenic protein-1 upregulates proteoglycan metabolism of human anulus fibrosus and nucleus pulposus cells. Spine (Phila Pa 1976) 32(12):1303–1309
115. Leckie SK, Bechara BP, Hartman RA, Sowa GA, Woods BI, Coelho JP, Witt WT, Dong QD, Bowman BW, Bell KM et al (2012) Injection of AAV2-BMP2 and AAV2-TIMP1 into the nucleus pulposus slows the course of intervertebral disc degeneration in an invivo rabbit model. Spine J 12(1):7–20
116. Masuda K, Imai Y, Okuma M, Muehleman C, Nakagawa K, Akeda K, Thonar E, Andersson G, An HS (2006) Osteogenic protein-1 injection into a degenerated disc induces the restoration of disc height and structural changes in the rabbit anular puncture model. Spine (Phila Pa 1976) 31(7):742–754
117. Kawakami M, Matsumoto T, Hashizume H, Kuribayashi K, Chubinskaya S, Yoshida M (2005) Osteogenic protein-1 (osteogenic protein-1/bone morphogenetic protein-7) inhibits degeneration and pain-related behavior induced by chronically compressed nucleus pulposus in the rat. Spine (Phila Pa 1976) 30(17):1933–1939
118. Huang KY, Yan JJ, Hsieh CC, Chang MS, Lin RM (2007) The in vivo biological effects of intradiscal recombinant human bone morphogenetic protein-2 on the injured intervertebral disc: an animal experiment. Spine (Phila Pa 1976) 32(11):1174–1180
119. An HS, Takegami K, Kamada H, Nguyen CM, Thonar EJ, Singh K, Andersson GB, Masuda K (2005) Intradiscal administration of osteogenic protein-1 increases intervertebral disc height and proteoglycan content in the nucleus pulposus in normal adolescent rabbits. Spine (Phila Pa 1976) 30(1):25–31 discussion 31-22
120. Imai Y, Okuma M, An HS, Nakagawa K, Yamada M, Muehleman C, Thonar E, Masuda K (2007) Restoration of disc height loss by recombinant human osteogenic protein-1injection into intervertebral discs undergoing degeneration induced by an intradiscal injection of chondroitinase ABC. Spine (Phila Pa 1976) 32(11):1197–1205
121. Chubinskaya S, Kawakami M, Rappoport L, Matsumoto T, Migita N, Rueger DC (2007) Anti-catabolic effect of OP-1 in chronically compressed intervertebral discs. J Orthop Res 25(4):517–530
122. Woods BI, Vo N, Sowa G, Kang JD (2011) Gene therapy for intervertebral disk degeneration. Orthop Clin North Am 42(4):563–574
123. Jeong CG, Zhang H, Hollister SJ (2012) Three-dimensional polycaprolactone scaffold-conjugated bone morphogenetic protein-2 promotes cartilage regeneration from primary chondrocytes in vitro and in vivo without accelerated endochondral ossification. J Biomed Mater Res A 100(8):2088–2096
124. Than KD, Rahman SU, Vanaman MJ, Wang AC, Lin CY, Zhang H, La Marca F, Park P (2012) Bone morphogenetic proteins and degenerative disk disease. Neurosurgery 70(4):996–1002 discussion 1002
125. Hunter CJ, Matyas JR, Duncan NA (2004) Cytomorphology of notochordal and chondrocytic cells from the nucleus pulposus: a species comparison. J Anat 205(5):357–362
126. Balk ML, Bray J, Day C et al (1997) Effect of rhBMP-2 on the osteogenic potential of bone marrow stromal cells from an osteogenesis imperfecta mouse (oim). Bone 21:7–15
127. Giannoudis PV, Einhorn TA, Marsh D (2007) Fracture healing: the diamond concept. Injury 38(Suppl. 4):S3–S6
128. Pountos I, Panteli M, Georgouli T, Giannoudis PV (2014 Nov) Neoplasia following use of BMPs: is there an increased risk? Expert Opin Drug Saf 13(11):1525–1534
129. Mognetti B, Marino S, Barberis A, Martin AS, Bala Y, Di Carlo F, Boivin G, Barbos MP (2011) Experimental stimulation of bone healing with teriparatide: histomorphometric and microhardness analysis in a mouse model of closed fracture. Calcif Tissue Int 89(2): 163–171

Osteogrow: A Novel Bone Graft Substitute for Orthopedic Reconstruction

Lovorka Grgurevic, Igor Erjavec, Ivo Dumic-Cule, Tatjana Bordukalo-Niksic, Martina Pauk, Vladimir Trkulja, Drazen Maticic, Marko Pecin, Marija Lipar, Mihaela Peric, and Slobodan Vukicevic

Abstract Complications associated with the clinical use of BMP2 and BMP7 result from the limited understanding of their molecular mechanisms in bone remodeling. Recently, a novel BMP6-based approach has been developed with superior healing results and reduced side effects in preclinical studies. BMP6-containing osteogenic medicinal product called Osteogrow, which is aimed to induce and accelerate bone formation, is currently being tested in clinical studies. It comprises of a biologically compatible autologous carrier made from the patient's peripheral blood and of rhBMP6 as an active ingredient. Such formulation circumvents the use of animal-derived materials, significantly limits inflammatory processes common in commercial bone devices, and renders the carrier flexible and injectable ensuring the ease of use. The ongoing clinical trial results will provide a more detailed insight into the safety, tolerability, pharmacokinetics, and bone healing effects in humans and hopefully provide novel and valuable therapeutic options in the field of bone regeneration.

Keywords BMP6 • Bone regeneration • Bone graft substitute • BMP complications • Osteogrow • Autologous blood BMP6 carrier

L. Grgurevic (✉) • I. Erjavec • I. Dumic-Cule • T. Bordukalo-Niksic
M. Pauk (✉) • S. Vukicevic (✉)
University of Zagreb School of Medicine, Center for Translational and Clinical Research, Laboratory for Mineralized Tissues, Salata 11, Zagreb, Croatia
e-mail: lgrgurev@mef.hr; vukicev@mef.hr

V. Trkulja
University of Zagreb School of Medicine, Department of Pharmacology, Salata 11, Zagreb, Croatia

D. Maticic • M. Pecin • M. Lipar
University of Zagreb School of Veterinary Medicine, Clinic for Surgery, Orthopedics and Ophthalmology, Heinzelova 55, Zagreb, Croatia

M. Peric (✉)
University of Zagreb School of Medicine, Center for Translational and Clinical Research, Department for Intercellular Communication, Salata 2, Zagreb, Croatia
e-mail: mihaela.peric@mef.hr

S. Vukicevic, K.T. Sampath (eds.), *Bone Morphogenetic Proteins: Systems Biology Regulators*, Progress in Inflammation Research,
DOI 10.1007/978-3-319-47507-3_9

1 Introduction

Molecular processes required for bone repair [36, 37] are a prerequisite for the development of new biological procedures for stimulation of bone healing. Bone fracture healing results in 10 % delayed or impaired registered cases out of currently six million fractures in the European Union (EU) [1, 20]. By 2050, it is predicted that around 12 million bone fractures will occur yearly in the EU.

2 Bone Fracture Repair

Bone healing process involves signals, cells, and substratum, divided into three stages: an early inflammatory and cell recruitment stage (callus formation), intermittent cell differentiation and formation of new bone (fracture repair), and late bone remodeling and formation of defined cortices (restoration) [16]. Most of the fractures heal without any consequences; however, compound or open fractures result in secondary healing due to incomplete mechanical stability of broken fragments. Intramembranous ossification produces bone directly under the periosteum within the first days after injury, overleaping chondrogenesis in the center leading to endochondral ossification [17]. Improper bone healing has potentially devastating consequences, ranging from disfigurement to the loss of function and eventually loss of limb [27]. In cases where normal bone fracture healing is not obtained, BMP containing bone devices might support induction of new bone formation locally and achieve the bridging [14]. In approximately 10 % of cases, fractured bones heal slowly (malunion) or fail to heal (nonunion) and require additional medical interventions to repair the fracture [14, 15].

3 BMP-Based Solutions for Fracture Healing

In efforts to develop BMP-based therapies to treat bone defects, it soon became clear that one way to treat a bone defect would be to implant into the defect site some type of implantable matrix carrying an effective amount of a human recombinant BMP (rhBMP). Currently, two therapeutic concepts have been introduced to the market in order to overcome nonhealing bone or complicated bone fractures. The bone devices consist of a bovine collagen matrix soaked with rhBMP2 (Infuse Bone Graft, lumbar tampered fusion device) or rhBMP7 (Osigraft) [5, 48] (Table 1).

Safety and clinical efficacy of these devices have been recently reviewed and reported [6, 19, 40].

In parallel with tibial fractures and nonunions approved by FDA and EMA, rhBMP2 and -7 have been used off-label for different bone repair indications with an aim to overcome the impaired healing [38, 48]. Small randomized controlled

Table 1 Approved BMP-based therapies

BMP/trade name/date of approval/ source	Presentation/dose	Approved indications in EU	Disadvantages
BMP2 InductOS (dibotermin alfa). Approved in EU 9/2002. Rec BMP2 is made in CHO cells	InductOs kit contains dibotermin alfa at the concentration of 1.5 mg/ml (12 mg per vial) and an absorbable collagen sponge (bovine collagen). Usually use one kit per fracture	1. For single-level anterior lumbar spine fusion as a substitute for autogenous bone graft in adults with degenerative disc disease who have had at least 6 months of nonoperative treatment for this condition 2. For the treatment of acute tibia fractures in adults, as an adjunct to standard care using open fracture reduction and intramedullary unreamed nail fixation	1. Ectopic bone formation is a "common" ADR[a] 2. Can cause bone remodeling where both bone resorption and formation occur – may lead to nerve compression or device migration[a] 3. Inflammation and swelling can occur[b] 4. Risk of using bovine collagen
BMP7 Osigraft: (eptotermin alfa). Approved in EU 5/2001. Rec BMP7 is made in CHO cells	Each vial of Osigraft contains 3.3 mg of OP-1 in 1 g dried bovine collagen. Use 1–2 vials per surgery	Treatment of nonunion of tibia of at least 9-month duration, secondary to trauma, in skeletally mature patients, in cases where previous treatment with autograft has failed or use of autograft is unfeasible	1. Heterotopic ossification is a common ADR[a] 2. Inflammation and swelling are common ADRs[a] 3. Risk of using bovine collagen

[a]From SmPCs (common is defined as ≥1/100 to <1/10)
[b]From Vukicevic et al. [47]

trials (RCTs) included, for example, a successful management of the proximal scaphoid pole nonunion by BMP7 alone and in combination with an autograft or allograft [4]. In addition, two studies with a total of 30 enrolled patients showed a full restoration of humeral nonunions when rhBMP7 was used with an autograft [7, 21]. rhBMP2 was efficacious in the same indication, and the union was accomplished in eight out of nine patients [12].

For both BMP devices, major side effects were reported, and their therapeutic value has been recently reevaluated [9, 10, 19, 25, 40, 47]. Local transient swelling, inflammation, heterotopic ossification as well as early osteolysis were among serious complications following long bone implantation and spinal fusion application, particularly in the cervical spine. Local swelling and inflammation can be easily overlooked if there is a sufficient amount of tissue envelope around the broken bone. The inflammation was mostly noticed in patients with distal radial and tibial fractures [18]. Swelling and inflammation were observed under the skin, in distal radial osteotomy patients treated with rhBMP7 where metaphyseal bone is predominantly present, resulting in bone resorption and skin redness. The bovine collagen

as a carrier for BMP2 and BMP7, noninjectable formulations for closed fractures and a high market price are preventing broader use of BMP containing devices for bone regeneration procedures. Bovine collagen has been subjected due to a potential bovine spongioform encephalopathy to new strict regulations when used as a medicinal product for human applications [34]. These side effects and the price restrict the routine use of current BMP devices in patients with osteoporotic fractures to prevent nonunions with bone defects. This especially applies to elderly patients with a high proportion of secondary interventions. Heterotopic ossification can be explained with an abundant quantity of rhBMP in currently used devices. The average amount of rhBMP incorporated into the collagen carrier is between 3.5 and 12.5 mg, sometimes up to 40 mg of rhBMP2 in patients with spinal fusion surgeries but depends on the site and size of the fracture gap, while entire human body normally contains only around 2 mg of rhBMPs (Fig. 1).

BMPs are not soluble at neutral pH and only about 75 μg of protein bind to 1 g of bovine collagen [11], while the rest precipitates, gets locally released, and represents a potential source for local and systemic side effects. According to previous pharmacokinetic and bioavailability studies of rhBMP7 and rhBMP2, it should be expected that ≤2–3 % of locally administered rhBMP will be present in the patient's circulation shortly after the application. We recently suggested that the skeletal impact of potentially systemically released BMP2 and -7 might rather have a positive effect on the skeletal volume via increasing the overall bone volume [13]. When

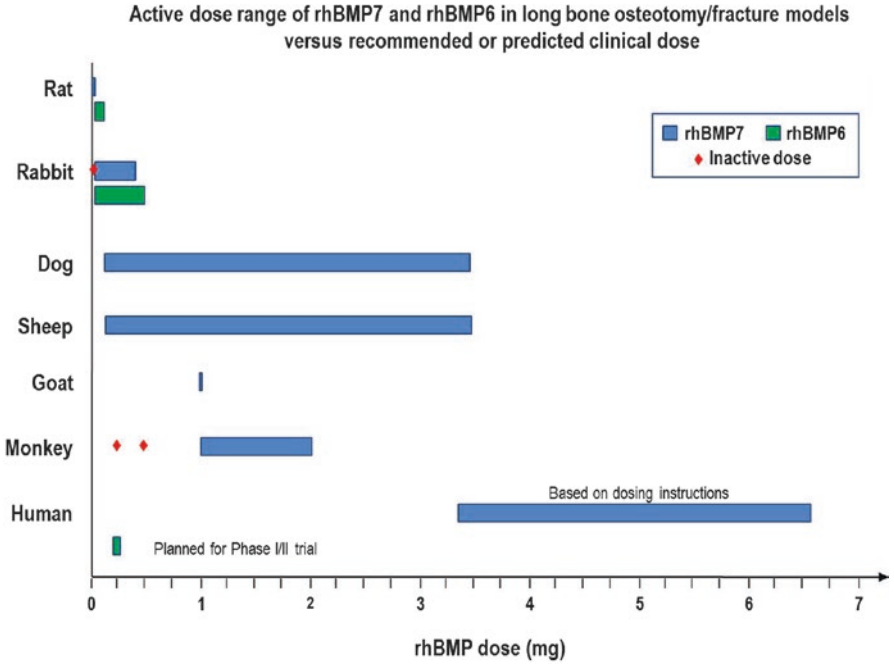


Fig. 1 Active dose range of rhBMP7 and rhBMP6 in animal long bone osteotomy fracture models versus recommended or used clinical dose

applied within the bone medullary canal, a pronounced bone resorption has been observed in sheep [32]. A rhBMP2-based device used in patients for the lower lumbar spine fusion resulted with complications like autonomic plexus injury, retrograde ejaculation, and heterotopic ossification [3, 9, 19, 26, 40]. In another study using rhBMP2-based device and autogenous bone in a laparoscopic anterior lumbar interbody fusion approach no adverse effects were detected [6, 28]. In patients undergoing posterior cervical fusion, complications like postoperative edema, dysphagia, and hematoma formation have not been observed [24].

Early osteolysis following the use of rhBMP2 and -7 devices might have caused the implant shift and subsequent fracture instability, especially if the periosteum was destroyed [18]. For example, in patients with unstable thoracolumbar fractures, the application of rhBMP7 resulted in substantial bone resorption, loss of reduction, and segmental collapse [22, 29, 39]. As previously clarified, rhBMP2 and rhBMP7 with their pronounced effect on osteoclasts in the vertebrae where surfaces are lined with coupled bone cells exert bone resorption at endosteal/trabecular surfaces. Upon retrospective analyses in several clinical studies, it was suggested that the observed initial resorption was of transient nature and that bone formation and bone repair subsequently occurred [19, 40]. This was initially overlooked due to insufficient knowledge about BMP mechanism of action on endosteal surfaces as a result of their predominant stimulation of osteoclasts in the early phase (Fig. 2) [32, 47]. Thus, complications associated with the clinical use of rhBMP2 and rhBMP7 bone devices were due to the limited understanding of their molecular mechanisms in bone turnover. There is therefore a need for the development of a new osteogenic device that will offer safe and cost-effective healing. Well-designed and performed studies are thus needed to better define the incidence of complications in regard to the type of rhBMP, region of fusion, surgical technique, dose, and carrier [33].

4 BMP6 Is a Novel Therapy for Bone Repair

New solutions for bone healing are therefore needed, taking into account the complexity of BMP signaling and different cellular and tissue effects. As BMPs exert different biological responses depending on the microenvironment, the specificities of bone fracture milieu should be considered. In bone, tissues surrounding the injury like periosteum, endosteum, bone marrow, vascular tissue, and muscles provide progenitor cells that initiate formation of bone callus and subsequently new bone.

4.1 BMP Receptors in Human Mesenchymal Stem Cells

Osteoinductive BMP activities in human mesenchymal stem cells (hMSCs) are elicited through the type I receptors ACVR1A and BMPR1A and the type II receptors ACVR2A and BMPR2. BMPR1B and ACVR2B are expressed at low levels, while type II receptor utilization differs significantly between BMP2/4 and BMP6/7. A

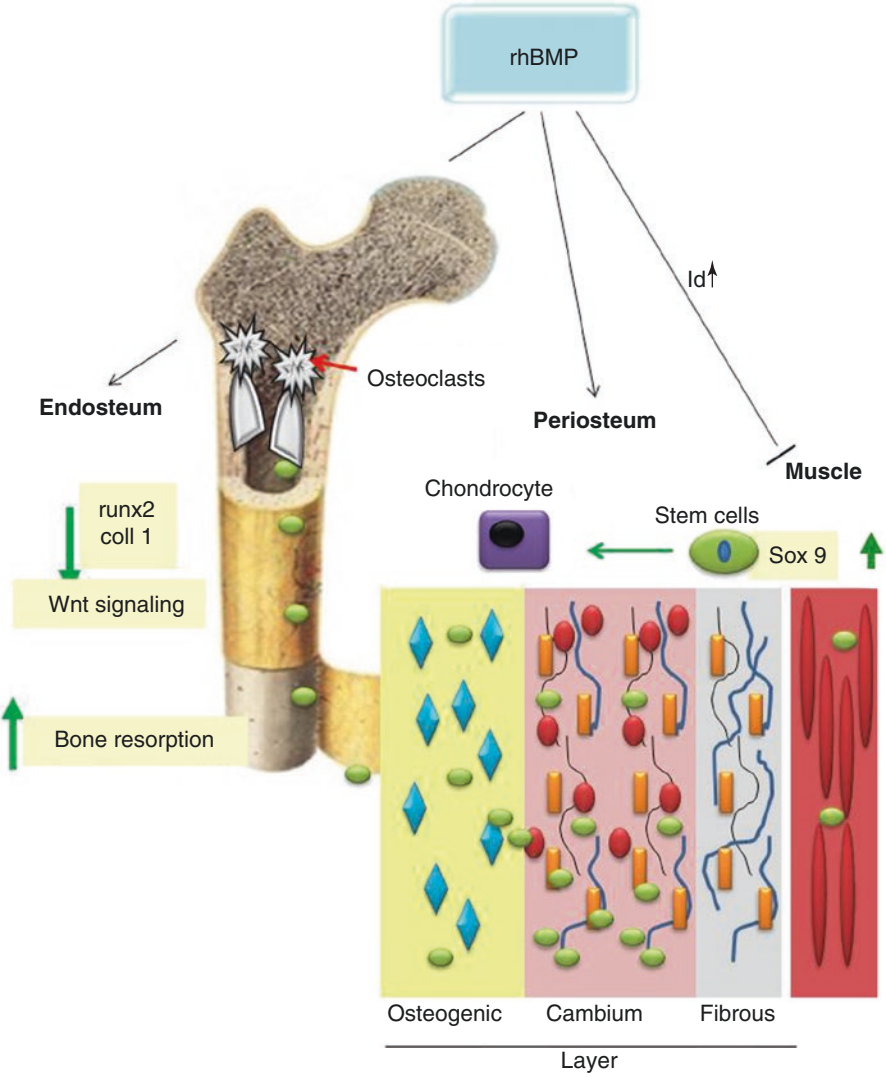


Fig. 2 *In vivo* effect of osteogenic BMPs on bone, periosteum, and muscle compartment. At the endosteal surface, BMPs affect both osteoclasts and osteoblasts with a net outcome of downregulation of Runx2, collagen I, and Wnt signaling; at the periosteum, BMPs stimulate differentiation of precursor cells into osteoblasts; and in surrounding muscle cells, BMPs upregulate Id genes and stimulate new osteoblasts and prechondrocytes to form cartilage and new bone around the cortical bone. The new bone then spreads into the medullar cavity (Modified from Vukicevic et al. [47])

greater reliance on BMPR2 exists for BMP2/4 relative to BMP6/7, whereas ACVR2A is more important to signaling by BMP6/7 than BMP2/4. Regarding the BMP type I receptor BMP2/4 used predominantly BMPR1A for signaling; however, ACVR1A is the preferred type I receptor for BMP6/7. Signaling by both

BMP2/4 and BMP6/7 is mediated by homodimers of ACVR1A or BMPR1A. A portion of BMP2/4 signaling requires concurrent BMPR1A and ACVR1A expression, suggesting that BMP2/4 signal in part through ACVR1A/BMPR1A heterodimers. Due to different receptor utilization of BMPs, different mechanisms for BMP6/7- and BMP2/4-induced osteoblastic differentiation in primary hMSC have been proposed [30]. Therefore, different mechanisms for BMP2/4- and BMP6/7-stimulated osteoblastic differentiation are present in primary hMSC from the bone marrow which actively participate in the bone healing process. Beyond bone, BMP receptors are broadly expressed in all tissues and organs with a variable density depending on the level of injury, since we and others have shown that their expression is significantly upregulated following acute and chronic kidney damage, injury of the liver, acute myocardial infarction, injury of the colon, etc. [8, 31, 42–45].

4.2 Osteogrow

The exact mechanism of BMP in bone remodeling was recently elucidated, resulting in novel BMP6-based clinical approach with superior healing results and reduced side effects in preclinical studies [47]. A novel rhBMP6 containing osteogenic medicinal product called Osteogrow aimed to accelerate bone regeneration was developed and is currently being tested in clinical studies. It comprises of a biologically compatible autologous carrier made from the patient's peripheral blood whole blood containing device (WBCD) and of rhBMP6 as an active ingredient. Such formulation circumvents the use of animal-derived materials, significantly limits inflammatory processes common in commercial bone devices, and renders the carrier flexible and injectable ensuring the ease of use (Fig. 3) [46]. Additionally, Osteogrow successfully rebridges critical size defects in animal models as well as enables physiological retention of rhBMP6 in the carrier upon binding to its extracellular matrix molecules and eventually to membrane receptors of cells constituting the WBCD as confirmed by negligible absolute bioavailability following local implantation in animals. Overall, nonclinical evaluation demonstrates a high safety margin for the use of Osteogrow in human bone defect indications. The ongoing clinical trial results will provide a more detailed insight into the safety, tolerability, pharmacokinetics, and bone healing effects in humans and hopefully provide novel and valuable therapeutic options in the field of bone regeneration.

5 BMP Mechanism of Action in Osteogrow

Unlike BMP2 and BMP7 knockout mice which die of placental deformation or renal insufficiency, respectively [43], BMP6 knockout mice show a delayed ossification with lower trabecular bone volume and suffer from a hereditary

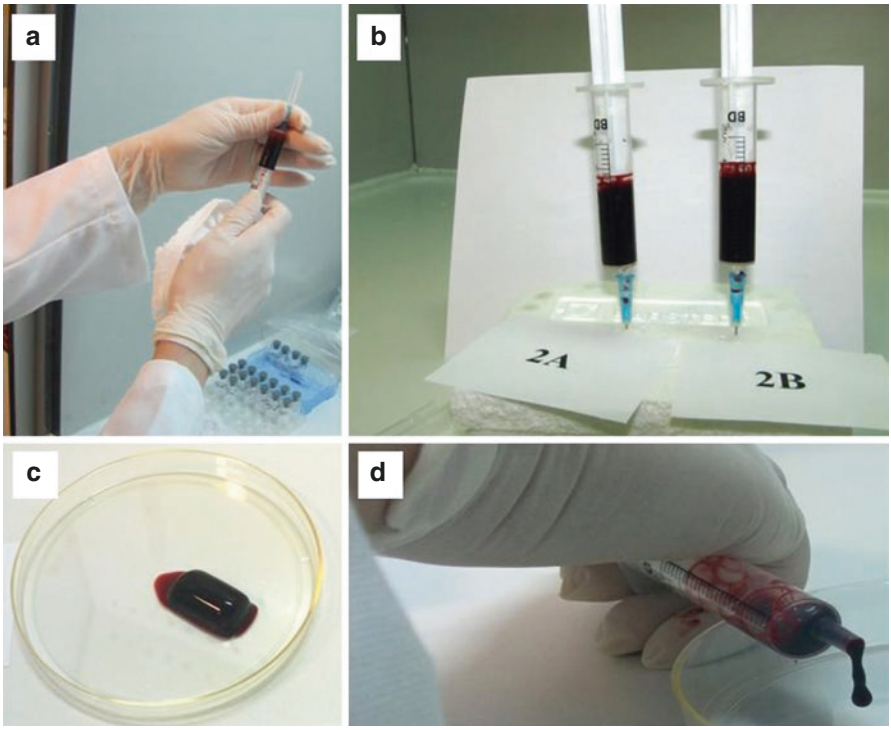


Fig. 3 Osteogrow preparation. (**a**) The rhBMP6 drug product is reconstituted with water for injection and mixed with the freshly sampled patient's own blood and with calcium chloride. (**b**) The blood mixture is incubated within the syringe at room temperature for 60–90 min. (**c**) The resulting coagulum or WBCD (Whole Blood Containing Device) is red to deep red in color and cylindrically shaped when ejected from the syringe. (**d**) The WBCD is easily injectable from the syringe

hemochromatosis phenotype [2]. BMP6 circulates in the plasma of normal human subjects [35] and is more active than its paralog BMP7 in stimulating bone regeneration in rabbits with critical size ulna defects [41]. BMP6 is resistant to noggin, the most abundant physiological BMP antagonist, due to the amino acid lysin in the position 60 of the mature BMP6 domain, while BMP2 and BMP7 contain prolin or aspartic acid in the same position, respectively, providing the structural basis for their irreversible binding to noggin [41]. Since noggin is abundantly present in the bone and its surrounding tissues, large amounts of rhBMP2 and rhBMP7 have been used in humans to achieve bone repair which also resulted in substantial osteolysis and other side effects related to robust bone formation in the soft tissues [9, 47]. The physiological bone repair is associated with the formation of hematoma and blood coagulum between fractured bone parts which eventually supports local bone formation. Accidentally, during characterization of pharmacokinetic properties of rhBMP6 we discovered that it binds to coagulating blood components, resulting in

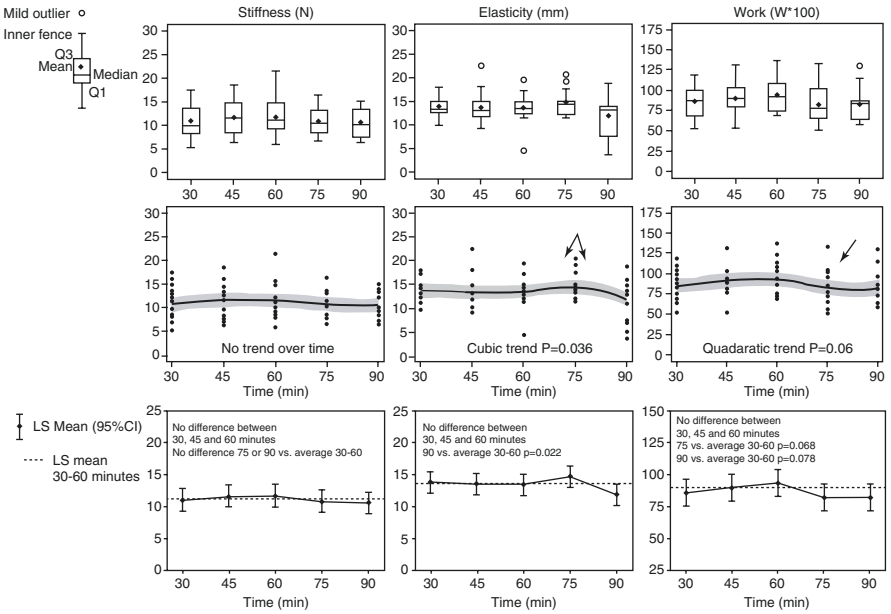


Fig. 4 Time effect on biomechanical properties of the coagulum in the FET (forward extrusion test) (n = 9 per time point). The only statistically significant effect appears to be a tendency of decreasing elasticity after 75 min. The test evaluates stiffness, elasticity, and work required for extrusion of the coagulum. Upper panel shows medians, quartiles, inner fences, and outliers (*dots*). The middle panel shows individual data with a cubic spline (shaded area = 95 % confidence interval). Lower panel shows least-square means with 95 % CI from a general linear model testing the effect of time. Dashed horizontal line depicts the average of values taken at 30, 45, and 60 min

almost full disappearance from the blood serum [46, 47], which has been demonstrated in experiments using rhBMP6 labeled with radioactive technetium (^{99m}Tc). The retention of rhBMP6 in the coagulum was above 99 % independent of the amount of protein used in blood samples from mouse, rat, and rabbit. These experiments proved that the whole-blood coagulum may potentially serve as a carrier or vehicle for applying rhBMP6 to bone fracture and/or defect [46].

Numerous experiments both *in vitro* and *in vivo* have been conducted to assure that an injectable blood coagulum following dilution of reconstituted rhBMP6 with water for injection will still remain homogenous, cohesive, syringable, injectable, and malleable for human use and maintain its biomechanical properties, including force, elasticity, and work of cutting measured by specifically designed CUT and forward extrusion tests (Fig. 4).

The influence of time, shaking, and calcium chloride on the coagulum biomechanical properties were also measured and showed that the coagulum maintained its biomechanical properties, structural characteristics, and the visual appearance within 90 min from mixing the blood with rhBMP6 in water for injection. Dilution of blood with up to 25 % with water did not impact the coagulum stiffness. The

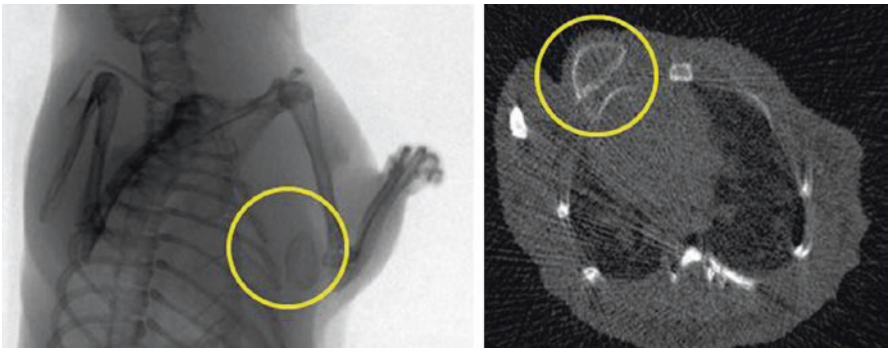


Fig. 5 Ectopic bone formation by μCT in mice 2 weeks after implantation of rhBMP6. *Circle* indicates the site of the ectopic bone formation (*insert*)

release of rhBMP6 from the coagulum was measured *in vitro* and indicated a slow release within a period of 5–7 days. *In vivo* pharmacokinetic studies in rats and rabbits showed that $t_{½}\alpha$ of the intravenously injected rhBMP6 was 1–10 min and it did not accumulate in organs. Pharmacokinetic measurements after orthotopic administration in the rat femur fracture model (paraosseous application) and in the rabbit ulna critical size defect (intraosseous application) indicated negligible absolute bioavailability of rhBMP6 administered within WBCD.

Preclinical rhBMP6 batches were tested for efficacy *in vitro* using the C2C12-BRE-Luc assay [23] and *in vivo* using an assay of subcutaneous implantation of Osteogrow in the pectoral region of rats (Fig. 5). Various doses have been tested and followed *in vivo* by microCT analyses to assess the bone formation activity and reproducibility of various production batches. Similar efficacy has been recorded between different batches produced for toxicology testing as well as for clinical trials.

In addition to induction of new bone formation, implanted coagula with different doses of rhBMP6 did not exhibit any swelling, edema, or inflammation at the site of implantation (Fig. 6).

Osteogrow was tested in rats and rabbits both for safety and for efficacy in the rabbit ulna critical size defect model in which 2.5 × bone diameter has been removed and filled in with an implant containing 100 μg rhBMP6/ml of blood used to form the coagulum (Fig. 7).

General toxicology studies were conducted in two species: rats and rabbits, while the local tolerance of the implant was tested in rabbits. Single doses of 30, 75, 150, and 450 μg/kg were safe, and similar amounts injected for 14 days did not cause any systemic toxicology signs. Administration of rhBMP6 within WBCD (concentration 500 μg/ml) after transcutaneous paraosseous injection or intraosseous implantation was well tolerated without any signs of local intolerance.

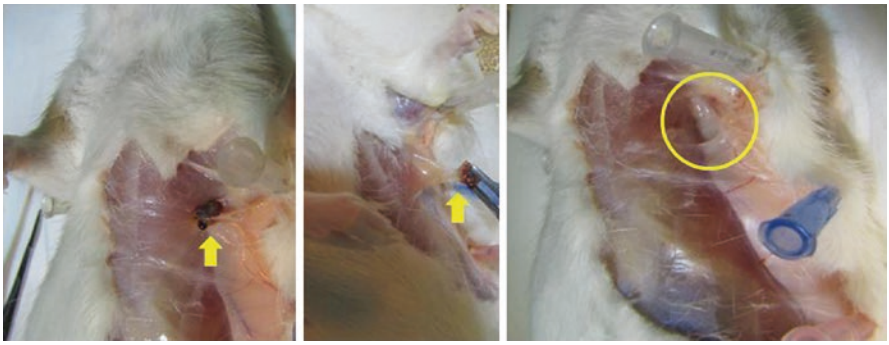


Fig. 6 *In vivo* testing of rhBMP6 activity in rat subcutaneous assay. WBCD containing rhBMP6 implanted in the pectoral region of rats was still visible after 14 days (arrows indicate the ossicle), while no inflammation of the surrounding tissue was observable (circle)

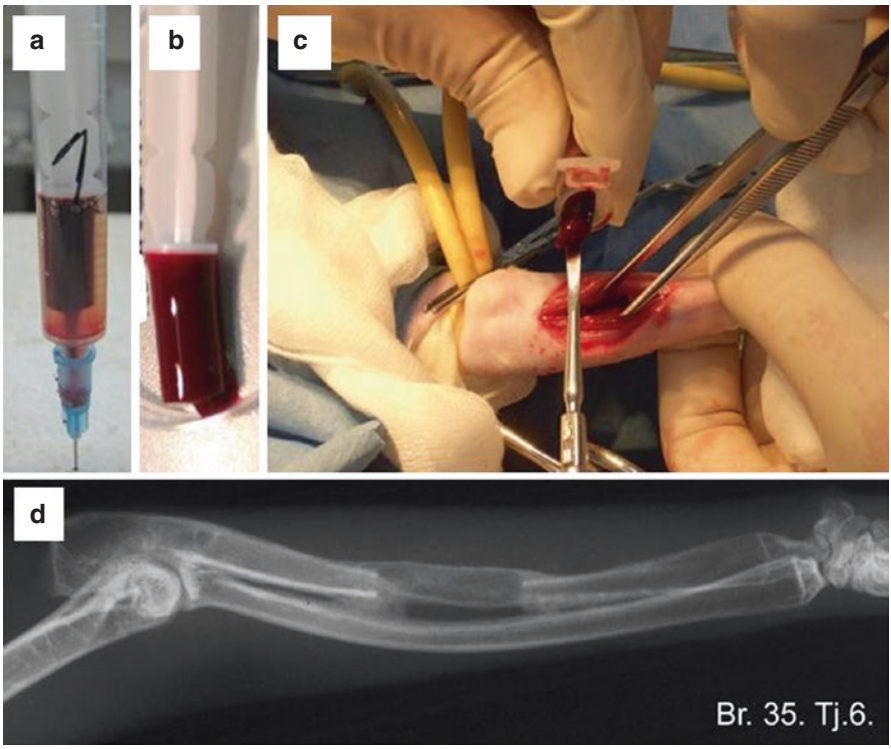


Fig. 7 Model of rabbit ulna critical size defect. Preparation of the WBCD containing rhBMP6 from autologous blood (**a**, **b**). Implantation of the WBCD at the defect site (**c**). Full rebridgement with cortical bone formation was observed 6 weeks after the surgery (**d**)

References

1. American Academy of Orthopaedic Surgeons (2000) Musculoskeletal injuries report: incidence, risk factors and prevention. AAOS, Rosemont, IL
2. Andriopoulos B Jr, Corradini E, Xia Y, Faasse SA, Chen S, Grgurevic L, Knutson MD, Pietrangelo A, Vukicevic S, Lin HY, Babitt JL (2009) BMP6 is a key endogenous regulator of hepcidin expression and iron metabolism. Nat Genet 41:482–487
3. Axelrad TW, Steen B, Lowenberg DW, Creevy WR, Einhorn TA (2008) Heterotopic ossification after the use of commercially available recombinant human bone morphogenetic proteins in four patients. J Bone Joint Surg Br 90:1617–1622
4. Bilic R, Simic P, Jelic M, Stern-Padovan R, Dodig D, van Meerdervoort HP, Martinovic S, Ivankovic D, Pecina M, Vukicevic S (2006) Osteogenic protein-1 (BMP-7) accelerates healing of scaphoid non-union with proximal pole sclerosis. Int Orthop 30:128–134
5. Bishop GB, Einhorn TA (2007) Current and future clinical applications of bone morphogenetic proteins in orthopaedic trauma surgery. Int Orthop 31:721–727
6. Boden SD, Zdeblick TA, Sandhu HS, Heim SE (2000) The use of rhBMP-2 in interbody fusion cages. Definitive evidence of osteoinduction in humans: a preliminary report. Spine 25:376–381. Phila Pa 1976
7. Bong MR, Capla EL, Egol KA, Sorkin AT, Distefano M, Buckle R, Chandler RW, Koval KJ (2005) Osteogenic protein-1 (bone morphogenic protein-7) combined with various adjuncts in the treatment of humeral diaphyseal nonunions. Bull Hosp Jt Dis 63:20–23
8. Bosukonda D, Shih MS, Sampath KT, Vukicevic S (2000) Characterization of receptors for osteogenic protein-1/bone morphogenetic protein-7 (OP-1/BMP-7) in rat kidneys. Kidney Int 58:1902–1911
9. Carragee EJ, Hurwitz EL, Weiner BK (2011a) A critical review of recombinant human bone morphogenetic protein-2 trials in spinal surgery: emerging safety concerns and lessons learned. Spine J 11:471–491
10. Carragee EJ, Mitsunaga KA, Hurwitz EL, Scuderi GJ (2011b) Retrograde ejaculation after anterior lumbar interbody fusion using rhBMP-2: a cohort controlled study. Spine J 11:511–516
11. Chatzinikolaidou M, Lichtinger TK, Müller RT, Jennissen HP (2010) Peri-implant reactivity and osteoinductive potential of immobilized rhBMP-2 on titanium carriers. Acta Biomater 6:4405–4421
12. Crawford CH 3rd, Seligson D (2009) Atrophic nonunion of humeral diaphysis treated with-locking plate and recombinant bone morphogenetic protein: nine cases. Am J Orthop (Belle Mead NJ) 38:567–570
13. Dumic-Cule I, Brkljacic J, Rogic D, Bordukalo Niksic T, Tikvica Luetic A, Draca N, Kufner V, Trkulja V, Grgurevic L, Vukicevic S (2014) Systemically available bone morphogenetic protein two and seven affect bone metabolism. Int Orthop 38:1979–1985
14. Dumic-Cule I, Pecina M, Jelic M, Jankolija M, Popek I, Grgurevic L, Vukicevic S (2015) Biological aspects of segmental bone defects management. Int Orthop 39:1005–1011
15. Einhorn TA (1995) Enhancement of fracture-healing. J Bone Joint Surg Am 77:940–956
16. Einhorn TA (1998) The cell and molecular biology of fracture healing. Clin Orthop Relat Res 355 Suppl:S7–21
17. Einhorn TA, Majeska RJ, Rush EB, Levine PM, Horowitz MC (1995) The expression of cytokine activity by fracture callus. J Bone Miner Res 10:1272–1281
18. Ekrol I, Hajducka C, Court-Brown C, McQueen MM (2008) A comparison of rhBMP-7 (OP-1) and autogenous graft for metaphyseal defects after osteotomy of the distal radius. Injury 39(Suppl 2):S73–S82
19. Fu R, Selph S, McDonagh M, Peterson K, Tiwari A, Chou R, Helfand M (2013) Effectiveness and harms of recombinant human bone morphogenetic protein-2 in spine fusion: a systematic review and meta-analysis. Ann Intern Med 158:890–902

20. Giannoudis PV, Kanakaris NK, Einhorn TA (2007) Interaction of bone morphogenetic proteins with cells of the osteoclast lineage: review of the existing evidence. Osteoporos Int 18:1565–1581
21. Giannoudis PV, Kanakaris NK, Dimitriou R, Gill I, Kolimarala V, Montgomery RJ (2009) The synergistic effect of autograft and BMP-7 in the treatment of atrophic nonunions. Clin Orthop Relat Res 467:3239–3248
22. Hansen SM, Sasso RC (2006) Resorptive response of rhBMP2 simulating infection in an anterior lumbar interbody fusion with a femoral ring. J Spinal Disord Tech 19:130–134
23. Herrera B, Inman GJ (2009) A rapid and sensitive bioassay for the simultaneous measurement of multiple bone morphogenetic proteins. Identification and quantification of BMP4, BMP6 and BMP9 in bovine and human serum. BMC Cell Biol 10:20
24. Hiremath GK, Steinmetz MP, Krishnaney AA (2009) Is it safe to use recombinant human bone morphogenetic protein in posterior cervical fusion? Spine 34:885–889. Phila Pa 1976
25. Hustedt JW, Blizzard DJ (2014) The controversy surrounding bone morphogenetic proteins in the spine: a review of current research. Yale J Biol Med 87:549–561
26. Kang JD (2011) Another complication associated with rhBMP-2? Spine J 11:517–519
27. Kenley RA, Yim K, Abrams J, Ron E, Turek T, Marden LJ, Hollinger JO (1993) Biotechnology and bone graft substitutes. Pharm Res 10:1393–1401
28. Kleeman TJ, Ahn UM, Talbot-Kleeman A (2001) Laparoscopic anterior lumbar interbody fusion with rhBMP-2: a prospective study of clinical and radiographic outcomes. Spine 26:2751–2756. Phila Pa 1976
29. Laursen M, Høy K, Hansen ES, Gelineck J, Christensen FB, Bünger CE (1999) Recombinant bone morphogenetic protein-7 as an intracorporal bone growth stimulator in unstable thoracolumbar burst fractures in humans: preliminary results. Eur Spine J 8:485–490
30. Lavery K, Swain P, Falb D, Alaoui-Ismaili MH (2008) BMP-2/4 and BMP-6/7 differentially utilize cell surface receptors to induce osteoblastic differentiation of human bone marrow-derived mesenchymal stem cells. J Biol Chem 283:20948–20958
31. Maric I, Poljak L, Zoricic S, Bobinac D, Bosukonda D, Sampath KT, Vukicevic S (2003) Bone morphogenetic protein-7 reduces the severity of colon tissue damage and accelerates the healing of inflammatory bowel disease in rats. J Cell Physiol 196:258–264
32. McGee MA, Findlay DM, Howie DW, Carbone A, Ward P, Stamenkov R, Page TT, Bruce WJ, Wildenauer CI, Toth C (2004) The use of OP-1 in femoral impaction grafting in a sheep model. J Orthop Res 22:1008–1015
33. Mroz TE, Wang JC, Hashimoto R, Norvell DC (2010) Complications related to osteobiologics use in spine surgery: a systematic review. Spine 35:S86–S104. Phila Pa 1976
34. Notices from European Union Institutions Bodies Offices and Agencies (2011) Note for guidance on minimising the risk of transmitting animal spongiform encephalopathy agents via human and veterinary medicinal products (EMA/410/01 rev.3). Official Journal of the European Union (2011/C 73/01)
35. Pauk M, Grgurevic L, Brkljacic J, Kufner V, Bordukalo-Niksic T, Grabusic K, Razdorov G, Rogic D, Zuvic M, Oppermann H, Babitt JL, Lin HY, Volarevic S, Vukicevic S (2015) Exogenous BMP7 corrects plasma iron overload and bone loss in Bmp6−/− mice. Int Orthop 39:161–172
36. Pecina M, Vukicevic S (2007) Biological aspects of bone, cartilage and tendon regeneration. Int Orthop 31:719–720
37. Pecina M, Vukicevic S (2014) Tissue engineering and regenerative orthopaedics (TERO). Int Orthop 38:1757–1760
38. Pecina M, Giltaij LR, Vukicevic S (2001) Orthopaedic applications of osteogenic protein-1 (BMP-7). Int Orthop 25:203–208
39. Pradhan BB, Bae HW, Dawson EG, Patel VV, Delamarter RB (2006) Graft resorption with the use of bone morphogenetic protein: lessons from anterior lumbar interbody fusion using femoral ring allografts and recombinant human bone morphogenetic protein-2. Spine (Phila Pa 1976) 31:E277–E284

40. Simmonds MC, Brown JV, Heirs MK, Higgins JP, Mannion RJ, Rodgers MA, Stewart LA (2013) Safety and effectiveness of recombinant human bonemorphogenetic protein-2 for spinal fusion: a meta-analysis of individual-participant data. Ann Intern Med 158:877–889
41. Song K, Krause C, Shi S, Patterson M, Suto R, Grgurevic L, Vukicevic S, van Dinther M, Falb D, Ten Dijke P (2010) Alaoui-Ismaili MH (2010) Identification of a key residue mediating bone morphogenetic protein (BMP)-6 resistance to noggin inhibition allows for engineered BMPs with superior agonist activity. J Biol Chem 285:12169–12180
42. Vukicevic S, Sampath TK (2002) Bone morphogenetic proteins: from laboratory to clinical practice. Birkhauser Verlag, Basel/Boston/Berlin
43. Vukicevic S, Sampath TK (2004) Bone morphogenetic proteins: regeneration of bone and beyond. Birkhauser Verlag, Basel/Boston/Berlin
44. Vukicevic S, Sampath TK (2008) Bone morphogenetic proteins: from local to systemic therapeutics. Birkauser Verlag AG, Basel/Boston/Berlin
45. Vukicevic S, Basic V, Rogic D, Basic N, Shih MS, Shepard A, Jin D, Dattatreyamurty B, Jones W, Dorai H, Ryan S, Griffiths D, Maliakal J, Jelic M, Pastorcic M, Stavljenic A, Sampath TK (1998) Osteogenic protein-1 (bone morphogenetic protein-7) reduces severity of injury after ischemic acute renal failure in rat. J Clin Invest 102:202–214
46. Vukicevic S, Grgurevic L, Oppermann H (2012) Whole blood-derived coagulum device for treating bone defects – US Patent 8, 197, 840
47. Vukicevic S, Oppermann H, Verbanac D, Jankolija M, Popek I, Curak J, Brkljacic J, Pauk M, Erjavec I, Francetic I, Dumic-Cule I, Jelic M, Durdevic D, Vlahovic T, Novak R, Kufner V, Bordukalo Niksic T, Kozlovic M, Banic Tomisic Z, Bubic-Spoljar J, Bastalic I, Vikic-Topic S, Peric M, Pecina M, Grgurevic L (2014) The clinical use of bone morphogenetic proteinsrevisited: a novel biocompatible carrier device OSTEOGROW for bone healing. Int Orthop 38:635–647
48. White AP, Vaccaro AR, Hall JA, Whang PG, Friel BC, McKee MD (2007) Clinicalapplications of BMP-7/OP-1 in fractures, nonunions and spinal fusion. Int Orthop 31:735–741

Biology of Spine Fusion and Application of Osteobiologics in Spine Surgery

Sachin Gupta, Vivek Mohan, and Munish C. Gupta

Abstract Bone healing and graft incorporation is a complex process that involves molecular, cellular, local, and mechanical factors. The interaction of these processes coordinate to allow successful fracture healing and bone formation. Our understanding of bone formation comes from studying the developmental process of bone formation during embryogenesis that mirrors during adult fracture healing. The cellular events of bone formation in combination with biomechanical stability are applied daily to successfully treat patients with various ailments. Several advances in biomedical devices and biologics have improved success rates, allowing surgeons to treat those patients with more options. Before the surgeon can appropriately select the methods and materials with which to treat their patients, they must clearly understand the biological processes that take place normally during bone formation and healing. Without this knowledge and understanding, the surgeon may not achieve optimal success rates in spinal fusions and also increased complications. In this chapter, we reviewed the available biologic options and bone morphogenetic proteins with reference to clinical application in spine surgery.

Keywords Fracture healing • Bone formation • Spinal fusion • Bone morphogenetic proteins • Spine surgery • rhBMP2 • Allograft • Autograft • Demineralized bone matrix • Autologous stem cells • rhBMP7 • Infuse • Infuse safety issues • GDF-5 • BMP6 • Platelet rich plasma

S. Gupta, BS, MD Cand
George Washington University, 900 23rd ST, NW, Washington DC, USA

V. Mohan, MD, MS
Orthopaedic Spine Surgeon, Spine Center at DuPage Medical Group, Naperville, IL 60540 USA

M.C. Gupta, MD (✉)
Department of Orthopedics, Washington University, St. Louis, MO, USA

S. Vukicevic, K.T. Sampath (eds.), *Bone Morphogenetic Proteins: Systems Biology Regulators*, Progress in Inflammation Research,
DOI 10.1007/978-3-319-47507-3_10

1 Introduction

Bone grafts and bone morphogenetic proteins (BMPs) have been used to aid in spinal fusion for many years now. The mechanisms and various bone morphogenetic proteins have been described in detail in the other chapters. This chapter is going to concentrate on the use of various bone grafts and BMPs for spinal fusion. Even today, there are controversies surrounding the use of bone graft substitutes as well as the use of BMP in spine surgery. There are several areas that are still being debated and researched. The dose of the rhBMP-2 used is considered very high compared to its natural occurrence in bone. The carrier used presently for rhBMP-2 is a collagen carrier. There is a debate regarding the use of bovine collagen as an ideal carrier. There is concern raised in the literature regarding the complications such as retrograde ejaculation and cancer with the use of rhBMP-2. In addition, the use of rhBMP-2 has been limited in many countries because of cost. In this chapter, we will describe the use of rhBMP-2 and discuss the various debates and controversies surrounding the use of rhBMP-2 in spinal fusion.

2 Biology of Spinal Fusions

Spinal fusion occurs through a very complex process. It is affected by many factors which have to be optimized to achieve a successful spinal fusion. The surgical technique is as important as the bone graft material. The host factors such as diabetes and hypothyroidism and many known conditions that can affect the ultimate outcome of bone healing. Since the biological process of a spinal fusion involves so many factors, the failure rate in single-level un-instrumented fusions can vary from 10–40 %, which only rises in multilevel fusion surgery [11]. Segmental pedicle screw instrumentation has increased the stability of the spinal constructs and have made a significant effect on the efficacy of spinal fusion. Due to the instrumentation, the nonunion rate significantly decreased but still occurs, down to 10–15 %, exemplifying the multifactorial process of fusions. As the process involves so many factors, often beyond the control of the patient and the surgeon, animal models were developed to better delineate the importance of each factor.

In the initial animal models, the fusion rate approached nearly 100 %, much higher than seen clinically, due to the fact that these fusions were interlaminar or inter-facet and the spine was stable. In contrast, human fusions are intertransverse process fusions. In 1995, Boden et al. developed a rabbit posterolateral fusion model, which was clinically relevant. Nonunions occurred spontaneously and at a rate similar to that seen clinically [11]. In this model, using iliac crest autograft, un-instrumented fusions had a 30–40 % nonunion rate, detected by radiographs. However, as in humans, the accuracy of detecting a fusion via radiographs is roughly 70 %. The fusion bed relies on a vascular supply to produce bone growth. Research via vascular injection have shown that the primary blood supply to the fusion mass comes from the decorticated transverse process. Failure of fusion without decortication shows the importance of thorough preparation of the fusion bed, providing the osteoprogenitor cells, blood supply, and cellular signals for bone formation [11].

In the healing process of spinal fusions, three temporally distinct histological phases occur, similar to that of endochondral fracture healing. Microscopic analysis shows that the fusion initially occurs in the periphery and proceeds centrally, with the most mature regions being the regions around the transverse processes. A similar delay in the osteoblast gene expression was seen in the central zone (1–2 weeks) than the outer zones. This lag is theorized to be the cause for failure of fusion in the central zone of fusion masses. The temporal and spatial variations seen in the healing process also correlates with the production of various bone morphogenetic proteins. The mRNA of BMP-2 is detected between weeks 2 through 6 and peaking in the third and fourth weeks. BMP-6 peaked on day 2 in the central and outer zones, but only peaked again in the outer zones during week 5. The lack of a second rise in the BMP-6 level later in the fusion process could explain the delay in central zone healing.

3 Patient Comorbidities

Outside of the surgical technique, many patient factors directly affect bone formation, which one should take into account during planning of any spinal fusion surgery. The nutritional status of the patient must be considered and maximized before elective procedures. Medical conditions, such as diabetes mellitus and HIV, have been shown to increase the rates of malunion, nonunion, and infection in bone healing. [31, 34]

Medications the patient may be taking can adversely affect the biologic process needed for healing: steroids and some chemotherapeutic agents have shown to be deleterious to spinal fusions. Nonsteroidal anti-inflammatory drugs have been linked to delayed bone healing. However, it is unclear whether cyclooxygenase-2-selective nonsteroidal anti-inflammatory drugs will have less effect on healing than nonselective drugs [13, 27]. These drugs usually affect the initial inflammatory stage that occurs in the first 14 days after surgery. Fluoroquinolones can decrease healing during the early stages of fracture healing [33, 50].Nicotine use, in any form, has been shown to increase nonunion rates in fractures and spinal fusions. Nicotine has been shown to decrease vascular ingrowth and capillary flow, which are fundamentally necessary to have bone formation [65].

4 Mechanical Factors

The structural integrity of the spinal segments plays an important role in healing of the fusion and bone graft maturation. The stability of the fixation will affect the healing that takes place. Primary bone healing in fractures without callous or cartilage intermediate requires direct bone apposition and absolute rigidity, usually in the form of internal fixation with compression plate or lag screw. Unlike direct primary cortical bone healing, the use of external fixators and unlocked intramedullary nails are load-sharing devices with relative stability. These devices allow

micromotion at the fracture site, which leads to indirect bone healing, evidenced by large callus formation. There has been a recent shift toward the use of less rigid fixation to allow load sharing, which results in callus formation. In spinal fusion, the rigidity of the segmental fixation is important. The rigid fixation allows for undisturbed bone healing of the spinal fusion. If there is too much motion, there is formation of a nonunion and ultimately a pseudarthrosis. Ultimately, if the local and systemic biology are satisfactory and the mechanical environment is stable, the spinal fusion will occur successfully.

5 Soft Tissue Conditions

The soft tissue surrounding the fracture or spinal fusion bed will have an impact on the biology of bone healing. Surgeons are aware of the importance of limiting iatrogenic soft tissue trauma during operative intervention. The advent of intramedullary nails and sliding plates with percutaneous fixation allows surgeons to avoid the injury zone, minimizing further compromise to the soft tissue and blood supply around the fracture. The value of early soft tissue coverage for open tibia fractures demonstrates the importance of the soft tissue envelope. Similarly, it is important to have a clean fusion bed with proper decortication of the bone as well as preservation of the paraspinal muscles that are going to aid in revascularization of the bone graft. The stages for fusion formation include (1) inflammation, (2) vascularization, (3) osteoinduction, (4) osteoconduction, and (5) remodeling. These steps closely resemble fracture healing and endochondral bone formation: inflammation, vascular ingrowth, callous formation, and remodeling to cortical lamellar bone. As the stages are similar, the factors involved in achieving a successful fusion are similar to the factors involved in fracture healing. These include minimal motion, adequate vascular supply, and osteoprogenitor cells with a bony substrate from which to create new bone. Over time, dynamic remodeling occurs as the bony fusion mass matures, usually by one year.

6 Osteobiologics for Spine Fusion

Any potential bone grafting material should possess the following properties that are important in bone healing.

1. An *osteoconductive* compound that provides the three-dimensional architecture to promote the ingrowth of sprouting capillaries, perivascular tissue, and osteoprogenitor cells, supporting the process of graft incorporation. This process is known as creeping substitution.
2. An *osteoinductive* substance that stimulates the recruitment and differentiation of mesenchymal stem cells (MSCs) into bone-forming cells. Specific BMPs are the primary known osteoinductive proteins BMP-2, BMP-4, BMP-6, BMP-7, and BMP-9.

3. An *osteogenic* graft that contains viable osteoblastic cells that are capable of directing bone formation. This potentially provides bone-forming cells that is characteristic of only fresh autogenous bone graft. Other grafts rely on recruitment of host progenitor cells to differentiate into bone-forming cells.
4. *Bone graft extenders* add bulk to a given amount of autogenous bone that is to be used over a larger surface area with a similar fusion rate (e.g., allograft bone).

Bone graft substitutes are substances that can entirely replace autogenous bone graft material with a similar or better fusion rate. Bone graft enhancers are used to increase the healing of potential of the fusion bed when added to autograft bone with the usual or smaller amount of bone graft.

7 Natural and Synthetic Osteobiologics

7.1 Autograft

Autograft has all the desirable properties for a bone graft option, including being osteoconductive and osteoinductive, having osteogenic cells, and having acceptable mechanical strength. Iliac crest bone graft (ICBG) is the "gold standard" for those reasons. The most significant drawback to iliac crest bone graft is the donor site morbidity, including the risk of chronic pain (3–50 %), neurovascular injury (2 %), hematoma (5 %), seroma (5 %), blood loss (1–5 %), iatrogenic fracture(1 %), bowel herniation(1–5 %), infection (2 %), and even cosmetic deformity. [2, 5, 36, 52, 58, 59] Also, iliac crest bone graft has a limited quantity; thus, in long fusions requiring large amounts of graft, autogenous bone graft cannot be the sole graft option. In addition, iliac crest bone harvest weakens the iliac fixation when instrumented fusions are performed to the pelvis. Pelvic fractures can also occur after exuberant harvest of the iliac crest.

7.2 Allograft

Allograft bone is the second most transplanted tissue only second to blood transfusions. Allograft is the most widely used substitute for autogenous bone graft material. It is osteoconductive as it has the structural framework upon which new bone can form. It is not osteoinductive because it is acellular due to tissue processing. It is usually prepared by freezing or lyophilization (i.e., freeze-drying). Frozen graft must be stored at −20 °C which allows to maintain the integrity of its structural properties for up to 1 year. Lyophilized allografts are vacuum packed and can be kept at room temperature. This process reduces its immunogenicity, but freeze-dried grafts are structurally weaker than frozen allografts, by almost 50 %. The use of cadaveric tissue also carries the risk of spreading infectious diseases, such as HIV and hepatitis. However, only two cases of infection transmission have been

documented, both of which were in unprocessed grafts; one was in a spine fusion. No infections were seen in freeze-dried grafts. The risk of transmission is less than one in a million [66]. Allograft bone is transplanted in one of three main forms: cortical (structural), cancellous (crushed), and demineralized. Each form has its advantages and their most common uses, which will be described in further detail.

Cortical Cortical allografts include femoral shaft, fibular shafts, humeral shafts, femoral rings, and fibular rings. These strong grafts are used in applications that require structural support in compression, such as anterior interbody fusions as well as corpectomy sites. However, these grafts incorporate slowly by means of a process of periosteal new bone formation around the allograft. Cortical allografts do not fully incorporate and remain a mixture of necrotic and viable bone at their site of implantation. Bridwell et al. examined their results in 24 patients with anterior thoracolumbar grafting with fresh-frozen cortical allograft and posterior instrumentation and fusion [12]. They had one pseudarthrosis and in two cases the graft's position changed. Samarztis et al. reviewed their fusion and outcome data with autograft versus allograft in multilevel anterior cervical spinal fusions with instrumentation [54]. In 80 patients (45 received autograft; 35 got allograft), 97.5 % fused with no significant difference in the fusion rate between the two groups. Good to excellent clinical outcomes were seen in 88.8 % of patients overall as well. As these studies demonstrate, cortical structural grafting with allograft femoral rings is a viable alternative to autograft in complex anterior spinal surgery, thus avoiding donor site morbidity.

Cancellous Cancellous allograft has both osteoconductive and osteoinductive properties. It provides significant surface area and stimulus for bone formation. Cancellous bone has a much faster rate of incorporation than cortical graft because of its large surface area and permitting rapid vascular ingrowth. The graft usually remodels completely with more rapid and complete revascularization compared to cortical allograft. Unlike cortical grafts, cancellous bone graft has little mechanical strength and cannot be used to maintain compressive or tensile loads. A disadvantage of cancellous allograft is its lack of osteogenic potential, as it does not carry with it bone-forming cells. Cancellous allograft is an excellent option for posterolateral fusions as they require little mechanical strength. With a large supply and relatively inexpensive option, cancellous allograft is very useful as a bone graft extender in spinal fusions that require a significant volume of bone graft (e.g., scoliosis, multilevel posterolateral fusions). Knapp et al. retrospectively reviewed their use of allograft in adolescent idiopathic scoliosis for posterior instrumented fusions in 111 patients with a 5-year minimum follow-up [39]. They had three pseudarthroses (2.7 %) with 5.9 % loss of correction in their cases, which is comparable to those in previous studies using autograft. Dodd et al. showed a 100 % fusion rate in adolescent idiopathic scoliosis patients with femoral head allograft and local autograft [24]. Adolescent idiopathic scoliosis patients are a very healthy spine population. Betz and colleagues found essentially no significant difference when using allograft or no graft. They randomized AIS patients into two groups: one group had posterior spinal fusion with allograft and the other had no bone graft at all. He had at least

2-year follow-up post-op patients. Only one pseudarthrosis was seen overall, in the allograft group [7]. However, there were patients that had loss of correction and their study. Posterior spinal fusions in adolescent idiopathic scoliosis can be successful in patients with local graft and allograft cancellous graft used as an extender.

Demineralized Bone Matrix (DBM) DBM is produced by a weak acid extraction process from allograft bone. DBM allograft has been stripped of its minerals, leaving behind only the organic materials, including type I collagen, non-collagenous proteins, and signaling cytokines. Marshall Urist first extracted BMP from demineralized bone back in 1965 [67]. DBM is used as a particulate graft, whose effectiveness depends on its localization and retention at the fusion site. Some of the advantages of DBM allograft include that it is cost-effective, readily available, attractive as an on-the-shelf graft extender, commercially available in multiple forms (powder, putty, chips, crushed granules, gel-filled syringes), and less immunogenic than mineralized allograft material. It can be used in combination with osteogenic precursor cells from bone marrow aspirate with DBM acting as the carrier. Human DBM requires a compatible carrier, which is often about 85 % of the product by weight. Osteoinductive capabilities vary based on the manufacturer and also between lots of a particular product [4]. Bae et al. showed via ELISA testing that this variance in BMP content is the probable cause for the variance in effectiveness of each product [3]. Since the first DBM product was introduced in 1991, it has become one of the most widely used fusion products. Many companies have their own formulations. Available carriers include glycerol, gelatin, calcium sulfate, lecithin, and hyaluronic acid (HA). Glycerol is primary carrier in Grafton™ (Medtronic, Memphis, TN). Osteofil™ (Regeneration Technologies Inc., Alachua, FL) uses porcine-derived gelatin, stored frozen and must be hydrated and heated before implantation. Accell™ (IsoTis Orthobiologics Inc., Irvine, CA) utilizes a gelatin from human DBM and can be stored at room temperatures. Allomatrix (Wright Medical Technology, Arlington, TN) uses a calcium sulfate hemihydrate mixed with carboxymethyl cellulose, and water is added before implantation. InterGro (Interpore Cross Inc., Irvine, CA) uses lecithin, which is a phospholipid derived from soybeans.

Peterson et al. studied the fusion rates in three groups of athymic rates based on DBM used: Grafton, DBX [from MTF or Synthes (Paoli, PA)], and Allomatrix. Grafton had the highest fusion rate and Allomatrix the lowest. The amount of BMP within DBM is often less than 0.1 % by weight. Though it is published as being osteoinductive, studies have shown limited improved outcomes with DBM. Cammisa et al. examined pseudarthrosis rates in posterolateral fusions using iliac crest autograft with and without DBM (Grafton™) [15]. No difference was found between the two groups. Price and colleagues also studied fusion rates using DBM in AIS patients with allograft and autograft and found no difference with or without DBM [51]. A study by Thalgott et al. showed that pseudarthrosis rates using DBM and HA were higher than HA alone [64]. These results show that the use of DBM as a bone graft substitute is controversial. Thus, although DBM might have some benefit as a bone graft extender or enhancer, especially when combined with autograft, bone marrow aspirate, or other forms of graft materials, its use as a stand-alone graft is unproven.

8 Bone Marrow Aspirate/Autologous Stem Cells

Bone marrow aspirate (BMA) was first used clinically in 1986 to treat a tibia fracture nonunion, which subsequently went on to heal fully [21]. Animal and laboratory studies have shown that bone marrow aspirate contains osteoprogenitor cells, enhancing bone formation and fracture union. However, further animal and clinical quantitative studies have shown that the actual number of osteoprogenitor cells in each sample varies widely between individuals and even among species. Majors et al. examined the number of osteoprogenitor cells in 30 patients of various ages and both genders. They found a much lower quantity of bone-forming cells in older patients as well as in females [45]. Earlier studies had found that the growth medium and harvest technique also contributed to the cell count and viability [41]. Since then, BMA has been used for nonunion surgery as well as spinal fusions. In the spine, BMA has typically been used in conjunction with allograft, bone graft substitutes, or even iliac crest autograft. Gupta et al. performed un-instrumented spinal fusions in an ovine model to compare stem cells from bone marrow aspirate to other graft types. They used a new method for obtaining a stem cell concentrate from bone marrow called selective cell retention (SCR) utilizing an affinity column; the stem cells will attach onto the graft, while the remaining hematopoietic cells pass through, broken down into four groups: (1) iliac crest autograft, (2) SCR with beta-tricalcium phosphate [TCP], (3) TCP soaked in whole bone marrow, and (4) TCP alone. With radiological and histological results collected at 3 and 6 months, the autograft and SCR-TCP groups were similar at 3 and 6 months. The TCP with whole bone marrow and TCP alone groups had produced significantly less bone at both intervals. This animal study displays the importance of using appropriate techniques when trying to obtain stem cells from bone marrow aspirate for spinal fusions [30].

In a retrospective cohort study of patients undergoing revision posterolateral lumbar fusions, Taghavi et al. examined patients who had iliac crest autograft compared to BMP-2 with allograft and to BMA with allograft. No significant difference in fusion rates was seen between the BMA/allograft and autograft groups; however, the BMP-2/allograft and the ICBG autograft groups had significantly higher fusion rates in multilevel fusions [63]. McLain et al. also showed that the vertebral body is a good source of osteoprogenitor cells, which can be accessed via the pedicle intraoperatively [47]. Utilizing their technique, they noted that the stem cells collected were more numerous from the vertebral body than the iliac crest, specifically in the first 2.5 cm from the pedicle-body junction. Aspirating through the pedicle avoids any need to use aspirate or structural iliac crest autograft.

As the fusion rates using bone marrow aspirate (BMA) are similar to iliac crest autograft, even in revision surgery, it is a viable alternative to iliac crest harvest for single-level fusions. In certain situation, BMA may also be more cost-effective as the price of commercially available bone morphogenetic protein adds a significant financial cost to surgery. Based on current studies, bone marrow aspirate has some use in certain situations. However, the number of cells obtained upon harvest varies widely from patient to patient and also the technique used [20, 41]. These factors currently hinder the widespread use of BMA in spinal fusion surgery.

9 Growth Factors

Bone Morphogenetic Proteins Bone morphogenetic proteins (BMPs) are soluble and low-molecular-weight glycoprotein signaling molecules belonging to the transforming growth factor beta superfamily. They have been discussed in great detail in the other chapters of this book. Marshall Urist first discovered the possibilities of bone-forming proteins in animals [67]. They have been extensively studied and found to initiate and regulate the osteoblastic and/or chondrogenic differentiation of mesenchymal stem cells in vitro. They are also the only signaling molecules that can produce ectopic bone in vivo. They bind to cell surface molecules and produce an intracellular cascade leading to cellular differentiation. Of the more than 20 BMPs that have been identified, five have osteogenic properties: BMP-2, BMP-4, BMP-6, BMP-7, and BMP-9. However, only two are currently used widely in spine fusions. Recombinant human BMP-2 (rhBMP-2; INFUSE, Medtronic Sofamor Danek, Memphis, TN) and recombinant human BMP-7 (rhBMP-7; OP-1, Stryker, Mahwah, NJ) both are osteogenic, but only rhBMP-2 has been shown to produce osteoblastic progenitor cells. rhBMP-2 (INFUSE) has been approved by the FDA for use in anterior interbody lumbar fusions as well as open tibia fractures. rhBMP-7 (OP-1, rhOP-1) is approved for long-bone nonunions.

One of the concerns in BMP use is the spatial and temporal diffusion in vivo. When used without an appropriate carrier, it has been shown to diffuse quickly into the surrounding tissue, thus decreasing its osteoinductive ability. The carrier's function is to restrict elution and also be an osteoconductive scaffold to which bone formation can occur via adhesion and vascular ingrowth. Many carriers have been tested, including autogenous bone graft, DBM, collagen, ceramics, and polylactic acid (PLA). The ideal carrier has not been identified, but an absorbable type I collagen sponge is currently used for rhBMP-2. As approved by the FDA, its use is limited to anterior lumbar interbody fusions within tapered, threaded cages (LT cage). However, rhBMP-2 is commonly used for posterolateral lumbar fusions. Whether the current collagen carrier is ideal in this environment, as compared to the anterior lumbar interbody region, has yet to be fully studied and optimized. Ideally, the carrier should have more structural integrity, similar to calcium phosphate or ceramic base material [29, 48, 53, 62]. Several studies also recommended wrapping the sponge around bone graft or a bone graft substitute, providing structural support to the sponge [6, 28, 40].

9.1 rhBMP-7 (OP-1/rhOP-1)

Preclinical Studies Several animal studies have shown the safety and efficacy of rhBMP-7. Cook and colleagues used a canine model divided in four groups to compare the effect of rhBMP-7 with its collagen carrier versus collagen only, with autogenous iliac crest bone graft only, and without any implants [22]. All four groups were implanted into each dog at different levels. The dogs were killed at 6,

12, and 26 weeks. All rhOP-1-treated levels had a complete fusion by 12 weeks. The ICBG group showed a slower fusion by 26 weeks. The carrier only and no implant groups failed to form any fusion mass. Both radiographic and histologic findings were consistent with those findings, indicating an improved fusion rate with rhOP-1 for posterolateral fusions in dogs. Cunningham et al. found rhBMP-7 to be more effective than autogenous iliac crest bone graft in a canine posterolateral fusion model [23], comparing ICBG, rhBMP-7, and ICBG + rhBMP-7, with fusion rates of 27 %, 72 %, and 87 %, respectively. They also demonstrated that the rhBMP-7 groups showed bone formation via intramembranous ossification, as opposed to endochondral ossification seen in the bone graft only group [23]. Magin et al. compared 3.5 mg rhOP-1 with 1 gm bovine bone collagen to autograft and an osteoconductive hydroxyapatite (HA), a bone graft substitute in a sheep posterolateral instrumented fusion model [44]. They demonstrated that the rhOP-1-treated group had greater bone formation and improved stiffness at 4 months, compared to the autograft or HA-treated groups. The autograft group fusion occurred much slower, and the HA-treated group failed to fuse at all.

Clinical Studies Preliminary clinical studies for rhOP-1 were performed in the setting of nonunions of open tibia fractures displaying its efficacy in forming a bony union [26]. The dose approved by the FDA is 3.5 mg of OP-1 in 1 gm of carboxymethyl cellulose resulting in 0.875 mg/mL of OP-1 concentration. Vaccaro et al. published a prospective, randomized, controlled multicenter trial of un-instrumented posterolateral fusion for lumbar spinal stenosis and degenerative spondylolisthesis with OP-1 putty compared to autograft [68]. The use of OP-1 was found to be safe, without any associated toxicity, ectopic bone formation, recurrent stenosis, or any other adverse event related to the product. They showed 55 % and 40 % fusion rates for OP-1 and ICBG, respectively, at minimum 2-year follow-up. Clinically, Short Form (SF)-36 scores were similar, and these fusion rates were comparable to those in the literature for un-instrumented arthrodesis with ICBG, with the benefit of no graft site morbidity. The fusion rates reported were lower than the general fusion rates with autograft in the literature for a posterolateral fusion in the lumbar spine. More importantly, Kanayama et al. performed a prospective, randomized controlled study with radiographic, surgical, and histologic assessment to evaluate the fusion rate of rhOP-1 compared to autograft with HA-TCP granules in instrumented posterolateral lumbar fusions [35]. Each patient in that study was taken back to the operating room for a biopsy of the fusion mass. In contrast to the study by Vaccaro et al., Kanayama's findings showed fusion in only 57 % rhOP-1 patients versus 78 % in the autograft/HA-TCP group. Histological analysis did show the presence of bone in the OP-1-treated group. Although the sample sizes were small (nine in the OP-1 group and ten in the autograft/HA-TCP group), these results illustrate that OP-1 fusion rates are, at best, equivalent to autograft. Given that there is known morbidity associated with distant autograft harvest, OP-1 may be considered when there is insufficient autograft present. Further clinical studies are needed to clearly delineate its efficacy in the setting of posterolateral spine fusions. OP-1 is not used clinically in the setting of spine fusion routinely.

9.2 rhBMP-2 (INFUSE)

Most studies have been performed on rhBMP-2 showing successful fusion rates in anterior and posterior spine fusion surgery. Initially, FDA approval was granted for its use based upon clinical studies in anterior lumbar interbody fusions and open tibia fractures. Many subsequent studies have shown significant efficacy in posterolateral spine fusion applications as well.

Preclinical Studies The first animal study comparing autograft to rhBMP-2 was performed in sheep by Sandhu [57]. Single-level anterior lumbar fusions with cylindrical threaded fusion cages were performed with either rhBMP-2 or autograft. The BMP carrier was bovine type I collagen. All the animals with rhBMP-2 had radiographic fusion at 6 months, as opposed to only 37 % in the autograft group. A dose-dependent response to rhBMP-2 was seen by Boden et al. in rhesus monkeys [9]. Cylindrical titanium cages with either 0.75 or 1.5 mg/mL on a collagen carrier were placed in the intervertebral levels. All levels fused but the 1.5 mg/mL dose showed denser and more rapid bone formation. The profound effects of rhBMP-2 were first described by Hecht and colleagues [32]. In six rhesus monkeys, they placed threaded cortical allograft dowels with rhBMP-2 on a collagen sponge placed at intervertebral locations. They compared this group with six monkeys who had allograft bone dowels packed with autograft bone only. In the rhBMP-2 group, all six fused, whereas only half of the monkeys with autograft fused. In addition, the radiographic and histologic analysis showed that in the rhBMP-2 group, the allograft dowels completely resorbed. This was the first study showing that rhBMP-2 not only stimulated and accelerated the osteoblast activity but also the osteoclastic activity, as no bone remodeling occurred in the autograft group. This first study helped to identify the appropriate dose for humans. To evaluate the effects of rhBMP-2 in the posterolateral fusion, Sandhu et al. used a radiographic and histologic canine model showing a 100 % fusion rate with rhBMP-2 and no bony fusion in the autograft group at 3 months [55]. In subsequent studies, Sandhu and his associates also found that a posterolateral fusion with rhBMP-2 could occur without decortication of the transverse process [56]. A significant step in identifying the appropriate dose and carrier for BMP in the posterolateral spine was identified by Martin et al. [46]. They made three important findings by performing posterolateral spinal fusions with rhBMP-2 in rhesus monkeys at varying doses and with different carriers. First, rhBMP-2 was safe around exposed dura after a laminectomy. Second, soft tissue compression prevented bone induction at standard rhBMP-2 doses, for which they felt was due to rapid elution from the pressure of soft tissue in the intertransverse process region. Third, after providing mechanical protection via a porous polyethylene shield and allowing longer rhBMP-2 loading times onto the collagen carrier, more bone formation was seen at lower doses of rhBMP-2. Finally, the 0.43 mg/mL dose used in lower animals did not induce bone formation in primates, identifying that rhBMP-2 is dose dependent and its effect even varies between species. There appeared to be a dose escalation required for the higher species. Boden et al. published their results using a newly developed calcium phosphate ceramic carrier in the posterolateral

spine in primates [10]. Within the carrier, the 40 % tricalcium phosphate was resorbed, while the remaining 60 % hydroxyapatite provided the structural scaffold on which the new bone was deposited.

Clinical Studies The first published clinical trial of rhBMP-2 (INFUSE) in humans was by Boden and associates. All 11 of their study patients who received BMP had solid fusions on radiographs by 6 months [8]. To note, they used 2 mg/mL of rhBMP-2 on the HA/TCP carrier. None of their patients developed measurable levels of antibodies to rhBMP-2. Within the same year, Burkus et al., in a prospectively randomized control study with 2-year follow-up, examined stand-alone anterior L5-S1 fusions using LT cages filled with either rhBMP-2 or iliac crest bone graft [14]. They showed a 100 % fusion rate using rhBMP-2 as opposed to 95.7 % with autograft at 2 years, with a clinical success rate of 94.5 % in the rhBMP-2 group and 88.7 % in the control group. About a third of the patients in the control group with iliac crest graft had donor site pain, with a 5.9 % rate of adverse events directly related to the harvest. The rhBMP-2 group also had shorter operative times with decreased blood loss. There were operative time savings when autograft was not harvested. A major finding from the human pilot study performed by Boden and colleagues was a failure of fusion in the face of instability [8]. One of the two patients with spondylolisthesis greater than Meyerding grade 1 who underwent an un-instrumented fusion with rhBMP-2 did not fuse. The authors felt that in the face of any instability, internal fixation and stability was a significant factor in posterolateral lumbar fusions. The biology alone was not enough; mechanical stability was necessary for a successful spine fusion.

Surgical treatment of adult spinal deformity often requires long segments of fusion and instrumentation. These patients have a high rate of pseudarthrosis. One study quoted 17 percent of these patients develop a pseudarthrosis and subsequent instrumentation failure. The pseudarthrosis can be apparent years after the surgical procedure. Forty percent of pseudarthrosis is discovered from the third year postoperatively and beyond [38]. A study by Kim et al. demonstrated increased fusion rates with the use of rhBMP-2 compared to iliac crest bone graft [37]. Maeda et al. also showed better fusion rates in adult spinal deformity patients which are the most challenging in getting a fusion [43]. In a case of 84-year-old female with degenerative scoliosis the patient was treated with a posterior-only approach with instrumentation to correct and stabilize the scoliosis. A combination of allograft cancellous chips, local bone, and two large kits (total of 24 mg of INFUSE) was used off label to aid in achieving a posterolateral fusion. The radiographs in follow-up displayed the large posterolateral fusion mass especially visible in the lateral gutter.

Adult spinal deformity patients undergoing a spinal fusion have a much lower rate of fusion compared to adolescents with idiopathic scoliosis. Dosing of the rhBMP-2 has only been studied in one- or two-level lumbar fusion models. The adult scoliosis patient frequently require fusion of five levels or more. These patients may benefit the most from the advances in safety and efficacy of bone graft

substitutes. The amount of rhBMP-2 used in the one level fusion cannot be translated into the many levels because it would be cost prohibitive. One kit of rhBMP-2 can cost $5000 or more. Many patients around the globe would benefit from the advances of rhBMPs if they were more affordable.

Safety Concerns Although, the FDA has approved the use of rhBMP-2 (INFUSE) for anterior lumbar interbody fusions, spine surgeons have clinically studied the use of rhBMP-2 in posterior lumbar and cervical fusions, with several alarming safety concerns being reported. The primary concerns with the use of rhBMP-2 are related to the regional edema and inflammatory reactions produced by the protein.

Smucker et al. showed increased risk of delayed postoperative swelling when rhBMP-2 was used in the anterior cervical spine, usually around postoperative day 4 on average [61]. The complications included dysphagia and airway obstruction, all secondary to anterior neck soft tissue swelling. Most patients required readmission and observation, with some patients needing reintubation. A few underwent washouts, none of which had fluid collections or hematomas, only edematous soft tissue, including the esophagus and strap muscles. The usual 1.5 mg/mL dose was used. These reports of adverse events have led to a warning issued from the FDA for the use of rhBMP-2 in the anterior cervical spine procedures [60, 61].

Bone formation in the spinal canal has been reported when the rhBMP-2 in the disc space with transforaminal lateral interbody fusion (TLIF) [1, 18]. The reports of bone formation adjacent to neural elements with INFUSE when placed in the lumbar intervertebral space via straight posterior or transforaminal approaches. It is unclear if it is the result of poor technique of placement of the rhBMP-2-soaked sponge or retrograde bone formation in the path of the cage placement. Several studies have shown radiculitis after rhBMP-2 use in transforaminal lateral interbody fusion (TLIF) and posterior lateral interbody fusion (PLIF) surgery in the lumbar spine.

Resorption of the vertebral body end plate has also been reported in conjunction with TLIF with subsidence of the cage into the vertebral body. The subsidence can lead to loss of sagittal plane correction and narrowing of the foramen. These reports have also documented cases of severe osteolysis of the vertebral body after placing rhBMP-2 in the intervertebral space. Lewandrowski et al. theorized three possible etiologies for the cause of osteolysis when placing rhBMP-2 in the interbody region: 1) end plate violation leading to rhBMP-2 being in contact with cancellous bone; 2) "overstuffing" rhBMP-2 into intervertebral space, providing too high a dose of BMP; and 3) dose-dependent biochemical sequence leading to greater osteoclast activation over osteoblasts [42]. This phenomenon may be related to the dose used.

The anterior approach has also been shown to have the same effects on the vertebral body end plate. Severe osteolysis has been shown with the use of allograft spacers as well as PEEK cages. Osteolysis is again hypothesized to be related to the dose, end plate violation with exposure of the cancellous bone, and increased osteoclastic activity. Retrograde ejaculation has also been reported by Carragee et al. as a complication of anterior lumbar interbody fusion with rhBMP-2. He not only reported increase rate of retrograde ejaculation in his patient but also reanalyzed the

data from other published studies and stated that there was an increased rate of retrograde ejaculation associated with the use of rhBMP-2 [19].

Carragee has also reported the incidence of new cancers in patients that had a higher dose of rhBMP-2 (AMPLIFY) used in the group data reported to the FDA. He stated that there were nine new malignancies reported in the rhBMP-2 group out of 239 patients compared to only two new malignancies reported to the control group of 224 patients [16]. This finding has been debated in the literature. Other reports have demonstrated that there was no increase in new malignancies with rhBMP-2. Glassman et al. reported no statistical significant increase in malignancies and no indication of causality related to the rhBMP-2. The rhBMP-2 was associated with basal cell carcinoma, lung cancer, lymphoma, ovarian cancer, pancreatic cancer, prostate cancer, squamous cell carcinoma, and vocal cord cancer. The iliac crest group was associated with colon cancer and lymphoma. Kelly et al. also agreed with their own report with no significant increase in malignancy associated with recombinant BMP use. Ref.

Growth and Differentiating Factor-5 (GDF-5) GDF-5 has many different names including MP-52, LAP-4, CDMP-1, BMP-14, and radotermin. This osteogenic factor originates from the TGF-beta/BMP superfamily and is required for proper skeletal patterning and limb development. It has also been found to promote tissue regeneration in bone, cartilage, soft tissue, and tendon in vivo. Increasing the dose too much may be counterproductive to bone formation. Magit et al. rabbit study showed 100 % fusion rate (GDF-5 with Healos) by anatomical and histological analysis at 8 weeks as compared to ICBG (38 %) or Healos (ceramic) alone (0 %). Gupta et al. presented data in sheep model at 3 months showing 100 % (6/6) fusion rates in anterior interbody (using carbon fiber-reinforced polymeric cages (DePuy Synthes, Raynham, MA)) fusions with 1 mg/ml GDF-5 + Healos; 5/6 fused with 0.5 mg/ml + Healos; 5/6 fused for ICBG alone; and 4/6 for empty cage. Currently GDF-5 is still undergoing preclinical trials to provide more evidence of its efficacy in spinal fusion or disc regeneration.

In addition to these reports, there was a review of the literature performed by the Cochrane and Yale group independently. In the review, they found equivalent rates of fusion with recombinant BMP-2 and autograft. They both conclude that there was no significant advantage in using recombinant BMP-2 to autograft.

9.3 BMP-6

rhBMP-6 has been used in spine studies as well. A porcine model was used where mesenchymal stem cells were infected with a BMP-6 gene. Mesenchymal stem cells were implanted in a bony defect. New bone formation much greater than the controls was seen in those defects at 12 weeks and 6 months. This model showed that implanting the mesenchymal stem cells that were overexpressing BMP-6 gene has increased bone formation compared to the controls. This study showed a normal

benefit in which BMP-6 activity can be enhanced by using delivery of mesenchymal stem cells to produce bone [49].

10 Cellular Biologics

Platelet Concentrates Platelet-rich plasma (PRP) has gained significant attention in the orthopedic community, as it is used in a wide variety of applications from joint replacement to muscle injuries. PRP is concentrate of platelets with a small amount of plasma derived from the patient's blood. The platelets release many inflammatory and growth factors after they are activated by an agonist, such as thrombin in vivo. Frechette identified these factors, including platelet-derived growth factor (PDGF), TGF-α, TGF-β, epidermal growth factor (EGF), bFGF/FGF-2, insulin-like growth factor (IGF), and vascular endothelial growth factor (VEGF), which then go on to participate in bone formation [25]. However, much of the data supporting PRP use for bone regeneration, such as the one by Frechette and colleagues, is from the dental and maxillofacial literature.

Studies regarding PRP use in spinal fusions are limited. Carreon et al. in a retrospective cohort study examined two groups of patients undergoing posterolateral spinal fusion with iliac crest autograft [17]. The study group had PRP with the iliac crest, and the control group had just iliac crest autograft. The nonunion rate in the study group was 25 %, whereas in the study group was only 17 %. In a similar study by Weiner et al. in 2003, PRP added to iliac crest bone graft showed decreased lumbar posterolateral arthrodesis rates as compared to iliac crest autograft alone. The fusions were examined via a "blinded" radiographic review. [69] PDGF and many other cytokines are not directly osteogenic, unlike bone morphogenetic proteins, even though they may be involved in the bone-forming cascade.

11 Conclusion

There are numerous spinal fusion procedures being performed daily for a spectrum of spinal conditions ranging from simple degenerative conditions to severe spinal deformities. The number of spinal fusion procedures being done are increasing with greater availability of spine surgery to more patients and the improvement in the medical facilities around the world. The growth of the minimally invasive spinal surgery approaches has also demonstrated the need for effective bone graft materials. There are many different products available; therefore, understanding the biological, chemical, and mechanical properties of the individual products is paramount as well as their clinical effectiveness. The goal of a safer and efficacious method in achieving spinal fusion with a bone graft substitute is closer today than ever before.

References

1. Ahn J, Jorgensen AY, Bohl DD, Tabaraee E, Rossi VJ, Aboushaala K, Singh K (2015). Neuroforaminal bone growth following minimally invasive transforaminal lumbar interbody fusion with BMP: a computed tomographic analysis. J Spinal Disord Tech. Doi: 10.1097/BSD.0000000000000347
2. Arrington ED, Smith WJ, Chambers HG, Bucknell AL, Davino NA (1996) Complications of iliac crest bone graft harvesting. Clin Orthop Relat Res:300–309
3. Bae HW, Zhao L, Kanim LE, Wong P, Delamarter RB, Dawson EG (2006) Intervariability and intravariability of bone morphogenetic proteins in commercially available demineralized bone matrix products, Spine (Phila Pa 1976) 31:1299–1306; discussion 307–8
4. Bae H, Zhao L, Zhu D, Kanim LE, Wang JC, Delamarter RB (2010) Variability across ten production lots of a single demineralized bone matrix product. J Bone Joint Surg Am 92:427–435
5. Banwart JC, Asher MA, Hassanein RS (1995) Iliac crest bone graft harvest donor site morbidity. A statistical evaluation. Spine (Phila Pa 1976) 20:1055–1060
6. Barnes B, Boden SD, Louis-Ugbo J, Tomak PR, Park JS, Park MS, Minamide A (2005) Lower dose of rhBMP-2 achieves spine fusion when combined with an osteoconductive bulking agent in non-human primates. Spine (Phila Pa 1976) 30:1127–1133
7. Betz RR, Petrizzo AM, Kerner PJ, Falatyn SP, Clements DH, Huss GK (2006) Allograft versus no graft with a posterior multisegmented hook system for the treatment of idiopathic scoliosis. Spine (Phila Pa 1976) 31:121–127
8. Boden SD, Kang J, Sandhu H, Heller JG (2002) Use of recombinant human bone morphogenetic protein-2 to achieve posterolateral lumbar spine fusion in humans: a prospective, randomized clinical pilot trial: 2002 Volvo Award in clinical studies. Spine (Phila Pa 1976) 27:2662–2673
9. Boden SD, Martin GJ Jr, Horton WC, Truss TL, Sandhu HS (1998) Laparoscopic anterior spinal arthrodesis with rhBMP-2 in a titanium interbody threaded cage. J Spinal Disord 11:95–101
10. Boden SD, Martin GJ Jr, Morone MA, Ugbo JL, Moskovitz PA (1999) Posterolateral lumbar intertransverse process spine arthrodesis with recombinant human bone morphogenetic protein 2/hydroxyapatite-tricalcium phosphate after laminectomy in the nonhuman primate. Spine (Phila Pa 1976) 24:1179–1185
11. Boden SD, Schimandle JH, Hutton WC (1995) An experimental lumbar intertransverse process spinal fusion model. Radiographic, histologic, and biomechanical healing characteristics. Spine (Phila Pa 1976) 20:412–420
12. Bridwell KH, Lenke LG, McEnery KW, Baldus C, Blanke K (1995) Anterior fresh frozen structural allografts in the thoracic and lumbar spine. Do they work if combined with posterior fusion and instrumentation in adult patients with kyphosis or anterior column defects? Spine (Phila Pa 1976) 20:1410–1418
13. Brown KM, Saunders MM, Kirsch T, Donahue HJ, Reid JS (2004) 'Effect of COX-2-specific inhibition on fracture-healing in the rat femur. J Bone Joint Surg Am 86-a:116–123
14. Burkus JK, Gornet MF, Dickman CA, Zdeblick TA (2002) Anterior lumbar interbody fusion using rhBMP-2 with tapered interbody cages. J Spinal Disord Tech 15:337–349
15. Cammisa FP Jr, Lowery G, Garfin SR, Geisler FH, Klara PM, McGuire RA, Sassard WR, Stubbs H, Block JE (2004) Two-year fusion rate equivalency between Grafton DBM gel and autograft in posterolateral spine fusion: a prospective controlled trial employing a side-by-side comparison in the same patient. Spine (Phila Pa 1976) 29:660–666
16. Carragee EJ, Chu G, Rohatgi R, Hurwitz EL, Weiner BK, Yoon ST, Comer G, Kopjar B (2013) Cancer risk after use of recombinant bone morphogenetic protein-2 for spinal arthrodesis. J Bone Joint Surg Am 95:1537–1545
17. Carreon LY, Glassman SD, Anekstein Y, Puno RM (2005) Platelet gel (AGF) fails to increase fusion rates in instrumented posterolateral fusions. Spine (Phila Pa 1976) 30:E243–E246 ; discussion E47

18. Chen NF, Smith ZA, Stiner E, Armin S, Sheikh H, Khoo LT (2010) Symptomatic ectopic bone formation after off-label use of recombinant human bone morphogenetic protein-2 in transforaminal lumbar interbody fusion. J Neurosurg Spine 12:40–46
19. Comer GC, Smith MW, Hurwitz EL, Mitsunaga KA, Kessler R, Carragee EJ (2012) Retrograde ejaculation after anterior lumbar interbody fusion with and without bone morphogenetic protein-2 augmentation: a 10-year cohort controlled study. Spine J 12:881–890
20. Connolly JF (1998) Clinical use of marrow osteoprogenitor cells to stimulate osteogenesis. Clin Orthop Relat Res:S257–S266
21. Connolly JF, Shindell R (1986) Percutaneous marrow injection for an ununited tibia. Nebr Med J 71:105–107
22. Cook SD, Dalton JE, Tan EH, Whitecloud TS 3rd, Rueger DC (1994) In vivo evaluation of recombinant human osteogenic protein (rhOP-1) implants as a bone graft substitute for spinal fusions. Spine (Phila Pa 1976) 19:1655–1663
23. Cunningham BW, Shimamoto N, Sefter JC, Dmitriev AE, Orbegoso CM, McCarthy EF, Fedder IL, McAfee PC (2002) Osseointegration of autograft versus osteogenic protein-1 in posterolateral spinal arthrodesis: emphasis on the comparative mechanisms of bone induction. Spine J 2:11–24
24. Dodd CA, Fergusson CM, Freedman L, Houghton GR, Thomas D (1988) Allograft versus autograft bone in scoliosis surgery. J Bone Joint Surg Br 70:431–434
25. Frechette JP, Martineau I, Gagnon G (2005) Platelet-rich plasmas: growth factor content and roles in wound healing. J Dent Res 84:434–439
26. Friedlaender GE, Perry CR, Cole JD, Cook SD, Cierny G, Muschler GF, Zych GA, Calhoun JH, LaForte AJ, Yin S (2001) Osteogenic protein-1 (bone morphogenetic protein-7) in the treatment of tibial nonunions. J Bone Joint Surg Am 83-A(Suppl 1):S151–S158
27. Gerstenfeld LC, Thiede M, Seibert K, Mielke C, Phippard D, Svagr B, Cullinane D, Einhorn TA (2003) Differential inhibition of fracture healing by non-selective and cyclooxygenase-2 selective non-steroidal anti-inflammatory drugs. J Orthop Res 21:670–675
28. Glassman SD, Carreon L, Djurasovic M, Campbell MJ, Puno RM, Johnson JR, Dimar JR (2007) Posterolateral lumbar spine fusion with INFUSE bone graft. Spine J 7:44–49
29. Glassman SD, Dimar JR, Carreon LY, Campbell MJ, Puno RM, Johnson JR (2005) Initial fusion rates with recombinant human bone morphogenetic protein-2/compression resistant matrix and a hydroxyapatite and tricalcium phosphate/collagen carrier in posterolateral spinal fusion. Spine (Phila Pa 1976) 30:1694–1698
30. Gupta MC, Theerajunyaporn T, Maitra S, Schmidt MB, Holy CE, Kadiyala S, Bruder SP (2007) Efficacy of mesenchymal stem cell enriched grafts in an ovine posterolateral lumbar spine model. *Spine (Phila Pa 1976)* 32:720–726 ; discussion 27
31. Harrison WJ, Lewis CP, Lavy CB (2004) Open fractures of the tibia in HIV positive patients: a prospective controlled single-blind study. Injury 35:852–856
32. Hecht BP, Fischgrund JS, Herkowitz HN, Penman L, Toth JM, Shirkhoda A (1999) The use of recombinant human bone morphogenetic protein 2 (rhBMP-2) to promote spinal fusion in a nonhuman primate anterior interbody fusion model. Spine (Phila Pa 1976) 24:629–636
33. Huddleston PM, Steckelberg JM, Hanssen AD, Rouse MS, Bolander ME, Patel R (2000) Ciprofloxacin inhibition of experimental fracture healing. J Bone Joint Surg Am 82:161–173
34. Jones KB, Maiers-Yelden KA, Marsh JL, Zimmerman MB, Estin M, Saltzman CL (2005) Ankle fractures in patients with diabetes mellitus. J Bone Joint Surg Br 87:489–495
35. Kanayama M, Hashimoto T, Shigenobu K, Yamane S, Bauer TW, Togawa D (2006) A prospective randomized study of posterolateral lumbar fusion using osteogenic protein-1 (OP-1) versus local autograft with ceramic bone substitute: emphasis of surgical exploration and histologic assessment. Spine (Phila Pa 1976) 31:1067–1074
36. Khanna G, Lewonowski K, Wood KB (2006) Initial results of anterior interbody fusion achieved with a less invasive bone harvesting technique. Spine (Phila Pa 1976) 31:111–114
37. Kim HJ, Buchowski JM, Zebala LP, Dickson DD, Koester L, Bridwell KH (2013) RhBMP-2 is superior to iliac crest bone graft for long fusions to the sacrum in adult spinal deformity: 4- to 14-year follow-up. Spine (Phila Pa 1976) 38:1209–1215

38. Kim YJ, Bridwell KH, Lenke LG, Rhim S, Cheh G (2006) Pseudarthrosis in long adult spinal deformity instrumentation and fusion to the sacrum: prevalence and risk factor analysis of 144 cases. Spine (Phila Pa 1976) 31:2329–2336
39. Knapp DR Jr, Jones ET, Blanco JS, Flynn JC, Price CT (2005) Allograft bone in spinal fusion for adolescent idiopathic scoliosis. J Spinal Disord Tech 18(Suppl):S73–S76
40. Kraiwattanapong C, Boden SD, Louis-Ugbo J, Attallah E, Barnes B, Hutton WC (2005) Comparison of Healos/bone marrow to INFUSE(rhBMP-2/ACS) with a collagen-ceramic sponge bulking agent as graft substitutes for lumbar spine fusion. Spine (Phila Pa 1976) 30:1001–1007 ; discussion 07
41. Lennon DP, Haynesworth SE, Young RG, Dennis JE, Caplan AI (1995) A chemically defined medium supports in vitro proliferation and maintains the osteochondral potential of rat marrow-derived mesenchymal stem cells. Exp Cell Res 219:211–222
42. Lewandrowski KU, Nanson C, Calderon R (2007) Vertebral osteolysis after posterior interbody lumbar fusion with recombinant human bone morphogenetic protein 2: a report of five cases. Spine J 7:609–614
43. Maeda T, Buchowski JM, Kim YJ, Mishiro T, Bridwell KH (2009) Long adult spinal deformity fusion to the sacrum using rhBMP-2 versus autogenous iliac crest bone graft. Spine (Phila Pa 1976) 34:2205–2212
44. Magin MN, Delling G (2001) Improved lumbar vertebral interbody fusion using rhOP-1: a comparison of autogenous bone graft, bovine hydroxylapatite (Bio-Oss), and BMP-7 (rhOP-1) in sheep. Spine (Phila Pa 1976) 26:469–478
45. Majors AK, Boehm CA, Nitto H, Midura RJ, Muschler GF (1997) Characterization of human bone marrow stromal cells with respect to osteoblastic differentiation. J Orthop Res 15:546–557
46. Martin GJ Jr, Boden SD, Marone MA, Marone MA, Moskovitz PA (1999) Posterolateral intertransverse process spinal arthrodesis with rhBMP-2 in a nonhuman primate: important lessons learned regarding dose, carrier, and safety. J Spinal Disord 12:179–186
47. McLain RF, Boehm CA, Rufo-Smith C, Muschler GF (2009) Transpedicular aspiration of osteoprogenitor cells from the vertebral body: progenitor cell concentrations affected by serial aspiration. Spine J 9:995–1002
48. Minamide A, Kawakami M, Hashizume H, Sakata R, Tamaki T (2001) Evaluation of carriers of bone morphogenetic protein for spinal fusion. Spine (Phila Pa 1976) 26:933–939
49. Pelled G, Sheyn D, Tawackoli W, Jun DS, Koh Y, Su S, Cohn Yakubovich D, Kallai I, Antebi B, Da X, Gazit Z, Bae H, Gazit D (2016) BMP6-Engineered MSCs Induce Vertebral Bone Repair in a Pig Model: a Pilot Study. Stem Cells Int 2016:6530624
50. Perry AC, Prpa B, Rouse MS, Piper KE, Hanssen AD, Steckelberg JM, Patel R (2003) Levofloxacin and trovafloxacin inhibition of experimental fracture-healing. Clin Orthop Relat Res:95–100
51. Price CT, Connolly JF, Carantzas AC, Ilyas I (2003) Comparison of bone grafts for posterior spinal fusion in adolescent idiopathic scoliosis. Spine (Phila Pa 1976) 28:793–798
52. Ransford AO, Morley T, Edgar MA, Webb P, Passuti N, Chopin D, Morin C, Michel F, Garin C, Pries D (1998) Synthetic porous ceramic compared with autograft in scoliosis surgery. A prospective, randomized study of 341 patients. J Bone Joint Surg Br 80:13–18
53. Rihn JA, Kirkpatrick K, Albert TJ (2010) Graft options in posterolateral and posterior interbody lumbar fusion. Spine (Phila Pa 1976) 35:1629–1639
54. Samartzis D, Shen FH, Matthews DK, Yoon ST, Goldberg EJ, An HS (2003) Comparison of allograft to autograft in multilevel anterior cervical discectomy and fusion with rigid plate fixation. Spine J 3:451–459
55. Sandhu HS, Kanim LE, Kabo JM, Toth JM, Zeegen EN, Liu D, Delamarter RB, Dawson EG (1996) Effective doses of recombinant human bone morphogenetic protein-2 in experimental spinal fusion. Spine (Phila Pa 1976) 21:2115–2122
56. Sandhu HS, Kanim LE, Toth JM, Kabo JM, Liu D, Delamarter RB, Dawson EG (1997) Experimental spinal fusion with recombinant human bone morphogenetic protein-2 without decortication of osseous elements. Spine (Phila Pa 1976) 22:1171–1180

57. Sandhu HS, Toth JM, Diwan AD, Seim HB 3rd, Kanim LE, Kabo JM, Turner AS (2002) Histologic evaluation of the efficacy of rhBMP-2 compared with autograft bone in sheep spinal anterior interbody fusion. Spine (Phila Pa 1976) 27:567–575
58. Sawin PD, Traynelis VC, Menezes AH (1998) A comparative analysis of fusion rates and donor-site morbidity for autogeneic rib and iliac crest bone grafts in posterior cervical fusions. J Neurosurg 88:255–265
59. Schnee CL, Freese A, Weil RJ, Marcotte PJ (1997) Analysis of harvest morbidity and radiographic outcome using autograft for anterior cervical fusion. Spine (Phila Pa 1976) 22:2222–2227
60. Shields LB, Raque GH, Glassman SD, Campbell M, Vitaz T, Harpring J, Shields CB (2006) Adverse effects associated with high-dose recombinant human bone morphogenetic protein-2 use in anterior cervical spine fusion. Spine (Phila Pa 1976) 31:542–547
61. Smucker JD, Rhee JM, Singh K, Yoon ST, Heller JG (2006) Increased swelling complications associated with off-label usage of rhBMP-2 in the anterior cervical spine. Spine (Phila Pa 1976) 31:2813–2819
62. Suh DY, Boden SD, Louis-Ugbo J, Mayr M, Murakami H, Kim HS, Minamide A, Hutton WC (2002) Delivery of recombinant human bone morphogenetic protein-2 using a compression-resistant matrix in posterolateral spine fusion in the rabbit and in the non-human primate. Spine (Phila Pa 1976) 27:353–360
63. Taghavi CE, Lee KB, Keorochana G, Tzeng ST, Yoo JH, Wang JC (2010) Bone morphogenetic protein-2 and bone marrow aspirate with allograft as alternatives to autograft in instrumented revision posterolateral lumbar spinal fusion: a minimum two-year follow-up study. Spine (Phila Pa 1976) 35:1144–1150
64. Thalgott JS, Giuffre JM, Fritts K, Timlin M, Klezl Z (2001) Instrumented posterolateral lumbar fusion using coralline hydroxyapatite with or without demineralized bone matrix, as an adjunct to autologous bone. Spine J 1:131–137
65. Theiss SM, Boden SD, Hair G, Titus L, Morone MA, Ugbo J (2000) The effect of nicotine on gene expression during spine fusion. Spine (Phila Pa 1976) 25:2588–2594
66. Tomford WW (1995) Transmission of disease through transplantation of musculoskeletal allografts. J Bone Joint Surg Am 77:1742–1754
67. Urist MR (2009) The classic : a morphogenetic matrix for differentiation of bone tissue. Clin Orthop Relat Res 467:3068–3070
68. Vaccaro AR, Anderson DG, Patel T, Fischgrund J, Truumees E, Herkowitz HN, Phillips F, Hilibrand A, Albert TJ, Wetzel T, McCulloch JA (2005) Comparison of OP-1 Putty (rhBMP-7) to iliac crest autograft for posterolateral lumbar arthrodesis: a minimum 2-year follow-up pilot study. Spine (Phila Pa 1976) 30:2709–2716
69. Weiner, B. K., and M. Walker. 2003. 'Efficacy of autologous growth factors in lumbar intertransverse fusions', Spine (Phila Pa 1976) 28:1968–1970; discussion 71

BMPs in Dental Medicine: Promises and Challenges

Ulf M.E. Wikesjö and Cristiano Susin

Abstract Regeneration of bone is critical to the rehabilitation of congenital malformations and defects resulting from trauma or tumor resection in the craniofacial skeleton, as well as defects resulting from periodontal disease or remodeling following tooth extractions. It is the objective of this text to reflect pioneering and significant preclinical and clinical observations, promises, and challenges, of bone morphogenetic proteins (BMPs) with focus on recombinant human BMP-2 (rhBMP-2) but also recombinant human BMP-7 (rhBMP-7) and recombinant human growth/differentiation factor-5 (rhGDF-5) in craniofacial settings to include alveolar bone augmentation for implant dentistry.

Keywords Recombinant human BMP-2 (rhBMP-2) • Recombinant human BMP-7 (rhBMP-7) • Recombinant human growth/differentiation factor-5 (rhGDF-5) • Alveolar augmentation • Sinus augmentation • Alveolar preservation • Osseointegration • Implant dentistry • Dental implants

1 Introduction

Regeneration of bone is vital to the rehabilitation of congenital malformations in the craniofacial skeleton, defects resulting from trauma or tumor resection and defects resulting from periodontal disease or remodeling following tooth extractions. Historically, autogenous bone grafts have been preferred for bone augmentation on craniofacial indications; however, demand for a second surgical site, finite intraoral sources, and associated morbidity has constrained their widespread acceptance and

U.M.E. Wikesjö, DDS, DMD, Dr Odont (✉) • C. Susin, DDS, MSD, Dr Odont
Laboratory for Applied Periodontal and Craniofacial Regeneration – The Dental College of Georgia, Augusta University, 1430 John Wesley Gilbert Drive, Augusta, 30912 GA, United States
e-mail: uwikesjo@augusta.edu; wikesjo@comcast.net

S. Vukicevic, K.T. Sampath (eds.), *Bone Morphogenetic Proteins: Systems Biology Regulators*, Progress in Inflammation Research,
DOI 10.1007/978-3-319-47507-3_11

use [2, 11, 51]. In consequence, the dental profession increasingly has embraced cadaver-sourced allogeneic and xenogeneic (bovine, porcine, equine, coral) or synthetic (polymeric, ceramic) bone biomaterials and in addition resorbable/non-resorbable devices (membranes) for guided tissue/guided bone regeneration (GTR/GBR) as stand-alone therapeutic interventions or in various combinations to meet clinical demands [1, 18, 61]. The global market for dental bone biomaterials and devices for GTR/GBR illustrates this trend, the US/North American market estimated to $363 M, the EU/Middle East/African market to $189 M, the Latin American market to $97 M, and the Asian Pacific market to $125 M in 2015 for a total estimated value of $773 M (iData Research). As the bone-anchored dental implant-based prosthesis progressively has become favored for oral rehabilitation replacing missing and compromised teeth, augmentation of the deficit alveolar ridge has become an even more significant prerequisite. In perspective, it is estimated that in excess of 14 M, dental implants are sold/placed annually worldwide, the US market alone estimated to approach 2.5 M units in 2015 (iData Research).

Intuitive observations of bone formation associated with implanted bone matrices [39, 46, 52] eventually led to the critical discovery of bone morphogenetic proteins (BMPs) [71]. Subsequent purification, characterization, and cloning [10, 30, 31, 53, 60, 72, 79] triggered research and development pursuing purified and recombinant forms of BMPs to induce bone formation in orthopedic, spine, and craniofacial settings [5, 16, 32, 40, 78]. Recombinant human BMP-2 in an absorbable collagen sponge carrier (rhBMP-2/ACS) became the first BMP technology approved for human use by the US Food and Drug Administration, approved for spine fusion in 2002 and in 2004 for open tibia fracture repair [48]. In 2007, rhBMP-2/ACS met approval for bone augmentation in conjunction with tooth extraction sockets and bone augmentation in the maxillary sinus to enable installation of bone-anchored (osseointegrated) dental implants in the rehabilitation of dilapidated dentitions. It is the objective of this text to reflect pioneering and significant preclinical and clinical observations, promises, and challenges of BMPs with focus on rhBMP-2 but also rhBMP-7 and recombinant human growth/differentiation factor-5 (rhGDF-5) in craniofacial settings to include alveolar bone and sinus augmentation for implant dentistry.

2 Setting the Stage

Alveolar augmentation may out of principle be divided into inlay and onlay indications translating to contained (inlay) and non-contained (onlay) defect sites. Tooth extraction sockets, intrabony defects, and maxillary sinus floor sites represent inlay defects, and width and height deficiencies of the alveolar ridge represent onlay defects.

In perspective, it is important to realize elementary biomechanical requirements for any compatible technology, BMP or other, tasked to support alveolar augmentation to challenges and constraints offered in inlay and onlay settings [28]. Whereas particulate or paste formulations may suffice to support/enhance bone formation in contained sites, structural integrity and geometry hardly offered by particulate

technologies become requisite characteristics for technologies considered for augmenting/expanding the width and height of the alveolar envelope. This is also true for compressible carriers such as the ACS which poorly withstands the challenges imposed by intraoral forces. Devices and membranes have long been used to provide containment and space provision for particulate and compressible carriers.

Slowly/non-resorbable biomaterials, which are often used alone or in combination for alveolar ridge augmentation, may actually compromise space-provision obstructing the site for bone formation. In the long-term, slowly/non-resorbable technologies may compromise mechanical properties of bone including dental implant fixation and load-bearing. Nevertheless, combined with successful space-providing delivery technologies or adjunctives, BMPs have shown significant promise to support bone formation in the craniofacial skeleton. A number of studies using rodent screening models, translational inlay and onlay defect models, and canine, porcine, or nonhuman primate platforms including discriminating critical-size defects and clinical modeling illustrate the potential of BMPs to augment alveolar bone in craniofacial settings. We herein separately review alveolar bone augmentation (inlay and onlay defects), maxillary sinus augmentation, and peri-implant defects in preclinical and clinical settings.

3 Alveolar Ridge Augmentation/Preservation

A concerted chain of events occur following tooth extraction leading to remodeling of the alveolar ridge and, ultimately, to the complete resorption of the alveolar bone. Whereas most of the efforts in implant dentistry have been directed at augmenting the resorbed alveolar ridge, alveolar bone preservation following extractions has become increasingly important. To that end, the application of BMPs at the time of tooth extraction – prior to bone loss due to remodeling – represents a compelling treatment option.

3.1 Observations from Preclinical Inlay Models

Preclinical studies using inlay defect models have evaluated rhBMP-2 and rhGDF-5 for alveolar augmentation. These early studies have primary focus on alternative delivery systems to present BMP to the defect site. Cochran and colleagues applied rhBMP-2/ACS (*rhBMP-2 at 0.2 mg/mL*) and rhBMP-2 in a polylactide/glycolide copolymer carrier (rhBMP-2/PLGA, *rhBMP-2 at 0.2 mg/mL*) to 1.5 × 4-mm (width × depth) gap defects circumscribing dental implants in dogs to evaluate bone formation following 4- and 12-week healing intervals [12, 13, 34]. Defect sites receiving rhBMP-2/ACS and rhBMP-2/PLGA showed significantly enhanced bone fill compared with control at 4 but not at 12 weeks. Comparing the ACS with the PLGA carrier, the ACS supported greater bone fill in this inlay defect model. Notably, sites additionally fitted with an occlusive expanded polytetrafluoroethylene (ePTFE) GBR membrane to exclude soft tissue infiltration showed delayed bone formation.

Commentary This early study points to accelerated bone formation in alveolar sites receiving rhBMP-2, and that tissue resources originating in adjoining mucosal tissues substantially contribute to rhBMP-2-induced bone formation if not blocked by an occlusive membrane.

In parallel studies using clinically advanced (~15 × 10 × 10 mm; length × depth × width) alveolar ridge saddle-type defects in dogs and a 12-week healing interval, Jovanovic and co-workers evaluated suitability of a volume-defining hyaluronan (Hy) sponge vs. the ACS technology to serve as delivery systems for rhBMP-2 (*rhBMP-2 at 0.2 mg/mL*). Both rhBMP-2/ACS- and rhBMP-2/Hy-induced bone formation filled the saddle-type defects to capacity suggesting that Hy may be used interchangeably with ACS in support of rhBMP-2-induced bone formation [33]. In separate studies, rhBMP-2/ACS (*rhBMP-2 at 0.2 mg/mL*) was benchmarked to GBR demonstrating superior bone fill over GBR following a 12-week healing interval [36]. Combining rhBMP-2/ACS with GBR did not offer additional benefits (Figs. 1 and 2). Of note, GBR sites often encountered suture-line dehiscences exposing the ePTFE membrane that readily became infected compromising wound healing/regeneration altogether in contrast to sites receiving rhBMP-2/ACS alone displaying uneventful healing potentially reflecting a beneficial effect of rhBMP-2 also on soft tissue healing. In still other studies, long-term stability of rhBMP-2/ACS-induced bone (*rhBMP-2 at 0.2 mg/mL*) vs. that of the pristine resident bone was compared [35] (Fig. 3). Dental implants were inserted into the rhBMP-2/ACS-induced and adjoining pristine resident bone, osseointegrated, and fitted with a fixed dental prosthesis. The animals were then returned to a solid dog-food diet for functional loading. Crestal bone levels and dental implant fixation evaluated following 12 months of functional loading showed limited, if any, differences between rhBMP-2/ACS-induced and pristine resident bone again substantiating unique properties of rhBMP-2/ACS rarely, if at all, reached using conventional allogeneic/xenogeneic bone derivatives or synthetic biomaterials.

Commentary Significant for this series of studies in addition to key observations of clinically meaningful bone formation for the benefit of fixation of dental implants following surgical implantation of rhBMP-2/ACS is the clinical swelling at the defect sites subsiding within 7–10 days as well as frequently occurring seroma formation, seromas constituted as serum-filled radiolucent vacuoles within the regenerate eventually filling with bone demonstrated in the radiographic and histologic evaluation.

Still other studies evaluated the clinical potential of rhGDF-5 in a resorbable particulate micro-/macroporous ß-tricalcium phosphate carrier (rhGDF-5/ß-TCP, *rhGDF-5 at 0.6 mg/g ß-TCP*) also using alveolar ridge saddle-type defects in dogs, sites receiving the rhGDF-5/ß-TCP technology showing enhanced bone formation compared with the autogenous bone graft control [73]. Studies in rodent screening models further substantiate the superiority of rhGDF-5/ß-TCP (*rhGDF-5 at 0.5 mg/g ß-TCP*) benchmarked to a market leader particulate bovine bone biomaterial [57].

Commentary Adverse events, i.e., local swelling or seroma formation, were not evident or reported with the use of rhGDF-5/ß-TCP.

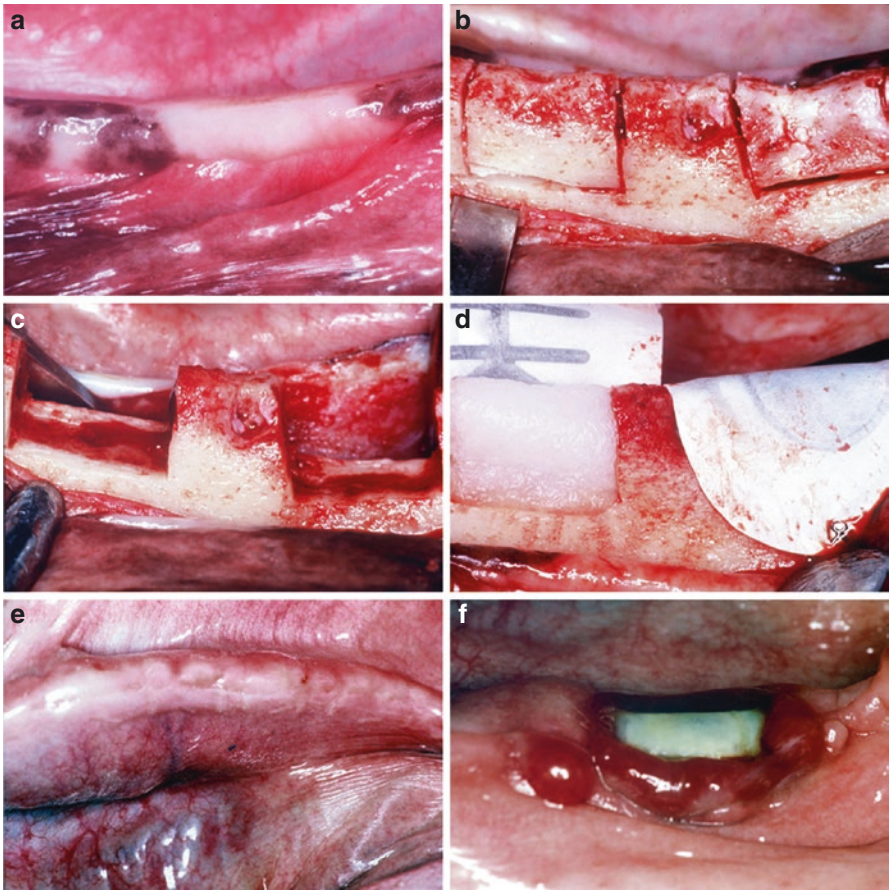


Fig. 1 Mandibular, alveolar ridge, saddle-type defect implanted with rhBMP-2/ACS and guided bone regeneration (GBR): presurgery baseline (**a**); surgical outline of the alveolar ridge defect (**b**); alveolar ridge saddle-type defect (**c**); application of rhBMP-2/ACS and GBR membranes (**d**); and clinical observations of sites implanted with rhBMP-2/ACS (**e**) and GBR (**f**). Note swelling of the site implanted with rhBMP-2/ACS and wound failure at the site receiving GBR (From Jovanovic et al.[36]; Figures copyrighted by and modified with permission from John Wiley & Sons Ltd.)

3.2 Observations from Preclinical Onlay Models

Our laboratories first showed that rhBMP-2/ACS (*rhBMP-2 at 0.4 mg/mL*) has potential to support clinically relevant bone formation for implant dentistry expanding the alveolar ridge [63] (Fig. 4). Using the critical-size supraalveolar peri-implant defect model [76], 10-mm dental implants were placed 5 mm into the edentulated mandibular alveolar crest leaving 5 mm of the implant extending above the crest covered with rhBMP-2/ACS or buffer/ACS (control) and submerged under the advanced

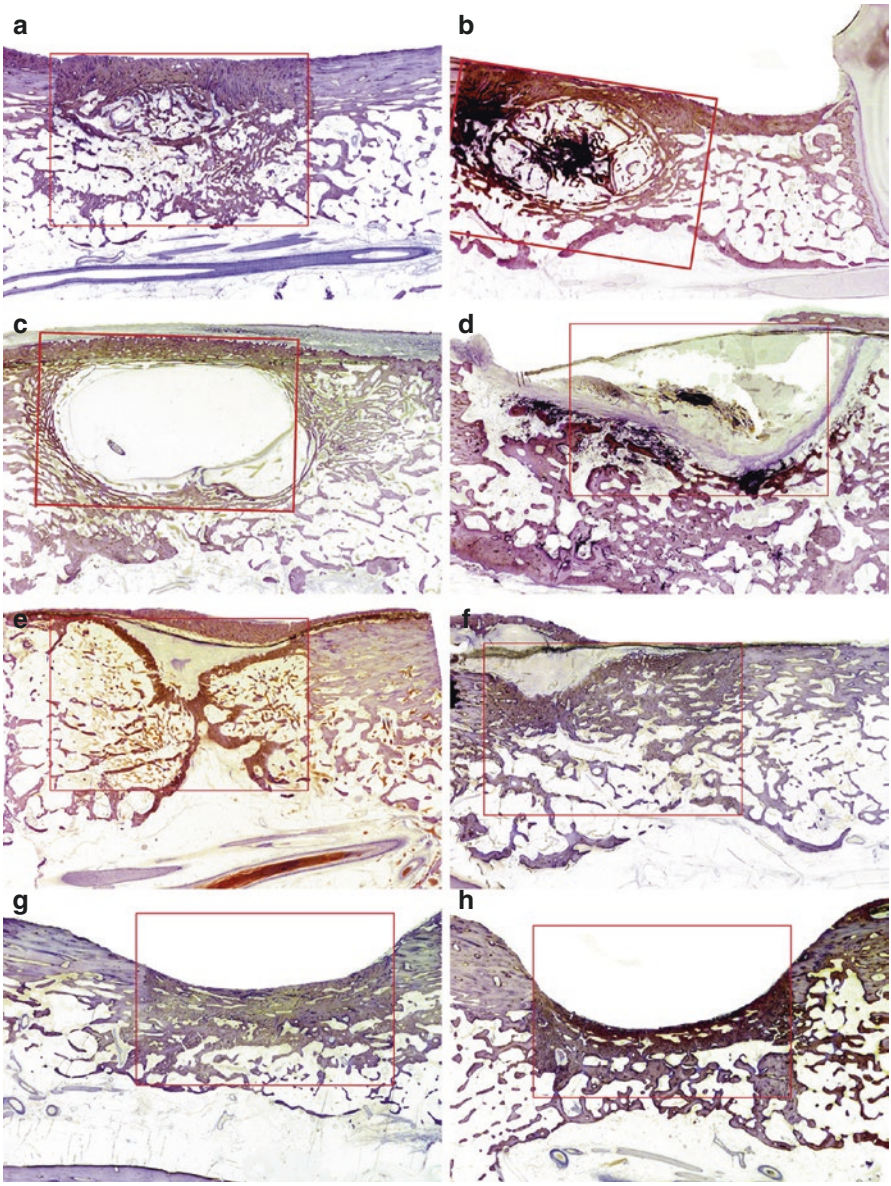


Fig. 2 Representative photomicrographs of defect sites receiving rhBMP-2/ACS (**a** cortex formation and complete trabecular bone fill; **b** cortex formation and resolving seroma filled with trabecular bone); rhBMP-2/GBR (**c** cortex formation and large seroma; **d** wound failure/membrane exposure; note cortex formation over part of the GBR barrier); GBR (**e** cortex formation; **f** limited, late(?) wound failure/membrane exposure; note cortex formation over part of the GBR barrier); and surgery controls with (**g**) or without (**h**) ACS. Red frames approximate the original defect sites. Healing interval 12 weeks (From Jovanovic et al. [36]; Figures copyrighted by and modified with permission from John Wiley & Sons Ltd.)

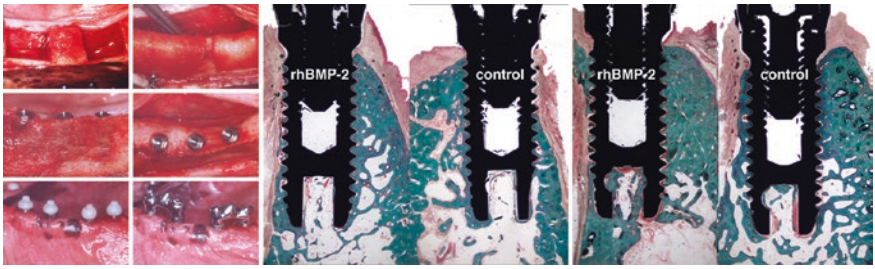


Fig. 3 Evaluation of titanium implants placed into rhBMP-2-induced bone subject to 12 months of functional loading. The clinical panels show surgically induced mandibular, saddle-type (~15 × 10 mm), full-thickness alveolar ridge defects (two per jaw quadrant). The defects were immediately implanted with rhBMP-2/ACS with or without a barrier membrane. Healing progressed for 3 months when endosseous oral implants were installed into the rhBMP-2/ACS-induced bone and adjoining resident bone (control). Following 4 months of osseointegration, the implants received abutments and prosthetic reconstruction. Prosthetic reconstructed implants were then subject to functional loading for 12 months. The photomicrographs show implants placed into rhBMP-2-induced and resident bone following 12 months of functional loading. There is no discernable difference in bone formation and osseointegration between rhBMP-2-induced and resident bone (From Jovanovic et al. [35]; Figures copyrighted by and modified with permission from John Wiley & Sons Ltd.)

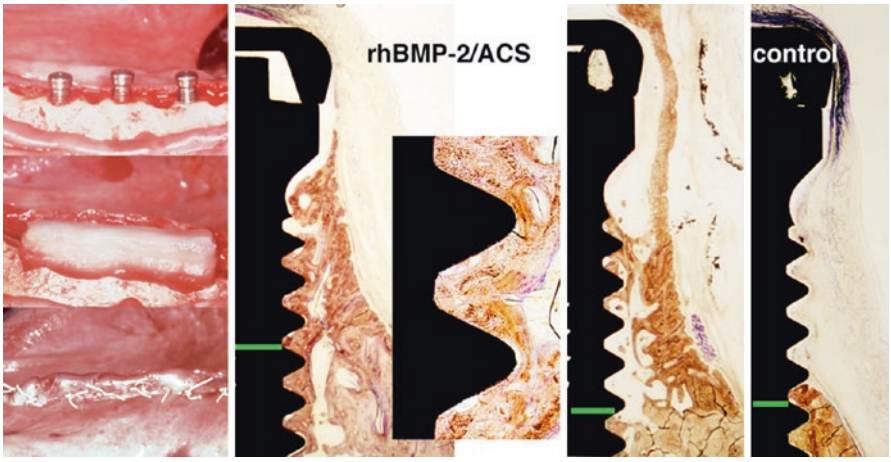


Fig. 4 Critical-size, supraalveolar, peri-implant defect implanted with rhBMP-2/ACS or ACS without rhBMP-2 (control). Clinical panels show the supraalveolar defect with rhBMP-2/ACS before and after wound closure for primary intention healing. The photomicrographs show defect sites implanted with rhBMP-2/ACS exhibiting bone formation reaching or exceeding the implant platform, the newly formed bone showing osseointegration to the titanium implant surface (high magnification insert). Control sites show limited, if any, bone formation. *Green lines* delineate the level of the surgically reduced alveolar crest. Healing interval 16 weeks (From Sigurdsson et al. [63]; Figures copyrighted by and modified with permission from John Wiley & Sons Ltd.)

mucoperiosteal flaps for primary intention healing. The histologic evaluation following a 16-week healing interval showed significant bone formation anchored to the previously naked implant surface reaching the top of the dental implants at sites receiving rhBMP-2/ACS, whereas controls displayed negligible bone formation. In comparison, parallel studies using space-providing membranes for GBR or membranes combined with an allogeneic demineralized bone matrix demonstrate the limited native regenerative potential of this defect model emphasizing the unique potential of rhBMP-2/ACS to stimulate local bone formation in support of implant dentistry [9, 75] (Fig. 5). Nevertheless, rhBMP-2/ACS-induced bone formation expressed considerable variability at times wallpapering the implant threads, at times showing bone formation of clinically relevant volume and geometry adjoining the implant. Apparently, the rhBMP-2/ACS technology appears ineffective to consistently support significant bone formation in onlay settings also shown in other studies using the canine supraalveolar peri-implant defect model, rhBMP-2, evaluated at concentrations of 0.05, 0.1, and 0.2 mg/mL [47, 68, 74] (Figs. 6 and 7).

Commentary Variable bone formation may rest with rhBMP-2 dose and/or bioavailability but also ACS structural integrity, biodegradation, soak-load, or any combination thereof.

Several routines have been considered to safeguard rhBMP-2/ACS performance for alveolar augmentation for implant dentistry. They include above mentioned rhBMP-2 dose variation ([68]; *rhBMP-2 at 0.05*, *0.1*, *and 0.2 mg/mL*) (Fig. 6), as well as the use of purpose-designed space-providing macroporous membranes/devices ([41, 74, 75]; *rhBMP-2 at 0.2 mg/mL*) (Fig. 7). Bulking agents including

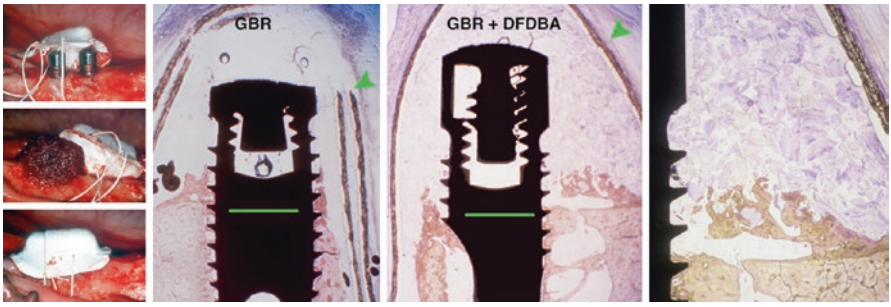


Fig. 5 Critical-size, supraalveolar, peri-implant defect treated with guided bone regeneration (*GBR*) using an occlusive space-providing ePTFE membrane (*green arrowheads*), with or without an allogeneic demineralized bone matrix (DBM). Clinical panels show the supraalveolar defect with the ePTFE membrane, with DBM rehydrated in autologous blood, and with the membrane in place prior to wound closure for primary intention healing. Note limited regeneration of alveolar bone in the absence and presence of DBM suggesting that the innate regenerative potential of alveolar bone is limited and that the DBM biomaterial has limited, if any, osteoinductive and/or osteoconductive properties to support bone regeneration. *Green lines* delineate the level of the surgically reduced alveolar crest. Healing interval 16 weeks (From Caplanis et al. [9]; Figures copyrighted by and modified with permission from Quintessence Publishing)

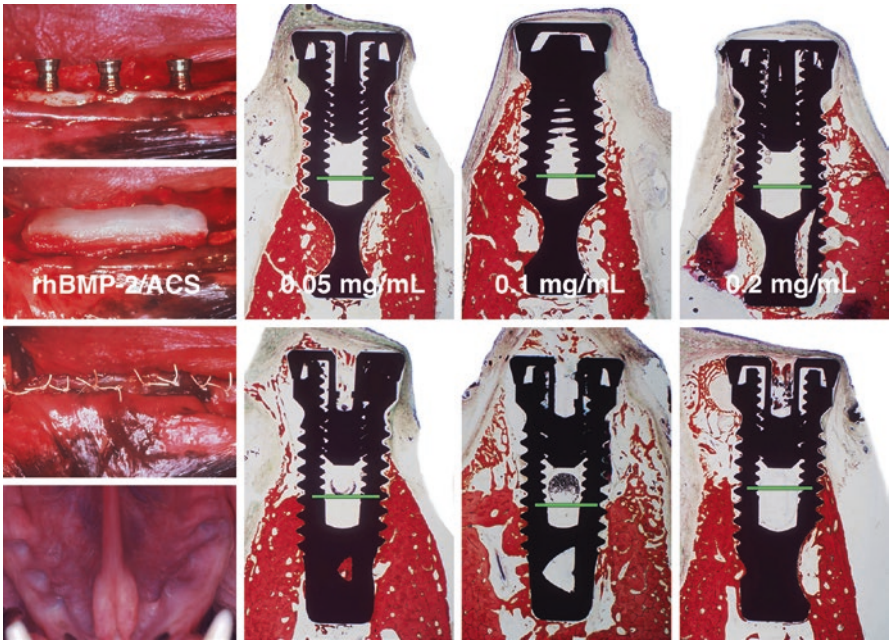


Fig. 6 Critical-size, supraalveolar, peri-implant defects treated with rhBMP-2/ACS; rhBMP-2 at *0.05*, *0.1*, and *0.2 mg/mL*. Clinical panels show a supraalveolar defect implanted with rhBMP-2/ACS before and after wound closure for primary intention healing, and clinical appearance at week 6 postsurgery; the right and left mandibular jaw quadrants of this animal received rhBMP-2 at 0.05 and 0.2 mg/mL, respectively. Representative photomicrographs show defect sites implanted with rhBMP-2/ACS exhibiting bone formation reaching or exceeding the implant platform. The newly formed, sparsely trabecular bone shows osseointegration to the machined titanium implant surface. The top photomicrographs show sites with the poorest bone induction for the various rhBMP-2 concentrations evaluated. The lower photomicrographs show corresponding sites with the best response. The *green lines* delineate the level of the surgically reduced alveolar crest. Healing interval 8 weeks (From Tatakis et al. [68]; Figures copyrighted by and modified with permission from John Wiley & Sons Ltd.)

granular hydroxyapatite, biphasic calcium phosphate, ß-tricalcium phosphate technologies, and others have likewise been considered to counter compressive forces onto the rhBMP-2/ACS as well as outlining desired bone volume and geometry. However, bulking agents may also introduce compromises related to their biodegradation; slowly or non-resorbable technologies may compromise the structural integrity of the newly formed bone including dental implant osseointegration ([3, 4, 47, 49]; *rhBMP-2 at 0.2 and 0.4 mg/mL*), while for bioresorbable conduits, the resorption process per se may solicit inflammatory reactions compromising bone formation and/or maintenance ([62]; *rhBMP-2 at 0.2 mg/mL*).

Commentary Whereas dose variation failed to influence rhBMP-2/ACS-induced bone formation, the use of macroporous space-providing devices allowed directed

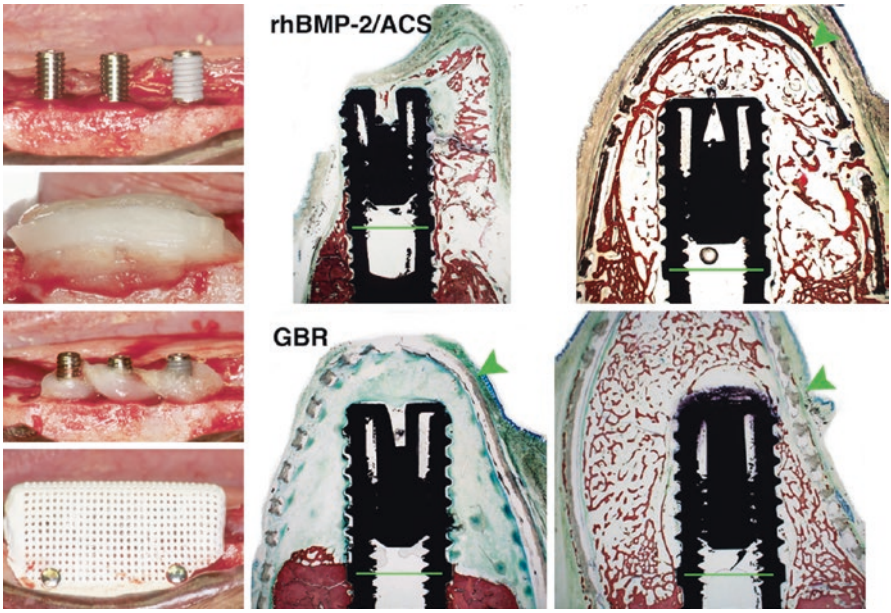


Fig. 7 Critical-size, supraalveolar, peri-implant defects treated with rhBMP-2/ACS, a porous, space-providing ePTFE membrane for guided bone regeneration (*GBR*), or rhBMP-2/ACS combined with the porous ePTFE membrane. The clinical panels show the supraalveolar defect with rhBMP-2/ACS and with the porous ePTFE membrane. Note how rhBMP-2-induced bone fills the space provided by the membrane (*green arrowheads*), whereas rhBMP-2/ACS alone provides very irregular bone formation (*top left*). The ePTFE membrane alone (*bottom left*) provides limited, if any, regeneration of alveolar bone. *Green lines* delineate the level of the surgically reduced alveolar crest. Healing interval 8 weeks (From Wikesjö et al. [74, 75]; Figures copyrighted by and modified with permission from John Wiley & Sons Ltd.)

rhBMP-2/ACS-induced bone formation/alveolar augmentation supporting the principle that the volume/geometry of new bone formation can be ascertained in the design of a space-providing device/matrix.

Pilot observations from rodent screening models suggest that a considerably lowered rhBMP-2 dose may effectively support bone formation/maturation [29, 54]. Using the critical-size supraalveolar peri-implant defect model, we evaluated the effect of rhBMP-2, rhBMP-7, and rhGDF-5 coated immediately onto dental implants on alveolar bone formation using a dose range protocol [43–45, 58, 66, 77]. Compared with control, BMP-coated implants yielded clinically relevant vertical bone gain (Fig. 8). Notably, rhBMP-2-coated implants displayed an inverse relationship between rhBMP-2 dose and induced bone formation/maturation [43, 77]. Whereas the low rhBMP-2 dose supported clinically relevant vertical/horizontal alveolar augmentation, in contrast, the high dose delayed bone maturation and in addition showed considerable clinical swelling and radiographic seroma formations.

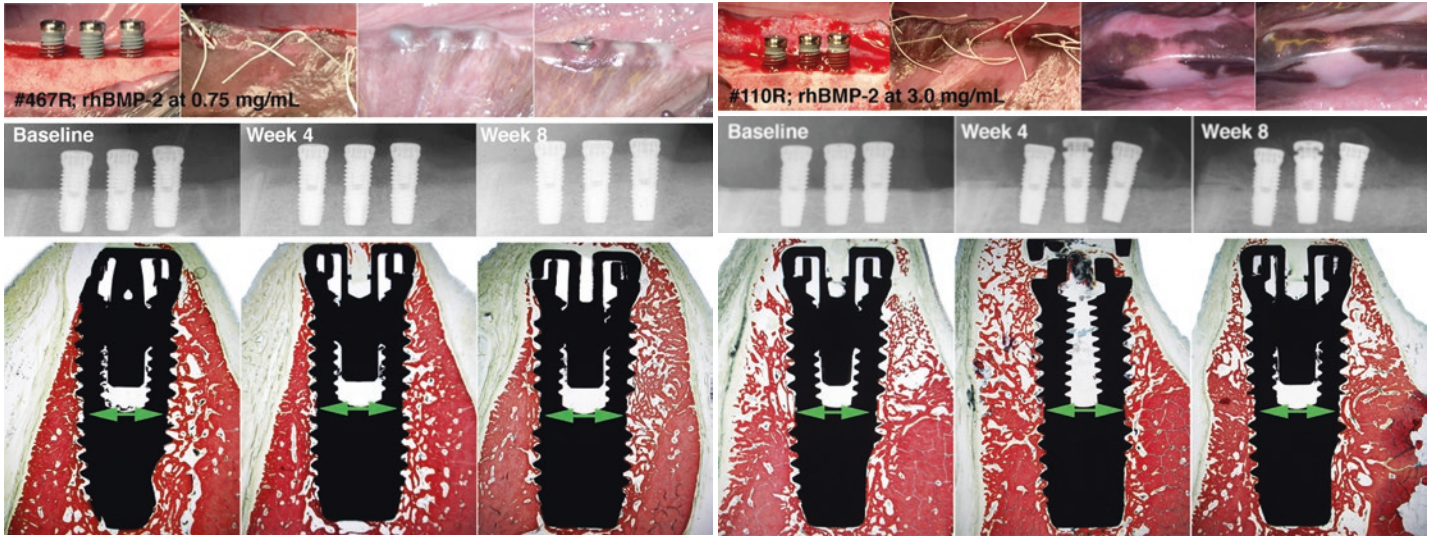


Fig. 8 Clinical panels showing ø4.0 × 10-mm dental implants coated with rhBMP-2 at *0.75 mg/mL* (*left*) and *3.0 mg/mL* (*right*) following placement and wound closure and healing at week 4 and 8. For implants coated with rhBMP-2 at *0.75 mg/mL* (*left*) the implant platforms (*cover screws*) can be visualized through the mucosa at week 4 and 8 when one implant becomes exposed. Radiographs show bone formation reaching the implant platform at week 4 and 8. Photomicrographs show bone formation with an established cortex reaching or exceeding the implant platform. Implants coated with rhBMP-2 at *3.0 mg/mL* (*right*) show significant swelling at week 4 somewhat resolving week 8. Radiographs show significant peri-implant radiolucencies (seromas) at week 4 apparently resolving week 8. Note the partial loosening of a cover screw within the tissues and implant displacements. The photomicrographs show immature bone formation exceeding the implant platform without an established cortex. Again note the partial loosening of the cover screw within the tissues at the central implant. *Green arrows* delineate the 5-mm notch placed level with the resident alveolar bone. Healing interval 8 weeks (From Wikesjö et al. [77]; Figures copyrighted by and modified with permission from John Wiley & Sons Ltd.)

Commentary Comparing bone formation/maturation at rhBMP-2/ACS ([47]; [68]; [74]) and rhBMP-2-coated dental implants suggests that the rhBMP-2-coated implant provides a more effective outcome than rhBMP-2/ACS and at a low dose. Such observations provide a rationale for developing novel delivery technologies with release kinetics profiling that of the rhBMP-2-coated implant for next generation BMP technologies for craniofacial indications and beyond.

3.3 Observations from Clinical Trials

A randomized controlled clinical trial evaluating rhBMP-2/ACS (*rhBMP-2 at 0.75 and 1.5 mg/mL*) for alveolar ridge augmentation following tooth extraction demonstrates that extraction socket sites receiving rhBMP-2/ACS (mean rhBMP-2 dose 1.9 mg/site) critically maintained alveolar crestal height, whereas control sites without this treatment projected a mean 1.2 mm crestal loss [20]. A recent randomized clinical trial expanded these findings by testing rhBMP-2/ACS (*rhBMP-2 at 1.5 mg/mL*) at extraction sites with large bone fenestrations. rhBMP-2/ACS yielded greater bone formation than ACS alone, rendering the resulting alveolar ridge more suitable to receive a dental implant [14].

rhBMP-2/ACS has also been evaluated in a randomized controlled clinical trial as alternative to autogenous bone grafts for alveolar augmentation and dental implant installation in the atrophic anterior maxilla [15]. Participating subjects either received rhBMP-2/ACS (*rhBMP-2 at 1.5 mg/mL*) or the "gold standard" particulated autogenous bone harvested from the mandibular retromolar region. A titanium mesh was used to define the regenerative space and provide wound stability. rhBMP-2/ACS yielded significantly greater radiographic horizontal bone gain compared with the autogenous bone graft at the critical immediate subcrestal level averaging 1.5 vs. 0.5 mm. No other significant differences in clinical/radiographic horizontal bone gain between rhBMP-2/ACS and autogenous bone graft were observed at 6 months allowing placement and osseointegration of dental implants.

Commentary The observations from this randomized clinical trial document and broaden the potential use of rhBMP-2/ACS in support bone augmentation beyond approved maxillary sinus and extraction socket augmentation indications.

4 Maxillary Sinus Augmentation

Prosthetic rehabilitation of the edentulated posterior maxilla presents considerable challenge. Remodeling following tooth loss not only produces decreased alveolar ridge width and height but also increased pneumatization significantly reducing potential housing for dental implant anchors. Modified Caldwell-Luc and transalveolar surgical approaches have thus been developed to access the subantral space with the intent to

increase the vertical dimension of the alveolar ridge through implantation of autogenous bone or bone biomaterials [6, 65]. Systematic reviews confirm the clinical efficacy of these approaches to fixation of dental implants [1, 19, 56, 67]. However, efficacious, present BMP technologies offer to expand the clinical protocol beyond autogenous bone grafting or the use of off-the-shelf cadaver-sourced or synthetic biomaterials.

4.1 Observations from Preclinical Studies

Hanisch and co-workers first evaluated rhBMP-2/ACS (*rhBMP-2 at 0.4 mg/mL*) for maxillary sinus augmentation and dental implant osseointegration using the modified Caldwell-Luc approach in nonhuman primates [24]. Dental implants were placed 3 months following implantation of rhBMP-2/ACS and allowed osseointegration over 3 months. The histometric evaluation showed sites receiving rhBMP-2/ACS exhibiting a clinically relevant two-fold increase in vertical bone augmentation compared with the ACS control (6.0 *vs.* 2.6 mm), newly formed bone exhibiting the same density and osseointegration as the adjoining native resident bone.

Commentary This first study provided the evidence for clinically relevant bone augmentation by rhBMP-2/ACS in maxillary sinus serving as a baseline for subsequent clinical evaluations and regulatory approval.

As autogenous cancellous bone maintains recognition as the "gold standard" for bone grafting, we compared local bone formation/osseointegration following sinus augmentation using rhBMP-2/ACS (*rhBMP-2 at 0.4 mg/mL*) vs. a particulated fresh autogenous cancellous bone graft harvested from the iliac crest in mini-pigs [42]. Dental implants were installed in conjunction with the augmentation procedure rather than using the staged protocol from our previous nonhuman primate study. Histologic evaluation at 8 weeks post-implantation revealed significant augmentation of the maxillary sinus following implantation of rhBMP-2/ACS approximating most of the dental implant bone-anchoring surfaces compared with irregular bone formation/active resorption in sites receiving autogenous bone grafting, rhBMP-2/ACS-induced bone exhibiting significantly greater density compared with the autogenous bone grafted sites (52 % *vs.* 33 %).

Commentary The observations in this study imply significant clinical time-savings using rhBMP-2/ACS due to the augmentation protocol that can be used in parallel with implant placement without need to access a donor site and associated morbidity; greater bone density of predicable volume and geometry without evidence of osteoclastic resorption overall suggests that rhBMP-2/ACS appears a realistic effective alternative to autogenous bone grafts for maxillary sinus augmentation and should thus be considered the new standard for this indication.

In similar evaluations, also using the mini-pig model, the Terheyden group applied rhBMP-7 (0.4 mg rhBMP-7 in 0.6 mL acetate buffer) with 1080 mg (3 mL) of a non-resorbable bovine bone mineral matrix vs. bovine bone mineral matrix with buffer only (control). Osseointegration at 6 months postsurgery averaged 80 %

for the rhBMP-7 sites vs. 39 % for the control [69]. In following, they compared the rhBMP-7 construct with a bovine bone mineral/autologous bone/platelet-rich plasma (PRP) composite. Osseointegration following a 6-week healing interval at sites receiving rhBMP-7 amounted to 46 % compared with 6 % for the PRP composite, whereas vertical bone gain averaged 8.3 *vs.* 3.6 mm, respectively [59].

rhGDF-5/ß-TCP (*rhGDF-5 at 0.4 mg/g ß-TCP or 0.8 mg/g ß-TCP*) has also successfully been considered in support of sinus augmentation using the mini-pig model. Control treatments included ß-TCP [22] or ß-TCP mixed with autogenous cortical bone chips (1:1) [23]. Healing intervals ranged up to 12 weeks. The authors concluded that rhGDF-5/ß-TCP significantly enhanced local bone formation (volume, density and osseointegration) compared with ß-TCP alone or combined with autogenous bone. Notably, there were no remarkable differences between rhGDF-5 concentrations.

Commentary Observations in the studies evaluating rhBMP-7 and rhGDF-5 suggest that both technologies present as viable alternatives to rhBMP-2 and should be considered as such. In comparison, the use of resorbable in front of non-resorbable technologies appears preferable relative to bone formation and osseointegration.

4.2 Observations from Clinical Trials

rhBMP-2/ACS has been scrutinized for sinus augmentation to meet regulatory approval [7, 8, 70]. Summarized in a systematic review (16) "rhBMP-2/ACS yielded clinically meaningful new bone formation for maxillary sinus augmentation – new bone height ranging between 7.8 and 10.2 mm" well meeting clinical requirements for dental implant installation although the statistical analysis showed average new bone height for the autogenous/allogeneic bone graft control exceeding the rhBMP-2/ACS by 1.6 mm. These studies used rhBMP-2 at 0.43, 0.75, and 1.5 mg/mL without consistent differences in bone formation, actual rhBMP-2 dose ranging between 2.9 and 20.8 mg/site.

Commentary It may be surprising that large rhBMP-2 dose differences do not reflect significant differences in bone formation, volume, or density; however, considering the maxillary sinus volume and geometry and rhBMP-2/ACS weak structural integrity vs. that of the autogenous bone graft, space provision and structural integrity become naturally limiting factors. Also lengthy observation intervals in these studies would allow considerable remodeling deflating any discernable differences in bone formation.

In separate studies, rhBMP-2/ACS was combined with particulate allogeneic mineralized bone or a commercial bovine bone preparation for maxillary sinus augmentation [21, 37]. Using core biopsies for a qualitative histologic analysis, sites receiving rhBMP-2/ACS (*rhBMP-2 at 1.5 mg/mL*) for a total of 4.2 or 8.4 mg/sinus combined with the allogeneic bone matrix could not demonstrate bone formation exceeding that of the allogeneic bone matrix as a stand-alone treatment [21]. Core biopsies featuring the rhBMP-2/ACS (*rhBMP-2 at 1.5 mg/mL*) bovine bone combination showed less bone formation than the bovine bone control prompting the authors to conclude "that the addition of rhBMP-2/ACS to Bio-Oss has a negative effect on bone formation" [37].

Commentary It must be noted that core biopsies only provide partial appreciation of rhBMP-2/ACS-induced bone formation. Observed from preclinical histology, rhBMP-2/ACS yields significant bone formation for dental implant osseointegration equal to if not surpassing autogenous bone grafts following sinus augmentation [42]. Moreover, non-resorbable matrices such as the bovine bone preparation have repeatedly been shown to displace/obstruct rhBMP-2/ACS-induced local bone formation ([3]; [4]; [47]; [49]) in part explaining the unexpected observations above.

A parallel group randomized clinical trial was used to evaluate rhGDF-5/ß-TCP for maxillary sinus augmentation [38, 64]. Using a staged protocol, the patients either received rhGDF-5/ß-TCP (rhGDF-5 at 500 mg/g ß-TCP) or an autogenous bone/ß-TCP (1:1) composite (control) using a modified Caldwell-Luc approach and a 16-week healing interval followed by installation of dental implants. The radiographic evaluation favored the rhGDF-5/ß-TCP construct; the histometric evaluation of trephine core biopsies showed similar fractions of bone formation at sites receiving rhGDF-5/ß-TCP (28 %) compared with sites receiving the autogenous bone/ß-TCP composite (32 %). In other words, the rhGDF-5/ß-TCP construct was as effective as the benchmark autogenous bone/ß-TCP composite, even though the rhGDF-5/ß-TCP construct does not provide viable bone cells at implantation, whereas the ß-TCP/autogenous bone composite does.

Commentary The observations herein suggest that the rhGDF-5/ß-TCP construct is worthy second-generation BMP candidate for regeneration of bone in the craniofacial skeleton, the ß-TCP structural integrity, and timely biodegradation presenting as advantages over present ACS technology.

5 Peri-implant Defect Repair

Peri-implantitis is defined as a biofilm-induced inflammatory lesion around a dental implant, which progressively causes alveolar bone resorption. The array of pathogens found at implants affected by peri-implantitis closely resembles the microbiota associated with periodontitis. The prevalence of peri-implantitis seems to be in the order of 10 % of the implants and 20 % of the patients within 5–10 years following implant placement though reported estimates are rather disperse [17, 50]. Even if favorable short-term treatment outcomes have been reported, failing disease resolution, disease progression or recurrence, and implant loss despite treatment have also been reported [27]. Importantly, predictable re-osseointegration of the exposed implant surface has not been achieved with current treatments [55].

Hanisch and co-workers used ligature-enhanced plaque accumulation to provoke peri-implantitis at hydroxyapatite-coated titanium dental implants in the posterior maxilla and mandible in four *Macaca mulatta* monkeys over 11 months [25]. Submucosal microbial samples revealed a large proportion of G-anaerobic rods, predominantly *Porphyromonas gingivalis*, *Bacteroides forsythus*, and *Fusobacterium* species as well as beta-hemolytic streptococci following ligature removal, microbiota associated with destructive periodontal disease and peri-implantitis in humans.

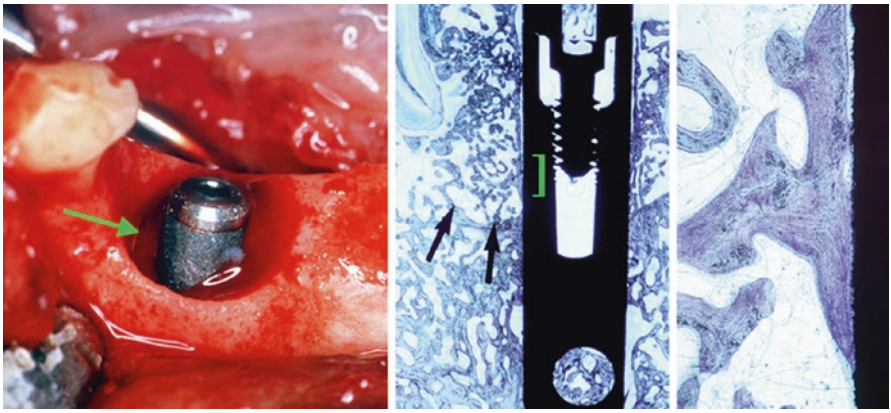


Fig. 9 Re-osseointegration following treatment of chronic peri-implantitis defect with rhBMP-2/ACS. The clinical panel shows the debrided peri-implantitis defect prior to treatment with rhBMP-2/ACS; the *green arrow* points to the aspect of the implant shown in the photomicrographs. *Black arrows* delineate the apical aspect of the peri-implantitis defect; the green bracket depicts a high magnification area (*right*) showing re-osseointegration. Note that the rhBMP-2-induced bone exhibits qualities of the contiguous resident bone. Healing interval 16 weeks (From Hanisch et al. [26]; Figures copyrighted by and modified with permission from Quintessence Publishing)

Resulting advanced inlay/onlay defects exhibited a mean depth of 3.3 ± 1.3 mm and width of 2.0 ± 0.5 mm. Subsequently the investigators implanted rhBMP-2/ACS (*rhBMP-2 at 0.4 mg/mL*) as a stand-alone therapy following defect soft tissue debridement and cleansing of the biofilm-contaminated denuded implant surfaces to resolve the peri-implantitis defects [26] (Fig. 9). rhBMP-2/ACS supported significant resolution of the advanced chronic peri-implantitis defects, defect fill averaging 77 % of the defect depth vs. 24 % for the sham surgery control following the 16-week healing interval. Importantly, the newly formed bone osseointegrated to a hydroxyapatite-coated titanium dental implant surface that had been exposed to a biofilm-induced inflammatory lesion over 11 months, osseointegration reaching clinically relevant 40 %.

Commentary The singularly unique observations gained in this "first" proof-of-concept study become even more critically important considering the increasing awareness of peri-implantitis and the up till now, almost two decades later, absence of effective clinical solutions.

6 Concluding Remarks

Bone regeneration has become a major objective of implant dentistry, dictated by functional and esthetic demands. rhBMP-2, rhBMP-7, and rhGDF-5 have been evaluated in independent- and industry-sponsored preclinical and clinical studies focused on craniofacial indications. Whereas rhBMP-2 is the only approved BMP

for craniofacial use, other members of the BMP family show clinical relevance and should be pursued. Clinically relevant bone augmentation for inlay defects including extraction sockets and the maxillary sinus has been demonstrated for rhBMP-2; however, dose optimization remains poorly understood. For onlay defects, there is a clear need for the development of BMP carrier technologies with easy-to-handle characteristics, structural integrity, and that allow timely replacement by bone.

Acknowledgments Earlier versions of this text have been published for reviews in journals and book chapters. The text is continuously subject to revisions and updating as new information becomes available in our laboratory. Studies elaborated herein conducted in our laboratories were supported by W.L. Gore & Associates, Genetics Institute, Wyeth Research, Medtronic, Daewoong Pharmaceuticals, and Nobel Biocare.

References

1. Al-Nawas B, Schiegnitz E (2014) Augmentation procedures using bone substitute materials or autogenous bone – a systematic review and meta-analysis. *Eur J Oral Implantol* 7(Suppl 2): S219–S234
2. Andersson L (2008) Patient self-evaluation of intra-oral bone grafting treatment to the maxillary frontal region. *Dent Traumatol* 24:164–169
3. Barboza EP, Leite Duarte ME, Geolás L, Sorensen RG, Riedel GE, Wikesjö UME (2000) Ridge augmentation following implantation of recombinant human bone morphogenetic protein-2 in the dog. *J Periodontol* 71:488–496
4. Barboza E, Caúla AL, Caúla F, Oliveira de Souza R, Neto LG, Sorensen RG, Li XJ, Wikesjö UME (2004) Effect of recombinant human bone morphogenetic protein-2 in an absorbable collagen sponge with space-providing biomaterials on the augmentation of chronic alveolar ridge defects. *J Periodontol* 75:702–708
5. Bishop GB, Einhorn TA (2007) Current and future clinical applications of bone morphogenetic proteins in orthopaedic trauma surgery. *Int Orthop* 31:721–727
6. Boyne PJ, James RA (1980) Grafting of the maxillary sinus floor with autogenous marrow and bone. *J Oral Surg* 38:613–616
7. Boyne PJ, Marx RE, Nevins M, Triplett G, Lazaro E, Lilly LC, Alder M, Nummikoski P (1997) A feasibility study evaluating rhBMP-2/absorbable collagen sponge for maxillary sinus floor augmentation. *Int J Periodontics Restorative Dent* 17:11–25
8. Boyne PJ, Lilly LC, Marx RE, Moy PK, Nevins M, Spagnoli DB, Triplett RG (2005) *De novo* bone induction by recombinant human bone morphogenetic protein-2 (rhBMP-2) in maxillary sinus floor augmentation. *J Oral Maxillofac Surg* 63:1693–1707
9. Caplanis N, Sigurdsson TJ, Rohrer MD, Wikesjö UME (1997) Effect of allogeneic, freeze-dried, demineralized bone matrix on guided bone regeneration in supraalveolar peri-implant defects in dogs. *Int J Oral Maxillofac Implants* 12:634–642
10. Celeste AJ, Iannazzi JA, Taylor RC, Hewick RM, Rosen V, Wang EA, Wozney JM (1990) Identification of transforming growth factor-ß family members present in bone-inductive protein purified from bovine bone. *Proc Natl Acad Sci USA* 87:9843–9847
11. Chiapasco M, Zaniboni M (2011) Failures in jaw reconstructive surgery with autogenous onlay bone grafts for pre-implant purposes: incidence, prevention and management of complications. *Oral Maxillofac Surg Clin North Am* 23:1–15
12. Cochran DL, Nummikoski PV, Jones AA, Makins SR, Turek TJ, Buser D (1997) Radiographic analysis of regenerated bone around endosseous implants in the canine using recombinant human bone morphogenetic protein-2. *Int J Oral Maxillofac Implants* 12:739–748

13. Cochran DL, Schenk R, Buser D, Wozney JM, Jones AA (1999) Recombinant human bone morphogenetic protein-2 stimulation of bone formation around endosseous dental implants. *J Periodontol* 70:139–150
14. Coomes AM, Mealey BL, Huynh-Ba G, Barboza-Arguello C, Moore WS, Cochran DL (2014) Buccal bone formation after flapless extraction: a randomized, controlled clinical trial comparing recombinant human bone morphogenetic protein 2/absorbable collagen carrier and collagen sponge alone. *J Periodontol* 85:525–535
15. de Freitas RM, Susin C, Spin-Neto R, Marcantonio C, Wikesjö UME, Violin Dias Pereira LA, Marcantonio E Jr (2013) Horizontal ridge augmentation of the atrophic anterior maxilla using rhBMP-2/ACS or autogenous bone grafts: a proof-of-concept randomized clinical trial. *J Clin Periodontol* 40:968–975
16. de Freitas RM, Spin-Neto R, Marcantonio E Jr, Pereira LAVD, Wikesjö UME, Susin C (2015) Alveolar ridge and maxillary sinus augmentation using rhBMP-2: a systematic review. *Clin Implant Dent Relat Res* 17(Suppl 1):e192–e201
17. Derks J, Tomasi C (2015) Peri-implant health and disease. A systematic review of current epidemiology. *J Clin Periodontol* 42(Suppl 16):S158–S171
18. Esposito M, Grusovin MG, Felice P, Karatzopoulos G, Worthington HV, Coulthard P (2009) The efficacy of horizontal and vertical bone augmentation procedures for dental implants – a Cochrane systematic review. *Eur J Oral Implantol* 2:167–184
19. Esposito M, Grusovin MG, Rees J, Karasoulos D, Felice P, Alissa R, Worthington H, Coulthard P (2010) Effectiveness of sinus lift procedures for dental implant rehabilitation: a Cochrane systematic review. *Eur J Oral Implantol* 3:7–26
20. Fiorellini JP, Howell TH, Cochran D, Malmquist J, Lilly LC, Spagnoli D, Toljanic J, Jones A, Nevins M (2005) Randomized study evaluating recombinant human bone morphogenetic protein-2 for extraction socket augmentation. *J Periodontol* 76:605–613
21. Froum SJ, Wallace S, Cho SC, Khouly I, Rosenberg E, Corby P, Froum S, Bromage T, Schoor R, Norman R, Tarnow DP (2013) Histomorphometric comparison of different concentrations of recombinant human bone morphogenetic protein with allogeneic bone compared to the use of 100 % mineralized cancellous bone allograft in maxillary sinus grafting. *Int J Periodontics Restorative Dent* 33:721–730
22. Gruber RM, Ludwig A, Merten HA, Achilles M, Pöhling S, Schliephake H (2008) Sinus floor augmentation with recombinant human growth and differentiation factor-5 (rhGDF-5): a histological and histomorphometric study in the Göttingen miniature pig. *Clin Oral Implants Res* 19:522–529
23. Gruber RM, Ludwig A, Merten HA, Pippig S, Kramer FJ, Schliephake H (2009) Sinus floor augmentation with recombinant human growth and differentiation factor-5 (rhGDF-5): a pilot study in the Göttingen miniature pig comparing autogenous bone and rhGDF-5. *Clin Oral Implants Res* 20:175–182
24. Hanisch O, Tatakis DN, Rohrer MD, Wöhrle PS, Wozney JM, Wikesjö UME (1997) Bone formation and osseointegration stimulated by rhBMP-2 following subantral augmentation procedures in nonhuman primates. *Int J Oral Maxillofac Implants* 12:785–792
25. Hanisch O, Cortella CA, Boskovic MM, James RA, Slots J, Wikesjö UME (1997) Experimental peri-implant tissue breakdown around hydroxyapatite-coated implants. *J Periodontol* 68:59–66
26. Hanisch O, Tatakis DN, Boskovic M,M, Rohrer MD, Wikesjö UME (1997c) Bone formation and reosseointegration in peri-implantitis defects following surgical implantation of rhBMP-2. *Int J Oral Maxillofac Implants* 12:604–610
27. Heitz-Mayfield LJ, Mombelli A (2014) The therapy of peri-implantitis: a systematic review. *Int J Oral Maxillofac Implants* 29(Suppl):325–345
28. Herberg S, Siedler M, Pippig S, Schuetz A, Dony C, Kim C-K, Wikesjö UME (2008) Development of an injectable composite as a carrier for growth factor enhanced periodontal regeneration. *J Clin Periodontol* 3:976–984
29. Herberg S, Susin C, Pelaez M, Howie N, Moreno de Freitas R, Lee J, Johnson MH, Elsalanty ME, Hamrick MW, Isales CM, Wikesjö UME, Hill WD (2014) Low-dose bone morphogenetic

protein-2 / stromal cell-derived factor-1β co-therapy induces bone in critical-size rat calvarial defects. *Tissue Eng Part A* 20(9–10):1444–1453
30. Hötten G, Neidhardt H, Jacobowsky B, Pohl J (1994) Cloning and expression of recombinant human growth/differentiation factor 5. *Biochem Biophys Res Commun* 204:646–652
31. Hötten GC, Matsumoto T, Kimura M, Bechtold RF, Kron R, Ohara T, Tanaka H, Satoh Y, Okazaki M, Shrai T, Pan H, Kawai S, Pohl JS, Kudo A (1996) Recombinant human growth/differentiation factor 5 stimulates mesenchyme aggregation and chondrogenesis responsible for the skeletal development of limbs. *Growth Factors* 13:65–74
32. Hsu WK, Wang JC (2008) The use of bone morphogenetic protein in spine fusion. *Spine J* 8:419–425
33. Hunt DR, Jovanovic SA, Wikesjö UME, Wozney JM, Bernard GW (2001) Hyaluronan supports recombinant human bone morphogenetic protein-2 induced bone reconstruction of advanced alveolar ridge defects in dogs. *J Periodontol* 72:651–658
34. Jones AA, Buser D, Schenk R, Wozney JM, Cochran DL (2006) The effect of rhBMP-2 around endosseous implants with and without membranes in the canine model. *J Periodontol* 77:1184–1193
35. Jovanovic SA, Hunt DR, Bernard GW, Spiekermann H, Nishimura R, Wozney JM, Wikesjö UME (2003) Long-term functional loading of dental implants in rhBMP-2 induced bone. A histologic study in the canine ridge augmentation model. *Clin Oral Implants Res* 14:793–803
36. Jovanovic SA, Hunt DR, Bernard GW, Spiekermann H, Wozney JM, Wikesjö UME (2007) Bone reconstruction following implantation of rhBMP-2 and guided bone regeneration in canine alveolar ridge defects. *Clin Oral Implants Res* 18:224–230
37. Kao DW, Kubota A, Nevins M, Fiorellini JP (2012) The negative effect of combining rhBMP-2 and Bio-Oss on bone formation for maxillary sinus augmentation. *Int J Periodontics Restorative Dent* 32:61–67
38. Koch FP, Becker J, Terheyden H, Capsius B, Wagner W (2010) A prospective, randomized pilot study on the safety and efficacy of recombinant human growth and differentiation factor-5 coated onto ß-tricalcium phosphate for sinus lift augmentation. *Clin Oral Implants Res* 21:1301–1308
39. Lacroix P (1945) Recent investigations on the growth of bone. *Nature* 156:576
40. Lee J, Wikesjö UME (2014) Growth/differentiation factor-5: pre-clinical and clinical evaluations of periodontal regeneration and alveolar augmentation - Review. *J Clin Periodontol* 41:797–805
41. Lee J, Lee EN, Yoon J, Chung S-M, Prasad H, Susin C, Wikesjö UME (2013) Comparative study of Chinese Hamster Ovary cell- versus *Escherichia coli*-derived bone morphogenetic protein-2 using the critical-size supraalveolar peri-implant defect model. *J Periodontol* 84:415–422
42. Lee J, Susin C, Rodriguez NA, de Stefano J, Prasad HS, Buxton AN, Wikesjö UME (2013) Sinus augmentation using rhBMP-2/ACS in a mini-pig model: Relative efficacy of autogenous fresh particulate iliac bone grafts. *Clin Oral Implants Res* 24:497–504
43. Leknes KN, Yang J, Qahash M, Polimeni G, Susin C, Wikesjö UME (2008) Alveolar ridge augmentation using implants coated with recombinant human bone morphogenetic protein-2 (rhBMP-2): radiographic observations. *Clin Oral Implants Res* 19:1027–1033
44. Leknes KN, Yang J, Qahash M, Polimeni G, Susin C, Wikesjö UME (2008) Alveolar ridge augmentation using implants coated with recombinant human bone morphogenetic protein-7 (rhBMP-7/rhOP-1): radiographic observations. *J Clin Periodontol* 35:914–919
45. Leknes KN, Yang J, Qahash M, Polimeni G, Susin C, Wikesjö UME (2013) Alveolar ridge augmentation using implants coated with recombinant human growth/differentiation factor −5 (rhGDF-5): radiographic observations. *Clin Oral Implants Res* 24:1185–1191
46. Levander G (1938) A study of bone regeneration. *J Sur Gynecol Obstet* 67:705–714
47. Lu SX, Fiorini T, Lee J, Prasad HS, Buxton AN, Bisch FC, Dixon DR, Susin C, Wikesjö UME (2013) Evaluation of a compression resistant carrier for recombinant human bone morphogenetic protein-2. *J Clin Periodontol* 40:688–697

48. McKay WF, Peckham SM, Badura JM (2007) A comprehensive clinical review of recombinant human bone morphogenetic protein-2 (INFUSE® Bone Graft). *Int Orthop* 31:729–734
49. Miranda DAO, Blumenthal NM, Sorensen RG, Wozney JM, Wikesjö UME (2005) Evaluation of recombinant human bone morphogenetic protein-2 on the repair of alveolar ridge defects in baboons. *J Periodontol* 76:210–220
50. Mombelli A, Müller N, Cionca N (2012) The epidemiology of peri-implantitis. *Clin Oral Implants Res* 23(Suppl 6):67–76
51. Nkenke E, Neukam FW (2014) Autogenous bone harvesting and grafting in advanced jaw resorption: morbidity, resorption and implant survival. *Eur J Oral Implantol* 7(Suppl 2): S203–S217
52. Orell, S. (1934) Studien über Knochenimplantation und Knochenneubildung, Implantation von 'Os Purum' sowie Transplantation von 'Os Novum.' *Acta Chirurgica Scandinavica* 74 Supplement 31.
53. Özkaynak E, Rueger DC, Drier EA, Corbett C, Ridge RJ, Sampath TK, Oppermann H (1990) OP-1 cDNA encodes an osteogenic protein in the TGF-ß family. *EMBO J* 9:2085–2093
54. Pelaez M, Susin C, Lee J, Fiorini T, Bisch FC, Dixon DR, McPherson JC III, Buxton AM, Wikesjö UME (2014) Effect of rhBMP-2 dose on bone formation/maturation in a rat critical-size calvarial defect model. *J Clin Periodontol* 41:827–836
55. Persson LG, Berglundh T, Lindhe J, Sennerby L (2001) Re-osseointegration after treatment of peri-implantitis at different implant surfaces. An experimental study in the dog. *Clin Oral Implants Res* 12:595–603
56. Pjetursson BE, Tan WC, Zwahlen M, Lang NP (2008) A systematic review of the success of sinus floor elevation and survival of implants inserted in combination with sinus floor elevation. *J Clin Periodontol* 35(8 Suppl):216–240
57. Pöhling S, Pippig SD, Hellerbrand K, Siedler M, Schütz A, Dony C (2006) Superior effect of MD05, beta-tricalcium phosphate coated with recombinant human growth/differentiation factor-5, compared to conventional bone substitutes in the rat calvarial defect model. *J Periodontol* 77:1582–1590
58. Polimeni G, Wikesjö UME, Susin C, Qahash M, Shanaman RH, Prasad H, Rohrer MD, Hall J (2010) Alveolar ridge augmentation using implants coated with recombinant human growth/differentiation factor-5: histologic observations. *J Clin Periodontol* 37:759–768
59. Roldán JC, Jepsen S, Schmidt C, Knüppel H, Rueger DC, Açil Y, Terheyden H (2004) Sinus floor augmentation with simultaneous placement of dental implants in the presence of platelet-rich plasma or recombinant human bone morphogenetic protein-7. *Clin Oral Implants Res* 15:716–723
60. Sampath TK, Maliakal JC, Hauschka PV, Jones WK, Sasak H, Tucker RF, White KH, Coughlin JE, Tucker MM, Pang RH, Corbett C, Özkaynak E, Oppermann H, Rueger D (1992) Recombinant human osteogenic protein-1 (hOP-1) induces new bone formation in vivo with a specific activity comparable with natural bovine osteogenic protein and stimulates osteoblast proliferation and differentiation in vitro. *J Biol Chem* 267:20352–20362
61. Sanz-Sánchez I, Ortiz-Vigón A, Sanz-Martín I, Figuero E, Sanz M (2015) Effectiveness of lateral bone augmentation on the alveolar crest dimension: a systematic review and meta-analysis. *J Dent Res* 94(9 Suppl):128S–142S
62. Sigurdsson TJ, Nygaard L, Tatakis DN, Fu E, Turek TJ, Jin L, Wozney JM, Wikesjö UME (1996) Periodontal repair in dogs: Evaluation of rhBMP-2 carriers. *Int J Periodontics Restorative Dent* 16:525–537
63. Sigurdsson TJ, Fu E, Tatakis DN, Rohrer MD, Wikesjö UME (1997) Bone morphogenetic protein-2 for peri-implant bone regeneration and osseointegration. *Clin Oral Implants Res* 8:367–374
64. Stavropoulos A, Becker J, Capsius B, Açil Y, Wagner W, Terheyden H (2011) Histological evaluation of maxillary sinus floor augmentation with recombinant human growth and differentiation factor-5-coated ß-tricalcium phosphate: results of a multicenter randomized clinical trial. *J Clin Periodontol* 38:966–974
65. Summers RB (1994) The osteotome technique: Part 3–Less invasive methods of elevating the sinus floor. *Compend Contin Educ Dent* 15:698–704

66. Susin C, Qahash M, Polimeni G, Lu PH, Prasad H, Rohrer MD, Hall J, Wikesjö UME (2010) Alveolar ridge augmentation using implants coated with recombinant human bone morphogenetic protein-7 (rhBMP-7/rhOP-1): histological observations. *J Clin Periodontol* 37:574–581
67. Tan WC, Lang NP, Zwahlen M, Pjetursson BE (2008) A systematic review of the success of sinus floor elevation and survival of implants inserted in combination with sinus floor elevation. Part II: transalveolar technique. *J Clin Periodontol* 35(8 Suppl):241–254
68. Tatakis DN, Koh A, Jin L, Wozney JM, Rohrer MD, Wikesjö UME (2002) Peri-implant bone regeneration using rhBMP-2/ACS in a canine model: a dose-response study. *J Periodontal Res* 37:93–100
69. Terheyden H, Jepsen S, Möller B, Tucker MM, Rueger DC (1999) Sinus floor augmentation with simultaneous placement of dental implants using a combination of deproteinized bone xenografts and recombinant human osteogenic protein-1. A histometric study in miniature pigs. *Clin Oral Implants Res* 10:510–521
70. Triplett RG, Nevins M, Marx RE, Spagnoli DB, Oates TW, Moy PK, Boyne PJ (2009) Pivotal, randomized, parallel evaluation of recombinant human bone morphogenetic protein-2/absorbable collagen sponge and autogenous bone graft for maxillary sinus floor augmentation. *J Oral Maxillofac Surg* 67:1947–1960
71. Urist MR (1965) Bone: formation by autoinduction. *Science* 150:893–899
72. Wang EA, Rosen V, D'Alessandro JS, Bauduy M, Cordes P, Harada T, Israel DI, Hewick RM, Kerns KM, LaPan P, Luxenburg DP, McQuaid D, Moutsatsos IK, Nove J, Wozney JM (1990) Recombinant human bone morphogenetic protein induces bone formation. *Proc Natl Acad Sci USA* 87:2220–2224
73. Weng D, Pöhling S, Pippig S, Bell M, Richter EJ, Zuhr O, Hürzeler MB (2009) The effects of recombinant human growth/differentiation factor-5 (rhGDF-5) on bone regeneration around titanium dental implants in barrier membrane-protected defects: a pilot study in the mandible of beagle dogs. *Int J Oral Maxillofac Implants* 24:31–37
74. Wikesjö UME, Qahash M, Thomson RC, Cook AD, Rohrer MD, Wozney JM, Hardwick WR (2003) Space-providing expanded polytetrafluoroethylene devices define alveolar augmentation at dental implants induced by recombinant human bone morphogenetic protein 2 in an absorbable collagen sponge carrier. *Clin Implant Dent Relat Res* 5:112–123
75. Wikesjö UME, Qahash M, Thomson RC, Cook AD, Rohrer MD, Wozney JM, Hardwick WR (2004) rhBMP-2 significantly enhances guided bone regeneration. *Clin Oral Implants Res* 15:194–204
76. Wikesjö UME, Susin C, Qahash M, Polimeni G, Leknes KN, Shanaman RH, Prasad HS, Rohrer MD, Hall J (2006) The critical-size supraalveolar peri-implant defect model: characteristics and use. *J Clin Periodontol* 33:846–854
77. Wikesjö UME, Qahash M, Polimeni G, Susin C, Shanaman RH, Rohrer MD, Wozney JM, Hall J (2008) Alveolar ridge augmentation using implants coated with recombinant human bone morphogenetic protein-2: histologic observations. *J Clin Periodontol* 35:1001–1010
78. Wikesjö UME, Qahash M, Huang Y-H, Xiropaidis AV, Polimeni G, Susin C (2009) Bone morphogenetic proteins for periodontal and alveolar indications; biological observations – clinical implications. *Orthod Craniofac Res* 12:263–270
79. Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Whitters MJ, Kriz RW, Hewick RM, Wang EA (1988) Novel regulators of bone formation: molecular clones and activities. *Science* 242:1528–1534

Bone Morphogenetic Protein-7 and Its Role in Acute Kidney Injury and Chronic Kidney Failure

Kuber T. Sampath, Lovorka Grgurevic, and Slobodan Vukicevic

Abstract Bone morphogenetic protein (BMP)-7 is required for embryonic kidney development, plays a functional role in the adult kidney as renal hormone for vascular and skeletal integrity, and modulates calcium and phosphate homeostasis. Preclinical studies have shown that systemic administration of recombinant BMP-7 provides tissue protection in models of acute kidney injury (AKI), glomerulosclerosis, diabetic nephropathy, chronic kidney disease (CKD), renal osteodystrophy, lupus nephropathy, and Alport's syndrome. The molecular mechanism of BMP-7 actions has been attributed to its role in suppression on inflammation, improvement of renal blood flow, preservation of tubular structure, reduction of interstitial fibrosis, maintenance of vascular smooth muscle cell (SMC) function, and reduction of serum phosphate and subsequently vascular calcification by improving disordered bone remodeling. As BMP-7 is a potent bone-inducing morphogenic protein and forms ectopic ossification at the injection sites, it presents with safety concerns as a viable therapy for repeated chronic administration. Approaches are therefore being attempted to enhance BMP-7 signaling by peptide mimetics designed based on crystal structure of BMP-7, by endogenous "active BMP-7 protein" pool in the kidney by preventing its interaction with specific anti-BMP-7 antagonists, and via secretagogues.

Keywords BMP-7 in kidney development • BMP-7 as a renal hormone • BMP-7 in acute and chronic kidney diseases • BMP-7 in rare renal disorders • Focal segmental glomerular-sclerosis • Alports syndrome • Polycystic kidney diseases and lupus nephritis • BMP-7 in diabetic nephropathy • BMP-7 in calcium and phosphate homeostasis • BMP-7 antagonist • USAG1 • BMP-7 mimetics

T.K. Sampath (✉)
perForm biologics Inc., Holliston, MA 01746, USA
e-mail: kuber.sampath@performbiologics.com; kuber.sampath@gmail.com

L. Grgurevic • S. Vukicevic
Center for Translational and Clinical Research, Laboratory for Mineralized Tissues, University of Zagreb School of Medicine, Salata 11, Zagreb 10000, Croatia
e-mail: vukicev@mef.hr

S. Vukicevic, K.T. Sampath (eds.), *Bone Morphogenetic Proteins: Systems Biology Regulators*, Progress in Inflammation Research,
DOI 10.1007/978-3-319-47507-3_12

1 Introduction

Bone morphogenetic proteins (BMPs) induce new bone when implanted with a collagenous substratum at ectopic non-bony sites [52, 53] and are members of TGF-β superfamily. BMPs are involved in the developmental process of many organs during embryogenesis [23, 26] and play a morphogenic role during tissue repair and protection in post-fetal life [5, 68]. The expression of BMP proteins in tissues other than bone and the induction of new bone by *Drosophila* BMP orthologs (*dpp* and *60A*) when implanted in rats [51] suggest the formation of new bone is dictated by the responding cell than the signal. Thus, BMP-induced new bone formation provides a prototype for tissue engineering and demonstrates the biological principles of regenerative medicine.

The highly purified BMP from bovine bone has been shown to compose of homodimers of BMP-2 and BMP-7 (OP-1). Though BMP-7 was purified from bone matrix, a high level of its expression was found in the kidney and shown to be available in circulation albeit at very low concentration. Systemically administered BMP-7/6 hybrid molecule is secreted into the urine and that its biological activity is preserved, suggesting that analysis of BMP in urine might reflect its presence in serum [21]. However, the native form of BMP-7 made in the kidney and available in circulation is currently unknown. BMP-7 exerts its function by binding to a specific Ser/Thr kinase receptor complex composed of one type I receptor (e.g., ALK-2, ALK-3, and ALK-6) and one type II receptor (e.g., BMPR-II, ActRII-A, and ActRII-B) and subsequently induces phosphorylation of SMAD-1/5/8 [62]. With engagement of co-SMAD-4, the P-SMAD-1/5/8 complex is then translocated into the nucleus and switches on/off of a set of genes that are involved in tissue protection, repair, and regeneration. The binding of a BMP to its receptor complex is tightly controlled at extracellular milieu by its interaction with anti-BMPs (e.g., follistatin, sclerostin, twisted gastrulation, gremlin, and USAG-1/Wise) and downstream intracellular signaling via interaction of P-SMAD-1/5/8 with anti-SMAD-6/7 and subsequently by ubiquitination through smurf1 and E2/E3 ubiquitine ligases [33].

2 BMP-7 and Embryonic Kidney Development

Ozkaynak and Oppermann showed for the first time that BMP-7 (OP-1) was expressed at high levels in the kidney obtained from 17-day embryo and 2-week-old mouse by Northern blot analysis by using mouse-specific BMP-7 probe (Fig. 1) [49]. The high level expression of BMP-7 was further confirmed in rat embryonic kidney and in human fetal kidney [23, 66] and found to be localized in basement membranes underlying the epithelium and convoluted tubules of developing kidneys and in the epithelium of the branching ureteric buds.

Two groups independently generated BMP-7-deficient mice and showed that mice that lack BMP-7 die shortly after birth because of poor kidney development. One group [45] showed that metanephric mesenchyme has failed to differentiate, resulting in a virtual absence of glomerulus in newborn kidneys. Besides, they showed BMP-7 (−/−) mice lack the expression of molecular markers of nephrogenesis, such as *Pax-2* and *Wnt-4* between 12.5- and 14.5-day postcoitum. The other group [14] suggested the

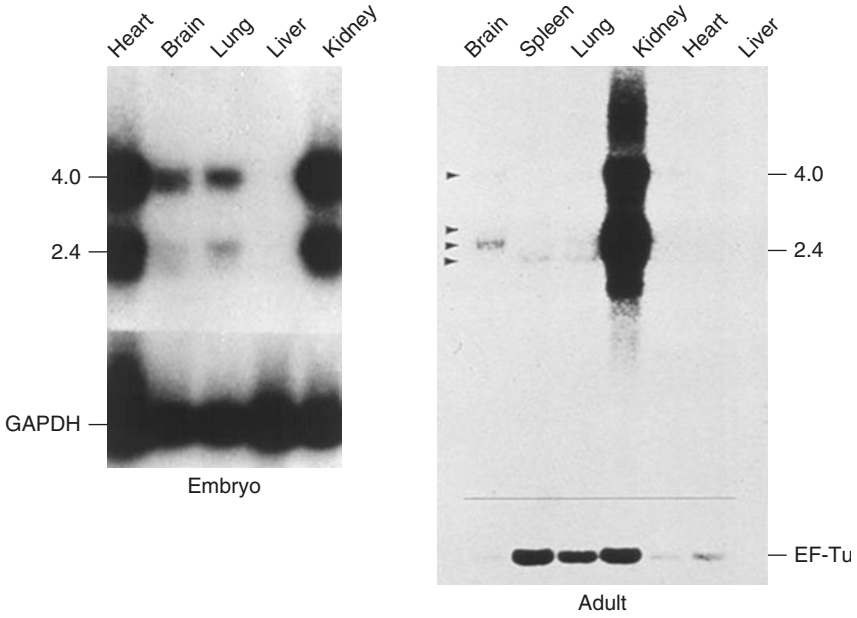


Fig. 1 Northern blot analysis of BMP-7 expression in different organs of embryo and adult mice. BMP-7 is expressed in several tissues associated with inductive interactions and is required for proper nephrogenesis. Maximal levels of BMP-7 mRNA were found in the kidney of a 17-day embryo (From Helder et al. [23]) and 2-week-old mice (From Ozkaynak et al. [49]) as the main site of BMP-7 synthesis

Fig. 2 Morphological analysis of *Bmp7* knockout mice. Rapid disappearance of the metanephric mesenchyme resulted in loss of kidney mass upon birth (right-atrophied kidney at day 19 of gestation; From Dudley et al. [14])

early inductive tissue interactions responsible for establishing nephrogenesis appeared largely unaffected, but subsequent cellular interactions required for their continued renal growth and development were affected; consequently, homozygous mutant animals exhibit a renal dysplasia at birth (Fig. 2). The apparent discrepancy observed by these two groups may likely be explained by genetic background of the BMP-7 (−/−)

mice. In a subsequent study, Vukicevic S et al. [67] showed unequivocally that BMP-7 produced in ureteric bud is required for nephrogenic mesenchymal condensation and differentiation during glomerulogenesis and further epithelization. Overall, these findings identified that BMP-7 is required for mammalian kidney development and suggests that it may have a functional role in the adult kidney [57].

3 Role of BMP-7 in Acute Kidney Injury

Bone morphogenic protein-7 has been demonstrated to provide cytoprotection, reduce inflammation and macrophage infiltration, and minimize tissue damage and improve kidney function in animal models of acute kidney injury (AKI) [69]. Acute kidney injury (AKI) is an important clinical syndrome and a global public health issue with high mortality rate and socioeconomic burden due to lack of effective therapy. AKI occurs as a result of a sudden loss of renal blood flow following ischemia and reperfusion injury associated with critical care medicine conditions and renal transplant or tubular necrosis associated with diagnostic use of radiocontrast agents in patients with compromised renal function or ureteral obstruction or sepsis associated with multi-organ failure. AKI results in acute cell death and necrosis of renal tubule epithelial cells accompanied with leakage of tubular fluid and inflammation [13, 63]. The target cell type in AKI is proximal tubule epithelial cell (PTEC), which is responsible for the production of chemokines and cytokines that signal the inflammatory response, migration of macrophages, resulting in transient loss of basement membrane and expression of epithelial phenotype and reduced glomerular filtration rate [16, 42]. In most part, PTECs have a capacity to repair and regenerate and attain full function following AKI, but this recovery is dependent on the degree and type of insult and healthy status of the kidney. A slow and abnormal repair following AKI in compromised renal function can lead to kidney fibrosis and pose a greater risk to the progression of CKD [6].

In an animal model of ischemia (60 min warm) and post-reperfusion injury, sepsis, radiocontrast agent-induced tubular necrosis, BMP-7 expression, and its downstream signaling were found to be reduced severalfold in the kidney [2, 58]. Systemic administration of BMP-7 protein in respective AKI models demonstrated to have suppressed inflammation, minimized tubular necrosis (Fig. 3b) and tissue infarction, regained the expression of epithelial phenotype, reduced the programmed cell death, and restored renal function (Fig. 3a) [47, 69, 75]. While there is little or no detectable expression of BMP-7 in PTECs, replenishing with protein speeds up the repair and regenerative processes of PTECs as they express BMP receptors including type I (ALK-2, ALK-3, and ALK-6) and type II (ActRII-A, ActRII-B, and BMPR-II). Furthermore, BMP-7 is a survival factor to podocytes and exerts a positive influence on proximal tubular epithelium, mesangium, and vascular endothelium to maintain the glomerular structure. BMP-7 was also shown to reduce the

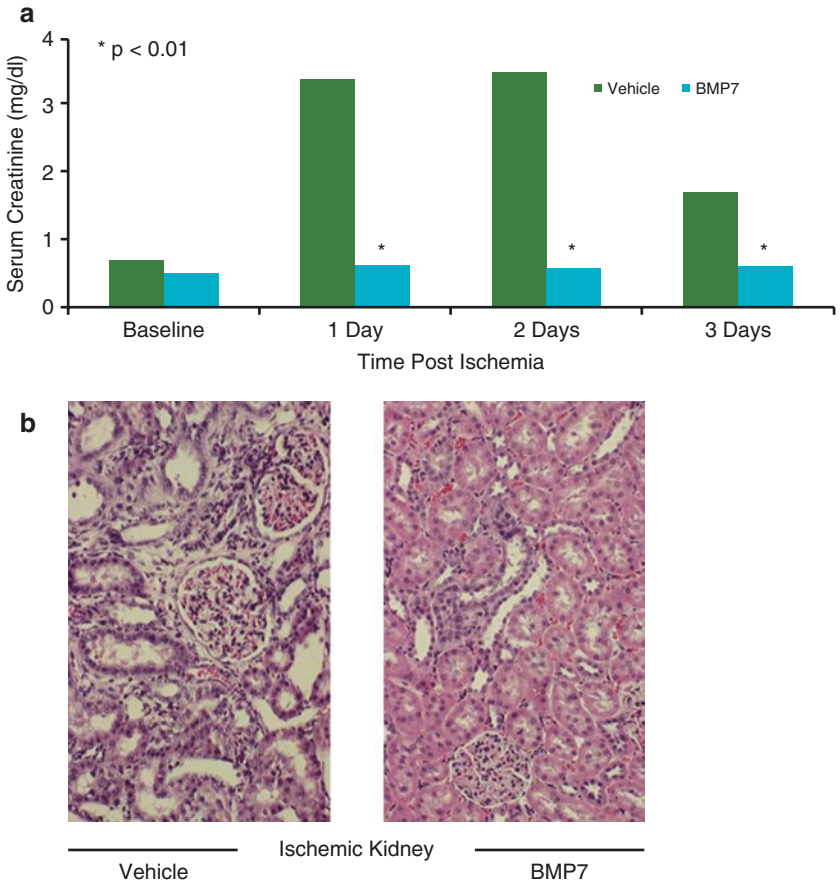


Fig. 3 Therapeutic effect of BMP-7 on serum creatinine values and kidney regeneration in the rat model of ischemic acute renal failure. (**a**) Serum creatinine levels in rats treated with vehicle and BMP-7 daily at 24-h intervals beginning 8 h following 60-min ischemic injury (data shown as mean ± SEM; $P < 0.01$). (**b**) Histological images of an ischemic rat kidney treated with vehicle or BMP-7. BMP-7 improves kidney function and maintains tubule phenotype in the clamping ischemia model (From Nguyen and Goldschmeding [47], Vukicevic et al. [69], and Xu et al. [75])

production of pro-inflammatory cytokines and chemokines and ICAM expression in PTECs (Fig. 4a) and to suppress the adherence of leukocytes and myeloperoxidase *in vivo* (Fig. 4b) [20]. The biological activity of BMP-7 in the kidney is tightly controlled. It is important to speed up the recovery process upon AKI injury; a delay in regeneration of proximal tubule epithelium can lead to tubulointerstitial fibrosis, a major event associated with the progression to chronic kidney failure and end-stage renal failure. As the regeneration of proximal epithelia occurs, expression of endogenous antagonists like gremlin, chordin-like proteins is also enhanced in order to titrate the action of BMP-7 [40].

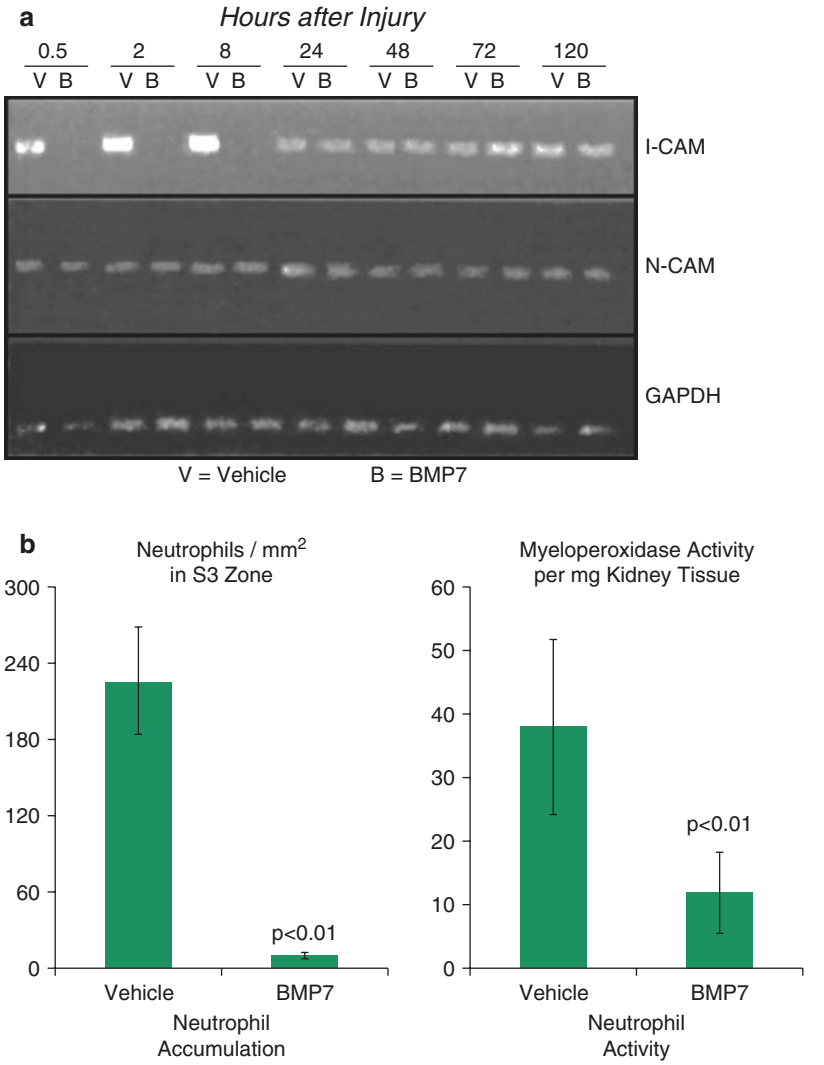


Fig. 4 BMP-7 attenuates expression of ICAM mRNA and suppresses inflammation after ischemic renal injury. (**a**) Intracellular adhesion molecule (ICAM) expression was reduced following BMP-7 treatment at 0.5, 2, and 8 h following injury. (**b**) Neutrophil accumulation and neutrophil activity, as measured by myeloperoxidase activity per μg of kidney tissue, are significantly decreased in animals treated with BMP-7 when compared to untreated rats in acute renal ischemia (From Vukicevic et al. [69])

4 BMP-7 and Chronic Kidney Disease

Chronic kidney disease (CKD) affects approximately one-seventh of adults above the age of 20 years. The recent discoveries of novel mechanisms underlying CKD progression opened the gate for more comprehensive understanding of the

pathophysiology of CKD progression and the development of new therapeutic strategies. The role of chemokines in the recruitment of inflammatory cells into the kidney of a variety underlying diseases has opened the gate for new promising therapeutic modalities [55].

In several preclinical models of chronic kidney diseases (CKDs), administration of BMP-7 has been shown to reduce glomerular sclerosis, maintain epithelial and endothelial phenotype and their integrity, minimize glomerular sclerosis and reverse tubulointerstitial fibrosis, and improve kidney function [29, 70]. BMP-7 exerts its positive influence against several pathological changes associated with CKD by (1) improving hemodynamic property of filtration, (2) reducing extracellular matrix synthesis and expansion of mesangium, (3) serving as survival factor for podocytes, (4) suppressing the production of inflammatory cytokines and chemokines, and (5) reversing TGF-beta-mediated epithelial-mesenchymal and endothelial-mesenchymal transition and interstitial mesenchyme into myofibroblast differentiation [20, 43, 79, 82].

4.1 Unilateral Urinary Obstruction

Unilateral ureteral obstruction (UUO) is a model of renal injury characterized by progressive tubulointerstitial fibrosis and renal damage, while relatively sparing the glomerulus and not producing hypertension (Fig. 5a) [38]. With administration of BMP-7 at the time of UUO and every other day thereafter, interstitial inflammation and fibrogenesis are prevented, leading to preservation of renal function during the first 5 days after obstruction [29]. Compared with angiotensin-converting enzyme inhibition with enalapril treatment, BMP-7 was more effective in preventing tubulointerstitial fibrosis and in preserving renal function (Fig. 5b). Approximately 50 % of the stimulation of this damage cascade, after UUO, is due to angiotensin II [15]. The mechanism of BMP-7-induced renal protection was associated with (1) prevention of tubular atrophy and (2) reduction in epithelial cell apoptosis produced by UUO by providing a survival signal to epithelial cells and preservation of renal blood flow (RBF) [69]. In a treatment protocol, when BMP-7 was administered 7 days after the release of UUO, it was found to significantly decrease the interstitial volume and tubule atrophy restoring GFR (Fig. 5c, d).

4.2 Glomerular Sclerosis

The glomerular apparatus is composed of Bowman's capsule and mesangium and capillaries located within them. The glomerulus is a spherical mass of specialized capillaries fed by an afferent arteriole and draining into an efferent arteriole. The glomerular filtration barrier (GFB), specialized to permit substantial filtration of water and solutes, is composed of three layers: glomerular endothelial cells, basement membrane (GBM), and podocytes, within Bowman's space. The capillaries

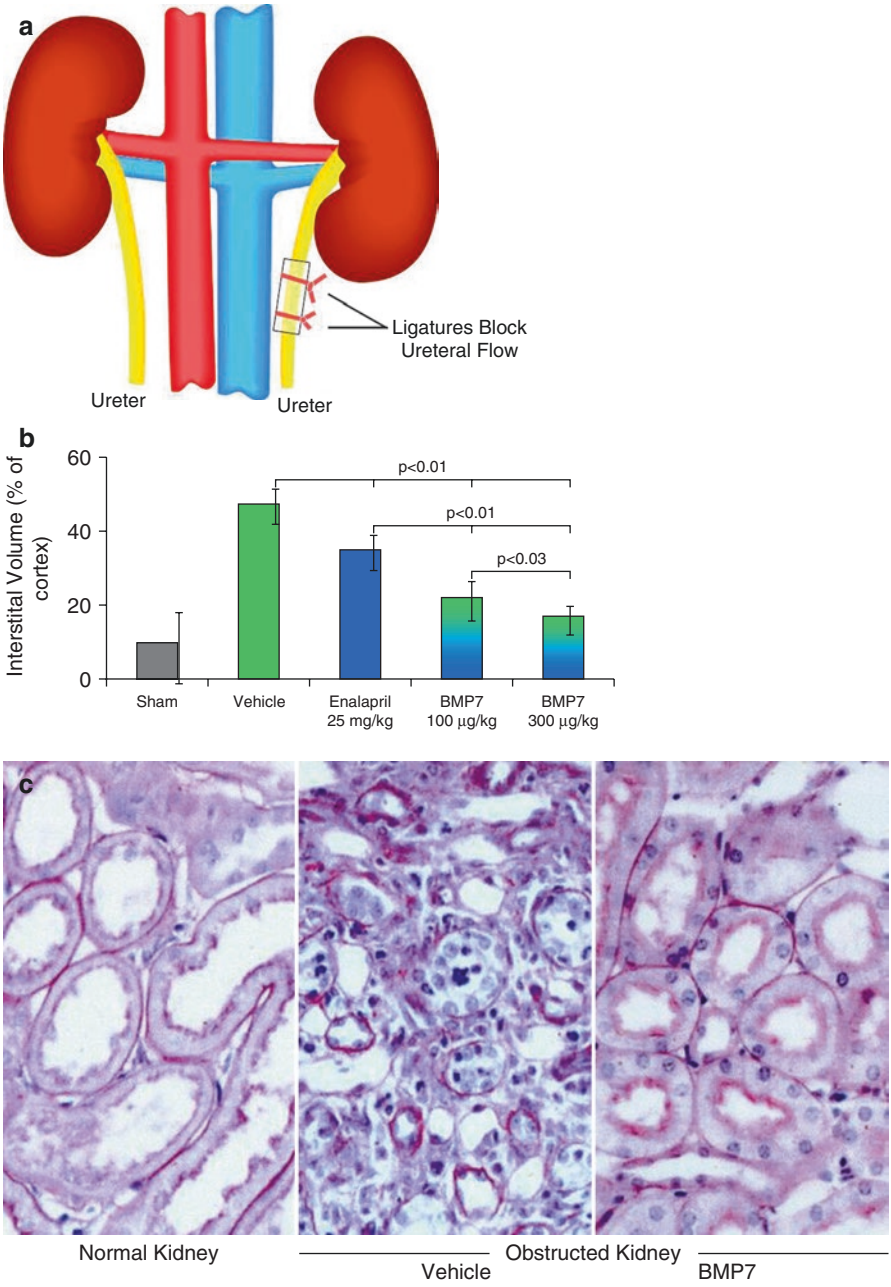


Fig. 5 Therapeutic effect of BMP-7 in unilateral ureteral obstruction (UUO) renal fibrosis rat model. (**a**) Rats were subjected to UUO, which was removed after 5 days. (**b**) Rats were subjected to i.v. application of enalapril (25 mg/kg), BMP-7 (100 or 300 µg/kg), or vehicle acetate buffer three times a week, and interstitial volume was measured at the end of the experiment. (**c**) Histological analysis of type IV collagen in normal rat kidney, rat kidney following UUO, and kidney following UUO treated with BMP-7. (**d**) BMP-7 inhibits renal fibrosis and maintains tubule phenotype and suppresses tubule atrophy in UUO model, while ACE inhibitors do not suppress tubule atrophy [29]

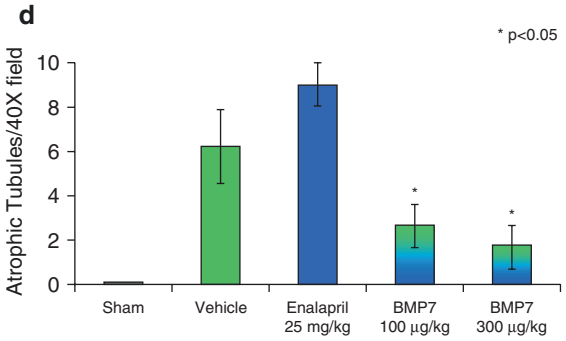


Fig. 5 (Continued)

are lined by a layer of cells (an endothelium) that has a unique structure that contains numerous fenestrae, allowing blood components to be filtered and resulting ultimately in the formation of urine. The glomerulus basement membrane is synthesized and secreted by endothelial cells that face outward from the capillary and podocytes that lined with folds of cytoplasm called foot processes or pedicles. These foot processes control the filtration of proteins from the capillary lumen into Bowman's space. They are not part of the filtration barrier but are specialized and participate indirectly in filtration by contracting and reducing the glomerular surface area and therefore filtration rate, in response mainly to stretch [37]. Angiotensin I and II and its receptors regulate the hemodynamic properties of renal capillary system. BMP-7 has been shown to influence positively the rheological properties of capillary upon change in systemic blood pressure in coordination with angiotensin-converting enzyme, ACE inhibitors, and AT receptor blockers. Mesangial cells are of monocyte or smooth muscle origin, typically covering 30 % of glomerular capillaries, responsible for filtration, structural support, and phagocytosis. Additionally, mesangial cells are able to monitor glucose levels via processes sent into the capillary lumen. Gremlin, a BMP antagonist, was observed to express abundantly in human diabetic nephropathy (DN); the expression was most prominent in areas of tubulointerstitial fibrosis, where it colocalized with TGF-beta expression [12, 39, 40]. There was a strong correlation between gremlin expression and tubulointerstitial fibrosis score. In an animal model of DN, administration of BMP-7 has been shown to reduce the production of extracellular matrix and expansion of mesangium in response to metabolic changes (e.g., high glucose) [72, 73].

5 BMP-7 and Rare Renal Disorders

Focal segmental glomerulosclerosis (FSGS) is a disease characterized by marked proteinuria and podocyte injury, largely due to alterations in structural genes of the podocyte [7]. Genetic risk alleles in apolipoprotein L1 are especially prevalent in

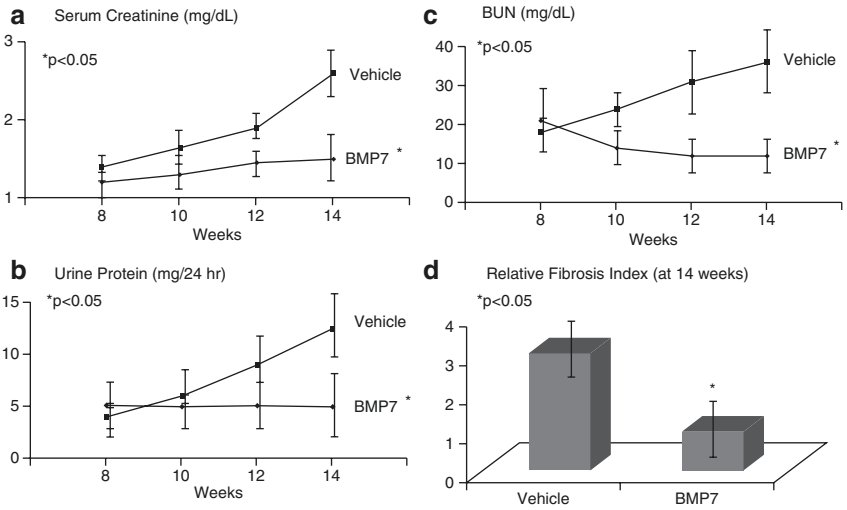


Fig. 6 Treatment of Col4A3$^{-/-}$ mice with BMP-7 results in decrease of renal disease. Col4A3$^{-/-}$ mice were treated with BMP-7 (300 μg/kg), and control mice were injected with vehicle buffer alone. Urine and blood were obtained every 2 weeks, and the study was terminated when animals were 14 weeks of age. (**a–c**) Renal function parameters: serum creatinine, blood urea nitrogen (BUN), and urine protein, $*P < 0.05$; (**d**) relative fibrosis index determined by morphometric analysis. Average values of each group are summarized. Treatment with BMP-7 (300 μg/kg) resulted in decreased relative interstitial volume and tubular atrophy [80]

African Americans and are linked not only to adult-onset FSGS but also to progression of chronic kidney diseases [18]. Infection, drug use, and secondary maladaptive responses after loss of nephrons from any cause may also cause FSGS. Biopsies from patients with FSGS exhibited an increased activation of TGF-β signaling and mitochondrial oxidative stress, which is associated with dysfunction in adjacent endothelial cells leading to podocyte apoptosis and mitochondrial DNA damage. Antagonizing TGF-beta activity using anti-TGF-beta antibody or TGF-beta type I receptor kinase inhibitors has been shown to reduce proteinuria and minimize damage to podocytes in preclinical models of glomerulosclerosis [3] and in FSGS African American patients [65]. Since BMP-7 is capable of overcoming TGF-beta-mediated epithelium- and endothelium-mesenchyme transition, extracellular matrix expansion and serves as a survival factor for podocytes; it remains to be seen whether administration of BMP-7 or enhancing endogenous BMP-7 downstream signaling could provide therapeutic benefits against proteinuria and podocyte loss associated with FSGS. New insights into glomerular cell injury response and repair may pave the way for possible therapeutic strategies.

Alport's syndrome is a progressive hereditary kidney disease associated with sensorineural deafness, caused by mutations in any one of genes encoding the α3, α4, and α5 chains of type IV collagen (*COL4A3*, *COL4A4*, and *COL4A5*), the major component of glomerular basement membrane (GBM) [34, 83]. Alport's syndrome (AS) occurs one in ~5000 people and is the more prevalent of known genetic

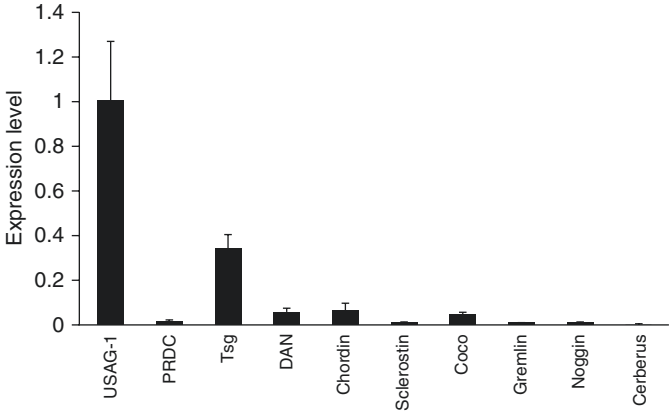


Fig. 7 Expression of uterine sensitization-associated gene-1 (USAG-1) in the kidney. USAG-1 which is predominately expressed in sensitized endometrium of the rat uterus has been shown to be abundantly expressed in the kidney and acts as a BMP antagonist [77]

disorders that affects predominantly male. Due to mutations in α3/α4/α5 (IV) collagen network, the GBM in AS retains the fetal α1/α1/α2 (IV) collagen network, which confers an increased susceptibility to proteolytic enzyme, leading to progressive destruction of the GBM with subsequent hematuria and proteinuria, glomerulosclerosis, and ultimately end-stage renal disease [36]. Endogenous BMP-7 expression and subsequently its downstream signaling (SMAD-1/5 phosphorylation) were found to be reduced significantly with enhanced epithelial-mesenchymal transition and myofibroblast fibrosis in AS kidney. Administration of BMP-7 in a therapeutic mode was shown to repair the damaged renal tubules, preserve renal function, and improve mortality in the Col4A3 knockout mice model of AS (Fig. 6a–d). BMP-7 was able to restore the epithelial phenotype and its polarity and reduced the interstitial fibrosis [30, 31, 46, 69, 80, 81]. While the exact role of BMP-7 and its mechanism of action remain unclear, BMP-7 was shown to inhibit the release of pro-inflammatory cytokines and chemokines by minimizing inflammation and reversing epithelial-to-mesenchymal transition by acting as an antagonist of TGF-β1 as it is shown to induce E-cadherin [79]. The upregulation of MMP-2 by BMP-7 as demonstrated in AS mice may also increase ECM degradation and potentially decreasing the amount scar tissue formed in the renal interstitium. The product of uterine sensitization-associated gene-1 (USAG-1), a kidney-specific BMP antagonist, is expressed and colocalized with BMP-7 in distal convoluted tubules and acts as a regulator of BMP-7 action (Fig. 7) [77]. As expected, the USAG knockout mouse was shown to be resistant to tubular injury and to reduce interstitial fibrosis in AKI models, and double USAG-1/ Col4A3 knockout mice was able to reverse renal fibrosis associated with AS mice [61]. Because in adults the expression of USAG-1 is confined to the kidneys, targeting it with anti-USAG-1 antibody may likely to enhance the endogenous "BMP-7 pool" and yield a safer and more kidney-specific therapy than the administration of BMP-7.

Polycystic kidney disease (*PKD*) is one of the most common monogenic disorders, with a prevalence of 1:400 to 1:1000. It is genetically heterogeneous and has been linked to two loci, *PKD1* (*polycystin*, *PC1*) and *PKD2* (*PC2*), mutated in approximately 85 % and 15 % of cases, respectively [8, 35]. Typically, the disease manifests with progressive bilateral cystic kidney enlargement, leading to end-stage renal disease (ESRD) in midlife. Cyst development has been strongly associated with defects to the primary cilia including length abnormalities, categorizing PKD as a ciliopathy [17, 25]. In addition to primary cilia defects, PKD cells exhibit many other cellular aberrations including dedifferentiation of epithelium and loss of polarity, increased proliferation and apoptosis, and altered gene expression that may be linked to increased intracellular cAMP and calcium [76]. Recently, treatments focused on CDK inhibitors, lowering cAMP by targeting the arginine V2 vasopressin receptor (AVPR2), which is mainly expressed in the thick ascending limb of Henle and the collecting duct (CD) [24, 64]. Unfortunately, the currently available PKD1 rodent models are not ideal for the analysis of PKD pathogenesis or therapeutic testing. *Pkd1*-null animals die embryonically, and heterozygotes develop only very mild disease in old age, while conditional models do not reflect the disease development in human PKD due to the loss of all functional protein at one time [27, 74]. TGF-beta signaling pathway as observed by nuclear accumulation of P-SMAD-2 in cyst lining epithelial cells was enhanced at mild and more advanced stages of PKD mice and in human kidneys with progressive PKD [22]. Though BMP signaling has been affected in animal models of PKD, it remains to be seen whether exogenous BMP-7 signaling or endogenously upregulating BMP-7 downstream signaling could provide a therapeutic benefit to PKD patients.

Lupus nephritis (LN) is prevalent in Asians (55 %), Africans (51 %), and Hispanics (43 %) than Caucasians (14 %). About 25 % of LN patients end up in ESRD in 10 years. LN is associated with immune complexes (IC) primarily with antibody against double-stranded (ds) DNA and subsequently with antibody against Clq complement, histone, and nucleosome and autoimmune response to IgG. IC once deposited fail to undergo phagocytosis in glomerulus, which then results in injury to glomeruli, mesangium, and basement membrane endothelium and proximal tubule epithelium, which causes the recruitment of PMN and release of pro-inflammatory cytokines and stimulation of complements and chemokines and overexpression of ICAM/VCAM, induction of proteinases and growth factors (PDGF, TGF-beta, gremlin, a BMP antagonist), proliferation of mesangial and endothelial cells and then fibrosis, and induction of innate and adaptive immune responses [10]. LN is an unmet need and currently managed by steroid and immunosuppressive agents (cyclophosphamide, mycophenolate mofetil, and azathioprine). Directed target therapy to regulate T- and B-cells is also being pursued as an off-label use with a limited success. The etiology of LN insult is ill defined and it is difficult to select a uniform diseased population for a clinical study and the outcome of study is long. In mouse model of LN (MRL/MpJ$^{lpr-/lpr-}$) a decreased expression of tubular endogenous BMP-7 was shown to correlate to the progression of renal disease in injured kidneys. Administration of BMP-7 ameliorates progression of chronic renal disease in MRL/MpJlpr/lpr mice [80]. BMP-7-treated mice displayed reduced relative interstitial volume as well as a reduced number of atrophic tubular structures as compared

to untreated control mice. Animals that were treated with BMP-7 displayed reduced glomerular crescents, markedly reduced glomerulosclerosis, and reduced glomerular hypercellularity. These findings also correlated with reduced interstitial staining for type I collagen in the treated mice. However, localization for IgG in glomeruli did not show substantial difference between untreated and treated mice. In addition, an upregulation of MMP-2 was observed upon administration of BMP-7 in MRL/MpJlpr/lpr mice in interstitium, suggesting resolution of fibrotic tissue by activated myofibroblasts expressing α-smooth muscle actin [59].

6 BMP-7 and Diabetic Nephropathy

Diabetic nephropathy (DN) is a renal-vascular complication of hyperglycemia, and frequent cause of it is end-stage renal disease (ESRD). It is estimated that about 40 % of all diabetic patients worldwide, expected to have DN. In its early stages, diabetic nephropathy is primarily a glomerular disease, and podocyte injury is an important component. The effects of hyperglycemia include mesangium expansion and podocyte foot process effacement leading to detachment of their cell body from the glomerular basement membrane (GBM) [11]. Synaptopodin and podocin, two podocyte-specific genes, contemplated in rare nephritic proteinuric have also shown to correlate with DN [54]. An altered ratio of these two genes may be a useful marker to predict podocyte damage and reversible response. Reduced endogenous BMP-7 expression was observed with high glucose and profibrotic effects in streptozotocin-induced diabetic model [28, 72, 73]. Studies in diabetic animals with targeted (transgenic) expression of BMP-7 in glomerular podocytes suggested to have a protective role [71]. It was further shown that BMP-7 inhibits the TGF-β1-activated signaling pathway in mesangial cells and podocytes in vitro [1, 56]. A high level of TGF-β1 is locally produced by damaged podocytes and is implicated in the pathogenesis of glomerulosclerosis. In the BMP-7 transgene, BMP-7 prevents podocyte dropout and reduction of nephrin and restores podocin and synaptopodin, indicating that endogenous BMP-7 may be a podocyte survival factor. As BMP-7 is produced by podocytes, it may likely function as an autocrine podocyte survival factor and perhaps restore structural proteins of the foot processes such as synaptopodin and podocin. BMP-7 may be useful in delaying diabetic glomerulosclerosis and reversing early podocyte injury. In preclinical models of DN, BMP-7 was shown to attenuate tubular pro-inflammatory responses by suppressing oxidative stress and multiple inflammatory signaling pathways in mesangium and proximal tubular epithelium and advanced glycosylation end products and reducing interstitial fibrosis. In the diabetic BMP-7 treated rats, GFR was preserved and higher than diabetic enalapril-treated rats. Kidney weights were reduced and proteinuria was reversed to normal (Fig. 8a–b). Glomerular area and interstitial volume were significantly decreased. Glomerular sclerosis was prevented more effectively than by enalapril. Enalapril controlled hypertension throughout the course of therapy, while BMP-7 did not affect blood pressure until the final 4 weeks of therapy [72]. Diabetic vehicle-treated rats lost BMP-7 expression in the kidney. BMP-7 and enalapril therapy restored BMP-7 expression at high levels.

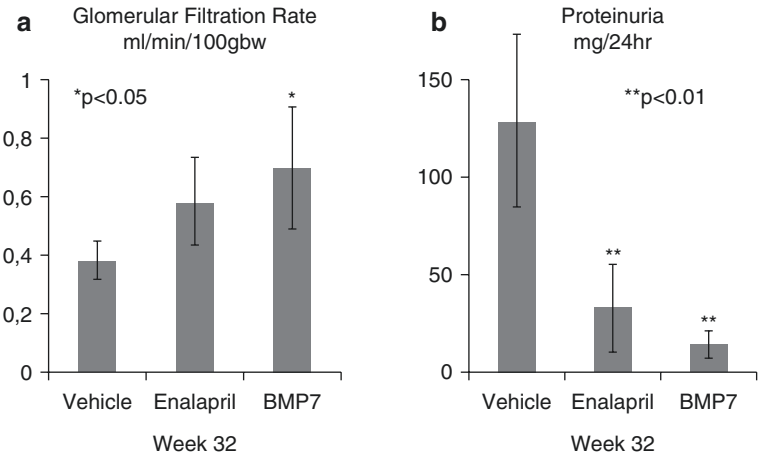


Fig. 8 Effects of BMP-7 and enalapril treatments in long-term streptozotocin (STZ)-induced model of diabetic nephropathy. (**a**) In diabetic rats BMP-7 and enalapril treatments restored GFR to normal or slightly above normal; (**b**) diabetic rats exhibited a pronounced increase in albumin excretion rate which was markedly reduced by BMP-7 and enalapril treatment.*$P < 0.05$ BMP-7 compared to 32-week diabetes mellitus vehicle treated,**$P < 0.01$ diabetes mellitus vs. BMP-7 and enalapril treatment [72]

7 BMP-7 in Calcium and Phosphate Homeostasis

The kidney is responsive to minute changes in serum calcium and phosphate levels, which are tightly regulated by the rates of glomerular filtration and tubular reabsorption and by dietary intake of calcium and phosphate. In addition, the kidney is responsible for the production of active 1,25-dihydroxyvitamin D3 from its precursor 25-dihroxyvitamin D3, and the loss of renal function results in renal osteodystrophy, which include (1) osteomalacia (osteoid formation without mineralization) due to vitamin deficiency, (2) osteitis fibrosa (high bone turnover) due to secondary hyperparathyroidism, and (3) adynamic bone disorder (low bone turnover) due to suppression of PTH [4].

Vitamin D deficiency (rickets) leads to secondary parathyroidism. The secondary hyperparathyroidism occurs in CKD, which produces a high turnover osteodystrophy that is associated with peritrabecular fibrosis. The nature of the cells involved in the development of peritrabecular fibrosis may represent osteoprogenitors expressing a fibroblastic phenotype and retarded from progressing through osteoblast differentiation. In animal models of CKD, BMP-7 treatment was shown to eliminate peritrabecular fibrosis, increased "active" osteoblast number, osteoblast surface, mineralizing surface, and significant decrease in the eroded surface induced [19, 44].

Loss of renal function is associated with hyperphosphatemia and elevated calcium x phosphate (Ca x P) product, leading to vascular stiffness, dysfunction, and calcification. Hyperphosphatemia has been a known predictor of

cardiovascular death, particularly in hemodialysis patients. Vascular smooth muscle cells (VSMCs) are very responsive to changes in elevated serum phosphate and undergo a loss of phenotypic expression and differentiate into cell types of the osteoblast lineage [41]. Although phosphate is managed through binders, it is becoming increasingly important to improve vascular tone and elastic modulus of vessel in ESRD patients. Hyperphosphatemia induces the loss of phenotype in VSMCs and induces dedifferentiation into myofibroblast and subsequently their proliferation in culture. In CKD models of hyperphosphatemia, BMP-7 treatment reduces the loss of VSMC phenotype and vascular calcification [9]. The effect of BMP-7 on osteoblast differentiation also reduces the systemic phosphate level and thus indirectly has a positive influence reducing phosphate levels in circulation. In summary, application of BMP-7 biology agonists may likely reduce hyperphosphatemia, secondary parathyroidism, associated osteodystrophy (osteitis fibrosa), and the loss of VSMC phenotype, thus reducing vascular stiffness, dysfunction and calcification, bone pain, and high fracture incidence in patients with loss of kidney function.

8 Mimicking BMP-7 Biology

While the molecular form of BMP-7 (free and bound) that circulates in the blood is currently unknown, chronic administration of recombinant mature BMP-7 in preclinical studies elicits ossification (ectopic bone formation) at the injection sites and also generates neutralizing antibodies upon repeated administration. It is therefore believed agents that mimic BMP-7 ligand-like biochemical property or enhancing existing endogenous BMP pool or upregulating the expression of BMP-7 expression by secretagogues may serve as safer therapeutics for CKD. Since the loss of renal function is directly related to GFR rate, one could envision intervening with peptide mimetics or anti-BMP-7 antagonist antibody or BMP-7 secretagogues, while still the kidney is partially preserved.

BMP-7 mimetic peptide: Recently a BMP-7 peptide agonist (THR-123) was identified by utilizing the structure-function analysis of BMP-7 ligand with type I receptor (ALK-3) and type II receptor (BMPR-II), data obtained from BMP-7 crystal structure and screening a small peptide library. This peptide was shown to suppress inflammation, apoptosis, and epithelial-mesenchymal and tubular fibrosis in preclinical models of acute and chronic kidney failures [60]. This compound is shown to signal by binding to ALK-3, a type I receptor. Since BMP-7 prefers ALK-2 and ALK-6 as well in proximal and collecting tubule epithelial cells and vascular endothelial cells, it is likely this peptide is not specific to BMP-7 and may provide some safety concerns for chronic administration.

Enhancing endogenous "BMP-7 pool": USAG-1 is a novel BMP-7 and Wnt-antagonist with significant amino acid identity to sclerostin (38 %) [78]. It is expressed predominantly in the kidney and overlaps with BMP-7 expression and modulates BMP-7 activity by binding to BMP-7 and Wnt signaling by binding to LRP-6, a Wnt

co-receptor. It is likely anti-USAG-1 antibody would provide therapeutic benefits in CKD patients by enhancing "active BMP-7" pool and promoting Wnt signaling in the kidney. Since there are several Wnt ligands, most of the therapeutic efforts for Wnt signaling have been focused on developing antibody to inhibitors of Wnt-receptor interaction (e.g., sclerostin for osteoporosis). It is believed that development of humanized anti-USAG-1 antibody could provide therapeutic utility for chronic kidney failure at stage 3 by enhancing BMP-7 and Wnt signaling in the kidney.

BMP-7 secretagogues: Since the kidney is highly vascularized and exposed to systemic vascular flow constantly, it is conceivable one could administer a small molecule that is safe and directly influences the expression and secretion of BMP-7 in the kidney locally. There are anecdotal reports that suggest such compound may be feasible. A recent study suggests that propofol (2,6-diisopropylphenol), containing phenol hydroxyl group, which confers antioxidant activity and is used for the induction and maintenance of anesthesia, was shown to increase BMP-7 expression and provide protection against sepsis-AKI model by suppressing inflammation [32]. Similarly, retinoic acid (RA) and prostaglandin E_2 (PGE_2) treatment has been shown to increase BMP-7 mRNA and protein levels, but does not transcriptionally activate the hBMP-7. Additionally, *in vivo* expression of BMP-7 in bone was increased upon PGE treatment. In conclusion, RA and PGE_2 upregulate BMP-7 protein expression both *in vitro* and *in vivo* [50]. A recent study linked the use of adrenoceptor agonist dexmedetomidine protection against septic acute kidney injury through increase of BMP-7 and inhibiting HDAC2 and HDAC5 [32]. The chronic administration of pitavastatin in STZ-induced diabetic mice exhibited reno- and podocyte-protective effects, which is accompanied by BMP-7 preservation and Rho suppression [48]. It remains to be seen whether this could be extended in human clinical studies.

9 Conclusion

BMP-7 was originally purified from bone matrix and later was shown that the kidney is a major site of its production in adult. Loss of function studies revealed that BMP-7 is required for embryonic kidney development and serves as renal hormone for vascular and skeletal integrity. Preclinical studies have shown that systemic administration of recombinant BMP-7 provides tissue protection in the models of acute kidney injury and chronic kidney diseases and renal osteodystrophy and Alport's syndrome, a rare x-linked renal disease. BMP-7 exerts its function by binding to a specific Ser-Thr kinase receptor and subsequently induces phosphorylation of SMAD-1/5/8. The binding of BMP-7 to its receptor complex is tightly controlled at extracellular by its interaction with anti-BMPs like USAG-1/Wise. As BMP-7 is a potent bone-inducing morphogenic protein and forms ectopic ossification at the injection site, it presents with safety issues as a viable therapy for repeated chronic administration. Approaches are therefore being investigated to mimic BMP-7 biology.

References

1. Abbate M, Zoja C, Morigi M, Rottoli D, Angioletti S, Tomasoni S, Zanchi C, Longaretti L, Donadelli R, Remuzzi G (2002) Transforming growth factorbeta1is up-regulated by podocytes in response to excess intraglomerular passage of proteins: a central pathway in progressive glomerulosclerosis. Am J Pathol 161:2179–2193
2. Almanzar M, Frazier KS, Dube PH, Piqueras AI, Jones WK, Charette MF, Paredes AL (1998) Osteogenic protein-1mRNA expression is selectively modulated after acute ischemic renal injury. J Am Soc Nephrol 9:1456–1463
3. Benigni A, Zoja C, Campana M, Corna D, Sangalli F, Rottoli D, Gagliardini E, Conti S, Ledbetter S, Remuzzi G (2006) Beneficial effect of TGFbeta antagonism in treating diabetic nephropathy depends on when treatment is started. Nephron Exp Nephrol 104:e158–e168
4. Blaine J, Chonchol M, Levi M (2015) Renal control of calcium, phosphate, and magnesium homeostasis. Clin J Am Soc Nephrol 10:1257–1272
5. Borovecki F, Jelic M, Grgurevic L, Sampath KT, Bosukonda D, Vukicevic S (2004) Bone morphogenetic protein-7 from serum of pregnant mice is available to the fetus through placental transfer during early stages of development. Nephron Exp Nephrol 97:e26–e32
6. Brandt S, Mertens PR (2016) The kidney regulates regeneration, but don't upset the balance. Int Urol Nephrol 48(8):1371–1376 . Apr 30
7. D'Agati VD, Kaskel FJ, Falk RJ (2011) Focal segmental glomerulosclerosis. N Engl J Med 365:2398–2411
8. Dalgaard OZ (1957) Bilateral polycystic disease of the kidneys; a follow-up of two hundred and eighty four patients and their families. Acta Med Scand Suppl 328:1–255
9. Davies MR, Lund RJ, Hruska KA (2003) BMP-7 is an efficacious treatment of vascular calcification in a murine model of atherosclerosis and chronic renal failure. Am Soc Nephrol 14:1559–1567
10. de Zubiria SA, Herrera-Diaz C (2012) Lupus Nephritis: an Overview of Recent Findings. Autoimmune Dis 2012:849684
11. Dolan V, Hensey C, Brady HR (2003) Diabetic nephropathy: renal development gone awry? Pediatr Nephrol 18:75–84
12. Droguett A, Krall P, Burgos ME, Valderrama G, Carpio D, Ardiles L, Rodriguez-Diez R, Kerr B, Walz K, Ruiz-Ortega M, Egido J, Mezzano S (2014) Tubular overexpression of gremlin induces renal damage susceptibility in mice. PLoS One 9(7):e101879
13. Duann P, Lianos EA, Ma J, Lin PH (2016) Autophagy, Innate Immunity and Tissue Repair in Acute Kidney Injury. Int J Mol Sci 3:17(5)
14. Dudley AT, Lyons KM, Robertson EJ (1995) A requirement for bone morphogenetic protein-7 during development of the mammalian kidney and eye. Genes Dev 9:2795–2807
15. Fern RJ, Yesko CM, Thornhill BA, Kim H-Y, Smithies O, Chevalier RL (1999) Reduced angiotensinogen expression attenuates renal interstitial fibrosis in obstructive nephropathy in mice. J Clin Invest 103:39–46
16. Franquesa M, Riera M, Herrero-Fresneda I, Sola A, Hotter G, Lloberas N, Cruzado JM, Torras J, Grinyó JM (2009) Tubular epithelial cells transfected with hHGF counteracts monocyte chemotactic protein-1 up-regulation after hypoxia/reoxygenation insult. Transplant Proc 41(6):2069–2072
17. Gascue C, Katsanis N, Badano JL (2011) Cystic diseases of the kidney: ciliary dysfunction and cystogenic mechanisms. Pediatr Nephrol 26:1181–1195
18. Genovese G, Friedman DJ, Ross MD et al (2010) Association of trypanolytic ApoL1 variants with kidney disease in African Americans. Science 329:841–845
19. González EA, Lund RJ, Martin KJ, McCartney JE, Tondravi MM, Sampath TK, Hruska KA (2002) Treatment of a murine model of high-turnover renal osteodystrophy by exogenous BMP-7. Kidney Int 61:1322–1331
20. Gould SE, Day M, Jones SS, Dorai H (2002) BMP-7regulates chemokine, cytokine, and hemodynamic gene expression in proximal tubule cells. Kidney Int 61:51–60

21. Grgurevic L, Macek B, Erjavec I, Mann M, Vukicevic S (2007) Urine release of systemically administered bone morphogenetic protein hybrid molecule. J Nephrol 20:311–319
22. Hassane S, Leonhard WN, van der Wal A, Hawinkels LJ, Lantinga-van Leeuwen IS, ten Dijke P, Breuning MH, de Heer E, Peters DJ (2010) Elevated TGF-beta-Smad signaling in experimental Pkd1 models and human patients with polycystic kidney disease. J Pathol 222:21–31
23. Helder MN, Ozkaynak E, Sampath KT, Luyten FP, Latin V, Oppermann H, Vukicevic S (1995) Expression pattern of osteogenic protein-1 (bone morphogenetic protein-7) in human and mouse development. J Histochem Cytochem 43:1035–1044
24. Higashihara E, Torres VE, Chapman AB, Grantham JJ, Bae K, Watnick TJ, Horie S, Nutahara K, Ouyang J, Krasa HB, Czerwiec FS, TEMPOFormula and 156-05-002 Study Investigators (2011) Tolvaptan in autosomal dominant polycystic kidney disease: three years' experience. Clin J Am Soc Nephrol 6:2499–2507
25. Hildebrandt F, Benzing T, Katsanis N (2011) Ciliopathies. N Engl J Med 364:1533–1543
26. Hogan BL (1996) Bone morphogenetic proteins in development. Curr Opin Genet Dev 6:432–438
27. Hopp K, Ward CJ, Hommerding CJ, Nasr SH, Tuan H-F, Gainullin VG, Rossetti S, Torres VE, Harris PC (2012) Functional polycystin-1 dosage governs autosomal dominant polycystic kidney disease severity. J Clin Invest 122:4257–4273
28. Hruska KA, De Petris L, Li T et al (2006) Contemporary diabetes: the diabetic kidney. In: Cortes P, Mogensen CE (eds) The prospect of a novel therapeutic, BMP7, in diabetic nephropathy. Chapter 18. Humana Press, Totowa
29. Hruska KA, Guo G, Wozniak M, Martin D, Miller S, Liapis H, Loveday K, Klahr S, Sampath TK, Morrissey J (2000) Osteogenic protein-1 prevents renal fibrogenesis associated with ureteral obstruction. Am J Physiol Renal Physiol 279:F130–F143
30. Hruska KA (2002) Treatment of chronic tubulointerstitial disease: a new concept. Kidney Int 61:1911–1922
31. Hruska KA, Saab G, Chaudhary LR, Quinn CO, Lund RJ, Surendran K (2004) Kidney-bone, bone-kidney, and cell-cell communications in renal osteodystrophy. Semin Nephrol 24:25–38
32. Hsing CH, Lin CF, So E, Sun DP, Chen TC, Li CF, Yeh CH (2012) α2-Adrenoceptor agonist dexmedetomidine protects septic acute kidney injury through increasing BMP-7 and inhibiting HDAC2 and HDAC5. Am J Physiol Renal Physiol 303:F1443–F1453
33. Hsu DR, Economides AN, Wang X, Eimon PM, Harland RM (1998) The Xenopus dorsalizing factor Gremlin identifies a novel family of secreted proteins that antagonize BMP activities. Mol Cell 1:673–683
34. Hudson BG, Tryggvason K, Sundaramoorthy M, Neilson EG (2003) Alport's syndrome Goodpasture's syndrome, and type IV collagen. N Engl J Med 348:2543–2556
35. Iglesias CG, Torres VE, Offord KP, Holley KE, Beard CM, Kurland LT (1983) Epidemiology of adult polycystic kidney disease, Olmsted County, Minnesota: 1935–1980. Am J Kidney Dis 2:630–639
36. Kalluri R, Shield CF, Todd P, Hudson BG, Neilson EG (1997) Isoform switching of type IV collagen is developmentally arrested in X-linked Alport syndrome leading to increased susceptibility of renal basement membranes to endoproteolysis. J Clin Invest 99(10):2470–2478
37. Kitching AR, Hutton HL (2016) The Players: Cells Involved in Glomerular Disease. Clin J Am Soc Nephrol 11(9):1664–1674
38. Klahr SS (1998) Nephrology forum: obstructive nephropathy. Kidney Int 54:286–300
39. Lappin DW, Hensey C, McMahon R, Godson C, Brasy HR (2000) Gremlins, glomeruli and diabetic nephropathy. Curr Opin Nephrol Hypertens 9:469–472
40. Lappin DW, McMahon R, Murphy M, Brady HR (2002) Gremlin: an example of the re-emergence of developmental programmes in diabetic nephropathy. Nephrol Dial Transplant 9:65–67
41. Lau WL, Pai A, Moe SM, Giachelli CM (2011) Direct effects of phosphate on vascular cell function. Adv Chronic Kidney Dis 18:105–112

42. Lee DW, Faubel S, Edelstein CL (2011) Cytokines in acute kidney injury (AKI). Clin Nephrol 76:165–173
43. Li M, Hering-Smith KS, Simon EE, Batuman V (2008) Myeloma light chains induce epithelial-mesenchymal transition in human renal proximal tubule epithelial cells. Nephrol Dial Transplant 23:860–870
44. Lund RJ, Davies MR, Hruska KA (2002) Bone morphogenetic protein-7: an anti-fibrotic morphogenetic protein with therapeutic importance in renal disease. Curr Opin Nephrol Hypertens 11:31–36
45. Luo G, Hofmann C, Bronckers AL, Sohocki M, Bradley A, Karsenty G (1995) BMP-7 is an inducer of nephrogenesis, and is also required for eye development and skeletal patterning. Genes Dev 9:2808–2820
46. Morrissey J, Hruska K, Guo G, Wang S, Chen Q, Klahr S (2002) Bone morphogenetic protein-7 improves renal fibrosis and accelerates the return of renal function. J Am Soc Nephrol 13(Suppl 1):S14–S21
47. Nguyen TQ, Goldschmeding R (2008) Bone morphogeneticprotein-7 and connective tissue growth factor: novel targets for treatment of renal fibrosis? Pharm Res 25:2416–2426
48. Ohigashi M, Imai N, Toba H, Kobara M, Nakata T (2016) Pitavastatin Exhibits Protective Effects on Podocytes Accompanied by BMP-7 Up-Regulation and Rho Suppression. Pharmacology 97:265–276
49. Ozkaynak E, Schnegelsberg PN, Oppermann H (1991) Murine osteogenic protein (OP-1): high levels of mRNA in kidney. Biochem Biophys Res Commun 179:116–123
50. Paralkar VM, Grasser WA, Mansolf AL, Baumann AP, Owen TA, Smock SL, Martinovic S, Borovecki F, Vukicevic S, Ke HZ, Thompson DD (2002) Regulation of BMP-7 expression by retinoic acid and prostaglandin E(2). J Cell Physiol 190:207–217
51. Sampath TK, Rashka KE, Doctor JS, Tucker RF, Hoffmann FM (1993) Drosophila transforming growth factor beta superfamily proteins induce endochondral bone formation in mammals. Proc Natl Acad Sci U S A 90:6004–6008
52. Sampath TK, Maliakal JC, Hauschka PV, Jones WK, Sasak H, Tucker RF, White KH, Coughlin JE, Tucker MM, Pang RH, Corbett C, Özkaynak E, Oppermann H, Rueger DC (1992) Recombinant human osteogenic protein-1 (hOP-1) induces new bone formation in vivo with a specific activity comparable with natural bovine osteogenic protein and stimulates osteoblast proliferation and differentiation in vitro. J Biol Chem 267:20352–20362
53. Sampath T, Rueger D (1994) Structure, function, and orthopaedic application of osteogenic protein (OP-1). Complications Ortho 9:101–107
54. Schmid H, Henger A, Cohen CD, Frach K, Gröne HJ, Schlöndorff D, Kretzler M (2003) Gene expression profiles of podocyte-associated molecules as diagnostic markers in acquired proteinuric diseases. J Am Soc Nephrol 14:2958–2966
55. Sharaf El Din UA, Salem MM, Abdulazim DO (2016) Stop chronic kidney disease progression: Time is approaching. World J Nephrol 5:258–273
56. Sharma K, Ziyadeh FN (1995) Hyperglycemia and diabetic kidney disease. The case for transforming growth factor-beta as a key mediator. Diabetes 44:1139–1146
57. Simic P, Vukicevic S (2005) Bone morphogenetic proteins in development and homeostasis of kidney. Cytokine Growth Factor Rev 16:299–308
58. Simon M, Maresh JG, Harris SE, Hernandez JD, Arar M, Olson MS, Abboud HE (1999) Expression of bone morphogeneticprotein-7 mRNA in normal and ischemic adult rat kidney. Am J Physiol 276(3 Pt 2):F382–F389
59. Strutz F, Zeisberg M, Renziehausen A, Raschke B, Becker V, van Kooten C, Muller G (2001) TGF-β1 induces proliferation in human renal fibroblasts via induction of basic fibroblast growth factor (FGF-2). Kidney Int 59:579–592
60. Sugimoto H, LeBleu VS, Bosukonda D, Keck P, Taduri G, Bechtel W, Okada H, Carlson W Jr, Bey P, Rusckowski M, Tampe B, Tampe D, Kanasaki K, Zeisberg M, Kalluri R (2012) Activin-like kinase 3 is important for kidney regeneration and reversal of fibrosis. Nat Med 18:396–404

61. Tanaka M, Asada M, Higashi AY, Nakamura J, Oguchi A, Tomita M, Yamada S, Asada N, Takase M, Okuda T, Kawachi H, Economides AN, Robertson E, Takahashi S, Sakurai T, Goldschmeding R, Muso E, Fukatsu A, Kita T, Yanagita M (2010) Loss of the BMP antagonist USAG-1 ameliorates disease in a mouse model of the progressive hereditary kidney disease Alport syndrome. J Clin Invest 120:768–777
62. ten Dijke P, Franzén P, Yamashita H, Ichijo H, Heldin CH, Miyazono K (1994) Serine/threonine kinase receptors. Prog Growth Factor Res 5:55–72
63. Thadhani R, Pascual M, Bonventre JV (1996) Acute renal failure. N Engl J Med 334:1448–1460
64. Torres VE, Wang X, Qian Q, Somlo S, Harris PC, Gattone VH 2nd (2004) Effective treatment of an orthologous model of autosomal dominant polycystic kidney disease. Nat Med 10:363–364
65. Trachtman H, Fervenza FC, Gipson DS, Heering P, Jayne DR, Peters H, Rota S, Remuzzi G, Rump LC, Sellin LK, Heaton JP, Streisand JB, Hard ML, Ledbetter SR, Vincenti F (2011) A phase 1, single-dose study of fresolimumab, an anti-TGF-β antibody, in treatment-resistant primary focal segmental glomerulosclerosis. Kidney Int 79:1236–1243
66. Vukicevic S, Latin V, Chen P, Batorsky R, Reddi AH, Sampath TK (1994) Localization of osteogenic protein-1 (bone morphogenetic protein-7) during human embryonic development: high affinity binding to basement membranes. Biochem Biophys Res Commun 198:693–700
67. Vukicevic S, Kopp JB, Luyten FP, Sampath TK (1996) Induction of nephrogenic mesenchyme by osteogenic protein 1 (bone morphogenetic protein 7). Proc Natl Acad Sci U S A 93:9021–9026
68. Vukicevic S, Stavljenic A, Pecina M (1995) Discovery and clinical applications of bone morphogenetic proteins. Eur J Clin Chem Clin Biochem 33:661–671
69. Vukicevic S, Basic V, Rogic D, basic N, Shih M, Shepard A, Jin D, dattatreyamurty B, Jones W, Dorai H et al (1998) Osteogenic protein-1 (bone morphogenetic protein-7) reduces severity of injury after ischemic acute renal failure in rat. J Clin Invest 102:202–214
70. Vukicevic S, Sampath KT (2004) Bone morphogenetic proteins: regeneration of Bone and Bayond. Birkhauser Verlag, Basel/Switzerland
71. Wang S, de Caestecker M, Kopp J et al (2006) Renal bone morphogenetic protein-7 protects against diabetic nephropathy. J Am Soc Nephrol 17:2504–2512
72. Wang S, Chen Q, Simon TC, Strebeck F, Chaudhary L, Morrissey J, Liapis H, Klahr S, Hruska KS (2003) Bone morphogenetic protein-7 (BMP-7), a novel therapy for diabetic nephropathy. Kidney Int 63:2037–2049
73. Wang SN, Lapage J, Hirschberg R (2001) Loss of tubular bone morphogenetic protein 7 in diabetic nephropathy. J Am Soc Nephrol 12:2392–2399
74. Wilson PD (2008) Mouse models of polycystic kidney disease. Curr Top Dev Biol 84:311–350
75. Xu Y, Wan J, Jiang D, Wu X (2009) BMP-7 counteracts TGF-beta1-induced epithelial-to-mesenchymal transition in human renal proximal tubular epithelial cells. J Nephrol 22:403–410
76. Yamaguchi T, Wallace DP, Magenheimer BS, Hempson SJ, Grantham JJ, Calvet JP (2004) Calcium restriction allows cAMP activation of the B-Raf/ERK pathway, switching cells to a cAMP-dependent growth-stimulated phenotype. J Biol Chem 279:40419–40430
77. Yanagita M (2004) USAG-1: a bone morphogenetic protein antagonist abundantly expressedin the kidney. Biochem Biophys Res Commun 316:490–500
78. Yanagita M (2006) Modulator of bone morphogenetic protein activity in the progression of kidney diseases. Kidney Int 70:989–993
79. Zeisberg M, Hanai J, Sugimoto H, Mammoto T, Charytan D, Strutz F, Kalluri R (2003a) BMP-7 counteracts TGF-beta1-induced epithelial-to-mesenchymal transition and reverses chronic renal injury. Nat Med 9:964–968
80. Zeisberg M, Bottiglio C, Kumar N, Maeshima Y, Strutz F, Müller GA, Kalluri R (2003b) Bone morphogenic protein-7 inhibits progression of chronic renal fibrosis associated with two genetic mouse models. Am J Physiol Renal Physiol 285:F1060–F1067

81. Zeisberg M, Shah AA, Kalluri R (2005) Bone morphogenicprotein-7 induces mesenchymal to epithelial transition in adult renal fibroblasts and facilitates regeneration of injured kidney. J Biol Chem 280:8094–8100
82. Zhang XL, Selbi W, de la Motte MC, Hascall V, Phillips AO (2005) Bone morphogenic protein-7 inhibits monocyte-stimulated TGF-beta1 generation in renal proximal tubular epithelial cells. J Am Soc Nephrol 16:79–89
83. Zoja C, Morigi M, Benigni A, Remuzzi G (2004) Genetics of rare diseases of the kidney: learning from mouse models. Cytogenet Genome Res 105:479–484

Bone Morphogenetic Protein Signaling in Pulmonary Arterial Hypertension

Peiran Yang and Paul B. Yu

Abstract A wealth of evidence from human genetics, developmental and cell biology, and translational science has implicated members of the bone morphogenetic protein (BMP) and transforming growth factor-β (TGF-β) signaling family in the pathogenesis of pulmonary arterial hypertension (PAH). The discovery of loss-of-function germline mutations in BMPR2 and in functionally related BMP/TGF-β signal transduction molecules as causes of heritable PAH and several overlapping congenital vascular syndromes has catalyzed work to elucidate how BMP signals critically regulate vascular development, vascular homeostasis, inflammation, metabolism and pathogenic remodeling. This work in vascular biology and experimental medicine has in turn led to a more nuanced understanding by which the structurally diverse family of BMP ligands and receptors achieve their tissue-specific and context-dependent functions. Recently this work has shed light on promising new strategies by which dysregulated BMP/TGF-β might be modulated for therapeutic benefit in PAH and related conditions.

Keywords Pulmonary artery • Pulmonary vascular disease • Pulmonary arterial hypertension • BMPR2 • Bone morphogenetic protein • Heritable pulmonary arterial hypertension • Hereditary hemorrhagic telangiectasia • Juvenile familial polyposis • ALK1 • BMP9 • Endoglin • Vascular endothelium • Vascular smooth muscle

1 BMP Signaling in the Cardiopulmonary System

BMP signaling plays a fundamental role in the development and homeostasis of the heart and the systemic and pulmonary circulation. Spatiotemporal specificity of BMP signaling in the cardiopulmonary system is achieved by selective expression

P. Yang, PhD • P.B. Yu, MD, PhD, FAHA (✉)
Brigham and Women's Hospital, Division of Cardiovascular Medicine, Department of Medicine, 20 Shattuck Street, Thorn Biosciences 1203, Boston, MA 02115, USA
e-mail: pbyu@partners.org

S. Vukicevic, K.T. Sampath (eds.), *Bone Morphogenetic Proteins: Systems Biology Regulators*, Progress in Inflammation Research,
DOI 10.1007/978-3-319-47507-3_13

of a particular BMP ligands, receptors, and modulatory proteins to facilitate context-dependent signaling. This signaling pathway regulates vasculogenesis and cardiomyogenesis during development. In the pulmonary circulation, BMP signaling controls the fate and function of vascular cells. Furthermore, the BMP pathway interacts with and influences other pathways to fine-tune their regulatory effects on vascular development and homeostasis.

1.1 BMP Ligands and Receptors in the Cardiopulmonary System

The BMP signaling pathway consists of a diverse range of ligand, receptors, co-receptors, antagonists, and downstream mediators. The functional roles of these components have been investigated individually by direct application of recombinant ligand or antagonist proteins of interest or by the transgenic overexpression of wild-type or constitutively active mutant receptor proteins [16], whereas the consequences of their removal have been studied by genetic ablation [137], neutralizing antibodies, ligand traps, and small molecule inhibitors [24, 47, 124]. The most relevant BMP ligands in the cardiopulmonary system are likely to be BMP9, BMP10, and BMP6 as they are secreted into the circulation at functionally relevant concentrations [40, 82, 108, 209]. BMP9 is expressed in the adult liver by non-parenchymal cells such as endothelial, stellate, and Kupffer cells [131]. BMP10 expression is restricted to the ventricular trabeculae during mid-gestation and the right atrium of the adult heart [29, 98]. BMP type I receptors are expressed in multiple cell types, except ALK1, which is predominantly expressed in endothelial cells [70, 179]. ALK2 is also found in the endothelium and is reported to modulate ALK1 expression in response to BMP stimuli [224]. Type II BMP receptors BMPR2 and ACTR2A are expressed in mesenchyme-derived tissues, while BMPR2 is expressed at high levels in the endothelium [137]. ALK2, ALK3, BMPR2, and ACTR2A are also found in pulmonary artery smooth muscle cells (PASMCs) and cardiomyocytes [154, 218, 226]. BMP ligands demonstrate different affinities to specific receptor complexes. For example, the BMP2 and BMP4 subgroup bind preferentially to BMPR2 in a complex with ALK3, whereas the BMP6 and BMP7 subgroup bind preferentially to ACTR2A with ALK2 [226]. Importantly, BMP9 and BMP10 bind to receptor complexes formed by BMPR2 with ALK1 or ALK2 in the endothelium [41, 110, 149, 169, 199] and specifically in pulmonary artery endothelial cells (PAECs) [205]. Signaling mediated by BMP9, BMP10, BMPR2, ALK1, and the type III co-receptor endoglin is specialized in the vascular endothelium and in embryonic endocardium owing to high abundance of these components [137]. Evidence suggests that BMPR2, ALK1, and endoglin are required for endothelial cell signaling and/or function in response to BMP9 or BMP10 [33, 34, 150, 205].

1.2 BMP Signaling and Pulmonary Vascular Cell Functions

The tissue-specific expression of receptors enables BMP ligands to exert differential effects on various vascular cell types including endothelial cells, smooth muscle cells, pericytes, and adventitial cells. BMP signaling modulates vasculogenesis, angiogenesis, and vascular integrity by regulating vascular cell survival, phenotype, and function in a ligand- and lineage-dependent manner [27].

1.2.1 Effects of BMPs on Endothelial Cells

In human PAECs, BMP9 signals through BMPR2 and ALK1 to induce SMAD phosphorylation and Id gene expression, leading to growth inhibition [205]. BMP9 has been shown to be a circulating vascular quiescence factor, inhibiting sprouting angiogenesis in vivo [40]. BMP9 prevents apoptosis and enhances monolayer integrity in PAECs [114]. However, low concentrations of BMP9 induce proliferation and migration of endothelial cells in vitro [193]. On the other hand, administration of the soluble extracellular domain of ALK1 expressed as an immunoglobulin Fc domain fusion protein (ALK1-Fc), which functions as a BMP9/BMP10 ligand trap, blocks tumor angiogenesis [39]. Therefore, the effect of BMP9 on angiogenesis is at least partly dependent on the ligand concentration. Besides BMP9, BMPs 2, 4, 6, and 7 promote endothelial cell proliferation, migration, and tube formation [172, 207]; protect endothelial cells from apoptosis [197]; and induce angiogenesis in vitro and in vivo [198]. In contrast, BMP10 limits endothelial cell number in and stabilizes the caliber of nascent arteries in embryonic vascular development [108]. Thus, the endothelial cell response to BMP signaling is ligand and context specific. The pro-angiogenic and pro-survival effects of BMPs are mediated at least in part by their ability to recruit the expression of Id transcriptional modulator proteins via the canonical SMAD1/SMAD5/SMAD8 signaling pathway [18, 35, 207].

1.3 Effects of BMPs on Mural Cells

In addition to endothelial cells, BMP signaling also regulates vascular smooth muscle cell survival and differentiation. The response of PASMCs to BMPs may depend on the anatomical origin of these cells [136]. BMPs 2, 4, and 7 are reported to inhibit proliferation and induce apoptosis of PASMCs isolated from proximal pulmonary arteries via the SMAD pathway [138, 223, 227, 231]. In contrast, BMP2 and BMP4 stimulate proliferation of PASMCs from peripheral pulmonary arteries via ERK1/ERK2 and p38MAPK [223]. Moreover, BMPs promote the contractile

phenotype of smooth muscle cells via microRNA-21 [100]. This effect on cell phenotype is mediated through induction of myocardin-related transcription factors [104] or suppression of microRNA-96 [103]. Less is known about the response of pericytes in pulmonary vessels to BMP signaling. ALK1 deficiency results in reduced pericyte coverage in the brain [30], whereas the ALK1-Fc ligand trap increases the pericyte coverage of tumor vessels [80]. Loss of ALK3 caused resistance to apoptosis in human brain microvascular pericytes [53].

1.4 Cross Talk with Other Signaling Pathways

In order to coordinate cardiovascular development and homeostasis, the BMP pathway interacts with other pathways, including Wnt, Notch, and tyrosine growth factor signaling [95]. BMP signaling via BMPR2 activates canonical and noncanonical Wnt signaling to regulate PAEC survival, proliferation, and migration [2, 43] and to promote motility and repress growth of smooth muscle cells [44]. In endothelial cells, BMP2 may modulate the expression of Wnt inhibitors Sost and Dkk1 via ALK3 [99]. BMP signaling regulates the expression of a Notch ligand to transactivate Notch signaling in neighboring cells [135]. In addition, BMP signaling co-regulates Notch transcriptional targets to modulate endothelial cell function [90] and determines the identity of tip versus stalk cells during angiogenesis via ALK1 [6, 101, 107, 142]. In addition, BMPs may interact with sonic hedgehog signaling during lung development [17, 23]. Furthermore, BMP9, via ALK1 and BMPR2, suppresses vascular endothelial growth factor (VEGF) expression [180] in endothelial cells and blocks VEGF-induced angiogenesis, while inhibiting basic fibroblast growth factor (bFGF)-stimulated proliferation and migration of endothelial cells [178]. BMP2 and BMP4 signaling via BMPR2 reduces platelet-derived growth factor (PDGF)-BB-induced proliferation of PASMCs [77], while signaling of these BMP ligands via ACTR2A is not able to mediate this effect [227]. The augmenting or opposing effects of BMP signaling on other pathways are not limited to vascular homeostasis but also contribute more broadly to cardiac and pulmonary development [83, 137] and are thus critical for normal development and homeostasis of the cardiopulmonary system.

2 BMP Signaling in Cardiopulmonary Disease

Impairment of BMP signaling perturbs the homeostasis of the pulmonary vasculature. Genetic defects in components of the BMP signaling pathway have been identified in cardiovascular diseases with pulmonary manifestations, such as heritable pulmonary arterial hypertension (HPAH) and hereditary hemorrhagic telangiectasia (HHT). Other cardiovascular abnormalities, such as vascular calcification [25], atherosclerosis [47], and coronary artery disease [190], have also been associated with aberrant or maladaptive BMP expression. This chapter focuses on the role of BMP signaling in PAH and discusses the advances in our knowledge in the past 15 years, since the first discovery of mutations in BMPR2 in this disease.

2.1 Introduction to PAH

2.1.1 Disease Pathology

PAH is a devastating disease characterized by elevated mean pulmonary arterial pressure of greater than 25 mmHg at rest with increased pulmonary vascular resistance [84]. Small pulmonary arterioles undergo remodeling in PAH including intimal hyperplasia, medial hypertrophy, and plexiform arteriopathy in the severe cases, as a result of excessive cell proliferation and insufficient apoptosis [128]. This obliteration of the vascular lumen restricts flow and increases pulmonary vascular resistance, leading to increased pulmonary arterial pressure and right ventricular afterload and consequently in right ventricular hypertrophy and failure [128]. Depending on the cohort, the prevalence of PAH is reported to be between 15 and 50 cases per million people [88, 156] and may be substantially higher in certain at-risk populations. Patients present with nonspecific symptoms such as fatigue, dyspnea on exertion, and syncope and therefore require confirmation of diagnosis with right heart catheterization [128, 162]. The National Institutes of Health PAH Registry originally reported a median survival of 2.8 years following diagnosis [167]. Current therapies do not reverse or cure PAH but generally improve functional status and in some cases improve composite survival and morbidity endpoints [161]. In the era of modern therapies for PAH, the 5-year survival rate has been reported to be better than 50 % [19, 55, 89], an improvement which may be as much due to improvements in recognition and supportive care over the past three decades as well as novel therapeutics.

2.1.2 Subtypes of PAH

Under the updated World Health Organization clinical classification system, Group 1 PAH describes precapillary pulmonary hypertension with left-sided or pulmonary capillary wedge pressures (PCWPs) of < 15 mm Hg and includes a heterogeneous group of conditions associated with PAH [84, 183]. Idiopathic PAH (IPAH) describes PAH that occurs in the absence of identifiable cardiac structural, pulmonary or airway disease, or systemic inflammatory of infectious illness as potential explanations of PAH. IPAH can include PAH that occurs in a familial pattern, inherited typically in an autosomal dominant fashion with reduced penetrance. Heritable PAH includes such cases of familial PAH, as well as sporadic cases of IPAH in which a disease-causing mutation is identified. The annual incidence of IPAH is 1–2 cases per million people [63] with a prevalence of 2.4–7.6 cases per million people [5]. IPAH is 10–15 times more common than HPAH [88], which is now known to include individuals with germline heterozygous mutations in *BMPR2*, *ACVRL1* (encoding ALK1), and *ENG* (encoding endoglin), the latter two occurring in hereditary hemorrhagic telangiectasia (HHT) syndromes which may include PAH as part of their phenotypic spectrum. Hereditary transmission of PAH occurs in 4–10 % of PAH patients [88, 128]. Additionally, WHO Group 1 disease also includes drug- (e.g., fenfluramine, amphetamine, and other stimulants) and other toxin-induced PAH and PAH associated with congenital heart disease, HIV infection, portal hypertension, schistosomiasis, pulmonary veno-occlusive disease, and pulmonary

capillary hemangiomatosis [183]. Collectively these subtypes of Group 1 PAH have a higher prevalence in the population than idiopathic and heritable PAH, despite representing small proportions of the patients carrying the associated diseases [63, 128]. The WHO classification scheme includes other types of pulmonary hypertension (PH) that are not necessarily precapillary, including Group 2 disease associated with left-sided heart dysfunction; Group 3 disease associated with airways, airway structural, or central nervous system-mediated breathing disorders; Group 4 disease describing chronic thromboembolic pulmonary hypertension (CTEPH); and Group 5 describing a number of miscellaneous conditions associated with PH.

2.2 BMP Pathway and Genetics of PAH

2.2.1 BMPR2 Mutations

Discovery of BMPR2 Mutations

Primary or idiopathic PAH (IPAH) was noted to occur in a familial form by Melmon and Braunwald [130] and was reported to transmit in families as an autosomal dominant disease before the identification of genetic mutations, suggesting that a heterozygous mutation might be responsible for the disease [116, 117]. However, not all individuals at risk develop PAH, due to reduced penetrance of approximately 20–30 % [116, 117]. Linkage analysis studies by two independent teams associated the chromosome region 2q31–33 in PAH families [139, 145]. Following a candidate-based sequencing effort, a diverse set of germline heterozygous loss-of-function mutations were identified in the BMPR2 locus, encoding the BMP type II receptor [46, 105].

Prevalence of BMPR2 Mutations in PAH Subtypes

Since the initial description, it has since been confirmed in other cohorts that mutations in BMPR2 are responsible for approximately 70 % (53–86 % reported) of cases of heritable PAH (HPAH, [118, 121]). In addition, approximately 10–20 % (6–40 % reported) of sporadic cases of idiopathic PAH in which there are no known related carriers or family history are caused by BMPR2 mutations [4, 62, 118, 201]. It is possible that some of these cases were the result of de novo mutations or alternatively could represent unrecognized cases of HPAH due to reduced penetrance and small family size [12]. BMPR2 gene mutations have been reported in other PAH subtypes. For example, approximately 10 % of subjects with drug (fenfluramine derivatives)-induced PAH carry BMPR2 gene mutations [87]. However, it is unclear to what degree these mutations may potentiate the development of PAH associated with stimulant usage, as the frequency among this population is not higher than that found in sporadic IPAH. Germline mutations in BMPR2 have been

detected in patients with pulmonary veno-occlusive disease [4, 134, 174], indicating that the mutations may cause this atypical subtype of PAH or this represents different manifestations of the same disease. Reports of BMPR2 mutations in PAH associated with congenital heart disease are not consistent as one study detected mutations [168], whereas the second study in a different and smaller cohort found no mutations [109]. To date, no mutations have been detected in PAH associated with scleroderma [140] or HIV infection [151].

Types of BMPR2 Mutations

The number of distinct BMPR2 mutations identified in PAH patients continues to increase since the initial discoveries owing to collaborative research effort in specialist PAH centers and the advance in screening technologies [118, 119]. A comprehensive analysis in 2015 documented a total of 668 germline variants of BMPR2, highlighting the major causal role of this gene [121]. Approximately 70 % of the mutations are predicted to introduce premature truncation of the BMPR2 open reading frame [118, 119], encompassing nonsense, frameshift, splice site defects and major gene rearrangements [119]. Gene rearrangements, exon deletions, and mutations affecting the 5-prime untranslated region are increasingly recognized as screening centers expand their analytic methods [121]. Missense variants account for the remaining 30 % of BMPR2 mutations [118]. Despite the identification of mutations across the entire coding sequence of the BMPR2 gene, the mutation load is not uniform across the 13 exons. Exon 12 harbors the largest number of mutations, whereas exon 9 contains the highest mutations per exon size [121]. Mutation hot spots are found in regions important to the function of BMPR2 protein. For example, missense mutations cluster in exons 2–3, 6–9, and 11–12 where the ligand-binding domain and the catalytic regions of the kinase domain are encoded [121].

Effect of Mutations on Expression and Activity of BMPR2

BMPR2 mutations affect its expression and activity by mechanisms that are heterogeneous and mutation specific [136]. Histological evidence suggests markedly downregulated BMPR2 expression in the pulmonary vasculature of patients with mutations and moderately decreased BMPR2 expression in that of IPAH patients even without known mutations [8], suggesting deficiency of BMPR2 signaling may be a wider phenomenon beyond HPAH. Premature terminations result in the activation of the nonsense-mediated decay pathway and cause disease due to functional haploinsufficiency [143, 212]. On the other hand, amino acid substitutions may lead to loss of kinase activity and aberrant trafficking of misfolded BMPR2 in the endoplasmic reticulum and cause disease by dominant-negative effects [60, 212]. Some BMPR2 mutants may reach the cell membrane but form clusters or otherwise exhibit altered associations with membrane

domains, including caveolae, lipid rafts, or clathrin-coated pits [94]. The correct conformation of the extracellular ligand-binding domain of BMPR2 requires the formation of five disulfide bridges between ten conserved cysteine residues [72], of which nine have been found mutated [121]. These cysteine mutants exhibit intracellular retention, likely to be due to a profound loss of conformational integrity, combined with a diminished activity of SMAD activation [147, 173]. In addition, a mutation of an asparagine residue adjacent to the cysteine has been documented [119]. In contrast, non-cysteine substitutions within the kinase domain traffic normally to the cell surface but typically fail to activate downstream SMAD signaling [136]. For example, mutations of an arginine residue (R491) disrupt its interaction with glutamic acid 386 and render the kinase inactive [119, 173]. Mutations in the cytoplasmic tail do not affect signaling through SMAD but may perturb noncanonical pathways involving LIM kinase-1, a regulator of the actin depolymerizing factor cofilin, and Tctex-1, a light chain of the motor complex dynein in the endothelium and smooth muscle [58, 120]. However, evidence suggests that missense mutations across the entire gene result in upregulation of p38MAPK [173].

2.2.2 Mutations in Other Components of the BMP Signaling Pathway

While BMPR2 is the major genetic determinant underlying PAH due to the high prevalence of mutations in this gene, less frequent mutations in additional genes have been discovered and facilitated by advances in DNA sequencing technologies. Many of these affected genes are receptors or intracellular components of the BMP signaling pathway, highlighting its central importance [12, 121]. Several of the mutations in these genes are found PAH or PAH associated with HHT [121], indicating a common cause and pathobiology of these pulmonary vascular diseases.

2.2.3 Hereditary Hemorrhagic Telangiectasia (HHT) Is an Overlap Syndrome with PAH

Most widely recognized for its systemic vascular phenotypes, HHT is considered to exist on a phenotypic spectrum with PAH, owing to the fact that all of the mutations associated with HHT have also been identified in subsets of HPAH. HHT is an autosomal dominant disease with a prevalence of more than 1 case per 10,000 [182]. Symptoms of HHT include frequent epistaxis, telangiectasias in the skin and mucosa, and importantly the development of arteriovenous malformations in the pulmonary, hepatic, and cerebral circulation [127]. A number of HHT-affected families and individuals have been diagnosed with PAH [1, 68, 203], where the precapillary pattern of pulmonary arterial hypertension is histologically indistinguishable between these diseases [208]. HHT is divided into subtypes such as HHT-1, HHT-2, and HHT-5 according to the underlying genetic mutations, in addition to HHT associated with juvenile polyposis [137].

Mutations of ACVRL1/ALK1 in PAH With and Without HHT-2

Mutations in the type I receptor ALK1 (gene *ACVRL1*) are known to cause HHT-2 [96]. A gene linkage analysis in 2001 identified mutations in the ALK1 gene, located at chromosome position 12q13, in patients with HHT-associated PAH, e.g., manifestations of the HHT syndrome accompanied by PAH [203]. To date, 57 loss-of-function mutations in ALK1 have been identified, mostly in HHT-associated PAH [121]. However, ALK1 mutations have also been documented in PAH patients without HHT [32, 62, 78, 79, 157]. The vast majority of these cases were diagnosed in childhood, leaving open the possibility of developing manifestations of HHT later in life [121]. The majority of ALK1 mutations are missense mutations resulting in pathogenic amino acid substitutions, in contrast to premature termination caused by most BMPR2 mutations [121]. An uneven mutation load is also observed across the ALK1 gene, where most mutations locate to exons 6–10, which encode the kinase domain of the receptor [121]. The effects of ALK1 mutations on expression and signaling of the receptor are less well characterized compared to BMPR2. In one study, six out of eight ALK1 mutations found in HHT-PAH resulted in retention of expressed receptor in the endoplasmic reticulum while two mutants reached the cell surface. One of these is a GS domain mutation predicted to disrupt conformational changes owing to the loss of a critical hydrogen bond [79]. A different study reported normal trafficking to the cell surface and BMP9 binding of most HHT-PAH-associated ALK1 mutants, but these mutants were defective in BMP9-induced signaling [166]. Of note, BMP9-induced SMAD1/SMAD5 phosphorylation and BMP9-mediated inhibition of capillary network formation are impaired in murine $ALK1^{-/-}$ and $ALK1^{+/-}$ pulmonary microvascular endothelial cells [33].

Mutations of ENG/Endoglin in PAH With or Without HHT-1

HHT-1 is caused by mutations in the type III receptor endoglin (gene ENG) [126]. Mutations in the ENG gene have also been discovered in patients with HHT-associated PAH [79]. The total number of independent ENG mutations in PAH and HHT-PAH has now reached 9 [121]. Interestingly, ENG mutations are found in a patient with PAH associated with HHT and dexfenfluramine use [28], a patient with idiopathic PAH, and a patient with congenital heart defect-associated PAH [157]. The non-HHT-PAH patients with ENG mutations were identified in childhood [157] and could manifest HHT in later life. ENG mutations documented in PAH and HHT-PAH include missense, frameshift, and branch-site mutations [121]. The predominant type of mutation, the existence of mutation hot spots within the gene, and the effects of the mutations on expression and signaling of the receptor are currently unknown. However, evidence suggests that depletion of endoglin impaired BMP9-induced SMAD1/SMAD5/SMAD8 phosphorylation in human pulmonary artery endothelial cells [150]. Recently we reported that a soluble form of endoglin that may function as an anti-angiogenic ligand trap of BMP9 is present at elevated levels in the circulation of individuals with Group 1 PAH, suggesting an additional mechanism by which abnormalities in this protein may attenuate BMP9 signaling [123].

Mutations of BMPR1B/ALK6

Two missense mutations of the type I receptor ALK6 have been reported in two idiopathic PAH patients [31]. One mutation (F392 L) is located in the kinase domain, whereas the other (S160 N) is not located within functional domains. Paradoxically, these mutations, particularly the former, result in increased SMAD8/9 phosphorylation and transcriptional activity. The authors suggested that the gain of function of signaling by ALK6 may play a pathogenic role in PAH [31]. It is known that loss of BMPR2 in PASMCs leads to the gain of signaling by a subset of BMP ligands transduced by a different receptor, ACTR2A [226], supporting the notion that loss-of-function mutations affecting individual receptors can paradoxically lead to gain of function in signaling due to the partially overlapping and redundant nature of ligand-receptor interactions in this pathway. Further functional analysis in the pathogenic mechanism of these ALK6 mutations is required.

SMAD8/SMAD9

In additional to the receptors, mutations of intracellular partners of the BMP signaling pathway have also been found in PAH. SMAD8 (a.k.a. SMAD9/*SMAD9*) is affected by one missense [144] and two nonsense [50, 181] mutations in idiopathic and heritable PAH patients. The amino acid substitution (K43E) within the MH1 domain of SMAD8/SMAD9 resulted in reduced SMAD-responsive reporter activity [144], while the truncation mutation (C202X) caused defects in response to receptor-mediated phosphorylation, impaired interaction with SMAD4, and decreased transcriptional activity [181]. In contrast, the R294X truncation abrogated microRNA induction by BMP9 in PAECs, but reduced canonical signaling only by one third. In spite of redundancy of the receptor-regulated SMADs in canonical BMP signaling, this noncanonical effect of SMAD9 mutation may explain its pathogenic role [50]. In addition to germline mutations, somatic chromosomal abnormalities in the lung of a BMPR2 mutation carrier were reported to result in the loss of SMAD9 and were felt to represent a potential example of a second hit acting upon the BMP pathway [3].

SMAD1

Similarly, the *SMAD1* gene is also mutated in one idiopathic PAH patient. This missense mutation causes an amino acid substitution (V3 A) and reduced activity of a SMAD-responsive reporter compared to wild type [144]. This remains the only SMAD1 mutation reported to date. However, it is known that the activated form of SMAD1 is deficient in the pulmonary vasculature of idiopathic and heritable PAH patients [223].

SMAD4 in PAH and (JP-)HHT

Two mutations in the common mediator *SMAD4* have been identified in two idiopathic PAH cases [144]. One of these is a missense (N13S) mutation resulting in the substitution of a conserved amino acid. However, no difference in the activity of a

SMAD-responsive reporter was observed between wild-type and this mutant [144]. It is possible that this mutation may affect SMAD-independent pathways or this is a rare population variant with no impact on PAH susceptibility [144]. On the other hand, the second mutation is a splice variant and may cause transcript loss due to compromised splicing efficiency [144]. Interestingly, *SMAD4* mutations are found to cause HHT with or without juvenile polyposis (JP, [64, 65]), leading some to postulate a phenotypic spectrum between PAH and JP-HHT syndromes.

BMP9 in HHT

A small proportion of HHT patients do not carry mutations in *ACVRL1*, *ENG*, or *SMAD4*. Missense mutations in BMP9 have been reported in three individuals with HHT-like presentations overlapping with JP in a novel syndrome that has been provisionally named HHT5. These three amino acid substitutions, two in the prodomain and one in the mature protein, negatively affect protein processing and/or function to varying degrees. This study confirms the role of impaired BMP signaling pathway in the pathogenesis of this vascular disorder [217]. No mutation in BMP ligands has been found in PAH as yet.

CAV1 (Interacts with BMPR2)

Whole-exome sequencing technology enabled the discovery of two frameshift mutations in the CAV1 gene, which encodes caveolin-1, in two patients with idiopathic and heritable PAH [10]. Caveolin-1 is the major protein constituent of flask-shaped invaginations of the cell membrane, caveolae [171], abundant in endothelial and mesenchymal cells [12]. The CAV1 mutation resulted in reduced caveolin-1 on endothelial cells of small pulmonary arteries [10]. Caveolin-1 is not normally classified as a member of the BMP signaling pathway, but it is known that caveolin-1 interacts directly and dynamically with BMP receptors BMPR2 and ALK3 [148] and colocalizes with BMP signaling complexes. In smooth muscle cells, the loss of caveolin-1 impaired BMPR2 membrane localization and association of BMPR2 with ALK3 [211]. A number of other possible effects of CAV1 mutations not involving BMPR2 have been proposed, but the precise mechanisms involving or not involving BMPR2 have yet to be fully elucidated [12].

2.3 Functional Consequences (i.e., Mechanistic Link from Mutation to Disease)

As described above, mutations of BMPR2 and other BMP signaling pathway components alter their expression and signaling. At the cellular level, regulation of the pulmonary vasculature by normal BMP signaling is lost, disrupting homeostasis and presumably promoting vascular remodeling. This is likely to be responsible for the PAH phenotypes or increased susceptibility to PAH as observed in animal models with genetic lesions in BMP pathway members. Additionally, gene mutations also alter the interaction between the BMP pathway and other signaling pathways and

systems, such as TGF-β and estrogen signaling, inflammation, and the immune system and metabolism. The combined effects of mutations and these other factors may determine the risk of developing PAH, thus connecting genotype to phenotype.

2.3.1 Vascular Homeostasis and Remodeling

Endothelial Cells

Mutations in members of the BMP signaling pathway, particularly in *BMPR2*, disrupt their function in maintaining homeostasis of the pulmonary vasculature in a cell type-specific manner. Overexpression of a mutation in the kinase domain of BMPR2 (D485G) in PAECs increased their susceptibility to apoptosis [222]. Similarly, siRNA knockdown of *BMPR2* also increased apoptosis of PAECs [197]. In addition, knockdown of *BMPR2*, or *SMAD1* and *SMAD5*, eliminated the anti-apoptotic effect of BMP9, demonstrating the effect of reduced canonical SMAD signaling [114]. Another proposed pathway involves a BMPR2-mediated transcriptional complex between peroxisome proliferator-activated receptor γ (PPARγ) and β-catenin, which is impaired with reduced *BMPR2* expression, thus reducing the induction of endothelial survival genes including apelin [2, 22]. Mice with endothelial deletion of PPARγ spontaneously develop PAH possibly due to increased platelet-derived growth factor receptor-beta expression and signaling [73]. Moreover, reduction in *BMPR2* expression occurs not only in mutation carriers but also in patients without detectable mutations [8]. This overall impairment BMP signaling in PAH renders endothelial cells prone to apoptosis, which is observed in early stages of disease pathogenesis, making endothelial cells the initiating cell type [114, 136]. This increased rate of apoptosis may trigger the development of apoptosis resistant clones of endothelial cells, contributing to plexiform lesions in later disease [136].

In addition to the survival of endothelial cells, reduced *BMPR2* expression compromises the integrity and barrier function of the pulmonary endothelium, enhancing leukocyte transmigration and endothelial secretion of cytokines, such as interleukins-8 and -6, upon inflammatory challenge [26]. Pulmonary endothelial cells with heterozygous null *BMPR2* mutations exhibit SRC-dependent caveolar trafficking defects, and this may contribute to pulmonary endothelial barrier dysfunction [160]. The interaction between BMP signaling and cytoskeleton is defective in *BMPR2* mutant PMVECs and is associated with activation of the Rho GTPase, Rac1 [97]. BMPR2-mediated phosphorylation of Tctex-1 is impaired by mutations in exon 12 of BMPR2 [120]. Mutations in the tail of BMPR2 also disrupt its function in inhibiting LIM kinase 1 [58]. In PAECs with *BMPR2* mutation or knockdown, BMPs fail to activate endothelial nitric-oxide synthase, resulting in reduced nitric oxide, a vasodilator and suppressor of smooth muscle proliferation [66]. Moreover, PAECs with mutant *BMPR2* and pulmonary endothelial cells from PAH patients release more growth factors such as TGF-β1 and fibroblast growth factor 2, impacting on the under-

lying smooth muscle cells [91, 222]. A pathway has been proposed where the reduction of BMPR2 expression results in increased expression of fibroblast growth factor 2 via microRNA intermediates and reduced expression of apelin [22, 102].

Smooth Muscle Cells

PAEC alterations precede muscularization of vessel but contribute to it by favoring smooth muscle cell proliferation [162]. PASMCs are also directly affected by impaired BMPR2 signaling, as BMPR2 is required for BMP-mediated growth arrest in PASMCs [227]. BMP/SMAD-mediated growth suppression is lost in PASMCs from patients with PAH and BMPR2 mutations [138, 219, 223]. The growth inhibitory effects of BMPs are mediated by the SMAD and Id pathway [219] and/or PPARγ and Src/STAT3 pathway possibly with microRNA intermediates [22, 77]. Mice with smooth muscle deletion of PPARγ spontaneously develop PAH [77]. Additionally, oxidative injury, in the form of increased reactive oxygen species, is observed in vascular smooth muscle cells expressing mutant *BMPR2* and in transgenic mice which overexpress mutant *BMPR2* in the vascular smooth muscle [106]. BMPR2-deficient PASMCs also exhibit increased proliferation in response to growth factors such as serotonin [113] and TGF-β1 [42, 138]. The levels of the latter are increased in the conditioned media from PAECs with a mutant BMPR2 [222]. Furthermore, TGF-β1, signaling via ALK5 and SMAD3, represses BMP4-mediated SMAD signaling and transcriptional response in BMPR2-mutant PASMCs [204]. These studies highlight the contribution of TGF-β to vascular remodeling and pathogenesis of PAH, particularly in the setting of *BMPR2* mutations or reduced BMPR2 signaling. It is possible that TGF-β and BMP signaling are opposing pathways in PAH, similar to fibrotic diseases where BMP is anti-fibrotic via TGF-β1 inhibition [45, 92].

Other Affected Cell Types in PAH

In addition to endothelial and smooth muscle cells, impaired BMP signaling plays a role in other cell types implicated in PAH. For example, increased numbers of smooth muscle actin-expressing cells are observed in PAH [225]. One possible source of these cells is endothelial-to-mesenchymal transition in conditions of reduced BMPR2 signaling [163]. Additionally, BMPR2 mutations have been associated with an increased number of endothelial progenitor cells in vascular lesions and circulation [202]. These endothelial progenitor cells while hyper-proliferative are less competent in forming capillary-like vascular networks in vitro suggesting dysregulated angiogenic activity. However, other researchers have reported reduced circulating endothelial progenitor cell numbers in PAH [48]. Further investigation is required to unravel the contribution of defective BMP signaling to the expansion of these smooth muscle-like cells/myofibroblasts [136]. Overall, the evidence would

suggest that impaired BMPR2 function leads to an imbalance between apoptosis and proliferation that may potentially contribute to vascular lesions and remodeling in PAH [128].

2.3.2 Phenotypes in Genetic Animal Models and Perturbation in Nongenetic Models

Animal models of PAH enable the investigation of impaired BMP signaling and disease pathogenesis to be extended from cellular experiments to in vivo phenotypes. Proof-of-concept studies based on genetic manipulation of *BMPR2* may not produce robust and reproducible models recapitulating all aspects of human PAH but generally show some disease phenotypes or increased susceptibility [176]. Global expression of homozygous *BMPR2*-null mutation causes mice to die during gastrulation [21]. Heterozygous *BMPR2* knockout mice develop minimally increased right ventricular systolic pressure (a surrogate for pulmonary arterial pressure) with very modest pulmonary vascular remodeling [20]. While PAH at baseline is not consistently reported in these haploinsufficient mice, under various stressors, they appear to be susceptible than wild-type mice in developing pulmonary hypertension and vascular remodeling in response to serotonin [113] or overexpression of 5-lipoxygenase [185]. Mice heterozygous for an N-terminal exon 2 deletion of BMPR2, resulting in an in-frame product missing a portion of the extracellular domain and thus potentially a hypomorphic allele, have increased susceptibility to hypoxia-induced PAH [59]. Conditional knockout mice have been generated to circumvent embryonic lethality and achieve tissue-specific ablation of BMPR2. Endothelial-targeted conditional BMPR2-null mice developed pulmonary hypertension, ventricular hypertrophy, and vascular remodeling spontaneously with variable penetrance [85]. Intriguingly, transgenic mice overexpressing siRNA targeting BMPR2 do not develop spontaneously PAH despite 90 % knockdown but display some phenotypes reminiscent of HHT [111]. In addition to haploinsufficient mutations, mice with smooth muscle [214] or endothelial-specific [122] expression of a dominant-negative BMPR2 mutation develop increased right ventricular systolic pressure and vascular remodeling. R899X is human disease-associated mutation in the carboxyl terminus of BMPR2 that causes premature truncation. Smooth muscle overexpressed R899X transgenic mice develop pulmonary hypertension with extensive pulmonary vascular remodeling [215]. Universal expression of this mutation also results in increased right ventricular systolic pressure and vascular remodeling at Denver altitude [97]. In another study, heterozygous R899X knock-in mice develop age-related PAH, displaying increased right ventricular systolic pressure and vascular remodeling but without right ventricular hypertrophy by 6 months of age [114]. Furthermore, the first reported rat genetic model with a deletion in exon 1 of BMPR2 shows vascular remodeling but no increase in pressure at 3 months of age [163]. It is clear that in a number of BMPR2-based models, an additional stimulus is required for PAH development, reminiscent of the 20 % penetrance of heritable PAH in humans.

BMPR2 has been the focus of genetic-based animal models of PAH since its major role in human PAH. Other components of or associated with the BMP signaling pathway have been manipulated. For example, homozygous knockout models of *CAV1* [232] or *SMAD9* [86] caused mice to spontaneously develop manifestation of pulmonary hypertension, supporting the pathogenicity of the human mutations at these loci. Similarly, mice with the heterozygous loss of *ACVRL1*/ALK1 are reported to have increased right ventricular systolic pressure, right ventricular hypertrophy, and pulmonary vascular remodeling [93]. BMPR2-R899X and SMAD1 compound heterozygous mice show right ventricular hypertrophy and more elevated right ventricular systolic pressure compared to mice heterozygous for BMPR2-R899X alone [114].

In addition to these animal models of PH resulting from genetic modifications of loci related to the BMP signaling pathway, nongenetic small animal models of PH exhibit features of diminished or defective BMP signaling in the pulmonary vasculature. Treatment of wild-type rats with monocrotaline, a plant alkaloid which exhibits broad cytotoxicity including against the vascular endothelium, decreases the expression of *BMPR2* and ALK3/*BMPR1B*, as well as SMAD activation and SMAD-responsive gene expression, accompanied by increased TGF-β signaling [112, 141]. Furthermore, *BMPR2* expression is also reduced in rats subjected to chronic hypoxia, a model of class III pulmonary hypertension [112]. Therefore, altered BMP signaling in nongenetic animal models underscores the importance of this pathway in PAH.

2.3.3 Interaction with Other Systems

Inflammation and Immunity

Inflammation and the immune system form an important part of the pathogenesis of PAH, as demonstrated by the presence of leukocytes in plexiform lesions, autoantibodies, increased levels of cytokines and chemokines, as well as the association of PAH with autoimmune disorders and infections such as schistosomiasis and HIV [5]. Emerging evidence suggests that dysfunctional BMP signaling is linked to a pro-inflammatory state. For example, heterozygous *BMPR2* mutant mice develop PAH in response to an inflammatory stimulus [185]. These mice also produce higher levels of interleukin-6 (IL-6) and IL-8 following lipopolysaccharide stimulation, compared with controls. Interestingly, mice expressing IL-6 under the Clara cell promoter in the lung develop severe PAH with vascular remodeling following exposure to chronic hypoxia [192]. Chronic lipopolysaccharide administration induces PAH in these *BMPR2* mutant mice, but not in wild-type controls [186]. Similarly, mutation or reduced expression of *BMPR2* in smooth muscle cells results in upregulation of IL-6 and IL-8 via p38 and/or NF-κB signaling [42, 74]. On the other hand, loss of *BMPR2* in PAECs is associated with increased expression of the chemokine granulocyte macrophage colony-stimulating factor in response to tumor necrosis factor-α [177]. In addition to cytokine and chemokine production, cells of the

immune system are also affected by BMPR2 mutations. For example, BMPR2 deficiency in the endothelium results in enhanced transmigration of leukocytes [26]. It is known that natural killer cell phenotype and function are impaired in human PAH patients [152]. Mice with heterozygous R899X mutation have reduced numbers circulating natural killer cells, possibly due to reduced levels of natural killer cell survival signal IL-15 [153]. Furthermore, defects are found in bone marrow-derived macrophages from transgenic mice expressing a dominant-negative mutation in *BMPR2*, including activation state, increased cytokine secretion [196], and higher levels of endothelin expression in response to lipopolysaccharide [195]. When challenged with schistosomiasis infection, heterozygous *BMPR2* mutant mice develop more marked pulmonary vascular remodeling, egg deposition, and cytokine production, indicating a link between dysfunctional BMPR2 signaling and response to infection [38]. One of the elevated cytokines, IL-13, mediates the activation of TGF-β signaling in pulmonary granulomas of schistosomiasis-infected mice [71].

Metabolism

PAH is increasingly recognized as a syndrome with metabolic dysfunction, as demonstrated by mitochondrial abnormalities, Warburg phenotype, and insulin resistance [162]. Impaired BMP signaling has been associated with metabolic defects from cellular to systemic level. Mutations in *BMPR2* are reported to influence cellular glucose homeostasis via with its link with PPARγ, which is the "master regulator" of insulin sensitivity [7]. The PPARγ downstream targets apolipoprotein E and adiponectin that are both regulators of metabolism and are implicated in PAH [162]. Transcriptomic analyses of *BMPR2* mutant pulmonary microvascular endothelial cells identified extensive alterations in expression of genes regulating metabolism including increased aerobic glycolysis and pentose phosphate pathway activation, decreases in carnitine and fatty acid oxidation pathways, and increased isocitrate dehydrogenase activity [57]. Besides the pulmonary vasculature, the right ventricles of *BMPR2* mutant mice demonstrate lipid deposition inside cardiomyocytes. Fatty acid oxidation is also suppressed in right ventricular tissue from human patients of heritable PAH [81]. At the whole-body level, BMPR2 mutant mice exhibit insulin resistance prior to development of PAH. These mice developed severe PAH with increased disease penetrance when fed a high-fat diet. Impaired glucocorticoid responses may contribute to the metabolic defects [216].

2.3.4 Effects of Mutations on Penetrance, Presentation, and Prognosis of PAH

Penetrance

Although the type of BMPR2 mutation has been associated with disease penetrance [14], the overall incomplete penetrance of PAH indicates that a mutation in *BMPR2* is required but insufficient for the development of PAH. Additional genetic and

environmental risk factors may be required as a "second hit" as they interact with BMP signaling and modify the risk of PAH in predisposed individuals.

As mutations in *BMPR2* are heterozygous, the unaffected wild-type allele controls the expression of *BMPR2* transcript and protein in carriers of haploinsufficient mutations. The levels of *BMPR2* transcript produced by the wild-type allele are lower in mutation carriers with PAH compared with PAH-free mutation carriers. Therefore the activity of the wild-type *BMPR2* allele has been associated with PAH disease penetrance in genetically susceptible mutation carriers [75]. In addition, the ratio between alternatively spliced isoforms of *BMPR2* is also associated with disease penetrance. Mutation-positive PAH patients have more isoform A, which is full-length BMPR2, relative to isoform B, which lacks the functionally important exon 12 [37].

Common genetic variations in the form of single-nucleotide polymorphisms may influence penetrance of PAH with carriers of nonsense-mediated decay-resistant BMPR2 mutations. Among these BMPR2 mutation carriers, those with polymorphisms of the TGF-β gene resulting in higher activity of TGF-β1 show increased penetrance of PAH [159]. This finding further supports the contribution of disequilibrium between BMP and TGF-β signaling to the pathogenesis of PAH.

PAH is known to preferentially affect females more than males [51, 167], possibly owing to the effects of estrogen and its impact on pulmonary vascular cell physiology and/or the BMP signaling pathway. Expression arrays from *BMPR2* mutation carriers with or without PAH enabled the identification of the estrogen-metabolizing enzyme CYP1B1, whose expression was tenfold lower in female mutation carriers with PAH compared with those without PAH [213], with the penetrance of PAH appearing fourfold higher in female subjects homozygous for the wild-type N/N genotype of the CYP1B1 N453S polymorphism [9]. Reduced CYP1B1 favors the synthesis of 16α-hydroxyestrone, a mitogenic and pro-proliferative estrogen metabolite [11]. Increased levels of 16α-hydroxyestrone are associated with increased penetrance of PAH in female [9] and male [56] PAH patients and in *BMPR2* mutant mice [56]. Estrogen and its metabolites directly reduce BMPR2 expression, possibly via binding of estrogen receptor alpha to the *BMPR2* promoter [13], representing one of the multiple avenues through which estrogen interacts with and regulates the BMP pathway and other pathways and systems [11].

Presentation

Mutations of BMP pathway components influence the disease phenotype, resulting in earlier onset and higher severity in general. *BMPR2* mutation carriers are diagnosed with PAH approximately 10 years earlier than *BMPR2* mutation-negative patients [69, 158, 194]. Patients with *BMPR2* mutations also present worse clinical phenotypes at diagnosis, including higher mean pulmonary artery pressure, lower cardiac index, higher pulmonary vascular resistance, and lower mixed venous oxygen saturation compared to mutation-free patients [194]. In addition, mutation-positive patients are less likely to respond in acute vasoreactivity testing [54, 170]. However, the presence of *BMPR2* mutations does not lead to worse exercise

capacity, possibly owing to their younger age [121]. *BMPR2* mutation type and position of the mutation may have an effect on PAH phenotype, as carriers of missense mutations are diagnosed at a younger age than truncation mutation carriers [14]. Patients with a point mutation within the cytoplasmic tail of BMPR2 display later onset, lower pulmonary vascular resistance and a higher proportion of response to acute vasodilator challenge as compared to patients with mutations located elsewhere. This observation might be explained by the preserved activation of the SMAD pathway in cytoplasmic tail mutants [67].

Mutations in *ACVRL1*/ALK1 have also been reported to affect PAH presentation. Carriers of *ACVRL1* mutations are diagnosed with PAH at a younger age than non-carriers and *BMPR2* mutation carriers. *ACVRL1* mutation carriers displayed better hemodynamic status at diagnosis, but none demonstrated acute vasodilator response [68]. In addition, *BMPR2* mutation carriers of more active TGF-β1 polymorphism genotypes demonstrate earlier age at diagnosis [159]. Similarly, digenic mutations, both *BMPR2* and *KCNA5*, which encode a protein that forms a part of voltage-gated potassium channels, may account for the earlier occurrence and increased severity in one patient [210].

Prognosis

The outcome of PAH patients may be affected by the presence of genetic mutations. Patients with *BMPR2* mutations have been reported to progress more rapidly with shorter time to death or lung transplantation compared with mutation-free patients [194]. However, the overall survival of *BMPR2* mutation-positive and mutation-negative patients are similar [69, 194], possibly due to the younger age of onset in mutation carriers [121]. Among the mutation types, missense mutation carriers demonstrate shorter survival and duration from diagnosis to death or lung transplantation than patients with truncating mutations [14]. Furthermore, patients with *ACVRL1* mutations also exhibit shorter survival compared with other patients with PAH [68].

2.4 Implications on Diagnosis: Genetic Testing

Discovery of mutations within the BMP pathway may facilitate the diagnosis of PAH in the form of genetic testing. It is recommended to offer genetic analysis to heritable PAH patients and possibly to idiopathic PAH patients due to the possibility that they carry a mutation [15, 128]. Asymptotic relatives of these patients may also benefit from genetic testing. The identification of a pathogenic mutation in a patient allows less costly testing for other family members [187]. Traditional methods have focused on *BMPR2*, *ACVRL1*, and *ENG* and have identified numerous mutations [4, 36]. Given the highest prevalence, genetic testing should begin with *BMPR2* unless there is a family history of HHT [12]. Additionally, it is now possible to screen for

SMAD9, *CAV1*, and *KCNK3* in North America and Europe [12]. A unified PAH mutation panel would be extremely useful. However, as the number of mutated gene expands, custom capture and next-generation sequencing should replace the expensive and labor-intensive traditional sequencing methods [121]. Genetic testing should be conducted with pretest informed consent and counseling, as well as post-test counseling, explaining the implications of the test results. The absence of mutations in the asymptotic member of an HPAH family with known mutations is reassuring as it reduces the PAH risk to near zero [12]. On the other hand, the presence of a mutation in an asymptotic individual does not necessary lead to PAH disease, owing to the reduced penetrance, but it doubles the pretest probability and increases risk yet higher in females [12]. Emerging evidence suggests that *BMPR2* mutations are associated with subtle pulmonary abnormalities in asymptotic carriers [155]. However, it is not currently possible to identify which carriers will develop PAH. Also, no interventions have yet been proven effective in preventing the development of PAH in mutation carriers. Some current PAH therapies, such as sildenafil, are also used in other conditions and may have potential in PAH prevention [12]. In order to ensure early diagnosis, asymptotic mutation carriers should undergo regular noninvasive echocardiographic screening [187]. Moreover, genetic mutations in parents have implications for reproductive planning, as one mutation carrier parent confers a 50 % chance of mutation inheritance in the offspring [12]. Preimplantation genetic diagnosis following in vitro fertilization allows the selection of mutation-free embryos and the birth of a healthy child [61].

2.5 Implications on Treatment and Proof-of-Concept Studies

Given the central importance of dysfunctional BMP signaling to PAH pathogenesis, a number of strategies correcting these defects have been tested. These approaches aim to rescue BMPR2 expression directly or enhance BMP signaling by targeting other components of the pathway [212]. Interestingly, current vasodilatory PAH drugs such as sildenafil [221] and prostacyclin analogues [220] partly rescue SMAD/Id signaling via cyclic adenosine monophosphate and cyclic guanosine monophosphate, supporting the therapeutic benefits of enhancing BMP signaling. In addition, a number of compounds showing benefits in proof-of-concept studies, such as chloroquine and tacrolimus, are also drugs approved for treatment of other conditions. This may facilitate the development of these drugs for use in PAH [137].

2.5.1 Approaches Targeting BMPR2

Owing to the high prevalence of *BMPR2* mutations and reduced BMPR2 expression with or without mutation in PAH patients, this gene has been a central focus of research in this field. One of the approaches toward enhancing BMPR2 expression has been via gene therapy vectors. Vector-targeted delivery of BMPR2 to the pulmonary

vascular endothelium was beneficial in animal models of pulmonary hypertension (PH, [164, 165]). These results are contradictory to a different study where adenoviral delivery of BMPR2 into the pulmonary vasculature did not improve monocrotaline-induced PAH [129]. This may be explained by differences in methodology, for example, in viral construction, time, and route of gene delivery. The effectiveness of single-dose gene delivery has been questioned [206], and the choice of vector and its ability to sustain expression without immunologic response is likely critical.

An alternative approach involves rescuing the expression of *BMPR2* mutants affected by premature truncation. Premature termination can be prevented by small molecules enhancing ribosomal read-through, resulting in increased expression of full-length protein, increased BMPR2 signaling, and inhibition of pulmonary vascular cell hyper-proliferation [49, 76]. On the other hand, aberrant trafficking of misfolded BMPR2 can be rescued by treatment of cells with chemical chaperones, demonstrated by enhanced cell surface BMPR2 expression and signaling [60, 184].

In order to prolong the cell surface expression of BMPR2, lysosomal degradation of BMPR2 can be inhibited by chloroquine and its analogues. Several studies have demonstrated restoration of BMPR2 signaling in vitro and prevention of PAH using chloroquine. Furthermore, chloroquine may block autophagy and promote apoptosis of PASMCs [52, 115, 175].

2.5.2 Approaches Directed on Other Components of BMP Signaling

In addition to BMPR2 expression, dysfunctional BMPR2 signaling is also amenable to modulation by targeting other components of the BMP signaling axis, including ligands, other receptors and associated proteins, and downstream mediators.

Systemic administration of BMP9, which binds BMPR2/ALK1 receptor complexes on endothelial cells, reverses established PAH in genetic and nongenetic animal models, without inducing ossification [114]. Intriguingly, BMP9 administration also increased *BMPR2* expression in a SMAD-dependent manner [114]. This study supports the therapeutic potential of BMP9, BMP10, and their analogues.

FK-binding protein 12 (FKBP12), a repressor of BMP signaling, has been targeted in PAH. Treatment of vascular cells with tacrolimus releases FKBP12 from type I BMP receptors, potentiating the activation of downstream signaling. In vivo treatment with tacrolimus was reported to reverse PH in the rat [189]. This drug is currently tested in a clinical trial and is reported to show benefit in initial patients [188]. BMPR2 signaling can be enhanced by the endogenous elastase inhibitor elafin, which stabilizes CAV1 on the cell surface and augments interaction between BMPR2 and CAV1. Elafin improves endothelial function, induces PASMC apoptosis, and reverses established PAH in rats [146]. Additionally, suppression of TGF-β signaling with various small molecule inhibitors of the activin/TGF-β type I receptor ALK4/ALK5/ALK6 kinases attenuates both PH and pulmonary vascular remod-

eling in monocrotaline-treated rats [112, 200, 230], indicating that the exaggerated TGF-β signaling seen in the presence of defective BMP signaling represents a bona fide target. Recently our group found that a more selective approach to inhibiting TGF-β1 and TGF-β3 signaling using a recombinant TGFBRII extracellular domain fused to the immunoglobulin Fc domain as a ligand trap was also effective in abrogating PH and pulmonary vascular remodeling not only in monocrotaline-treated rats but also markedly improved survival in these animals, as well as improving PH and related endpoints in mice and rats treated with the combination of VEGFR1/VEGFR2 inhibitor SU5416 and hypoxia ([229], accepted).

Inside the cell, the functions of SMAD8 (SMAD9) affected by a nonsense mutation can be rescued by the read-through-promoting molecule ataluren, similar to BMPR2 mutants [49]. PPARγ and its downstream targets such as platelet-derived growth factor receptor have been targeted in PAH models and have shown benefits [162]. As BMP signaling plays a role in regulation of cytoskeleton, correction of cytoskeletal impairment using human recombinant angiotensin-converting enzyme 2, possibly acting by correcting Rac1 defects, has reversed pulmonary hypertension in mice with universal expression of the heterozygous R899X mutation [97].

3 Remaining Questions and Directions for Future Research

In conclusion, our understanding of the BMP signaling pathway and its roles in PAH has advanced significantly in the last 15 years, since the initial discovery of mutations in *BMPR2* as the most common explanation for cases of heritable PAH. More recently, improved sequencing technologies have enabled the association of additional components of the BMP pathway as well as other gene loci with PAH, as well as numerous other polymorphisms and genomic and epigenetic alterations. The functional consequences of these genetic lesions are beginning to be understood at molecular, cellular, and system levels but will require substantial further elucidation. This growing body of knowledge supports the potential for therapeutic intervention aiming to rectify dysfunctional BMP signaling in PAH and may also provide opportunities for correcting aberrant BMP signaling in other conditions such as HHT, vascular inflammation, calcification and atherosclerosis [47], anemia of chronic disease [125, 191], and heterotopic ossification [132, 133, 228]. However, fundamental questions still remain on the mechanisms linking the mutations to disease, the involvement of a necessary "second hit," genetic, epigenetic, environmental, infectious, endocrine, or otherwise, to fully explain the phenomena of reduced penetrance and gender bias. Future investigations in the next few years will continue to search for the answers and will also test the exploitation of BMP signaling in the treatment of human cardiovascular diseases including but not limited to PAH.

References

1. Abdalla SA, Gallione CJ, Barst RJ, Horn EM, Knowles JA, Marchuk DA et al (2004) Primary pulmonary hypertension in families with hereditary haemorrhagic telangiectasia. Eur Respir J. 23(3):373–377
2. Alastalo TP, Li M, de Jesus Perez VA, Pham D, Sawada H, Wang JK et al (2011) Disruption of PPARγ/β-catenin-mediated regulation of apelin impairs BMP-induced mouse and human pulmonary arterial EC survival. J Clin Invest. 121(9):3735–3746
3. Aldred MA, Comhair SA, Varella-Garcia M, Asosingh K, Xu W, Noon GP et al (2010) Somatic chromosome abnormalities in the lungs of patients with pulmonary arterial hypertension. Am J Respir Crit Care Med. 182(9):1153–1160
4. Aldred MA, Vijayakrishnan J, James V, Soubrier F, Gomez-Sanchez MA, Martensson G et al (2006) BMPR2 gene rearrangements account for a significant proportion of mutations in familial and idiopathic pulmonary arterial hypertension. Hum Mutat. 27(2):212–213
5. Archer SL, Weir EK, Wilkins MR (2010) Basic science of pulmonary arterial hypertension for clinicians: new concepts and experimental therapies. Circulation. 121(18):2045–2066
6. Aspalter IM, Gordon E, Dubrac A, Ragab A, Narloch J, Vizán P et al (2015) Alk1 and Alk5 inhibition by Nrp1 controls vascular sprouting downstream of Notch. Nat Commun. 6:7264
7. Assad TR, Hemnes AR (2015) Metabolic Dysfunction in Pulmonary Arterial Hypertension. Curr Hypertens Rep. 17(3):20
8. Atkinson C, Stewart S, Upton PD, Machado R, Thomson JR, Trembath RC et al (2002) Primary pulmonary hypertension is associated with reduced pulmonary vascular expression of type II bone morphogenetic protein receptor. Circulation. 105(14):1672–1678
9. Austin ED, Cogan JD, West JD, Hedges LK, Hamid R, Dawson EP et al (2009) Alterations in oestrogen metabolism: implications for higher penetrance of familial pulmonary arterial hypertension in females. Eur Respir J. 34(5):1093–1099
10. Austin ED, Hamid R, Hemnes AR, Loyd JE, Blackwell T, Yu C et al (2012) BMPR2 expression is suppressed by signaling through the estrogen receptor. Biol Sex Differ. 3(1):6
11. Austin ED, Lahm T, West J, Tofovic SP, Johansen AK, Maclean MR et al (2013) Gender, sex hormones and pulmonary hypertension. Pulm Circ. 3(2):294–314
12. Austin ED, Loyd JE (2014) The genetics of pulmonary arterial hypertension. Circ Res. 115(1):189–202
13. Austin ED, Ma L, LeDuc C, Berman Rosenzweig E, Borczuk A, Phillips JA et al (2012) Whole exome sequencing to identify a novel gene (caveolin-1) associated with human pulmonary arterial hypertension. Circ Cardiovasc Genet. 5(3):336–343
14. Austin ED, Phillips JA, Cogan JD, Hamid R, Yu C, Stanton KC et al (2009) Truncating and missense BMPR2 mutations differentially affect the severity of heritable pulmonary arterial hypertension. Respir Res. 10:87
15. Badesch DB, Abman SH, Simonneau G, Rubin LJ, McLaughlin VV (2007) Medical therapy for pulmonary arterial hypertension: updated ACCP evidence-based clinical practice guidelines. Chest. 131(6):1917–1928
16. Bagarova J, Vonner AJ, Armstrong KA, Börgermann J, Lai CS, Deng DY et al (2013) Constitutively active ALK2 receptor mutants require type II receptor cooperation. Mol Cell Biol. 33(12):2413–2424
17. Bellusci S, Henderson R, Winnier G, Oikawa T, Hogan BL (1996) Evidence from normal expression and targeted misexpression that bone morphogenetic protein (Bmp-4) plays a role in mouse embryonic lung morphogenesis. Development. 122(6):1693–1702
18. Benezra R, Rafii S, Lyden D (2001) The Id proteins and angiogenesis. Oncogene. 20(58):8334–8341
19. Benza RL, Miller DP, Barst RJ, Badesch DB, Frost AE, McGoon MD (2012) An evaluation of long-term survival from time of diagnosis in pulmonary arterial hypertension from the REVEAL Registry. Chest. 142(2):448–456

20. Beppu H, Ichinose F, Kawai N, Jones RC, Yu PB, Zapol WM et al (2004) BMPR-II heterozygous mice have mild pulmonary hypertension and an impaired pulmonary vascular remodeling response to prolonged hypoxia. Am J Physiol Lung Cell Mol Physiol. 287(6):L1241–L1247
21. Beppu H, Kawabata M, Hamamoto T, Chytil A, Minowa O, Noda T et al (2000) BMP type II receptor is required for gastrulation and early development of mouse embryos. Dev Biol. 221(1):249–258
22. Bertero T, Lu Y, Annis S, Hale A, Bhat B, Saggar R et al (2014) Systems-level regulation of microRNA networks by miR-130/301 promotes pulmonary hypertension. J Clin Invest. 124(8):3514–3528
23. Bitgood MJ, McMahon AP (1995) Hedgehog and Bmp genes are coexpressed at many diverse sites of cell-cell interaction in the mouse embryo. Dev Biol. 172(1):126–138
24. Boergermann JH, Kopf J, Yu PB, Knaus P (2010) Dorsomorphin and LDN-193189 inhibit BMP-mediated Smad, p38 and Akt signalling in C2C12 cells. Int J Biochem Cell Biol. 42(11):1802–1807
25. Boström K, Watson KE, Horn S, Wortham C, Herman IM, Demer LL (1993) Bone morphogenetic protein expression in human atherosclerotic lesions. J Clin Invest. 91(4):1800–1809
26. Burton VJ, Ciuclan LI, Holmes AM, Rodman DM, Walker C, Budd DC (2011) Bone morphogenetic protein receptor II regulates pulmonary artery endothelial cell barrier function. Blood. 117(1):333–341
27. Cai J, Pardali E, Sánchez-Duffhues G, ten Dijke P (2012) BMP signaling in vascular diseases. FEBS Lett. 586(14):1993–2002
28. Chaouat A, Coulet F, Favre C, Simonneau G, Weitzenblum E, Soubrier F et al (2004) Endoglin germline mutation in a patient with hereditary haemorrhagic telangiectasia and dexfenfluramine associated pulmonary arterial hypertension. Thorax. 59(5):446–448
29. Chen H, Shi S, Acosta L, Li W, Lu J, Bao S et al (2004) BMP10 is essential for maintaining cardiac growth during murine cardiogenesis. Development. 131(9):2219–2231
30. Chen W, Guo Y, Walker EJ, Shen F, Jun K, Oh SP et al (2013) Reduced mural cell coverage and impaired vessel integrity after angiogenic stimulation in the Alk1-deficient brain. Arterioscler Thromb Vasc Biol. 33(2):305–310
31. Chida A, Shintani M, Nakayama T, Furutani Y, Hayama E, Inai K et al (2012) Missense mutations of the BMPR1B (ALK6) gene in childhood idiopathic pulmonary arterial hypertension. Circ J. 76(6):1501–1508
32. Chida A, Shintani M, Yagi H, Fujiwara M, Kojima Y, Sato H et al (2012) Outcomes of childhood pulmonary arterial hypertension in BMPR2 and ALK1 mutation carriers. Am J Cardiol. 110(4):586–593
33. Choi EJ, Kim YH, Choe SW, Tak YG, Garrido-Martin EM, Chang M et al (2013) Enhanced responses to angiogenic cues underlie the pathogenesis of hereditary hemorrhagic telangiectasia 2. PLoS One. 8(5):e63138
34. Choi EJ, Walker EJ, Shen F, Oh SP, Arthur HM, Young WL et al (2012) Minimal homozygous endothelial deletion of Eng with VEGF stimulation is sufficient to cause cerebrovascular dysplasia in the adult mouse. Cerebrovasc Dis. 33(6):540–547
35. Ciumas M, Eyries M, Poirier O, Maugenre S, Dierick F, Gambaryan N et al (2013) Bone morphogenetic proteins protect pulmonary microvascular endothelial cells from apoptosis by upregulating α-B-crystallin. Arterioscler Thromb Vasc Biol. 33(11):2577–2584
36. Cogan JD, Pauciulo MW, Batchman AP, Prince MA, Robbins IM, Hedges LK et al (2006) High frequency of BMPR2 exonic deletions/duplications in familial pulmonary arterial hypertension. Am J Respir Crit Care Med. 174(5):590–598
37. Cogan J, Austin E, Hedges L, Womack B, West J, Loyd J et al (2012) Role of BMPR2 alternative splicing in heritable pulmonary arterial hypertension penetrance. Circulation. 126(15):1907–1916
38. Crosby A, Soon E, Jones FM, Southwood MR, Haghighat L, Toshner MR et al (2015) Hepatic shunting of eggs and pulmonary vascular remodeling in Bmpr2(+/−) mice with schistosomiasis. Am J Respir Crit Care Med 192:1355

39. Cunha SI, Pardali E, Thorikay M, Anderberg C, Hawinkels L, Goumans MJ et al (2010) Genetic and pharmacological targeting of activin receptor-like kinase 1 impairs tumor growth and angiogenesis. J Exp Med. 207(1):85–100
40. David L, Mallet C, Keramidas M, Lamandé N, Gasc JM, Dupuis-Girod S et al (2008) Bone morphogenetic protein-9 is a circulating vascular quiescence factor. Circ Res. 102(8): 914–922
41. David L, Mallet C, Mazerbourg S, Feige JJ, Bailly S (2007) Identification of BMP9 and BMP10 as functional activators of the orphanation of BMP9 and BMP10 as functional activators of the orphan activin receptor-like kinase 1 (ALK1) in endothelial cells. Blood 109(5):1953–1961
42. Davies RJ, Holmes AM, Deighton J, Long L, Yang X, Barker L et al (2012) BMP type II receptor deficiency confers resistance to growth inhibition by TGF-β in pulmonary artery smooth muscle cells: role of proinflammatory cytokines. Am J Physiol Lung Cell Mol Physiol. 302(6):L604–L615
43. de Jesus Perez VA, Alastalo TP, Wu JC, Axelrod JD, Cooke JP, Amieva M et al (2009) Bone morphogenetic protein 2 induces pulmonary angiogenesis via Wnt-beta-catenin and Wnt-RhoA-Rac1 pathways. J Cell Biol. 184(1):83–99
44. de Jesus Perez VA, Ali Z, Alastalo TP, Ikeno F, Sawada H, Lai YJ et al (2011) BMP promotes motility and represses growth of smooth muscle cells by activation of tandem Wnt pathways. J Cell Biol. 192(1):171–188
45. De Langhe E, Cailotto F, De Vooght V, Aznar-Lopez C, Vanoirbeek JA, Luyten FP et al (2015) Enhanced endogenous bone morphogenetic protein signaling protects against bleomycin induced pulmonary fibrosis. Respir Res. 16:38
46. Deng Z, Morse JH, Slager SL, Cuervo N, Moore KJ, Venetos G et al (2000) Familial primary pulmonary hypertension (gene PPH1) is caused by mutations in the bone morphogenetic protein receptor-II gene. Am J Hum Genet. 67(3):737–744
47. Derwall M, Malhotra R, Lai CS, Beppu Y, Aikawa E, Seehra JS et al (2012) Inhibition of bone morphogenetic protein signaling reduces vascular calcification and atherosclerosis. Arterioscler Thromb Vasc Biol. 32(3):613–622
48. Diller GP, Thum T, Wilkins MR, Wharton J (2010) Endothelial progenitor cells in pulmonary arterial hypertension. Trends Cardiovasc Med. 20(1):22–29
49. Drake KM, Dunmore BJ, McNelly LN, Morrell NW, Aldred MA (2013) Correction of nonsense BMPR2 and SMAD9 mutations by ataluren in pulmonary arterial hypertension. Am J Respir Cell Mol Biol. 49(3):403–409
50. Drake KM, Zygmunt D, Mavrakis L, Harbor P, Wang L, Comhair SA et al (2011) Altered MicroRNA processing in heritable pulmonary arterial hypertension: an important role for Smad-8. Am J Respir Crit Care Med. 184(12):1400–1408
51. Dresdale DT, Schultz M, Michtom RJ (1951) Primary pulmonary hypertension. I. Clinical and hemodynamic study. Am J Med. 11(6):686–705
52. Dunmore BJ, Drake KM, Upton PD, Toshner MR, Aldred MA, Morrell NW (2013) The lysosomal inhibitor, chloroquine, increases cell surface BMPR-II levels and restores BMP9 signalling in endothelial cells harbouring BMPR-II mutations. Hum Mol Genet. 22(18):3667–3679
53. El-Bizri N, Guignabert C, Wang L, Cheng A, Stankunas K, Chang CP et al (2008) SM22alpha-targeted deletion of bone morphogenetic protein receptor 1 A in mice impairs cardiac and vascular development, and influences organogenesis. Development. 135(17):2981–2991
54. Elliott CG, Glissmeyer EW, Havlena GT, Carlquist J, McKinney JT, Rich S et al (2006) Relationship of BMPR2 mutations to vasoreactivity in pulmonary arterial hypertension. Circulation. 113(21):2509–2515
55. Farber HW, Miller DP, Poms AD, Badesch DB, Frost AE, Muros-Le Rouzic E et al (2015) Five-Year Outcomes of Patients Enrolled in the REVEAL Registry. Chest. 148(4):1043–1054
56. Fessel JP, Chen X, Frump A, Gladson S, Blackwell T, Kang C et al (2013) Interaction between bone morphogenetic protein receptor type 2 and estrogenic compounds in pulmonary arterial hypertension. Pulm Circ. 3(3):564–577

57. Fessel JP, Hamid R, Wittmann BM, Robinson LJ, Blackwell T, Tada Y et al (2012) Metabolomic analysis of bone morphogenetic protein receptor type 2 mutations in human pulmonary endothelium reveals widespread metabolic reprogramming. Pulm Circ. 2(2):201–213
58. Foletta VC, Lim MA, Soosairajah J, Kelly AP, Stanley EG, Shannon M et al (2003) Direct signaling by the BMP type II receptor via the cytoskeletal regulator LIMK1. J Cell Biol. 162(6):1089–1098
59. Frank DB, Lowery J, Anderson L, Brink M, Reese J, de Caestecker M (2008) Increased susceptibility to hypoxic pulmonary hypertension in Bmpr2 mutant mice is associated with endothelial dysfunction in the pulmonary vasculature. Am J Physiol Lung Cell Mol Physiol. 294(1):L98–109
60. Frump AL, Lowery JW, Hamid R, Austin ED, de Caestecker M (2013) Abnormal trafficking of endogenously expressed BMPR2 mutant allelic products in patients with heritable pulmonary arterial hypertension. PLoS One. 8(11):e80319
61. Frydman N, Steffann J, Girerd B, Frydman R, Munnich A, Simonneau G et al (2012) Preimplantation genetic diagnosis in pulmonary arterial hypertension due to BMPR2 mutation. Eur Respir J. 39(6):1534–1535
62. Fujiwara M, Yagi H, Matsuoka R, Akimoto K, Furutani M, Imamura S et al (2008) Implications of mutations of activin receptor-like kinase 1 gene (ALK1) in addition to bone morphogenetic protein receptor II gene (BMPR2) in children with pulmonary arterial hypertension. Circ J. 72(1):127–133
63. Gaine SP, Rubin LJ (1998) Primary pulmonary hypertension. Lancet. 352(9129):719–725
64. Gallione CJ, Repetto GM, Legius E, Rustgi AK, Schelley SL, Tejpar S et al (2004) A combined syndrome of juvenile polyposis and hereditary haemorrhagic telangiectasia associated with mutations in MADH4 (SMAD4). Lancet. 363(9412):852–859
65. Gallione CJ, Richards JA, Letteboer TG, Rushlow D, Prigoda NL, Leedom TP et al (2006) SMAD4 mutations found in unselected HHT patients. J Med Genet. 43(10):793–797
66. Gangopahyay A, Oran M, Bauer EM, Wertz JW, Comhair SA, Erzurum SC et al (2011) Bone morphogenetic protein receptor II is a novel mediator of endothelial nitric-oxide synthase activation. J Biol Chem. 286(38):33134–33140
67. Girerd B, Coulet F, Jaïs X, Eyries M, Van Der Bruggen C, De Man F et al (2015) Characteristics of pulmonary arterial hypertension in affected carriers of a mutation located in the cytoplasmic tail of bone morphogenetic protein receptor type 2. Chest. 147(5):1385–1394
68. Girerd B, Montani D, Coulet F, Sztrymf B, Yaici A, Jaïs X et al (2010) Clinical outcomes of pulmonary arterial hypertension in patients carrying an ACVRL1 (ALK1) mutation. Am J Respir Crit Care Med. 181(8):851–861
69. Girerd B, Montani D, Eyries M, Yaici A, Sztrymf B, Coulet F et al (2010) Absence of influence of gender and BMPR2 mutation type on clinical phenotypes of pulmonary arterial hypertension. Respir Res. 11:73
70. González-Núñez M, Muñoz-Félix JM, López-Novoa JM (2013) The ALK-1/Smad1 pathway in cardiovascular physiopathology. A new target for therapy? Biochim Biophys Acta. 1832(10):1492–1510
71. Graham BB, Mentink-Kane MM, El-Haddad H, Purnell S, Zhang L, Zaiman A et al (2010) Schistosomiasis-induced experimental pulmonary hypertension: role of interleukin-13 signaling. Am J Pathol. 177(3):1549–1561
72. Greenwald J, Fischer WH, Vale WW, Choe S (1999) Three-finger toxin fold for the extracellular ligand-binding domain of the type II activin receptor serine kinase. Nat Struct Biol. 6(1):18–22
73. Guignabert C, Alvira CM, Alastalo TP, Sawada H, Hansmann G, Zhao M et al (2009) Tie2-mediated loss of peroxisome proliferator-activated receptor-gamma in mice causes PDGF receptor-beta-dependent pulmonary arterial muscularization. Am J Physiol Lung Cell Mol Physiol. 297(6):L1082–L1090
74. Hagen M, Fagan K, Steudel W, Carr M, Lane K, Rodman DM et al (2007) Interaction of interleukin-6 and the BMP pathway in pulmonary smooth muscle. Am J Physiol Lung Cell Mol Physiol. 292(6):L1473–L1479

75. Hamid R, Cogan JD, Hedges LK, Austin E, Phillips JA, Newman JH et al (2009) Penetrance of pulmonary arterial hypertension is modulated by the expression of normal BMPR2 allele. Hum Mutat. 30(4):649–654
76. Hamid R, Hedges LK, Austin E, Phillips JA, Loyd JE, Cogan JD (2010) Transcripts from a novel BMPR2 termination mutation escape nonsense mediated decay by downstream translation re-initiation: implications for treating pulmonary hypertension. Clin Genet. 77(3):280–286
77. Hansmann G, de Jesus Perez VA, Alastalo TP, Alvira CM, Guignabert C, Bekker JM et al (2008) An antiproliferative BMP-2/PPARgamma/apoE axis in human and murine SMCs and its role in pulmonary hypertension. J Clin Invest. 118(5):1846–1857
78. Harrison RE, Berger R, Haworth SG, Tulloh R, Mache CJ, Morrell NW et al (2005) Transforming growth factor-beta receptor mutations and pulmonary arterial hypertension in childhood. Circulation. 111(4):435–441
79. Harrison RE, Flanagan JA, Sankelo M, Abdalla SA, Rowell J, Machado RD et al (2003) Molecular and functional analysis identifies ALK-1 as the predominant cause of pulmonary hypertension related to hereditary haemorrhagic telangiectasia. J Med Genet. 40(12):865–871
80. Hawinkels LJ, de Vinuesa AG, Paauwe M, Kruithof-de Julio M, Wiercinska E, Pardali E, et al. Activin receptor-like kinase 1 ligand trap reduces microvascular density and improves chemotherapy efficiency to various solid tumors. Clin Cancer Res. 2015;22:96.
81. Hemnes AR, Brittain EL, Trammell AW, Fessel JP, Austin ED, Penner N et al (2014) Evidence for right ventricular lipotoxicity in heritable pulmonary arterial hypertension. Am J Respir Crit Care Med. 189(3):325–334
82. Herrera B, Inman GJ (2009) A rapid and sensitive bioassay for the simultaneous measurement of multiple bone morphogenetic proteins. Identification and quantification of BMP4, BMP6 and BMP9 in bovine and human serum. BMC Cell Biol. 10:20
83. Hines EA, Sun X (2014) Tissue crosstalk in lung development. J Cell Biochem. 115(9):1469–1477
84. Hoeper MM, Bogaard HJ, Condliffe R, Frantz R, Khanna D, Kurzyna M et al (2013) Definitions and diagnosis of pulmonary hypertension. J Am Coll Cardiol. 62(25 Suppl):D42–D50
85. Hong KH, Lee YJ, Lee E, Park SO, Han C, Beppu H et al (2008) Genetic ablation of the BMPR2 gene in pulmonary endothelium is sufficient to predispose to pulmonary arterial hypertension. Circulation. 118(7):722–730
86. Huang Z, Wang D, Ihida-Stansbury K, Jones PL, Martin JF (2009) Defective pulmonary vascular remodeling in Smad8 mutant mice. Hum Mol Genet. 18(15):2791–2801
87. Humbert M, Deng Z, Simonneau G, Barst RJ, Sitbon O, Wolf M et al (2002) BMPR2 germline mutations in pulmonary hypertension associated with fenfluramine derivatives. Eur Respir J. 20(3):518–523
88. Humbert M, Sitbon O, Chaouat A, Bertocchi M, Habib G, Gressin V et al (2006) Pulmonary arterial hypertension in France: results from a national registry. Am J Respir Crit Care Med. 173(9):1023–1030
89. Humbert M, Sitbon O, Chaouat A, Bertocchi M, Habib G, Gressin V et al (2010) Survival in patients with idiopathic, familial, and anorexigen-associated pulmonary arterial hypertension in the modern management era. Circulation. 122(2):156–163
90. Itoh F, Itoh S, Goumans MJ, Valdimarsdottir G, Iso T, Dotto GP et al (2004) Synergy and antagonism between Notch and BMP receptor signaling pathways in endothelial cells. EMBO J. 23(3):541–551
91. Izikki M, Guignabert C, Fadel E, Humbert M, Tu L, Zadigue P et al (2009) Endothelial-derived FGF2 contributes to the progression of pulmonary hypertension in humans and rodents. J Clin Invest. 119(3):512–523
92. Izumi N, Mizuguchi S, Inagaki Y, Saika S, Kawada N, Nakajima Y et al (2006) BMP-7 opposes TGF-beta1-mediated collagen induction in mouse pulmonary myofibroblasts through Id2. Am J Physiol Lung Cell Mol Physiol. 290(1):L120–L126
93. Jerkic M, Kabir MG, Davies A, Yu LX, McIntyre BA, Husain NW et al (2011) Pulmonary hypertension in adult Alk1 heterozygous mice due to oxidative stress. Cardiovasc Res. 92(3):375–384

94. Jiang Y, Nohe A, Bragdon B, Tian C, Rudarakanchana N, Morrell NW et al (2011) Trapping of BMP receptors in distinct membrane domains inhibits their function in pulmonary arterial hypertension. Am J Physiol Lung Cell Mol Physiol 301(2):L218–L227
95. Jin Y, Kaluza D, Jakobsson L (2014) VEGF, Notch and TGFβ/BMPs in regulation of sprouting angiogenesis and vascular patterning. Biochem Soc Trans. 42(6):1576–1583
96. Johnson DW, Berg JN, Gallione CJ, McAllister KA, Warner JP, Helmbold EA et al (1995) A second locus for hereditary hemorrhagic telangiectasia maps to chromosome 12. Genome Res. 5(1):21–28
97. Johnson JA, Hemnes AR, Perrien DS, Schuster M, Robinson LJ, Gladson S et al (2012) Cytoskeletal defects in Bmpr2-associated pulmonary arterial hypertension. Am J Physiol Lung Cell Mol Physiol. 302(5):L474–L484
98. Kahr PC, Piccini I, Fabritz L, Greber B, Schöler H, Scheld HH et al (2011) Systematic analysis of gene expression differences between left and right atria in different mouse strains and in human atrial tissue. PLoS One. 6(10):e26389
99. Kamiya N, Kobayashi T, Mochida Y, Yu PB, Yamauchi M, Kronenberg HM et al (2010) Wnt inhibitors Dkk1 and Sost are downstream targets of BMP signaling through the type IA receptor (BMPRIA) in osteoblasts. J Bone Miner Res. 25(2):200–210
100. Kang H, Davis-Dusenbery BN, Nguyen PH, Lal A, Lieberman J, Van Aelst L et al (2012) Bone morphogenetic protein 4 promotes vascular smooth muscle contractility by activating microRNA-21 (miR-21), which down-regulates expression of family of dedicator of cytokinesis (DOCK) proteins. J Biol Chem. 287(6):3976–3986
101. Kerr G, Sheldon H, Chaikuad A, Alfano I, von Delft F, Bullock AN et al (2015) A small molecule targeting ALK1 prevents Notch cooperativity and inhibits functional angiogenesis. Angiogenesis. 18(2):209–217
102. Kim J, Kang Y, Kojima Y, Lighthouse JK, Hu X, Aldred MA et al (2013) An endothelial apelin-FGF link mediated by miR-424 and miR-503 is disrupted in pulmonary arterial hypertension. Nat Med. 19(1):74–82
103. Kim S, Hata A, Kang H (2014) Down-regulation of miR-96 by bone morphogenetic protein signaling is critical for vascular smooth muscle cell phenotype modulation. J Cell Biochem. 115(5):889–895
104. Lagna G, Ku MM, Nguyen PH, Neuman NA, Davis BN, Hata A (2007) Control of phenotypic plasticity of smooth muscle cells by bone morphogenetic protein signaling through the myocardin-related transcription factors. J Biol Chem. 282(51):37244–37255
105. KB L, RD M, MW P, JR T, JA P, International PPH Consortium et al (2000) Heterozygous germline mutations in BMPR2, encoding a TGF-beta receptor, cause familial primary pulmonary hypertension. Nat Genet. 26(1):81–84
106. Lane KL, Talati M, Austin E, Hemnes AR, Johnson JA, Fessel JP et al (2011) Oxidative injury is a common consequence of BMPR2 mutations. Pulm Circ. 1(1):72–83
107. Larrivée B, Prahst C, Gordon E, del Toro R, Mathivet T, Duarte A et al (2012) ALK1 signaling inhibits angiogenesis by cooperating with the Notch pathway. Dev Cell. 22(3):489–500
108. Laux DW, Young S, Donovan JP, Mansfield CJ, Upton PD, Roman BL (2013) Circulating Bmp10 acts through endothelial Alk1 to mediate flow-dependent arterial quiescence. Development. 140(16):3403–3412
109. Limsuwan A, Choubtum L, Wattanasirichaigoon D (2013) 5'UTR repeat polymorphisms of the BMPR2 gene in children with pulmonary hypertension associated with congenital heart disease. Heart Lung Circ. 22(3):204–210
110. Little SC, Mullins MC (2009) Bone morphogenetic protein heterodimers assemble heteromeric type I receptor complexes to pattern the dorsoventral axis. Nat Cell Biol. 11(5):637–643
111. Liu D, Wang J, Kinzel B, Müeller M, Mao X, Valdez R et al (2007) Dosage-dependent requirement of BMP type II receptor for maintenance of vascular integrity. Blood. 110(5):1502–1510
112. Long L, Crosby A, Yang X, Southwood M, Upton PD, Kim DK et al (2009) Altered bone morphogenetic protein and transforming growth factor-beta signaling in rat models of pulmonary hypertension: potential for activin receptor-like kinase-5 inhibition in prevention and progression of disease. Circulation. 119(4):566–576

113. Long L, MacLean MR, Jeffery TK, Morecroft I, Yang X, Rudarakanchana N et al (2006) Serotonin increases susceptibility to pulmonary hypertension in BMPR2-deficient mice. Circ Res. 98(6):818–827
114. Long L, Ormiston ML, Yang X, Southwood M, Gräf S, Machado RD et al (2015) Selective enhancement of endothelial BMPR-II with BMP9 reverses pulmonary arterial hypertension. Nat Med. 21(7):777–785
115. Long L, Yang X, Southwood M, Lu J, Marciniak SJ, Dunmore BJ et al (2013) Chloroquine prevents progression of experimental pulmonary hypertension via inhibition of autophagy and lysosomal bone morphogenetic protein type II receptor degradation. Circ Res. 112(8):1159–1170
116. Loyd JE, Primm RK, Newman JH (1984) Familial primary pulmonary hypertension: clinical patterns. Am Rev Respir Dis. 129(1):194–197
117. Loyd JE, Slovis B, Phillips JA, Butler MG, Foroud TM, Conneally PM et al (1997) The presence of genetic anticipation suggests that the molecular basis of familial primary pulmonary hypertension may be trinucleotide repeat expansion. Chest. 111(6 Suppl): 82S–83S
118. Machado RD, Aldred MA, James V, Harrison RE, Patel B, Schwalbe EC et al (2006) Mutations of the TGF-beta type II receptor BMPR2 in pulmonary arterial hypertension. Hum Mutat. 27(2):121–132
119. Machado RD, Eickelberg O, Elliott CG, Geraci MW, Hanaoka M, Loyd JE et al (2009) Genetics and genomics of pulmonary arterial hypertension. J Am Coll Cardiol. 54(1 Suppl):S32–S42
120. Machado RD, Rudarakanchana N, Atkinson C, Flanagan JA, Harrison R, Morrell NW et al (2003) Functional interaction between BMPR-II and Tctex-1, a light chain of Dynein, is isoform-specific and disrupted by mutations underlying primary pulmonary hypertension. Hum Mol Genet. 12(24):3277–3286
121. Machado RD, Southgate L, Eichstaedt CA, Aldred MA, Austin ED, Best DH et al (2015) Pulmonary Arterial Hypertension: A Current Perspective on Established and Emerging Molecular Genetic Defects. Hum Mutat. 36(12):1113–1127
122. Majka S, Hagen M, Blackwell T, Harral J, Johnson JA, Gendron R et al (2011) Physiologic and molecular consequences of endothelial Bmpr2 mutation. Respir Res. 12:84
123. Malhotra R, Paskin-Flerlage S, Zamanian RT, Zimmerman P, Schmidt JW, Deng DY, Southwood M, Spencer R, Lai CS, Parker W, Channick RN, Morrell NW, Elliott CG, Yu PB (2013) Circulating angiogenic modulatory factors predict survival and functional class in pulmonary arterial hypertension. Pulm Circ. 3(2):369–380
124. Malhotra R, Burke MF, Martyn T, Shakartzi HR, Thayer TE, O'Rourke C et al (2015) Inhibition of bone morphogenetic protein signal transduction prevents the medial vascular calcification associated with matrix Gla protein deficiency. PLoS One. 10(1):e0117098
125. Mayeur C, Kolodziej SA, Wang A, Xu X, Lee A, Yu PB et al (2015) Oral administration of a bone morphogenetic protein type I receptor inhibitor prevents the development of anemia of inflammation. Haematologica. 100(2):e68–e71
126. McAllister KA, Grogg KM, Johnson DW, Gallione CJ, Baldwin MA, Jackson CE et al (1994) Endoglin, a TGF-beta binding protein of endothelial cells, is the gene for hereditary haemorrhagic telangiectasia type 1. Nat Genet. 8(4):345–351
127. McDonald J, Bayrak-Toydemir P, Pyeritz RE (2011) Hereditary hemorrhagic telangiectasia: an overview of diagnosis, management, and pathogenesis. Genet Med. 13(7):607–616
128. McLaughlin VV, Archer SL, Badesch DB, Barst RJ, Farber HW, Lindner JR et al (2009) ACCF/AHA 2009 expert consensus document on pulmonary hypertension: a report of the American College of Cardiology Foundation Task Force on Expert Consensus Documents and the American Heart Association: developed in collaboration with the American College of Chest Physicians, American Thoracic Society, Inc., and the Pulmonary Hypertension Association. Circulation. 119(16):2250–2294

129. McMurtry MS, Moudgil R, Hashimoto K, Bonnet S, Michelakis ED, Archer SL (2007) Overexpression of human bone morphogenetic protein receptor 2 does not ameliorate monocrotaline pulmonary arterial hypertension. Am J Physiol Lung Cell Mol Physiol. 292(4):L872–L878
130. Melmon KL, Braunwald E (1963) Familial pulmonary hypertension. N Engl J Med. 269:770–775
131. Miller AF, Harvey SA, Thies RS, Olson MS (2000) Bone morphogenetic protein-9. An autocrine/paracrine cytokine in the liver. J Biol Chem. 275(24):17937–17945
132. Mohedas AH, Wang Y, Sanvitale CE, Canning P, Choi S, Xing X et al (2014) Structure-activity relationship of 3,5-diaryl-2-aminopyridine ALK2 inhibitors reveals unaltered binding affinity for fibrodysplasia ossificans progressiva causing mutants. J Med Chem. 57(19): 7900–7915
133. Mohedas AH, Xing X, Armstrong KA, Bullock AN, Cuny GD, Yu PB (2013) Development of an ALK2-biased BMP type I receptor kinase inhibitor. ACS Chem Biol. 8(6): 1291–1302
134. Montani D, Achouh L, Dorfmüller P, Le Pavec J, Sztrymf B, Tchérakian C et al (2008) Pulmonary veno-occlusive disease: clinical, functional, radiologic, and hemodynamic characteristics and outcome of 24 cases confirmed by histology. Medicine (Baltimore). 87(4):220–233
135. Morikawa M, Koinuma D, Tsutsumi S, Vasilaki E, Kanki Y, Heldin CH et al (2011) ChIP-seq reveals cell type-specific binding patterns of BMP-specific Smads and a novel binding motif. Nucleic Acids Res. 39(20):8712–8727
136. Morrell NW, Adnot S, Archer SL, Dupuis J, Jones PL, MacLean MR et al (2009) Cellular and molecular basis of pulmonary arterial hypertension. J Am Coll Cardiol. 54(1 Suppl):S20–S31
137. Morrell NW, Bloch DB, Ten Dijke P, Goumans MT, Hata A, Smith J et al (2015) Targeting BMP signalling in cardiovascular disease and anaemia. Nat Rev Cardiol 13(2):–106
138. Morrell NW, Yang X, Upton PD, Jourdan KB, Morgan N, Sheares KK et al (2001) Altered growth responses of pulmonary artery smooth muscle cells from patients with primary pulmonary hypertension to transforming growth factor-beta(1) and bone morphogenetic proteins. Circulation. 104(7):790–795
139. Morse JH, Jones AC, Barst RJ, Hodge SE, Wilhelmsen KC, Nygaard TG (1997) Mapping of familial primary pulmonary hypertension locus (PPH1) to chromosome 2q31-q32. Circulation. 95(12):2603–2606
140. Morse J, Barst R, Horn E, Cuervo N, Deng Z, Knowles J (2002) Pulmonary hypertension in scleroderma spectrum of disease: lack of bone morphogenetic protein receptor 2 mutations. J Rheumatol. 29(11):2379–2381
141. Morty RE, Nejman B, Kwapiszewska G, Hecker M, Zakrzewicz A, Kouri FM et al (2007) Dysregulated bone morphogenetic protein signaling in monocrotaline-induced pulmonary arterial hypertension. Arterioscler Thromb Vasc Biol. 27(5):1072–1078
142. Moya IM, Umans L, Maas E, Pereira PN, Beets K, Francis A et al (2012) Stalk cell phenotype depends on integration of Notch and Smad1/5 signaling cascades. Dev Cell. 22(3):501–514
143. Nasim MT, Ghouri A, Patel B, James V, Rudarakanchana N, Morrell NW et al (2008) Stoichiometric imbalance in the receptor complex contributes to dysfunctional BMPR-II mediated signalling in pulmonary arterial hypertension. Hum Mol Genet. 17(11): 1683–1694
144. Nasim MT, Ogo T, Ahmed M, Randall R, Chowdhury HM, Snape KM et al (2011) Molecular genetic characterization of SMAD signaling molecules in pulmonary arterial hypertension. Hum Mutat. 32(12):1385–1389
145. Nichols WC, Koller DL, Slovis B, Foroud T, Terry VH, Arnold ND et al (1997) Localization of the gene for familial primary pulmonary hypertension to chromosome 2q31-32. Nat Genet. 15(3):277–280

146. Nickel NP, Spiekerkoetter E, Gu M, Li CG, Li H, Kaschwich M et al (2015) Elafin reverses pulmonary hypertension via caveolin-1-dependent bone morphogenetic protein signaling. Am J Respir Crit Care Med. 191(11):1273–1286
147. Nishihara A, Watabe T, Imamura T, Miyazono K (2002) Functional heterogeneity of bone morphogenetic protein receptor-II mutants found in patients with primary pulmonary hypertension. Mol Biol Cell. 13(9):3055–3063
148. Nohe A, Keating E, Underhill TM, Knaus P, Petersen NO (2005) Dynamics and interaction of caveolin-1 isoforms with BMP-receptors. J Cell Sci. 118(Pt 3):643–650
149. Nohno T, Ishikawa T, Saito T, Hosokawa K, Noji S, Wolsing DH et al (1995) Identification of a human type II receptor for bone morphogenetic protein-4 that forms differential heteromeric complexes with bone morphogenetic protein type I receptors. J Biol Chem. 270(38):22522–22526
150. Nolan-Stevaux O, Zhong W, Culp S, Shaffer K, Hoover J, Wickramasinghe D et al (2012) Endoglin requirement for BMP9 signaling in endothelial cells reveals new mechanism of action for selective anti-endoglin antibodies. PLoS One. 7(12):e50920
151. Nunes H, Humbert M, Sitbon O, Morse JH, Deng Z, Knowles JA et al (2003) Prognostic factors for survival in human immunodeficiency virus-associated pulmonary arterial hypertension. Am J Respir Crit Care Med. 167(10):1433–1439
152. Ormiston ML, Chang C, Long LL, Soon E, Jones D, Machado R et al (2012) Impaired natural killer cell phenotype and function in idiopathic and heritable pulmonary arterial hypertension. Circulation. 126(9):1099–1109
153. Ormiston ML, Moore SD, Long L, Colucci F, Morrell NW. Reduced natural killer cells in BMPR2-R899X knock in mice. American Thoracic Society International Conference Abstracts 2014: A4142
154. Pachori AS, Custer L, Hansen D, Clapp S, Kemppa E, Klingensmith J (2010) Bone morphogenetic protein 4 mediates myocardial ischemic injury through JNK-dependent signaling pathway. J Mol Cell Cardiol. 48(6):1255–1265
155. Pavelescu A, Vanderpool R, Vachiéry JL, Grunig E, Naeije R (2012) Echocardiography of pulmonary vascular function in asymptomatic carriers of BMPR2 mutations. Eur Respir J. 40(5):1287–1289
156. Peacock AJ, Murphy NF, McMurray JJ, Caballero L, Stewart S (2007) An epidemiological study of pulmonary arterial hypertension. Eur Respir J. 30(1):104–109
157. Pfarr N, Fischer C, Ehlken N, Becker-Grünig T, López-González V, Gorenflo M et al (2013) Hemodynamic and genetic analysis in children with idiopathic, heritable, and congenital heart disease associated pulmonary arterial hypertension. Respir Res. 14:3
158. Pfarr N, Szamalek-Hoegel J, Fischer C, Hinderhofer K, Nagel C, Ehlken N et al (2011) Hemodynamic and clinical onset in patients with hereditary pulmonary arterial hypertension and BMPR2 mutations. Respir Res. 12:99
159. Phillips JA, Poling JS, Phillips CA, Stanton KC, Austin ED, Cogan JD et al (2008) Synergistic heterozygosity for TGFbeta1 SNPs and BMPR2 mutations modulates the age at diagnosis and penetrance of familial pulmonary arterial hypertension. Genet Med. 10(5): 359–365
160. Prewitt AR, Ghose S, Frump AL, Datta A, Austin ED, Kenworthy AK et al (2015) Heterozygous null bone morphogenetic protein receptor type 2 mutations promote SRC kinase-dependent caveolar trafficking defects and endothelial dysfunction in pulmonary arterial hypertension. J Biol Chem. 290(2):960–971
161. Pulido T, Adzerikho I, Channick RN, Delcroix M, Galiè N, Ghofrani HA et al (2013) Macitentan and morbidity and mortality in pulmonary arterial hypertension. N Engl J Med. 369(9):809–818
162. Rabinovitch M (2012) Molecular pathogenesis of pulmonary arterial hypertension. J Clin Invest. 122(12):4306–4313
163. Ranchoux B, Antigny F, Rucker-Martin C, Hautefort A, Péchoux C, Bogaard HJ et al (2015) Endothelial-to-mesenchymal transition in pulmonary hypertension. Circulation. 131(11):1006–1018

164. Reynolds AM, Holmes MD, Danilov SM, Reynolds PN (2012) Targeted gene delivery of BMPR2 attenuates pulmonary hypertension. Eur Respir J. 39(2):329–343
165. Reynolds AM, Xia W, Holmes MD, Hodge SJ, Danilov S, Curiel DT et al (2007) Bone morphogenetic protein type 2 receptor gene therapy attenuates hypoxic pulmonary hypertension. Am J Physiol Lung Cell Mol Physiol. 292(5):L1182–L1192
166. Ricard N, Bidart M, Mallet C, Lesca G, Giraud S, Prudent R et al (2010) Functional analysis of the BMP9 response of ALK1 mutants from HHT2 patients: a diagnostic tool for novel ACVRL1 mutations. Blood. 116(9):1604–1612
167. Rich S, Dantzker DR, Ayres SM, Bergofsky EH, Brundage BH, Detre KM et al (1987) Primary pulmonary hypertension. A national prospective study. Ann Intern Med. 107(2):216–223
168. Roberts KE, McElroy JJ, Wong WP, Yen E, Widlitz A, Barst RJ et al (2004) BMPR2 mutations in pulmonary arterial hypertension with congenital heart disease. Eur Respir J. 24(3):371–374
169. Rosenzweig BL, Imamura T, Okadome T, Cox GN, Yamashita H, ten Dijke P et al (1995) Cloning and characterization of a human type II receptor for bone morphogenetic proteins. Proc Natl Acad Sci U S A. 92(17):7632–7636
170. Rosenzweig EB, Morse JH, Knowles JA, Chada KK, Khan AM, Roberts KE et al (2008) Clinical implications of determining BMPR2 mutation status in a large cohort of children and adults with pulmonary arterial hypertension. J Heart Lung Transplant. 27(6):668–674
171. Rothberg KG, Heuser JE, Donzell WC, Ying YS, Glenney JR, Anderson RG (1992) Caveolin, a protein component of caveolae membrane coats. Cell. 68(4):673–682
172. Rothhammer T, Bataille F, Spruss T, Eissner G, Bosserhoff AK (2007) Functional implication of BMP4 expression on angiogenesis in malignant melanoma. Oncogene. 26(28):4158–4170
173. Rudarakanchana N, Flanagan JA, Chen H, Upton PD, Machado R, Patel D et al (2012) Functional analysis of bone morphogenetic protein type II receptor mutations underlying primary pulmonary hypertension. Hum Mol Genet. 11(13):1517–1525
174. Runo JR, Vnencak-Jones CL, Prince M, Loyd JE, Wheeler L, Robbins IM et al (2003) Pulmonary veno-occlusive disease caused by an inherited mutation in bone morphogenetic protein receptor II. Am J Respir Crit Care Med. 167(6):889–894
175. Ryan JJ (2013) Chloroquine in pulmonary arterial hypertension: a new role for an old drug? Circ Cardiovasc Genet. 6(3):310–311
176. Ryan J, Bloch K, Archer SL (2011) Rodent models of pulmonary hypertension: harmonisation with the world health organisation's categorisation of human PH. Int J Clin Pract Suppl. 172:15–34
177. Sawada H, Saito T, Nickel NP, Alastalo TP, Glotzbach JP, Chan R et al (2014) Reduced BMPR2 expression induces GM-CSF translation and macrophage recruitment in humans and mice to exacerbate pulmonary hypertension. J Exp Med. 211(2):263–280
178. Scharpfenecker M, van Dinther M, Liu Z, van Bezooijen RL, Zhao Q, Pukac L et al (2007) BMP-9 signals via ALK1 and inhibits bFGF-induced endothelial cell proliferation and VEGF-stimulated angiogenesis. J Cell Sci. 120(Pt 6):964–972
179. Seki T, Hong KH, Oh SP (2006) Nonoverlapping expression patterns of ALK1 and ALK5 reveal distinct roles of each receptor in vascular development. Lab Invest. 86(2):116–129
180. Shao ES, Lin L, Yao Y, Boström KI (2009) Expression of vascular endothelial growth factor is coordinately regulated by the activin-like kinase receptors 1 and 5 in endothelial cells. Blood. 114(10):2197–2206
181. Shintani M, Yagi H, Nakayama T, Saji T, Matsuoka R (2009) A new nonsense mutation of SMAD8 associated with pulmonary arterial hypertension. J Med Genet. 46(5):331–337
182. Shovlin CL, Letarte M (1999) Hereditary haemorrhagic telangiectasia and pulmonary arteriovenous malformations: issues in clinical management and review of pathogenic mechanisms. Thorax. 54(8):714–729
183. Simonneau G, Gatzoulis MA, Adatia I, Celermajer D, Denton C, Ghofrani A et al (2013) Updated clinical classification of pulmonary hypertension. J Am Coll Cardiol. 62(25 Suppl):D34–D41

184. Sobolewski A, Rudarakanchana N, Upton PD, Yang J, Crilley TK, Trembath RC et al (2008) Failure of bone morphogenetic protein receptor trafficking in pulmonary arterial hypertension: potential for rescue. Hum Mol Genet. 17(20):3180–3190
185. Song Y, Jones JE, Beppu H, Keaney JF, Loscalzo J, Zhang YY (2005) Increased susceptibility to pulmonary hypertension in heterozygous BMPR2-mutant mice. Circulation. 112(4):553–562
186. Soon E, Crosby A, Southwood M, Yang P, Tajsic T, Toshner M et al (2015) Bone Morphogenetic Protein Receptor Type II Deficiency and Increased Inflammatory Cytokine Production. A Gateway to Pulmonary Arterial Hypertension. Am J Respir Crit Care Med. 192(7):859–872
187. Soubrier F, Chung WK, Machado R, Grünig E, Aldred M, Geraci M et al (2013) Genetics and genomics of pulmonary arterial hypertension. J Am Coll Cardiol. 62(25 Suppl):D13–D21
188. Spiekerkoetter E, Sung YK, Sudheendra D, Bill M, Aldred MA, van de Veerdonk MC et al (2015) Low-Dose FK506 (Tacrolimus) in End-Stage Pulmonary Arterial Hypertension. Am J Respir Crit Care Med. 192(2):254–257
189. Spiekerkoetter E, Tian X, Cai J, Hopper RK, Sudheendra D, Li CG et al (2013) FK506 activates BMPR2, rescues endothelial dysfunction, and reverses pulmonary hypertension. J Clin Invest. 123(8):3600–3613
190. Stahls PF, Lightell DJ, Moss SC, Goldman CK, Woods TC (2013) Elevated serum bone morphogenetic protein 4 in patients with chronic kidney disease and coronary artery disease. J Cardiovasc Transl Res. 6(2):232–238
191. Steinbicker AU, Sachidanandan C, Vonner AJ, Yusuf RZ, Deng DY, Lai CS et al (2011) Inhibition of bone morphogenetic protein signaling attenuates anemia associated with inflammation. Blood. 117(18):4915–4923
192. Steiner MK, Syrkina OL, Kolliputi N, Mark EJ, Hales CA, Waxman AB (2009) Interleukin-6 overexpression induces pulmonary hypertension. Circ Res 104(2):236–244 , 28p following 44
193. Suzuki Y, Ohga N, Morishita Y, Hida K, Miyazono K, Watabe T (2010) BMP-9 induces proliferation of multiple types of endothelial cells in vitro and in vivo. J Cell Sci. 123(Pt 10):1684–1692
194. Sztrymf B, Coulet F, Girerd B, Yaici A, Jais X, Sitbon O et al (2008) Clinical outcomes of pulmonary arterial hypertension in carriers of BMPR2 mutation. Am J Respir Crit Care Med. 177(12):1377–1383
195. Talati M, West J, Blackwell TR, Loyd JE, Meyrick B (2010) BMPR2 mutation alters the lung macrophage endothelin-1 cascade in a mouse model and patients with heritable pulmonary artery hypertension. Am J Physiol Lung Cell Mol Physiol. 299(3):L363–L373
196. Talati M, West J, Zaynagetdinov R, Hong CC, Han W, Blackwell T et al (2014) BMP pathway regulation of and by macrophages. PLoS One. 9(4):e94119
197. Teichert-Kuliszewska K, Kutryk MJ, Kuliszewski MA, Karoubi G, Courtman DW, Zucco L et al (2006) Bone morphogenetic protein receptor-2 signaling promotes pulmonary arterial endothelial cell survival: implications for loss-of-function mutations in the pathogenesis of pulmonary hypertension. Circ Res. 98(2):209–217
198. ten Dijke P, Goumans MJ, Pardali E (2008) Endoglin in angiogenesis and vascular diseases. Angiogenesis. 11(1):79–89
199. ten Dijke P, Yamashita H, Sampath TK, Reddi AH, Estevez M, Riddle DL et al (1994) Identification of type I receptors for osteogenic protein-1 and bone morphogenetic protein-4. J Biol Chem. 269(25):16985–16988
200. Thomas M, Docx C, Holmes AM, Beach S, Duggan N, England K et al (2009) Activin-like kinase 5 (ALK5) mediates abnormal proliferation of vascular smooth muscle cells from patients with familial pulmonary arterial hypertension and is involved in the progression of experimental pulmonary arterial hypertension induced by monocrotaline. Am J Pathol. 174(2):380–389

201. Thomson JR, Machado RD, Pauciulo MW, Morgan NV, Humbert M, Elliott GC et al (2000) Sporadic primary pulmonary hypertension is associated with germline mutations of the gene encoding BMPR-II, a receptor member of the TGF-beta family. J Med Genet. 37(10):741–745
202. Toshner M, Voswinckel R, Southwood M, Al-Lamki R, Howard LS, Marchesan D et al (2009) Evidence of dysfunction of endothelial progenitors in pulmonary arterial hypertension. Am J Respir Crit Care Med. 180(8):780–787
203. Trembath RC, Thomson JR, Machado RD, Morgan NV, Atkinson C, Winship I et al (2001) Clinical and molecular genetic features of pulmonary hypertension in patients with hereditary hemorrhagic telangiectasia. N Engl J Med. 345(5):325–334
204. Upton PD, Davies RJ, Tajsic T, Morrell NW (2013) Transforming growth factor-β(1) represses bone morphogenetic protein-mediated Smad signaling in pulmonary artery smooth muscle cells via Smad3. Am J Respir Cell Mol Biol. 49(6):1135–1145
205. Upton PD, Davies RJ, Trembath RC, Morrell NW (2009) Bone morphogenetic protein (BMP) and activin type II receptors balance BMP9 signals mediated by activin receptor-like kinase-1 in human pulmonary artery endothelial cells. J Biol Chem. 284(23):15794–15804
206. Upton PD, Morrell NW (2013) The transforming growth factor-β-bone morphogenetic protein type signalling pathway in pulmonary vascular homeostasis and disease. Exp Physiol. 98(8):1262–1266
207. Valdimarsdottir G, Goumans MJ, Rosendahl A, Brugman M, Itoh S, Lebrin F et al (2002) Stimulation of Id1 expression by bone morphogenetic protein is sufficient and necessary for bone morphogenetic protein-induced activation of endothelial cells. Circulation. 106(17):2263–2270
208. Vorselaars VM, Velthuis S, Snijder RJ, Vos JA, Mager JJ, Post MC (2015) Pulmonary hypertension in hereditary haemorrhagic telangiectasia. World J Cardiol. 7(5):230–237
209. Vukicevic S, Grgurevic L (2009) BMP-6 and mesenchymal stem cell differentiation. Cytokine Growth Factor Rev. 20(5–6):441–448
210. Wang G, Knight L, Ji R, Lawrence P, Kanaan U, Li L et al (2014) Early onset severe pulmonary arterial hypertension with 'two-hit' digenic mutations in both BMPR2 and KCNA5 genes. Int J Cardiol. 177(3):e167–e169
211. Wertz JW, Bauer PM (2008) Caveolin-1 regulates BMPRII localization and signaling in vascular smooth muscle cells. Biochem Biophys Res Commun. 375(4):557–561
212. West J, Austin E, Fessel JP, Loyd J, Hamid R (2014) Rescuing the BMPR2 signaling axis in pulmonary arterial hypertension. Drug Discov Today. 19(8):1241–1245
213. West J, Cogan J, Geraci M, Robinson L, Newman J, Phillips JA et al (2008) Gene expression in BMPR2 mutation carriers with and without evidence of pulmonary arterial hypertension suggests pathways relevant to disease penetrance. BMC Med Genomics. 1:45
214. West J, Fagan K, Steudel W, Fouty B, Lane K, Harral J et al (2004) Pulmonary hypertension in transgenic mice expressing a dominant-negative BMPRII gene in smooth muscle. Circ Res. 94(8):1109–1114
215. West J, Harral J, Lane K, Deng Y, Ickes B, Crona D et al (2008) Mice expressing BMPR2R899X transgene in smooth muscle develop pulmonary vascular lesions. Am J Physiol Lung Cell Mol Physiol. 295(5):L744–L755
216. West J, Niswender KD, Johnson JA, Pugh ME, Gleaves L, Fessel JP et al (2013) A potential role for insulin resistance in experimental pulmonary hypertension. Eur Respir J. 41(4):861–871
217. Wooderchak-Donahue WL, McDonald J, O'Fallon B, Upton PD, Li W, Roman BL et al (2013) BMP9 mutations cause a vascular-anomaly syndrome with phenotypic overlap with hereditary hemorrhagic telangiectasia. Am J Hum Genet. 93(3):530–537
218. Wu X, Sagave J, Rutkovskiy A, Haugen F, Baysa A, Nygård S et al (2014) Expression of bone morphogenetic protein 4 and its receptors in the remodeling heart. Life Sci. 97(2):145–154

219. Yang J, Davies RJ, Southwood M, Long L, Yang X, Sobolewski A et al (2008) Mutations in bone morphogenetic protein type II receptor cause dysregulation of Id gene expression in pulmonary artery smooth muscle cells: implications for familial pulmonary arterial hypertension. Circ Res. 102(10):1212–1221
220. Yang J, Li X, Al-Lamki RS, Southwood M, Zhao J, Lever AM et al (2010) Smad-dependent and smad-independent induction of id1 by prostacyclin analogues inhibits proliferation of pulmonary artery smooth muscle cells in vitro and in vivo. Circ Res. 107(2):252–262
221. Yang J, Li X, Al-Lamki RS, Wu C, Weiss A, Berk J et al (2013) Sildenafil potentiates bone morphogenetic protein signaling in pulmonary arterial smooth muscle cells and in experimental pulmonary hypertension. Arterioscler Thromb Vasc Biol. 33(1):34–42
222. Yang X, Long L, Reynolds PN, Morrell NW (2011) Expression of mutant BMPR-II in pulmonary endothelial cells promotes apoptosis and a release of factors that stimulate proliferation of pulmonary arterial smooth muscle cells. Pulm Circ. 1(1):103–110
223. Yang X, Long L, Southwood M, Rudarakanchana N, Upton PD, Jeffery TK et al (2005) Dysfunctional Smad signaling contributes to abnormal smooth muscle cell proliferation in familial pulmonary arterial hypertension. Circ Res. 96(10):1053–1063
224. Yao Y, Jumabay M, Ly A, Radparvar M, Wang AH, Abdmaulen R et al (2012) Crossveinless 2 regulates bone morphogenetic protein 9 in human and mouse vascular endothelium. Blood. 119(21):5037–5047
225. Yi SE, Daluiski A, Pederson R, Rosen V, Lyons KM (2000) The type I BMP receptor BMPRIB is required for chondrogenesis in the mouse limb. Development. 127(3):621–630
226. Yu PB, Beppu H, Kawai N, Li E, Bloch KD (2005) Bone morphogenetic protein (BMP) type II receptor deletion reveals BMP ligand-specific gain of signaling in pulmonary artery smooth muscle cells. J Biol Chem. 280(26):24443–24450
227. Yu PB, Deng DY, Beppu H, Hong CC, Lai C, Hoyng SA et al (2008) Bone morphogenetic protein (BMP) type II receptor is required for BMP-mediated growth arrest and differentiation in pulmonary artery smooth muscle cells. J Biol Chem. 283(7):3877–3888
228. Yu PB, Hong CC, Sachidanandan C, Babitt JL, Deng DY, Hoyng SA et al (2008) Dorsomorphin inhibits BMP signals required for embryogenesis and iron metabolism. Nat Chem Biol. 4(1):33–41
229. Yung LM, Nikolic I, Paskin-Flerlage SD, Pearsall RS, Kumar R, Yu PB (2016) A selective transforming growth factor-β ligand trap attenuates pulmonary hypertension. Am J Respir Crit Care Med. 194(9):1140–1151
230. Zaiman AL, Podowski M, Medicherla S, Gordy K, Xu F, Zhen L et al (2008) Role of the TGF-beta/Alk5 signaling pathway in monocrotaline-induced pulmonary hypertension. Am J Respir Crit Care Med. 177(8):896–905
231. Zhang S, Fantozzi I, Tigno DD, Yi ES, Platoshyn O, Thistlethwaite PA et al (2003) Bone morphogenetic proteins induce apoptosis in human pulmonary vascular smooth muscle cells. Am J Physiol Lung Cell Mol Physiol. 285(3):L740–L754
232. Zhao YY, Liu Y, Stan RV, Fan L, Gu Y, Dalton N et al (2002) Defects in caveolin-1 cause dilated cardiomyopathy and pulmonary hypertension in knockout mice. Proc Natl Acad Sci U S A. 99(17):11375–11380

BMP Signaling in Fibrodysplasia Ossificans Progressiva, a Rare Genetic Disorder of Heterotopic Ossification

Eileen M. Shore and Frederick S. Kaplan

Abstract Heterotopic ossification (HO), the formation of extraskeletal bone, is most frequently associated with severe tissue injury. However, predicting who will be susceptible to HO and when HO will form has been challenging, resulting in a paucity of information about the causes and progression of this heterotopic bone formation. Fibrodysplasia ossificans progressiva (FOP) is a rare genetic disorder in which heterotopic bone forms in soft connective tissues during childhood and throughout adult life, frequently in response to tissue trauma. The discovery that FOP is caused by gain-of-function mutations in *ACVR1*, the gene encoding the ALK2 BMP type I receptor, established that perturbation in the bone morphogenetic protein (BMP) signaling pathway is an underlying cellular mechanism for HO. The identification of the responsible gene for FOP, together with the development of animal models for HO and FOP, is now leading to advances in understanding the cellular and molecular mechanisms of bone formation and the induction of HO.

E.M. Shore, PhD (✉)
University of Pennsylvania, Department of Orthopaedic Surgery,
424 Stemmler Hall, 3450 Hamilton Walk, Philadelphia, PA 19104-6081, USA

Department of Orthopaedic Surgery, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104, USA

Department of Genetics, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104, USA

Department of Center for Research in FOP and Related Disorders, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104, USA
e-mail: shore@mail.med.upenn.edu

F.S. Kaplan
Department of Orthopaedic Surgery, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104, USA

Department of Medicine, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104, USA

Department of Center for Research in FOP and Related Disorders, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104, USA

S. Vukicevic, K.T. Sampath (eds.), *Bone Morphogenetic Proteins: Systems Biology Regulators*, Progress in Inflammation Research,
DOI 10.1007/978-3-319-47507-3_14

Keywords Fibrodysplasia ossificans progressiva • FOP • ACVR1 • ALK2 • Heterotopic ossification • BMP signaling • Rare genetic disease

1 Introduction

Fibrodysplasia ossificans progressiva (FOP) is a human genetic disorder (MIM #135100; http://omim.org/entry/135100) in which bone forms in soft connective tissues, beginning during childhood and continuing throughout adult life, often in response to injury [1]. Extraskeletal bone formation, or heterotopic ossification (HO), is an extremely rare clinical finding in children, mainly associated with genetic disorders such as FOP or progressive osseous heteroplasia (POH) [1]. In adults, more common, nongenetic forms of heterotopic ossification are frequently associated with a range of conditions that involve severe trauma including spinal cord and head injuries, hip replacement surgery, and high-impact blast injuries [2].

Although the bone morphogenetic protein (BMP) signaling pathway had been implicated in heterotopic ossification and FOP [3–6], it was the discovery of mutations in the *ACVR1* gene that confirmed that alterations of BMP signaling are the primary cause of FOP [1] and that enhanced signaling from the ACVR1/ALK2 type I receptor is sufficient to cause heterotopic bone formation [7–10]. In this chapter, we will review advances in our understanding of heterotopic ossification and BMP signaling in FOP.

2 Clinical Features of Fibrodysplasia Ossificans Progressiva (FOP)

Fibrodysplasia ossificans progressiva (FOP) is clinically diagnosed on the basis of two characteristic features: progressive formation of extraskeletal bone, or heterotopic ossification (HO), and congenital malformations of the great toes [1].

Skeletal Development in FOP Patients with FOP show specific skeletal malformations, indicating that the causative ACVR1 gene mutation influences bone formation during embryogenesis in addition to directing chondrogenesis and osteogenesis in soft connective tissues after birth [11].

Malformation of the great toes is the most consistent skeletal anomaly and is usually the first indication that a child has FOP. This feature has been used to diagnose FOP prior to the onset of heterotopic bone formation [12–14]. These malformations result from abnormal first metatarsal, proximal phalange, and interphalangeal joint formation. Typically, the proximal phalanx of the first toe is aberrantly shaped [12] and is broader than normal and often fused with the distal phalanx [15]. Some children have an intact interphalangeal joint of the great toe at birth that fuses early in life.

About half of patients with a classic clinical presentation of FOP have malformed thumbs, although the severity of thumb malformation is less than in the great toe [15]. Malformations of other skeletal elements are more variably observed [11, 16], the most common of which are short, broad femoral necks and narrow cervical vertebrae. Osteochondromas (benign osteochondral neoplasms or orthotopic lesions of skeletal remodeling) also are a common feature of FOP [17]. Proximal medial tibial osteochondromas are most frequently observed; however, osteochondromas are often detected at other skeletal sites. The osteochondromas are usually asymptomatic, frequently bilateral, and most often pedunculated [18].

Heterotopic Ossification in FOP Onset of heterotopic ossification usually occurs before the age of 10, although episodes as early as the first year after birth as well as onset occurring much later in life have been reported [11, 19–21]. In children, HO formation is preceded by painful, highly inflammatory soft tissue swellings [22]. These swellings, known as flare-ups, appear suddenly, expand rapidly, and are highly vascular [23]. In some cases, a flare-up subsides without residual bone formation, but most cases result in HO [22]. Although skeletal muscle is the tissue most often affected, heterotopic ossification also forms in other connective tissues such as aponeuroses, fascia, ligaments, and tendons [24]. Heterotopic bone formation in FOP is progressive, cumulative, and severely disabling [22].

In FOP, HO can be triggered by minor tissue injury such as intramuscular immunizations, mandibular blocks for dental work, severe muscle fatigue, blunt muscle trauma from bumps and falls, or surgical attempts to remove heterotopic bone [25–28]. In the absence of trauma in FOP, HO forms in a characteristic anatomic and temporal progression [29]. This ectopic bone formation in FOP, which generally is asymmetrically distributed, is usually seen first in dorsal, axial, cranial, and proximal regions of the body with early HO most commonly in the neck, spine, and shoulders and then later in ventral, appendicular, caudal, and distal regions. By the third decade of life, most body regions are affected [22, 30].

Histopathology of FOP Lesions Tissue trauma carries a risk of triggering episodes of FOP HO formation; therefore, biopsies are not obtained following diagnosis. However, early stages of FOP have been frequently misdiagnosed and biopsied, providing tissue samples that have been examined to define histological progression. FOP lesions involve an initial catabolic phase of tissue inflammation and destruction followed by an anabolic phase involving tissue formation and replacement by extraskeletal bone [23, 27, 31–33].

3 ACVR1/ALK2 Gene Mutations Cause FOP

Genetic transmission occurs rarely for FOP due to low reproductive fitness; most cases of FOP are caused by de novo mutations [34, 35]. The population frequency of FOP is estimated to be about one in 1.5–2 million [30, 34]. No gender, racial, ethnic, or geographic differences or clustering is observed [34]. Occasional families with genetic transmission of FOP have been reported [11, 34, 35] and show an autosomal dominant pattern of inheritance.

ACVR1 (ALK2) R206H Mutations in FOP Genetic linkage analysis and positional cloning using five families with a classic clinical presentation of FOP (progressive heterotopic ossification that initiates during childhood or adolescence and characteristic great toe malformation) identified mutation of the *ACVR1* (activin A type I receptor) gene [35]. The *ACVR1* gene, also known as activin-like kinase 2 (ALK2), encodes a type I receptor for bone morphogenetic proteins (BMPs). All patients with classic clinical features of FOP have the same heterozygous single-nucleotide substitution (c.617G>A) that changes amino acid 206 from arginine to histidine (R206H) [11, 35]. Codon 206, within the glycine-serine (GS) region of the cytoplasmic domain of ALK2, is highly conserved among species. Although the three human BMP type I receptors (ALK2, ALK3, and ALK6) have a high level of amino acid sequence conservation, ALK2 codon 206 uniquely encodes arginine, while the corresponding amino acids in ALK3 and ALK6 are lysine.

Atypical Forms of FOP Occasional cases of FOP are associated with unusual clinical features [11]. Some of these variant cases clinically present with differences in one or both of the two classic defining features of FOP, most notably more or less severe malformations of the digits. As with patients with classic features of FOP, all have heterozygous *ACVR1* missense mutations in conserved amino acids [11, 20, 36–42]. However, instead of the common recurrent c.617G>A; R206H mutation, non-R206H *ACVR1* mutations have been identified in many of these cases. All patients with FOP-type heterotopic endochondral ossification so far examined have a mutation in the *ACVR1* gene [11] and our additional unpublished data.

All of these variant *ACVR1* mutations are single-nucleotide substitutions causing missense mutations, with the exception of a three-nucleotide deletion spanning two codons that results in replacement of two amino acids with a single amino acid [11]. The identified mutations occur in either the glycine-serine (GS) activation domain or the protein kinase domain, regions of the ALK2 receptor important in downstream signal transduction [11, 43, 44]. Protein structure homology modeling predicts that these amino acid substitutions, as well as the R206H mutation, activate the ALK2 protein to enhance receptor signaling [11, 37].

ACVR1 Mutations in Diffuse Intrinsic Pontine Gliomas (*DIPGs*) As an additional note of interest, *ACVR1* mutations have been identified outside of the context of FOP in patients with diffuse intrinsic pontine gliomas (DIPGs) [45, 46]. DIPGs are a rare class of brainstem gliomas and the leading cause of death among all pediatric brain tumor patients [47]. Recent studies identified recurrent heterozygous somatic missense mutations in *ACVR1* in about 33 % of DIPGs [48–51]. Seven mutations were identified and all except one have also been identified in FOP patients [48–51]. The *ACVR1* mutation alone does not induce a tumorigenic phenotype, and ongoing studies are investigating the roles of *ACVR1* mutations in DIPG pathogenesis.

4 Acvr1 R206H Knock-In Mouse Models

An *Acvr1* R206H (c.617G>A) knock-in mouse ($Acvr1^{R206H/+}$) provided the first direct in vivo evidence that the R206H mutation in ACVR1 causes FOP [7]. Although expression of $Acvr1^{R206H}$ from its endogenous promoter in mice is

perinatal lethal, phenotypic characterization of mice that are chimeric for *Acvr1*^{R206H} cells identified every clinical feature of patients with classic FOP, including embryonic skeletal malformations (and specific hind limb first digit malformations) and postnatal heterotopic endochondral bone formation [7]. Histological analyses of regions undergoing heterotopic ossification further demonstrated the same progression of cellular events seen in patient lesions, including inflammation-associated destruction and turnover of connective tissues followed by tissue replacement by cartilage and bone.

A conditional knock-in mouse (*Acvr1*$^{cR206H/+}$) with expression of *Acvr1*^{R206H} under the regulation of Cre-inducible recombination has been recently reported [52, 53]. This conditional *Acvr1*^{R206H} mouse model can be used to avoid the perinatal lethality of the germline mutation transmission and can be used as a reliable model for postnatal injury-induced heterotopic ossification [52, 53]. The Cre-regulated mutation also permits cell-specific expression of the R206H mutation. Mice expressing *Acvr1*^{R206H} only in limb mesenchymal progenitor cells (*Prrx1*$^{+}$) formed skeletal malformations similar to those seen in patients due to altered chondrocyte development in the growth plates of long bones and developed heterotopic ossification postnatally in the absence of injury [52].

5 BMP Signaling and ACVR1/ALK2

BMP ligands signal through tetrameric complexes of two type I and two type II serine-threonine kinase receptors on the cell surface [54, 55]. When the ligand binds the extracellular region, type II receptors phosphorylate the glycine-serine (GS) domain of type I receptors. Activated type I receptors transduce downstream signaling through BMP pathway-specific SMAD1/5/8 proteins as well as through MAP kinase pathways [54, 55]. In addition to ALK2 (the BMP type I receptor mutated in FOP), BMP signal transduction is mediated through the BMPR1A/ALK3, BMPRIB/ALK6, AL1, and ALK4 type I receptors [54, 55].

The TGFβ/BMP family [56–58] regulates a wide range of cellular activities including differentiation, proliferation, apoptosis, migration, positional information, and stem cell renewal [59–63]. Unlike other members of the family, many members of the BMP subgroup can induce the complete process of endochondral bone formation [59]. BMP ligands and their receptors are expressed throughout development and in many adult tissues including skeletal muscle and cartilage.

Structural Homology Modeling of Mutant ALK2 Receptors Structural homology modeling was used to provide initial information regarding the functional effects of the R206H mutation in ALK2 on BMP signaling [64]. The cytoplasmic domains of all TGFβ/BMP type I receptors are highly conserved, allowing for modeling of ALK2 based on the structure of ALK5. Structural modeling of FOP variant mutations in ALK2 supports that these amino acid substitutions also lead to receptor malfunction of the kinase domain [11, 20, 37, 38, 44, 55]. Multiple ALK2 mutations, including the classic R206H mutation, disrupt key interactions with the BMP signaling regulatory protein FKBP12 that normally stabilize the inactive site of the receptor [44]. Glycine 328 mutations (in the kinase domain) have been identified in

FOP and may affect binding of Smad proteins or alter binding of FKBP12 [11, 38]. The Q207E variant mutation was initially predicted to function similarly to the engineered Q207D mutation, a constitutively active mutation that results in irreversible relocation of the GS domain into an activating position. Surprisingly, the Q207E mutation functioned similarly to the classic R206H mutation and retained some ability to be inhibited by FKBP12 [65]. Other mutations identified in the protein kinase domain of ALK2 (G356D and R375P) may disrupt ion pair formation and promote phosphorylation of the receptor, leading to constitutive activity [11].

BMP Signaling in FOP Patient Cells In vitro (no italics - it is the start of the first sentence) experiments using lymphoblastoid cell lines (LCLs) and stem cells from human exfoliated deciduous teeth (SHED cells) from FOP patients showed a consistent pattern of aberrant BMP signaling [3–5]. Although LCLs do not express detectable levels of BMP-SMAD-responsive proteins, FOP LCLs have increased p38 MAPK protein phosphorylation, indicating activation of a noncanonical BMP signaling pathway [4, 5]. In addition, expression of *ID1* and *ID3*, both direct transcriptional targets of BMP signaling, was increased in FOP LCLs [4, 5]. Similar experiments conducted using SHED cells isolated from FOP patients revealed dysregulation of both the canonical Smad-dependent and the noncanonical p38 MAPK BMP signaling pathways [3].

Elevated and prolonged cell surface expression of the BMP type I receptor BMPRIA/ALK3 was observed in FOP LCLs as a consequence of reduced receptor degradation and internalization [4]. The mechanism through which mutations in one BMP type I receptor (ALK2) affects another (ALK3) is not yet understood.

Effects of ACVR1/ALK2 Mutations on BMP Pathway Signaling Studies using FOP patient cells [4–6] revealed elevated BMP pathway signaling in response to exogenous BMP ligand compared to normal cells, indicating that $ALK2^{R206H}$ has increased ligand sensitivity. These investigations also revealed elevated canonical BMP signaling in the absence of exogenous BMP ligand, supporting that $ALK2^{R206H}$ is a mild gain-of-function mutation that remains ligand-responsive. Several in vitro *ALK2* overexpression assays have demonstrated enhanced BMP pathway signaling by FOP *ALK2* mutations [55, 66, 67], [8, 9], consistent with the results from patient cells, demonstrating that the enhanced activity of $ALK2^{R206H}$ is not cell type specific.

Mouse embryonic fibroblasts (MEFs) from $Acvr1^{R206H}$ mouse models [7, 53] have been used as an in vitro mesenchymal cell system to study elevated BMP pathway signaling conferred by the R206H mutation on a molecular level [68]. These cells recapitulate the increased levels of SMAD1/5/8 phosphorylation and BMP target gene expression seen in patient LCL cells and SHED cells, and can be differentiated to adipogenic, chondrogenic, and osteogenic lineages, demonstrating their utility as a mesenchymal cell model system to study the effects of the FOP mutation [68, 69]. Control cells were used to demonstrate that the ALK2 receptor is necessary for the earliest stages for chondrogenesis and that ALK2 gain-of-function mutations in FOP patients enhanced chondrogenic differentiation [60, 68, 69].

BMP ligand independence of $ALK2^{R206H}$ was also demonstrated using genetic approaches in an in vivo BMP-null zebrafish model [9]. Early zebrafish embryos

require both BMP2b and BMP7 for proper dorsal-ventral patterning [70]. However, excess BMP signaling induces ventralization in developing zebrafish embryos [70, 71] providing a screening method for elevated BMP signaling. Zebrafish lacking both BMP2b and BMP7 exhibited moderate to severe ventralization following transfection of *ACVR1*^{R206H} mRNA [9], confirming that the BMP pathway is active in the absence of ligand in vivo.

The activity of ALK2^{R206H} in the absence of BMP ligands is consistent with predictions based on structural homology modeling [44, 64]. The R206H mutation has been predicted to reduce binding of FKBP12, an inhibitor of TGFβ superfamily type I receptors that binds to the GS domain, preventing leaky activation of receptors in the absence of ligand [64, 72]. Co-immunoprecipitation experiments showed that in the absence of BMP ligand, FKBP12 shows reduced binding to ALK2^{R206H} compared to wild-type ALK2 [9], results supported by additional assays [10, 73]. A threefold decrease in binding of FKBP12 to ALK2^{R206H} compared to wild-type has been reported [74]. Structural homology investigation of the L196P nonclassical mutation also identified decreased receptor binding affinity for FKBP12 [44] and may contribute to enhanced receptor activity in the absence of ligand activation.

BMP signaling assays of some FOP variant mutations have been reported [44, 65, 75, 76]. Signaling assays in C2C12 cells transfected with either the G356D mutation [75] or the L196P mutation [76] showed that each increased phosphorylated SMAD1/5/8 levels and enhanced *ID1* promoter activity in the absence of BMP ligand, similar to the effects of the R206H mutation.

Activin A Induction of TGFβ/BMP Signaling Pathway Activins are potent regulators of inflammation and participate in positive feedback loops that potentiate expression of pro-inflammatory cytokines in many immune cell types [77–81]. Recently, activin A was identified as a ligand that binds to ALK2^{R206H}, but not wild-type ALK2, resulting in enhanced BMP signaling by the mutant receptor, demonstrated by an increase in phosphorylation of SMAD1/5/8 [53]. Similar results were found using human-induced pluripotent stem cells obtained from FOP patients that were subsequently differentiated to mesenchymal stromal cells [82]. These findings are also notable because activin A is normally associated with increased phosphorylation of SMAD2 and SMAD3, the downstream effectors of activated TGFβ signaling, although they have been also reported to bind BMP type I and type II receptors during receptor complex formation [83].

6 Effects of FOP ACVR1/ALK2 Mutation on Lesion Progression

Development of heterotopic bone-forming lesions in FOP involves an initial tissue turnover phase that includes inflammation and tissue degeneration, followed by a tissue formation phase during which cells differentiate to cartilage and bone and form endochondral bone tissue [1]. Early lesions begin with extensive soft tissue swelling (especially noted in children) that is associated with neutrophil,

macrophage, mast cell, and lymphocyte infiltration [33, 84–86]. Connective tissue degeneration follows immune cell infiltration; however, instead of the regeneration that is expected in response to injury, robust fibroproliferation is followed by chondrogenesis and osteogenesis leading to mature heterotopic bone [31, 87]. The *ACVR1*^{R206H} mutation may affect each stage of lesion development.

Immunological Contributions to Heterotopic Ossification Flare-ups of HO in patients with FOP can occur following inflammatory stimuli [84], suggesting that an immune response contributes to early HO events. A recent review of studies investigating immunological contributions to genetic and nongenetic forms of HO discussed the roles of multiple immune cell types and signaling pathways in this process [88]. The BMP pathway has a functional role in the immune system, suggesting the possibility that elevated BMP pathway signaling from ALK2^{R206H} enhances an inflammatory response. As an example, in response to BMP6, macrophages are induced to a pro-inflammatory state similar to the macrophage-lipopolysaccharide (LPS) immune response [89].

Specific immune cell types have been shown to participate in the development of HO in genetic and implant models of ectopic bone. Macrophages are present in the early FOP lesion [7], and mast cells are increased at all stages of lesion development, with vast increases in mast cell density (upward of 40- to 150-fold) in FOP compared to unaffected individuals [86]. Ablation of macrophages via clodronate liposomes [90] or diphtheria toxin [91] in the *Nse-BMP4* mouse model of HO significantly reduced HO volume [92]. Similarly, a mast cell-deficient *Nse-BMP4; c-kit*$^{W\text{-}sh/W\text{-}sh}$ mouse model also had reduced HO volume [93]. The pro-inflammatory neuropeptide substance P (SP), which stimulates mast cell function, was elevated in early HO lesions of patients with FOP and non-hereditary forms of HO, as well as in *Nse-BMP4* transgenic mice, and inhibition of the SP receptor NK1r, or ablation of mast cells, which express high levels of NK1r, inhibited the formation of HO [93].

Role of Activin A in FOP The recent report that activin A preferentially binds ALK2^{R206H}, but not wild-type ALK2, activating the BMP-pSMAD1/5/8 pathway in addition to the pSMAD2/3 pathway, identified activin A as candidate for understanding FOP lesion pathology and as a potential therapeutic target [53]. *Acvr1*$^{cR206H/+}$ mice treated with a humanized antibody against activin A inhibited HO formation, suggesting that activin A is a key factor in the development of HO in FOP [53]. The mechanism through which activin A leads to heterotopic ossification and how the activin A ligand differentiates between wild-type and mutant ALK2 is of great interest in ongoing investigations. Since the ligand-binding domain (LBD) of ALK2 is unchanged by the R206H mutation, the mechanism may require that the mutation alters the conformation and specificity of the ALK2 LBD or that the mutant ALK2 binds atypical type I and/or type II receptor partners to confer altered ligand binding. Little is currently known about the expression and function of ALK2 in various cell types or the identity of the cells that mediate an activin A-ALK2^{R206H}-HO response. It remains to be confirmed whether the absence of wild-type ALK2-pSMAD1/5/8 signaling in response to activin A is cell type specific and whether the differential response of mutant and wild-type ALK2 occurs in all cell types at

endogenous levels of receptor expression. Importantly, it also remains to be established whether the mechanism of differential activation of wild-type and mutant ALK2 is specific to activin A or if activin A functions similarly to other BMP ligands in their ability to trigger a more sensitive response by $ALK2^{R206H}$.

Role of Hypoxia in Heterotopic Ossification Pathology Recent investigations examined the interaction of elevated BMP pathway signaling in FOP with activation of the hypoxia-sensing HIF1α pathway [67]. Inhibition of the HIF1α pathway by genetic or pharmacologic means restored BMP-pSMAD1/5/8 signaling to normoxic levels in human FOP SHED cells and reduced HO in a constitutively active $Acvr1^{Q207D/+}$ mouse model of FOP-like HO [67]. This finding is consistent with previous reports that inhibition of the HIF1α pathway prevents nongenetic and genetic HO [94] and supports that cellular oxygen-sensing mechanisms modulate BMP signaling and contribute to HO development in FOP [67, 84, 88, 95].

Origin of HO Progenitor Cells Many cell types have chondrogenic and osteogenic potential in vitro; however, the specific identity of the cells that aberrantly differentiate to cartilage and bone during HO in vivo is not yet fully defined. TIE2 was identified in mouse models of HO as a marker for ~50 % of cells contributing to heterotopic bone and cartilage, and $TIE2^+$ cells are also present in FOP patient biopsies [87, 96]. Endothelial cells expressing $ALK2^{R206H}$ induced mesenchymal cell marker expression suggesting that these cells dedifferentiated through endothelial-to-mesenchymal transition (EndMT) in response to the FOP mutation, including the ability to differentiate into adipocytes, chondrocytes, or osteoblasts and demonstrating mesenchymal multipotency [68, 96]. A $TIE2^+$ progenitor cell population of non-endothelial lineage with osteogenic potential has also been identified [97]. This mesenchymal cell population ($TIE2^+$, $PDGFR\alpha^+$, $SCA\text{-}1^+$) localizes to the interstitium of skeletal muscle and other tissues [97]. Whether $TIE2^+$ endothelial and non-endothelial cells contribute to HO in vivo, along with contributions from additional cell populations, remains to be clarified.

Chondrogenesis and Osteogenesis BMP pathway signaling and ALK2 are regulators of chondrogenesis and osteogenesis during endochondral ossification [68, 98–101]. ALK2 is a BMP type I receptor that is expressed in skeletal tissues, chondrocytes, and osteoblasts [98]. Mouse embryonic fibroblasts (MEFs) expressing $Acvr1^{R206H/+}$ showed accelerated chondrogenesis compared to wild-type cells and increased sensitivity to low levels of BMP ligand, with upregulation of early chondrogenic marker genes *Sox9*, *Col2* (collagen type II), and *Acan* (Aggrecan) by $Acvr1^{R206H}$ MEFs [9, 68]. By contrast, overexpression of $ALK2^{Q207D}$, a constitutively active form of ALK2, induced a dramatic increase of the late-stage chondrogenic markers *IHH* and *Collagen type X*, while *Aggrecan* expression is only slightly enhanced and *Collagen type II* is significantly downregulated by $ALK2^{Q207D}$ [9]. The BMP antagonist Noggin caused no inhibition of chondrogenesis induced by $ALK2^{Q207D}$, but partially inhibited the enhanced differentiation by $ALK2^{R206H}$. These data are consistent with signaling assays showing that the R206H mutation is mildly activating with partial BMP ligand independence.

During heterotopic endochondral bone formation, hypertrophic chondrocytes provide a template for infiltrating osteoblasts. Patient-derived SHED cells in vitro

show higher basal expression of the osteogenic markers *RUNX2* and *ALP* and mineralize more rapidly than control SHED cells under osteogenic conditions without BMP ligand [3]. Human bone marrow mesenchymal stem cells infected with lentiviral *ACVR1*^{R206H} are similarly more sensitive to osteogenic differentiation [10]. ALK2^{R206H}-increased synthesis of ALP and mineralization, however, required BMP6 ligand [10]. Similar to chondrogenic differentiation, ALK2^{R206H} showed a milder effect on osteogenesis when compared to ALK2^{Q207D} [9, 10]. ALK2^{R206H} stimulation of both chondrogenic and osteogenic differentiation supports that this mutation contributes to both processes of endochondral bone formation in FOP patients.

7 Counseling and Treatment

Presently, there are no effective therapeutic options that prevent or reverse the formation of heterotopic bone in FOP. Surgery is discouraged given that surgical removal of lesions is often followed by significant recurrence [19, 22, 30]. Surgical release of joint contractures has been unsuccessful and also risks new, trauma-induced heterotopic ossification [30, 102–104]. Medical management is currently supportive [104], and current treatment of FOP involves early diagnosis, prevention of trauma, and other interventions that risk activating heterotopic ossification and symptomatic treatment of pain associated with flare-ups. The initial stages of heterotopic bone formation are associated with inflammation, and glucocorticoids seem effective in managing symptomatic new flare-ups affecting major joints of the appendicular skeleton, especially when used during early stage of onset. Guidelines for symptomatic management of FOP are available through the International Fibrodysplasia Ossificans Progressiva Association (IFOPA) website (www.ifopa.org).

Flare-ups of FOP are sporadic and unpredictable, with wide individual variability in the age of onset and rate of disease severity and progression [11]. Several large studies investigating the natural history of FOP illustrate the difficulty in predicting the occurrence, duration, or severity of an FOP flare-up, although characteristic anatomic patterning has been described [19, 22]. The rarity of FOP and the unpredictable nature of the condition make it extremely difficult to assess therapeutic interventions.

The most useful treatments for FOP would prevent or reverse heterotopic bone formation. The prevention and treatment of HO in FOP, as well as approaches for treating more common forms of heterotopic ossification, will likely target multiple stages that could be used in combination therapies or specifically directed as warranted. With emerging insights into the pathophysiology of ACVR1/ALK2-mediated heterotopic ossification, several strategies for the treatment and/or prevention of FOP have been proposed [105]. These approaches include blocking activity of the mutant FOP receptor and dysregulated BMP signaling pathway, inhibiting the inflammatory triggers and early-stage mediators of FOP flare-ups, altering the

inductive and/or conducive microenvironments that promote the formation of FOP lesions, and diverting the responding chondro-osseous progenitor cells to a soft tissue fate [105].

Preclinical data identified RARγ agonists as inhibitors of the BMP-induced chondrogenesis required for endochondral bone formation [52, 106]. One of these compounds, Palovarotene, is currently being tested in an FDA-approved phase 2 clinical trial for FOP (ClinicalTrials.gov identifier NCT02190747) to evaluate whether the drug will prevent HO development during and following flare-ups in FOP patients. This clinical trial represents a significant milestone in the ongoing efforts to treat HO disorders.

8 Summary

FOP is a rare autosomal dominant disorder caused by gain-of-function mutations in the *ACVR1* gene that increase signaling by the BMP type I receptor ALK2, causing progressive endochondral bone formation in extraskeletal connective tissues. The $ACVR1^{R206H}$ mutation is a recurrent mutation found in nearly all patients with FOP. In vivo models of heterotopic bone formation, along with in vitro assays, will continue to provide important insight into the cellular and molecular mechanisms of cell differentiation and bone formation and provide the basis for developing therapeutic strategies for FOP and other forms of heterotopic ossification.

Acknowledgments We thank the members of our research laboratory and many colleagues for the work reported. Our work was supported through the Center for Research in FOP and Related Disorders, the International FOP Association (IFOPA), the Ian Cali Endowment for FOP Research, the Whitney Weldon Endowment for FOP Research, the Ashley Martucci FOP Research Fund, the NIH/NIAMS-supported Penn Center for Musculoskeletal Disorders, the Isaac and Rose Nassau Professorship of Orthopaedic Molecular Medicine (FSK), and the Cali/Weldon Professorship for FOP Research (EMS) and by grants from the National Institutes of Health (R01-AR41916 and R01-AR046831).

References

1. Shore EM, Kaplan FS (2010) Inherited human diseases of heterotopic bone formation. Nat Rev Rheumatol 6(9):518–527
2. Pignolo RJ, Foley KL (2005) Nonhereditary heterotopic ossification: implications for injury, arthroplasty, and aging. Clin Rev Bone Miner Metab 3(3–4):261–266
3. Billings PC, Fiori JL, Bentwood JL, O'Connell MP, Jiao X, Nussbaum B, Caron RJ, Shore EM, Kaplan FS (2008) Dysregulated BMP signaling and enhanced osteogenic differentiation of connective tissue progenitor cells from patients with fibrodysplasia ossificans progressiva (FOP). J Bone Miner Res 23(3):305–313
4. de la Pena LS, Billings PC, Fiori JL, Ahn J, Kaplan FS, Shore EM (2005) Fibrodysplasia ossificans progressiva (FOP), a disorder of ectopic osteogenesis, misregulates cell surface expression and trafficking of BMPRIA. J Bone Miner Res 20(7):1168–1176

5. Fiori JL, Billings PC, de la Pena LS, Kaplan FS, Shore EM (2006) Dysregulation of the BMP-p38 MAPK signaling pathway in cells from patients with fibrodysplasia ossificans progressiva (FOP). J Bone Miner Res 21(6):902–909
6. Shafritz AB, Shore EM, Gannon FH, Zasloff MA, Taub R, Muenke M, Kaplan FS (1996) Overexpression of an osteogenic morphogen in fibrodysplasia ossificans progressiva. N Engl J Med 335(8):555–561
7. Chakkalakal SA, Zhang D, Culbert AL, Convente MR, Caron RJ, Wright AC, Maidment AD, Kaplan FS, Shore EM (2012) An Acvr1 R206H knock-in mouse has fibrodysplasia ossificans progressiva. J Bone Miner Res 27(8):1746–1756
8. Fukuda T, Kohda M, Kanomata K, Nojima J, Nakamura A, Kamizono J, Noguchi Y, Iwakiri K, Kondo T, Kurose J, Endo K, Awakura T, Fukushi J, Nakashima Y, Chiyonobu T, Kawara A, Nishida Y, Wada I, Akita M, Komori T, Nakayama K, Nanba A, Maruki Y, Yoda T, Tomoda H, Yu PB, Shore EM, Kaplan FS, Miyazono K, Matsuoka M, Ikebuchi K, Ohtake A, Oda H, Jimi E, Owan I, Okazaki Y, Katagiri T (2009) Constitutively activated ALK2 and increased SMAD1/5 cooperatively induce bone morphogenetic protein signaling in fibrodysplasia ossificans progressiva. J Biol Chem 284(11):7149–7156
9. Shen Q, Little SC, Xu M, Haupt J, Ast C, Katagiri T, Mundlos S, Seemann P, Kaplan FS, Mullins MC, Shore EM (2009) The fibrodysplasia ossificans progressiva R206H ACVR1 mutation activates BMP-independent chondrogenesis and zebrafish embryo ventralization. J Clin Invest 119(11):3462–3472
10. van Dinther M, Visser N, de Gorter DJ, Doorn J, Goumans MJ, de Boer J, ten Dijke P (2010) ALK2 R206H mutation linked to fibrodysplasia ossificans progressiva confers constitutive activity to the BMP type I receptor and sensitizes mesenchymal cells to BMP-induced osteoblast differentiation and bone formation. J Bone Miner Res 25(6):1208–1215
11. Kaplan FS, Xu M, Seemann P, Connor JM, Glaser DL, Carroll L, Delai P, Urban EF, Forman SJ, Gillessen-Kaesbach G, Hoover-Fong J, Koster B, Pauli RM, Reardon W, Zaidi SA, Zasloff M, Morhart R, Mundlos S, Groppe J, Shore EM (2009) Classic and atypical fibrodysplasia ossificans progressiva (FOP) phenotypes are caused by mutations in the bone morphogenetic protein (BMP) type I receptor ACVR1. Hum Mutat 30(3):379–390
12. Harrison RJ, Pitcher JD, Mizel MS, Temple HT, Scully SP (2005) The radiographic morphology of foot deformities in patients with fibrodysplasia ossificans progressiva. Foot Ankle Int 26(11):937–941
13. Kaplan FS, Xu M, Glaser DL, Collins F, Connor M, Kitterman J, Sillence D, Zackai E, Ravitsky V, Zasloff M, Ganguly A, Shore EM (2008a) Early diagnosis of fibrodysplasia ossificans progressiva. Pediatrics 121(5):e1295–e1300
14. Maftei C, Rypens F, Thiffault I, Dube J, Laberge AM, Lemyre E (2015) Fibrodysplasia ossificans progressiva: bilateral hallux valgus on ultrasound a clue for the first prenatal diagnosis for this condition-clinical report and review of the literature. Prenat Diagn 35(3):305–307
15. Schroeder HW Jr, Zasloff M (1980) The hand and foot malformations in fibrodysplasia ossificans progressiva. Johns Hopkins Med J 147(2):73–78
16. Schaffer AA, Kaplan FS, Tracy MR, O'Brien ML, Dormans JP, Shore EM, Harland RM, Kusumi K (2005) Developmental anomalies of the cervical spine in patients with fibrodysplasia ossificans progressiva are distinctly different from those in patients with Klippel-Feil syndrome: clues from the BMP signaling pathway. Spine (Phila Pa 1976) 30(12):1379–1385
17. Deirmengian GK, Hebela NM, O'Connell M, Glaser DL, Shore EM, Kaplan FS (2008) Proximal tibial osteochondromas in patients with fibrodysplasia ossificans progressiva. J Bone Joint Surg Am 90(2):366–374
18. Morales-Piga A, Bachiller-Corral J, Gonzalez-Herranz P, Medrano-SanIldelfonso M, Olmedo-Garzon J, Sanchez-Duffhues G (2015) Osteochondromas in fibrodysplasia ossificans progressiva: a widespread trait with a streaking but overlooked appearance when arising at femoral bone end. Rheumatol Int 35(10):1759–1767
19. Cohen RB, Hahn GV, Tabas JA, Peeper J, Levitz CL, Sando A, Sando N, Zasloff M, Kaplan FS (1993) The natural history of heterotopic ossification in patients who have fibrodysplasia ossificans progressiva. A study of forty-four patients. J Bone Joint Surg Am 75(2):215–219

20. Gregson CL, Hollingworth P, Williams M, Petrie KA, Bullock AN, Brown MA, Tobias JH, Triffitt JT (2011) A novel ACVR1 mutation in the glycine/serine-rich domain found in the most benign case of a fibrodysplasia ossificans progressiva variant reported to date. Bone 48(3):654–658
21. Whyte MP, Wenkert D, Demertzis JL, DiCarlo EF, Westenberg E, Mumm S (2012) Fibrodysplasia ossificans progressiva: middle-age onset of heterotopic ossification from a unique missense mutation (c.974G>C, p.G325 A) in ACVR1. J Bone Miner Res 27(3):729–737
22. Pignolo RJ, Bedford-Gay C, Liljesthrom M, Durbin-Johnson BP, Shore EM, Rocke DM, Kaplan FS (2016) The Natural History of Flare-Ups in Fibrodysplasia Ossificans Progressiva (FOP): A Comprehensive Global Assessment. J Bone Miner Res 31(3):650–656
23. Kaplan FS, Tabas JA, Gannon FH, Finkel G, Hahn GV, Zasloff MA (1993) The histopathology of fibrodysplasia ossificans progressiva. An endochondral process. J Bone Joint Surg Am 75(2):220–230
24. McKusick V (1972) Heritable disorders of connective tissue, 4 edn. Mosby, St Louis
25. Lanchoney TF, Cohen RB, Rocke DM, Zasloff MA, Kaplan FS (1995) Permanent heterotopic ossification at the injection site after diphtheria-tetanus-pertussis immunizations in children who have fibrodysplasia ossificans progressiva. J Pediatr 126(5 Pt 1):762–764
26. Luchetti W, Cohen RB, Hahn GV, Rocke DM, Helpin M, Zasloff M, Kaplan FS (1996) Severe restriction in jaw movement after routine injection of local anesthetic in patients who have fibrodysplasia ossificans progressiva. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 81(1):21–25
27. Gannon, FH, BA Valentine, EM Shore, MA Zasloff, and FS Kaplan (1998) Acute lymphocytic infiltration in an extremely early lesion of fibrodysplasia ossificans progressiva. Clin Orthop Relat Res 346:19–25.
28. Scarlett, RF, DM Rocke, S Kantanie, JB Patel, EM Shore, and FS Kaplan (2004) Influenza-like viral illnesses and flare-ups of fibrodysplasia ossificans progressiva. Clin Orthop Relat Res 423:275–279.
29. Kaplan FS, Tabas JA, Zasloff MA (1990) Fibrodysplasia ossificans progressiva: a clue from the fly? Calcif Tissue Int 47(2):117–125
30. Connor JM, Evans DA (1982) Fibrodysplasia ossificans progressiva. The clinical features and natural history of 34 patients. J Bone Joint Surg Br 64(1):76–83
31. Pignolo RJ, Suda RK, Kaplan FS (2005) The fibrodysplasia ossificans progressiva lesion. Clin Rev Bone Miner Metab 3(3–4):195–200
32. Glaser DL, Economides AN, Wang L, Liu X, Kimble RD, Fandl JP, Wilson JM, Stahl N, Kaplan FS, Shore EM (2003) In vivo somatic cell gene transfer of an engineered Noggin mutein prevents BMP4-induced heterotopic ossification. J Bone Joint Surg Am 85-A(12):2332–2342
33. Hegyi L, Gannon FH, Glaser DL, Shore EM, Kaplan FS, Shanahan CM (2003) Stromal cells of fibrodysplasia ossificans progressiva lesions express smooth muscle lineage markers and the osteogenic transcription factor Runx2/Cbfa-1: clues to a vascular origin of heterotopic ossification? J Pathol 201(1):141–148
34. Shore E, Feldman GJ, Xu M, Kaplan F (2005) The genetics of fibrodysplasia ossificans progressiva. Clin Rev Bone Miner Metab 3(3–4):201–204
35. Shore EM, Xu M, Feldman GJ, Fenstermacher DA, Cho TJ, Choi IH, Connor JM, Delai P, Glaser DL, LeMerrer M, Morhart R, Rogers JG, Smith R, Triffitt JT, Urtizberea JA, Zasloff M, Brown MA, Kaplan FS (2006) A recurrent mutation in the BMP type I receptor ACVR1 causes inherited and sporadic fibrodysplasia ossificans progressiva. Nat Genet 38(5):525–527
36. Furuya H, Ikezoe K, Wang L, Ohyagi Y, Motomura K, Fujii N, Kira J, Fukumaki Y (2008) A unique case of fibrodysplasia ossificans progressiva with an ACVR1 mutation, G356D, other than the common mutation (R206H). Am J Med Genet A 146A(4):459–463
37. Bocciardi R, Bordo D, Di Duca M, Di Rocco M, Ravazzolo R (2009) Mutational analysis of the ACVR1 gene in Italian patients affected with fibrodysplasia ossificans progressiva: confirmations and advancements. Eur J Hum Genet 17(3):311–318

38. Petrie KA, Lee WH, Bullock AN, Pointon JJ, Smith R, Russell RG, Brown MA, Wordsworth BP, Triffitt JT (2009) Novel mutations in ACVR1 result in atypical features in two fibrodysplasia ossificans progressiva patients. PLoS One 4(3):e5005
39. Carvalho DR, Navarro MM, Martins BJ, Coelho KE, Mello WD, Takata RI, Speck-Martins CE (2010) Mutational screening of ACVR1 gene in Brazilian fibrodysplasia ossificans progressiva patients. Clin Genet 77(2):171–176
40. Ratbi I, Borcciadi R, Regragui A, Ravazzolo R, Sefiani A (2010) Rarely occurring mutation of ACVR1 gene in Moroccan patient with fibrodysplasia ossificans progressiva. Clin Rheumatol 29(1):119–121
41. Barnett CP, Dugar M, Haan EA (2011) Late-onset variant fibrodysplasia ossificans progressiva leading to misdiagnosis of ankylosing spondylitis. Am J Med Genet A 155A(6): 1492–1495
42. Kaplan FS, Kobori JA, Orellana C, Calvo I, Rosello M, Martinez F, Lopez B, Xu M, Pignolo RJ, Shore EM, Groppe JC (2015) Multi-system involvement in a severe variant of fibrodysplasia ossificans progressiva (ACVR1 c.772G>A; R258G): a report of two patients. Am J Med Genet A 167A(10):2265–2271
43. Huse M, Chen YG, Massague J, Kuriyan J (1999) Crystal structure of the cytoplasmic domain of the type I TGF beta receptor in complex with FKBP12. Cell 96(3):425–436
44. Chaikuad A, Alfano I, Kerr G, Sanvitale CE, Boergermann JH, Triffitt JT, von Delft F, Knapp S, Knaus P, Bullock AN (2012) Structure of the bone morphogenetic protein receptor ALK2 and implications for fibrodysplasia ossificans progressiva. J Biol Chem 287(44): 36990–36998
45. Hennika T, Becher OJ (2016) Diffuse intrinsic pontine glioma: time for cautious optimism. J Child Neurol 31:1377–1385
46. Pacifici M, Shore EM (2016) Common mutations in ALK2/ACVR1, a multi-faceted receptor, have roles in distinct pediatric musculoskeletal and neural orphan disorders. Cytokine Growth Factor Rev 27:93–104
47. Hargrave D, Bartels U, Bouffet E (2006) Diffuse brainstem glioma in children: critical review of clinical trials. Lancet Oncol 7(3):241–248
48. Buczkowicz P, Hoeman C, Rakopoulos P, Pajovic S, Letourneau L, Dzamba M, Morrison A, Lewis P, Bouffet E, Bartels U, Zuccaro J, Agnihotri S, Ryall S, Barszczyk M, Chornenkyy Y, Bourgey M, Bourque G, Montpetit A, Cordero F, Castelo-Branco P, Mangerel J, Tabori U, Ho KC, Huang A, Taylor KR, Mackay A, Bendel AE, Nazarian J, Fangusaro JR, Karajannis MA, Zagzag D, Foreman NK, Donson A, Hegert JV, Smith A, Chan J, Lafay-Cousin L, Dunn S, Hukin J, Dunham C, Scheinemann K, Michaud J, Zelcer S, Ramsay D, Cain J, Brennan C, Souweidane MM, Jones C, Allis CD, Brudno M, Becher O, Hawkins C (2014) Genomic analysis of diffuse intrinsic pontine gliomas identifies three molecular subgroups and recurrent activating ACVR1 mutations. Nat Genet 46(5):451–456
49. Fontebasso AM, Papillon-Cavanagh S, Schwartzentruber J, Nikbakht H, Gerges N, Fiset PO, Bechet D, Faury D, De Jay N, Ramkissoon LA, Corcoran A, Jones DT, Sturm D, Johann P, Tomita T, Goldman S, Nagib M, Bendel A, Goumnerova L, Bowers DC, Leonard JR, Rubin JB, Alden T, Browd S, Geyer JR, Leary S, Jallo G, Cohen K, Gupta N, Prados MD, Carret AS, Ellezam B, Crevier L, Klekner A, Bognar L, Hauser P, Garami M, Myseros J, Dong Z, Siegel PM, Malkin H, Ligon AH, Albrecht S, Pfister SM, Ligon KL, Majewski J, Jabado N, Kieran MW (2014) Recurrent somatic mutations in ACVR1 in pediatric midline high-grade astrocytoma. Nat Genet 46(5):462–466
50. Taylor KR, Mackay A, Truffaux N, Butterfield YS, Morozova O, Philippe C, Castel D, Grasso CS, Vinci M, Carvalho D, Carcaboso AM, de Torres C, Cruz O, Mora J, Werle NE, Ingram WJ, Monje M, Hargrave D, Bullock AN, Puget S, Yip S, Jones C, Grill J (2014) Recurrent activating ACVR1 mutations in diffuse intrinsic pontine glioma. Nat Genet 46(5):457–461
51. Wu G, Diaz AK, Paugh BS, Rankin SL, Ju B, Li Y, Zhu X, Qu C, Chen X, Zhang J, Easton J, Edmonson M, Ma X, Lu C, Nagahawatte P, Hedlund E, Rusch M, Pounds S, Lin T, Onar-Thomas A, Huether R, Kriwacki R, Parker M, Gupta P, Becksfort J, Wei L, Mulder HL, Boggs K, Vadodaria B, Yergeau D, Russell JC, Ochoa K, Fulton RS, Fulton LL, Jones C,

Boop FA, Broniscer A, Wetmore C, Gajjar A, Ding L, Mardis ER, Wilson RK, Taylor MR, Downing JR, Ellison DW, Zhang J, Baker SJ (2014) The genomic landscape of diffuse intrinsic pontine glioma and pediatric non-brainstem high- grade glioma. Nat Genet 46(5): 444–450
52. Chakkalakal SA, Uchibe K, Convente MR, Zhang D, Economides AN, Kaplan FS, Pacifici M, Iwamoto M, Shore EM (2016) Palovarotene inhibits heterotopic ossification and maintains limb mobility and growth in mice with the human ACVR1 Fibrodysplasia Ossificans Progressiva (FOP) mutation. J Bone Miner Res 31:1647–1651
53. Hatsell SJ, Idone V, Wolken DM, Huang L, Kim HJ, Wang L, Wen X, Nannuru KC, Jimenez J, Xie L, Das N, Makhoul G, Chernomorsky R, D'Ambrosio D, Corpina RA, Schoenherr CJ, Feeley K, Yu PB, Yancopoulos GD, Murphy AJ, Economides AN (2015) ACVR1R206H receptor mutation causes fibrodysplasia ossificans progressiva by imparting responsiveness to activin A. Sci Transl Med 7(303):303ra137
54. Salazar VS, Gamer LW, Rosen V (2016) BMP signalling in skeletal development, disease and repair. Nat Rev Endocrinol 12(4):203–221
55. Yadin D, Knaus P, Mueller TD (2016) Structural insights into BMP receptors: Specificity, activation and inhibition. Cytokine Growth Factor Rev 27:13–34
56. Heldin CH, Moustakas A (2012) Role of smads in TGFbeta signaling. Cell Tissue Res 347(1):21–36
57. Shimmi O, Newfeld SJ (2013) New insights into extracellular and post-translational regulation of TGF-beta family signalling pathways. J Biochem 154(1):11–19
58. Weiss A, Attisano L (2013) The TGFbeta superfamily signaling pathway. Wiley Interdiscip Rev Dev Biol 2(1):47–63
59. Rahman MS, Akhtar N, Jamil HM, Banik RS, Asaduzzaman SM (2015) TGF- beta/BMP signaling and other molecular events: regulation of osteoblastogenesis and bone formation. Bone Res 3:15005
60. Rigueur D, Brugger S, Anbarchian T, Kim JK, Lee Y, Lyons KM (2015) The type I BMP receptor ACVR1/ALK2 is required for chondrogenesis during development. J Bone Miner Res 30(4):733–741
61. Sanchez-Duffhues G, Hiepen C, Knaus P, Ten Dijke P (2015) Bone morphogenetic protein signaling in bone homeostasis. Bone 80:43–59
62. Rys JP, Monteiro DA, Alliston T (2016) Mechanobiology of TGFbeta signaling in the skeleton. Matrix Biol 54-54:413–425
63. Scarfi S (2016) Use of bone morphogenetic proteins in mesenchymal stem cell stimulation of cartilage and bone repair. World J Stem Cells 8(1):1–12
64. Groppe JC, Shore EM, Kaplan FS (2007) Functional modeling of the ACVR1 (R206H) mutation in FOP. Clin Orthop Relat Res 462:87–92
65. Haupt J, Deichsel A, Stange K, Ast C, Bocciardi R, Ravazzolo R, Di Rocco M, Ferrari P, Landi A, Kaplan FS, Shore EM, Reissner C, Seemann P (2014) ACVR1 p.Q207E causes classic fibrodysplasia ossificans progressiva and is functionally distinct from the engineered constitutively active ACVR1 p.Q207D variant. Hum Mol Genet 23:5364–5377
66. Miyazono K, Kamiya Y, Morikawa M (2010) Bone morphogenetic protein receptors and signal transduction. J Biochem 147(1):35–51
67. Wang H, Lindborg C, Lounev V, Kim JH, McCarrick-Walmsley R, Xu M, Mangiavini L, Groppe JC, Shore EM, Schipani E, Kaplan FS, Pignolo RJ (2016) Cellular hypoxia promotes heterotopic ossification by amplifying BMP signaling. J Bone Miner Res 31:1652–1665
68. Culbert AL, Chakkalakal SA, Theosmy EG, Brennan TA, Kaplan FS, Shore EM (2014) Alk2 regulates early chondrogenic fate in fibrodysplasia ossificans progressiva heterotopic endochondral ossification. Stem Cells 32(5):1289–1300
69. de Kroon LM, Narcisi R, Blaney Davidson EN, Cleary MA, van Beuningen HM, Koevoet WJ, van Osch GJ, van der Kraan PM (2015) Activin receptor-like kinase receptors ALK5 and ALK1 are both required for TGFbeta-induced chondrogenic differentiation of human bone marrow-derived mesenchymal stem cells. PLoS One 10(12):e0146124

70. Little SC, Mullins MC (2009) Bone morphogenetic protein heterodimers assemble heteromeric type I receptor complexes to pattern the dorsoventral axis. Nat Cell Biol 11(5):637–643
71. Little SC, Mullins MC (2006) Extracellular modulation of BMP activity in patterning the dorsoventral axis. Birth Defects Res C Embryo Today 78(3):224–242
72. Wang T, Li BY, Danielson PD, Shah PC, Rockwell S, Lechleider RJ, Martin J, Manganaro T, Donahoe PK (1996) The immunophilin FKBP12 functions as a common inhibitor of the TGF beta family type I receptors. Cell 86(3):435–444
73. Song GA, Kim HJ, Woo KM, Baek JH, Kim GS, Choi JY, Ryoo HM (2010) Molecular consequences of the ACVR1(R206H) mutation of fibrodysplasia ossificans progressiva. J Biol Chem 285(29):22542–22553
74. Groppe JC, Wu J, Shore EM, Kaplan FS (2011) In vitro analyses of the dysregulated R206H ALK2 kinase-FKBP12 interaction associated with heterotopic ossification in FOP. Cells Tissues Organs 194(2–4):291–295
75. Fukuda T, Kanomata K, Nojima J, Kokabu S, Akita M, Ikebuchi K, Jimi E, Komori T, Maruki Y, Matsuoka M, Miyazono K, Nakayama K, Nanba A, Tomoda H, Okazaki Y, Ohtake A, Oda H, Owan I, Yoda T, Haga N, Furuya H, Katagiri T (2008) A unique mutation of ALK2, G356D, found in a patient with fibrodysplasia ossificans progressiva is a moderately activated BMP type I receptor. Biochem Biophys Res Commun 377(3):905–909
76. Ohte S, Shin M, Sasanuma H, Yoneyama K, Akita M, Ikebuchi K, Jimi E, Maruki Y, Matsuoka M, Namba A, Tomoda H, Okazaki Y, Ohtake A, Oda H, Owan I, Yoda T, Furuya H, Kamizono J, Kitoh H, Nakashima Y, Susami T, Haga N, Komori T, Katagiri T (2011) A novel mutation of ALK2, L196P, found in the most benign case of fibrodysplasia ossificans progressiva activates BMP-specific intracellular signaling equivalent to a typical mutation, R206H. Biochem Biophys Res Commun 407(1):213–218
77. Hedger MP, Winnall WR, Phillips DJ, de Kretser DM (2011) The regulation and functions of activin and follistatin in inflammation and immunity. Vitam Horm 85:255–297
78. Aleman-Muench GR, Soldevila G (2012) When versatility matters: activins/inhibins as key regulators of immunity. Immunol Cell Biol 90(2):137–148
79. Kaplan FS, Pignolo RJ, Shore EM (2016) Granting immunity to FOP and catching heterotopic ossification in the Act. Semin Cell Dev Biol 49:30–36
80. Marino FE, Risbridger G, Gold E (2013) The therapeutic potential of blocking the activin signalling pathway. Cytokine Growth Factor Rev 24(5):477–484
81. Aykul, S and E Martinez-Hackert (2016) Transforming Growth Factor-beta family ligands can function as antagonists by competing for type II receptor binding. J Biol Chem 291:10792–10804
82. Hino K, Ikeya M, Horigome K, Matsumoto Y, Ebise H, Nishio M, Sekiguchi K, Shibata M, Nagata S, Matsuda S, Toguchida J (2015) Neofunction of ACVR1 in fibrodysplasia ossificans progressiva. Proc Natl Acad Sci U S A 112(50):15438–15443
83. Massague J (2012) TGFbeta signalling in context. Nat Rev Mol Cell Biol 13(10):616–630
84. Kaplan FS, Shore EM, Gupta R, Billings PC, Glaser DL, Pignolo RJ, Graf D, Kamoun M (2005) Immunological features of fibrodysplasia ossificans progressiva and the dysregulated BMP4 pathway. Clin Rev Bone Miner Metab 3(3–4):189–193
85. Moriatis, JM, FH Gannon, EM Shore, W Bilker, MA Zasloff, and FS Kaplan (1997) Limb swelling in patients who have fibrodysplasia ossificans progressiva. Clin Orthop Relat Res 336:247–253.
86. Gannon FH, Glaser D, Caron R, Thompson LD, Shore EM, Kaplan FS (2001) Mast cell involvement in fibrodysplasia ossificans progressiva. Hum Pathol 32(8):842–848
87. Lounev VY, Ramachandran R, Wosczyna MN, Yamamoto M, Maidment AD, Shore EM, Glaser DL, Goldhamer DJ, Kaplan FS (2009) Identification of progenitor cells that contribute to heterotopic skeletogenesis. J Bone Joint Surg Am 91(3):652–663
88. Convente MR, Wang H, Pignolo RJ, Kaplan FS, Shore EM (2015) The immunological contribution to heterotopic ossification disorders. Curr Osteoporos Rep 13(2):116–124

89. Hong JH, Lee GT, Lee JH, Kwon SJ, Park SH, Kim SJ, Kim IY (2009) Effect of bone morphogenetic protein-6 on macrophages. Immunology 128(1 Suppl):e442–e450
90. Van Rooijen N (1989) The liposome-mediated macrophage 'suicide' technique. J Immunol Methods 124(1):1–6
91. Duffield JS, Forbes SJ, Constandinou CM, Clay S, Partolina M, Vuthoori S, Wu S, Lang R, Iredale JP (2005) Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair. J Clin Invest 115(1):56–65
92. Kan L, Liu Y, McGuire TL, Berger DM, Awatramani RB, Dymecki SM, Kessler JA (2009) Dysregulation of local stem/progenitor cells as a common cellular mechanism for heterotopic ossification. Stem Cells 27(1):150–156
93. Kan L, Lounev VY, Pignolo RJ, Duan L, Liu Y, Stock SR, McGuire TL, Lu B, Gerard NP, Shore EM, Kaplan FS, Kessler JA (2011) Substance P signaling mediates BMP-dependent heterotopic ossification. J Cell Biochem 112(10):2759–2772
94. Agarwal S, Loder S, Brownley C, Cholok D, Mangiavini L, Li J, Breuler C, Sung HH, Li S, Ranganathan K, Peterson J, Tompkins R, Herndon D, Xiao W, Jumlongras D, Olsen BR, Davis TA, Mishina Y, Schipani E, Levi B (2016) Inhibition of Hif1alpha prevents both trauma-induced and genetic heterotopic ossification. Proc Natl Acad Sci U S A 113:E338–E347
95. Gonzalez DM, Medici D (2014) Signaling mechanisms of the epithelial- mesenchymal transition. Sci Signal 7(344):re8
96. Medici D, Shore EM, Lounev VY, Kaplan FS, Kalluri R, Olsen BR (2010) Conversion of vascular endothelial cells into multipotent stem-like cells. Nat Med 16(12):1400–1406
97. Wosczyna MN, Biswas AA, Cogswell CA, Goldhamer DJ (2012) Multipotent progenitors resident in the skeletal muscle interstitium exhibit robust BMP-dependent osteogenic activity and mediate heterotopic ossification. J Bone Miner Res 27(5):1004–1017
98. Zhang D, Schwarz EM, Rosier RN, Zuscik MJ, Puzas JE, O'Keefe RJ (2003) ALK2 functions as a BMP type I receptor and induces Indian hedgehog in chondrocytes during skeletal development. J Bone Miner Res 18(9):1593–1604
99. Chen D, Zhao M, Mundy GR (2004) Bone morphogenetic proteins. Growth Factors 22(4):233–241
100. Goldring MB, Tsuchimochi K, Ijiri K (2006) The control of chondrogenesis. J Cell Biochem 97(1):33–44
101. Lim J, Tu X, Choi K, Akiyama H, Mishina Y, Long F (2015) BMP-Smad4 signaling is required for precartilaginous mesenchymal condensation independent of Sox9 in the mouse. Dev Biol 400(1):132–138
102. Shah PB, Zasloff MA, Drummond D, Kaplan FS (1994) Spinal deformity in patients who have fibrodysplasia ossificans progressiva. J Bone Joint Surg Am 76(10):1442–1450
103. Kitterman JA, Kantanie S, Rocke DM, Kaplan FS (2005) Iatrogenic harm caused by diagnostic errors in fibrodysplasia ossificans progressiva. Pediatrics 116(5):e654–e661
104. Kaplan FS, Le Merrer M, Glaser DL, Pignolo RJ, Goldsby RE, Kitterman JA, Groppe J, Shore EM (2008b) Fibrodysplasia ossificans progressiva. Best Pract Res Clin Rheumatol 22(1):191–205
105. Kaplan FS, Groppe J, Shore EM (2008c) When one skeleton is enough: approaches and strategies for the treatment of fibrodysplasia ossificans progressiva (FOP). Drug Discov Today Ther Strateg 5(4):255–262
106. Shimono K, Tung WE, Macolino C, Chi AH, Didizian JH, Mundy C, Chandraratna RA, Mishina Y, Enomoto-Iwamoto M, Pacifici M, Iwamoto M (2011) Potent inhibition of heterotopic ossification by nuclear retinoic acid receptor-gamma agonists. Nat Med 17(4):454–460

The Central Role of BMP Signaling in Regulating Iron Homeostasis

Herbert Y. Lin

Abstract Bone morphogenetic proteins (BMPs)/growth and differentiation factors (GDFs) are involved in a wide variety of embryologic, developmental, and physiologic processes. One important area of physiology that requires BMP signaling is the homeostatic regulation of iron in the body. Iron is an essential nutrient that is critical for several fundamental cellular processes including oxygen delivery to tissues and generation of adenosine triphosphate (ATP) in mitochondria. However, excess iron can lead to the generation of reactive oxygen species (ROS) that are highly damaging to cells, and insufficient iron is the major source of anemia worldwide. Therefore, the homeostatic regulation of total body iron content is an important physiologic process that must be exquisitely controlled to prevent the pathologic states of iron excess or iron deficiency. BMP signaling in the liver by the BMP ligands and receptors including the co-receptor hemojuvelin/RGMc regulates the expression of the iron hormone hepcidin to maintain iron homeostasis.

Keywords Bone morphogenetic proteins (BMPs) • BMP6 • Growth and differentiation factors (GDFs) • Hemochromatosis • Iron deficiency anemia • Hepcidin regulation • Iron regulation • Hemojuvelin • Smad signaling

1 Iron Metabolism and Genetic Hemochromatosis

Iron is an essential and critical nutrient required by most life forms on earth. In mammals, iron homeostasis is tightly regulated to provide this important element for growth and survival and to prevent the toxicity resulting from iron excess. Total body iron content is exquisitely and tightly controlled, and in normal adults there is no net loss or gain of iron on a daily basis. Plasma iron levels are maintained by intestinal absorption in the duodenum, reticuloendothelial cell recycling of senescent red cells, and mobilization of hepatocyte iron stores. Circulating iron is loaded

H.Y. Lin
Division of Nephrology, Program in Membrane Biology, and Center for Systems Biology, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, USA
e-mail: Lin.Herbert@mgh.harvard.edu

S. Vukicevic, K.T. Sampath (eds.), *Bone Morphogenetic Proteins: Systems Biology Regulators*, Progress in Inflammation Research,
DOI 10.1007/978-3-319-47507-3_15

onto serum transferrin and delivered primarily to the bone marrow for erythropoiesis. Sloughing of enterocytes and blood loss (e.g. through menstruation in women) are the only significant means for the removal of excess iron from the body, while the remaining excess iron is stored primarily in hepatocytes and macrophages [1].

Since there is no known regulated mechanism for iron excretion in mammals, systemic iron homeostasis is maintained by tight regulation of iron absorption from the intestine and release from macrophages and hepatocytes through the only known iron exporter protein, ferroportin [1]. Hepcidin, a soluble protein secreted by the liver [2], appears to be a key regulatory effector hormone for maintaining iron balance [3]. Hepcidin promotes internalization and degradation of ferroportin, an iron exporter located on the surface of enterocytes, macrophages, and hepatocytes [4]. When hepcidin is present in the circulation, it decreases intestinal iron absorption and inhibits the release of iron by macrophages.

The mechanisms by which hepcidin expression itself is regulated are complicated but are beginning to be understood at the molecular level. An abundance of data suggested that hepcidin expression is enhanced by iron overload and by states of inflammation [1, 5]. Thus, iron and inflammatory cytokines (e.g., interleukin-6) stimulate hepcidin expression, leading to reduced ferroportin levels at the cell surface and to reduced plasma iron levels. In contrast, hepcidin is inhibited by iron deficiency, hypoxia, and high erythropoietic activity [6]. This leads to enhanced ferroportin levels at the cell surface of cells and subsequently to increased serum iron levels. This physiologic regulation of hepcidin is consistent with a compensatory role for hepcidin to limit intestinal absorption during states of iron overload and to increase iron availability when needed for erythropoiesis during states of anemia [3].

When hepcidin is absent or abnormally low, a pathologic state is created leading to iron overload over a period of time. This condition is called hemochromatosis [7]. Mice and humans with null genetic mutations in the hepcidin gene develop severe iron overload at an early age, thus defining the first discovered cause of juvenile hemochromatosis [8]. Severe and early onset of iron overload is also seen in humans and in mice with mutations of the other genes in this pathway. For example, mutation or loss of the hemojuvelin gene (HFE2, also known as HJV or RGMc) causes juvenile hemochromatosis with an indistinguishable phenotype from juvenile hemochromatosis caused by mutations or loss of HAMP (encoding hepcidin) itself, in both human patients and in mice [9].

2 The Central Role of the BMP Co-Receptor Hemojuvelin and BMP6 in the Regulation of Hepcidin Expression

Since hepcidin is crucial for iron homeostasis and HJV is critical for hepcidin regulation, an understanding of the function of HJV would lead to insights into the regulation of iron metabolism. Hemojuvelin (HJV/HFE2/RGMc) was identified as the gene mutated in most cases of juvenile hemochromatosis [8, 9], resulting in an

indistinguishable phenotype from hemochromatosis caused by mutations in the hepcidin gene itself. HJV protein is most highly expressed in the liver, heart, and skeletal muscle [8, 10]. Although the function of HJV was unknown at the time, mice and humans with HJV mutations were known to have low hepcidin levels, and siRNA inhibition of HJV in liver cells *in vitro* decreased hepcidin expression, suggesting that HJV is involved in the positive regulation of hepcidin expression.

2.1 HJV Is a Member of the RGM Family

Hemojuvelin (also known as RGMc/DL2) is a member of the repulsive guidance molecule (RGM) family, which includes RGMa and DRAGON (also known as RGMb) [11]. RGMa was originally discovered to be a repulsive guidance molecule important during development for the guidance of chick retinal axons [12]. DRAGON/RGMb was independently discovered and found to be expressed in dorsal root ganglion cells and subsequently found to also be an axonal guidance molecule [13]. HJV shares 50–60 % sequence homology and key structural features with RGMa and DRAGON (RGMb), including an N-terminal signal sequence, proteolytic cleavage site, partial von Willebrand factor type D domain, and glycophosphatidylinositol (GPI) anchor [11]. RGMa and RGMb were found to interact with neogenin, a homologue of the netrin receptor DCC (deleted in colorectal cancer), and that this cell nonautonomous interaction was important for the guidance activity of RGMa and RGMb [11]. Subsequently, it was shown that both DRAGON and RGMa function as co-receptors to enhance bone morphogenetic protein (BMP) signaling [14, 15].

2.2 Hemojuvelin Is a BMP Co-receptor

Importantly, hemojuvelin was shown to function as a BMP co-receptor that can bind directly to BMP2 and BMP4 and BMP6 and enhances cellular responses to BMP ligands but not to BMP7 or BMP9 or other TGF-β superfamily ligands [10, 16]. Further evidence that BMP signaling was important in hepcidin regulation comes from data showing that BMP2 positively regulates hepcidin expression both *in vitro* and *in vivo* [16]. In addition, hemojuvelin increased hepcidin induction in response to BMP2 [17]. Hemojuvelin mutants associated with juvenile hemochromatosis have impaired BMP signaling ability, and hepatocytes from Hfe2-/- mice demonstrate blunted hepcidin induction in response to BMP2 [10]. This suggests that the mechanism for iron overload in patients with hemojuvelin mutations is due to decreased BMP signaling in the liver leading to decreased hepcidin expression. Thus, BMP signaling by the BMP co-receptor hemojuvelin appears to be central to hepcidin expression.

2.3 BMP6 Is a Major BMP Ligand in Hepcidin Regulation

The major native ligand for hemojuvelin in the liver appears to be BMP6, whose mRNA expression is regulated by iron *in vivo* [18]. BMP6 appears to be critical in mice to activate the BMP signaling cascade that leads to hepcidin expression [19, 20]. Global BMP6 KO mice have severe iron overload that is indistinguishable from the iron overload seen in mice with hepcidin and HJV mutations.

In humans, three heterozygous missense mutations were found in BMP6 in patients with unexplained iron overload [21]. These mutations lead to loss of signaling to the SMAD proteins and to reduced hepcidin production and to increased susceptibility to mild-to-moderate late-onset iron overload in these patients.

BMP6 binds type I and type II BMP receptors (BMPR-I and BMPR-II) in the presence of the BMP co-receptor hemojuvelin, inducing the phosphorylation of BMPR-I by BMPR-II. The activated receptor complex, in turn, phosphorylates a subset of SMAD proteins (SMAD1/SMAD5/SMAD8). These receptor-activated SMADs then form heteromeric complexes with the common mediator SMAD4, and these translocate to the nucleus where they regulate transcription of specific targets, such as hepcidin. Finally, there is also feedback regulation of this system, since serum iron and tissue iron can also regulate BMP6 mRNA expression [18].

2.4 Smad Signaling Is Important for Hepcidin Regulation

A liver-specific conditional knockout of Smad4 abrogated the transcriptional activation of hepcidin in response to iron overload, TGF-β, BMP, or IL-6 [22] and resulted in a similar iron overload phenotype in mice that is indistinguishable from the iron overload seen in hepcidin, HJV, or BMP6 KO mice. In addition, it was demonstrated that ectopic overexpression of SMAD4 in hepatocytes activated the hepcidin promoter and was associated with epigenetic modification of histone H3 to a transcriptionally active form.

Smad6 and Smad7, the inhibitory Smads, also appear to be involved in hepcidin regulation [23]. By using high-throughput siRNA screening, SMAD7 was identified as a potent hepcidin suppressor. SMAD7 was shown to be coregulated with hepcidin by BMPs in primary murine hepatocytes and that SMAD7 overexpression completely abolished hepcidin activation by BMPs. A distinct SMAD regulatory motif (GTCAAGAC) within the hepcidin promoter was identified that was involved in SMAD7-dependent hepcidin suppression, demonstrating that SMAD7 does not simply antagonize the previously reported hemojuvelin-/BMP-responsive elements. In addition, SMAD7 was shown to be coregulated with hepcidin via SMAD4 in response to altered iron availability in vivo [18]. Smad6 expression is similarly coordinated in response to iron as Smad7 [24].

More recently, it was determined that endofin, a SMAD anchor, is involved in hepcidin expression. Experiments showed that knockdown of endofin in liver cells inhibits basal and BMP-induced hepcidin expression along with other BMP-regulated genes, ID1 and SMAD7. Endofin was shown to interact *in situ* with SMAD proteins and to significantly reduce SMAD phosphorylation when endofin levels were knocked down, suggesting that endofin modulated hepcidin through the BMP-SMAD signaling pathway. Characterization of naturally occurring SNPs in the endofin gene showed that mutations in the conserved FYVE domain resulted in cellular mislocalization of endofin, potentially affecting downstream BMP signaling and modulating hepcidin expression [25].

3 Other BMP Ligands Involved in Hepcidin and Iron Regulation

While BMP6 appears to be the major BMP ligand involved in iron homeostasis under normal physiologic conditions, other BMPs have been shown to be able to upregulate hepcidin, both *in vivo* and *in vitro* [16, 17]. It is possible that under pathologic conditions, these other BMPs may play a prominent role in regulation of hepcidin and iron metabolism.

In anemia of multiple myeloma, hepcidin is induced by increased BMP2 [26]. Patients with multiple myeloma (MM) frequently present with anemia. It was shown that MM patients had increased serum hepcidin, which inversely correlated with hemoglobin, suggesting that hepcidin contributed to MM-related anemia. MM sera activated the hepcidin promoter significantly more than did sera from normal patients. Mutations in both BMP-responsive elements abrogated the activation by BMP or IL-6 dramatically, while mutations in the IL-6-responsive signal transducer and activator of transcription 3-binding site (STAT3-BS) had only a minor effect. Cotreatment with anti-BMP2/BMP4 or noggin-Fc blocked the promoter induction caused by all MM sera. Anti-IL-6 antibody blocked it with a minority of sera, whereas anti-BMP4, BMP6, or BMP9 antibodies had no effect. BMP2-immunodepleted MM sera had decreased promoter stimulatory capacity, and BMP2 concentrations in MM sera were significantly higher than in normal sera. These results support the hypothesis that BMP2 is a major mediator of the hepcidin stimulatory activity of MM sera.

Other BMPs that do not bind HJV such as BMP7 and BMP9 can also upregulate hepcidin [16]. Exogenous BMP7 has been shown to correct the iron overload seen in mouse models of hemochromatosis [27]. Therefore, it is not unreasonable to suggest that as yet undiscovered pathophysiologic states may exist where these other BMPs may be important contributors to hepcidin regulation, just as BMP2 has been shown to be important in the anemia of multiple myeloma.

4 Interactions of the BMP Signaling Pathway with Other Signaling Pathways in the Regulation of Hepcidin

4.1 Inflammation Is an Important Mediator of Hepcidin Expression and Requires BMP Signaling

Inflammation is associated with host defense mechanisms to infections. Since hepcidin is highly upregulated during infection and inflammation, it is thought that hepcidin may play a role in host defense against certain organisms. Supporting this notion are experiments in animals showing that siderophilic bacterium such as *Vibrio vulnificus* thrives in the presence of iron and that hepcidin deficiency results in increased bacteremia and decreased survival of infected mice [28]. Additionally, treatment with hepcidin agonists in hepcidin-deficient mice induced low iron levels that lead to decreased bacterial loads and rescued the infected mice from death. These findings demonstrated that hepcidin-mediated hypoferremia is a host defense mechanism against siderophilic pathogens, and evolution has selected this pathway for hepcidin regulation in mammals.

The IL-6/Stat3 pathway intersects with the BMP signaling pathway on the molecular level at the human hepcidin promoter, where a canonical BMP-responsive element is adjacent to a Stat3-binding element. When the Stat3 element is mutated in a hepcidin gene promoter construct, there is a blunted inductive response to IL-6 ligand [5]. Surprisingly, when the BMP-responsive element (BRE) is mutated instead, there is similar blunting of the inductive response of the hepcidin reporter gene to IL-6 stimulation. This result suggests that the BRE is required for the full effect of IL-6/Stat3 on hepcidin gene expression. If the BRE element is missing, then the effect of the IL-6/Stat3 pathway on hepcidin expression is highly muted.

To further corroborate these findings, it was demonstrated that eliminating BMP signaling, by sequestering BMP ligands with a soluble HJV. Fc protein [16], or blocking BMP receptor kinase activity directly using a small molecule chemical inhibitor [29], also leads to blunting of hepcidin expression by IL-6/Stat3. In mice, a liver-specific knockout of Smad4 leads to elimination of IL-6 induction of hepcidin [22], and IL-6 induction of hepcidin in HJV-KO [10] and BMP6-KO [19] mice is also impaired. Together, these data provide compelling evidence that BMP signaling is required for the full response of hepcidin expression to IL-6/Stat3, a key inflammatory mediator. Whether BMP signaling is required for the effects of other inflammatory pathways on hepcidin is not known.

4.2 Other Signaling Pathways that Interact with the BMP Pathway to Regulate Hepcidin

Matriptase-2, a liver-specific membrane protease encoded by the TMPRSS6 gene, has been hypothesized to cleave HJV on the cell surface of hepatocytes [30]. This would theoretically decrease BMP signaling, leading to dampening of hepcidin

expression. In patients with TMPRSS6 mutations, there is excess hepcidin present, and these patients develop iron refractory iron deficiency anemia (IRIDA) [31]. However, it has yet to be demonstrated that matriptase-2/TMPRSS6 actually cleaves HJV in hepatocytes *in vivo*. Alternatively, there is evidence that TMPRSS6 acts through an as yet unidentified inflammatory pathway to regulate hepcidin expression [32]. Interestingly, both iron and BMP6 can regulate the expression of the TMPRSS6 gene [33], providing feedback regulation of TMPRSS6 activity.

Erythroferrone (Erfe) has been identified as an erythroid regulator of hepcidin expression [34]. Erfe is expressed by erythroid cells during erythropoiesis and acts on the liver to suppress hepcidin expression. Erfe is thought to contribute to recovery from anemia of inflammation [35]. However, Erfe's ability to suppress hepcidin appears to only be effective under conditions of low or absent BMP signaling, since limiting hepatic Bmp-Smad signaling by matriptase-2 is required for erythropoietin-mediated hepcidin suppression in mice [36]. Thus, there is an intricate interplay between the BMP signaling pathway, matriptase-2/TMPRSS6, and the erythroferrone signaling pathways to finely tune hepcidin expression to control the availability of iron for erythropoiesis.

HFE is the most prevalent hemochromatosis gene and is responsible for the vast majority of adult hemochromatosis [7]. Bone morphogenetic protein signaling is impaired in an HFE knockout mouse model of hemochromatosis [37], and BMP signaling is impaired in human hepatocytes [38]. Furthermore, exogenous BMP6 treatment can compensate for the molecular defect and ameliorates hemochromatosis in Hfe knockout mice [39]. The exact mechanism by which HFE interacts with the BMP pathway is unknown, since the exact function of HFE remains unknown [9].

Neogenin, a homologue of the netrin receptor DCC (deleted in Colon Cancer), may interact with hemojuvelin in hepatocytes and may play a role in modifying HJV action, but the exact mechanisms of action on hemojuvelin and hepcidin are not yet clear [40, 41].

5 Therapeutic Potential of Targeting the BMP Pathway in the Treatment of Iron Disorders

Regulating the hepcidin-ferroportin axis may be useful in treating common diseases such as anemia of chronic disease and hemochromatosis. Since the BMP signaling pathway is critical for hepcidin expression, it is a prime therapeutic target for regulating hepcidin and consequently for regulating iron metabolism.

5.1 Strategies for Treating Hemochromatosis Using BMPs

Experiments have shown that injection of BMP ligands into mice with hemochromatosis can increase hepcidin levels and lower serum iron levels. BMP6 treatment compensates for the molecular defect and ameliorates hemochromatosis in Hfe

knockout mice [39]. Exogenous BMP7 corrects plasma iron overload and bone loss in Bmp6-/- mice [27]. However, there are several caveats to consider when contemplating use of BMPs to treat hemochromatosis and other iron overload disorders. First, BMP injection leads to calcification and bone formation at the injection site. Second, while increasing BMP signaling will lead to increased hepcidin levels and decreased serum iron levels, it does not lead to effective elimination of the excess iron that has already been accumulated in tissues. An adjunct iron chelation strategy must be used to remove previously stored excess iron. Because of these limitations, the direct use of BMPs remains a hypothetical strategy.

5.2 Anemia of Chronic Disease

Lowering BMP ligand levels and decreasing BMP signaling in hepatocytes can lead to lowering hepcidin levels and to increased serum iron levels, which would provide iron for erythropoeisis and thus treat anemia of chronic disease [42].

Several strategies have been employed, including sHJV. Fc [43], and anti-RGMc antibodies [44] to remove BMP ligands. In animal models of anemia caused by high hepcidin levels, these agents appear to be effective. Other strategies include the use of anti-BMP6 antibodies. One caveat with lowering BMP signaling is that elimination of BMP signaling may lead to as yet uncharacterized deleterious effects. Currently, several human clinic trials are underway to test these therapeutic strategies.

6 Conclusion/Perspectives

BMP signaling has been discovered to be central to iron metabolism by regulating the expression of the iron hormone hepcidin (Fig. 1). Dysregulation of the BMP signaling pathway components leads to iron disorders such as hemochromatosis and anemia in both animals and humans. Several other signaling pathways including the inflammatory pathway interact with the BMP signaling system to modulate hepcidin expression. Therapeutic strategies based on augmenting or inhibiting the BMP pathway may be useful in treating iron disorders and are being tested both in animals and in the clinic.

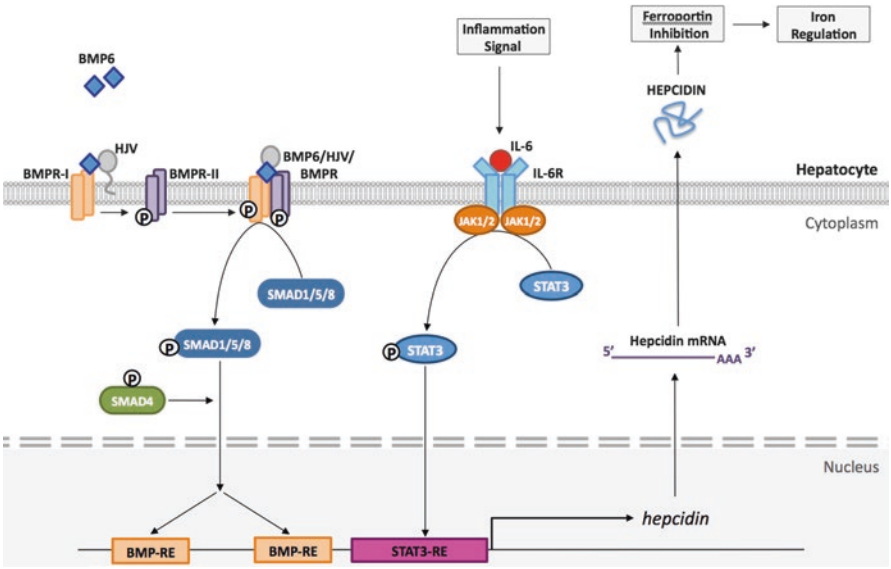


Fig. 1 Schematic representation of BMP signaling in a liver cell leading to hepcidin gene expression. The BMP6-HJV-SMAD and IL-6-STAT3 signaling pathways both activate hepcidin transcription in the liver (black arrows). In response to iron sufficiency, circulating bone morphogenetic protein 6 (BMP6) binds transmembrane BMP receptors type I (BMP-RI) and type II (BMP-RII) and BMP co-receptor hemojuvelin (HJV) to create a complex on the hepatocyte membrane to generate the SMAD signaling cascade. Phosphorylated SMAD1/SMAD5/SMAD8 proteins then bind to SMAD4 and translocate to the nucleus to induce hepcidin expression through BMP-responsive elements (BMP-REs) located on the hepcidin promoter. During inflammation, pro-inflammatory cytokines like IL-6 are released. Upon binding to its receptor, IL-6 initiates signaling through activated JAK1/JAK2 proteins to phosphorylate the transcription factor STAT3. Phosphorylated STAT3 then binds to a STAT3-responsive element (STAT3-RE) on the hepcidin promoter. Both STAT3-RE and the adjacent BMPR-RE are required for IL-6-mediated hepcidin expression. Hepcidin protein is secreted into the bloodstream leading to ferroportin inhibition, toresulting in iron retention in the reticuloendothelial macrophages and reduced iron absorption in the intestinal epithelia

Acknowledgments I would like to thank the members of the Division of Nephrology, Program in Membrane Biology, and Center for Systems Biology at the Massachusetts General Hospital for their continued support. This work was funded in part by NIH grant RO1DK071837. I own equity in Ferrumax sPharmaceuticals, Inc., a start-up company that has licensed technology from the Massachusetts General Hospital.

References

1. Ganz T (2013) Systemic iron homeostasis. Physiol Rev 93(4):1721–1741. doi:10.1152/physrev.00008.2013 Review
2. Ganz T, Nemeth E (2011) Hepcidin and disorders of iron metabolism. Annu Rev Med 62:347–360. doi:10.1146/annurev-med-050109-142444 Review
3. Meynard D, Babitt JL, Lin HY (2014) The liver: conductor of systemic iron balance. Blood 123(2):168–176. doi:10.1182/blood-2013-06-427757 Epub 2013 Nov 7. Review
4. Nemeth E, Tuttle MS, Powelson J, Vaughn MB, Donovan A, Ward DM, Ganz T, Kaplan J (2004) Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science 306(5704):2090–2093 Epub 2004 Oct 28
5. Verga Falzacappa MV, Vujic Spasic M, Kessler R, Stolte J, Hentze MW, Muckenthaler MU (2007) STAT3 mediates hepatic hepcidin expression and its inflammatory stimulation. Blood 109(1):353–358 Epub 2006 Aug 31
6. Nicolas G, Chauvet C, Viatte L, Danan JL, Bigard X, Devaux I, Beaumont C, Kahn A, Vaulont S (2002) The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. J Clin Invest 110:1037–1044
7. Pietrangelo A (2004) Hereditary hemochromatosis--a new look at an old disease. N Engl J Med 350(23):2383–2397
8. Papanikolaou G, Samuels ME, Ludwig EH, MacDonald ML, Franchini PL, Dube MP, Andres L, MacFarlane J, Sakellaropoulos N, Politou M et al (2004) Mutations in HFE2 cause iron overload in chromosome 1q-linked juvenile hemochromatosis. Nat Genet 36:77–82
9. Babitt JL, Lin HY (2011) The molecular pathogenesis of hereditary hemochromatosis. Semin Liver Dis 31(3):280–292. doi:10.1055/s-0031-1286059 Epub 2011 Sep 7. Review
10. Babitt JL, Huang FW, Wrighting DM, Xia Y, Sidis Y, Samad TA, Campagna JA, Chung RT, Schneyer AL, Woolf CJ, Andrews NC, Lin HY (2006) Bone morphogenetic protein signaling by hemojuvelin regulates hepcidin expression. Nat Genet 38(5):531–539 Epub 2006 Apr 9
11. Corradini E, Babitt JL, Lin HY (2009) The RGM/DRAGON family of BMP co-receptors. Cytokine Growth Factor Rev 20(5–6):389–398 Epub 2009 Nov 7. Review
12. Monnier PP, Sierra A, Macchi P, Deitinghoff L, Andersen JS, Mann M, Flad M, Hornberger MR, Stahl B, Bonhoeffer F, Mueller BK (2002) RGM is a repulsive guidance molecule for retinal axons. Nature 419(6905):392–395
13. Samad TA, Srinivasan A, Karchewski LA, Jeong SJ, Campagna JA, Ji RR, Fabrizio DA, Zhang Y, Lin HY, Bell E, Woolf CJ (2004) DRAGON: a member of the repulsive guidance molecule-related family of neuronal- and muscle-expressed membrane proteins is regulated by DRG11 and has neuronal adhesive properties. J Neurosci 24(8):2027–2036
14. Samad TA, Rebbapragada A, Bell E, Zhang Y, Sidis Y, Jeong SJ, Campagna JA, Perusini S, Fabrizio DA, Schneyer AL, Lin HY, Brivanlou AH, Attisano L, Woolf CJ (2005) DRAGON, a bone morphogenetic protein co-receptor. J Biol Chem 280(14):14122–14129 Epub 2005 Jan 25
15. Babitt JL, Zhang Y, Samad TA, Xia Y, Tang J, Campagna JA, Schneyer AL, Woolf CJ, Lin HY (2005) Repulsive guidance molecule (RGMa), a DRAGON homologue, is a bone morphogenetic protein co-receptor. J Biol Chem 280(33):29820–29827 Epub 2005 Jun 23
16. Babitt JL, Huang FW, Xia Y, Sidis Y, Andrews NC, Lin HY (2007) Modulation of bone morphogenetic protein signaling in vivo regulates systemic iron balance. J Clin Invest 117(7):1933–1939
17. Xia Y, Babitt JL, Sidis Y, Chung RT, Lin HY (2008) Hemojuvelin regulates hepcidin expression via a selective subset of BMP ligands and receptors independently of neogenin. Blood 111(10):5195–5204. doi:10.1182/blood-2007-09-111567 Epub 2008 Mar 7
18. Kautz L, Meynard D, Monnier A, Darnaud V, Bouvet R, Wang RH, Deng C, Vaulont S, Mosser J, Coppin H, Roth MP (2008) Iron regulates phosphorylation of Smad1/5/8 and gene expression of Bmp6, Smad7, Id1, and Atoh8 in the mouse liver. Blood 112(4):1503–1509. doi:10.1182/blood-2008-03-143354 Epub 2008 Jun 6

19. Meynard D, Kautz L, Darnaud V, Canonne-Hergaux F, Coppin H, Roth MP (2009) Lack of the bone morphogenetic protein BMP6 induces massive iron overload. Nat Genet 41(4):478–481. doi:10.1038/ng.320 Epub 2009 Mar 1
20. Andriopoulos B Jr, Corradini E, Xia Y, Faasse SA, Chen S, Grgurevic L, Knutson MD, Pietrangelo A, Vukicevic S, Lin HY, Babitt JL (2009) BMP6 is a key endogenous regulator of hepcidin expression and iron metabolism. Nat Genet 41(4):482–487. doi:10.1038/ng.335 Epub 2009 Mar 1
21. Daher R, Kannengiesser C, Houamel D, Lefebvre T, Bardou-Jacquet E, Ducrot N, de Kerguenec C, Jouanolle AM, Robreau AM, Oudin C, Le Gac G, Moulouel B, Loustaud-Ratti V, Bedossa P, Valla D, Gouya L, Beaumont C, Brissot P, Puy H, Karim Z, Tchernitchko D (2016) Heterozygous mutations in BMP6 pro-peptide lead to inappropriate hepcidin synthesis and moderate iron overload in humans. Gastroenterology 150(3):672–683.e4. doi:10.1053/j.gastro.2015.10.049 Epub 2015 Nov 12
22. Wang RH, Li C, Xu X, Zheng Y, Xiao C, Zerfas P, Cooperman S, Eckhaus M, Rouault T, Mishra L, Deng CX (2005) A role of SMAD4 in iron metabolism through the positive regulation of hepcidin expression. Cell Metab 2(6):399–409
23. Mleczko-Sanecka K, Casanovas G, Ragab A, Breitkopf K, Müller A, Boutros M, Dooley S, Hentze MW, Muckenthaler MU (2010) SMAD7 controls iron metabolism as a potent inhibitor of hepcidin expression. Blood 115(13):2657–2665. doi:10.1182/blood-2009-09-238105 Epub 2009 Dec 29
24. Vujić Spasić M, Sparla R, Mleczko-Sanecka K, Migas MC, Breitkopf-Heinlein K, Dooley S, Vaulont S, Fleming RE, Muckenthaler MU (2013) Smad6 and Smad7 are co-regulated with hepcidin in mouse models of iron overload. Biochim Biophys Acta 1832(1):76–84. doi:10.1016/j.bbadis.2012.08.013 Epub 2012 Aug 31
25. Goh JB, Wallace DF, Hong W, Subramaniam VN (2015) Endofin, a novel BMP-SMAD regulator of the iron-regulatory hormone, hepcidin. Sci Rep 5:13986. doi:10.1038/srep13986
26. Maes K, Nemeth E, Roodman GD, Huston A, Esteve F, Freytes C, Callander N, Katodritou E, Tussing-Humphreys L, Rivera S, Vanderkerken K, Lichtenstein A, Ganz T (2010) In anemia of multiple myeloma, hepcidin is induced by increased bone morphogenetic protein 2. Blood 116(18):3635–3644. doi:10.1182/blood-2010-03-274571 Epub 2010 Aug 2
27. Pauk M, Grgurevic L, Brkljacic J, Kufner V, Bordukalo-Niksic T, Grabusic K, Razdorov G, Rogic D, Zuvic M, Oppermann H, Babitt JL, Lin HY, Volarevic S, Vukicevic S (2015) Exogenous BMP7 corrects plasma iron overload and bone loss in Bmp6−/− mice. Int Orthop 39(1):161–172. doi:10.1007/s00264-014-2550-4 Epub 2014 Oct 11
28. Arezes J, Jung G, Gabayan V, Valore E, Ruchala P, Gulig PA, Ganz T, Nemeth E, Bulut Y (2015) Hepcidin-induced hypoferremia is a critical host defense mechanism against the siderophilic bacterium Vibrio vulnificus. Cell Host Microbe 17(1):47–57. doi:10.1016/j.chom.2014.12.001
29. Yu PB, Hong CC, Sachidanandan C, Babitt JL, Deng DY, Hoyng SA, Lin HY, Bloch KD, Peterson RT (2008) Dorsomorphin inhibits BMP signals required for embryogenesis and iron metabolism. Nat Chem Biol 4(1):33–41 Epub 2007 Nov 18
30. Silvestri L, Pagani A, Nai A, De Domenico I, Kaplan J, Camaschella C (2008) The serine protease matriptase-2 (TMPRSS6) inhibits hepcidin activation by cleaving membrane hemojuvelin. Cell Metab 8(6):502–511. doi:10.1016/j.cmet.2008.09.012 Epub 2008 Oct 30
31. Wang CY, Meynard D, Lin HY (2014) The role of TMPRSS6/matriptase-2 in iron regulation and anemia. Front Pharmacol 5:114. doi:10.3389/fphar.2014.00114 eCollection 2014. Review
32. Riba M, Rausa M, Sorosina M, Cittaro D, Garcia Manteiga JM, Nai A, Pagani A, Martinelli-Boneschi F, Stupka E, Camaschella C, Silvestri L (2013) A strong anti-inflammatory signature revealed by liver transcription profiling of Tmprss6−/− mice. PLoS One 8(7):e69694. doi:10.1371/journal.pone.0069694. Print 2013
33. Meynard D, Vaja V, Sun CC, Corradini E, Chen S, López-Otín C, Grgurevic L, Hong CC, Stirnberg M, Gütschow M, Vukicevic S, Babitt JL, Lin HY (2011) Regulation of TMPRSS6 by BMP6 and iron in human cells and mice. Blood 118(3):747–756. doi:10.1182/blood-2011-04-348698 Epub 2011 May 26

34. Kautz L, Jung G, Valore EV, Rivella S, Nemeth E, Ganz T (2014) Identification of erythroferrone as an erythroid regulator of iron metabolism. Nat Genet 46(7):678–684. doi:10.1038/ng.2996 Epub 2014 Jun 1
35. Kautz L, Jung G, Nemeth E, Ganz T (2014) Erythroferrone contributes to recovery from anemia of inflammation. Blood 124(16):2569–2574. doi:10.1182/blood-2014-06-584607 Epub 2014 Sep 5
36. Nai A, Rubio A, Campanella A, Gourbeyre O, Artuso I, Bordini J, Gineste A, Latour C, Besson-Fournier C, Lin HY, Coppin H, Roth MP, Camaschella C, Silvestri L, Meynard D (2016) Limiting hepatic Bmp-Smad signaling by matriptase-2 is required for erythropoietin-mediated hepcidin suppression in mice. Blood 127(19):2327–2336. doi:10.1182/blood-2015-11-681494 Epub 2016 Jan 11
37. Corradini E, Garuti C, Montosi G, Ventura P, Andriopoulos B Jr, Lin HY, Pietrangelo A, Babitt JL (2009) Bone morphogenetic protein signaling is impaired in an HFE knockout mouse model of hemochromatosis. Gastroenterology 137(4):1489–1497. doi:10.1053/j.gastro.2009.06.057 Epub 2009 Jul 7
38. Bolondi G, Garuti C, Corradini E, Zoller H, Vogel W, Finkenstedt A, Babitt JL, Lin HY, Pietrangelo A (2010) Altered hepatic BMP signaling pathway in human HFE hemochromatosis. Blood Cells Mol Dis 45(4):308–312. doi:10.1016/j.bcmd.2010.08.010 Epub 2010 Sep 21
39. Corradini E, Schmidt PJ, Meynard D, Garuti C, Montosi G, Chen S, Vukicevic S, Pietrangelo A, Lin HY, Babitt JL (2010) BMP6 treatment compensates for the molecular defect and ameliorates hemochromatosis in Hfe knockout mice. Gastroenterology 139(5):1721–1729. doi:10.1053/j.gastro.2010.07.044 Epub 2010 Aug 1
40. Zhang AS, West AP Jr, Wyman AE, Bjorkman PJ, Enns CA (2005) Interaction of hemojuvelin with neogenin results in iron accumulation in human embryonic kidney 293 cells. J Biol Chem 280(40):33885–33894 Epub 2005 Aug 15
41. Zhang AS, Anderson SA, Meyers KR, Hernandez C, Eisenstein RS, Enns CA (2007) Evidence that inhibition of hemojuvelin shedding in response to iron is mediated through neogenin. J Biol Chem 282(17):12547–12556 Epub 2007 Mar 1
42. Sun CC, Vaja V, Babitt JL, Lin HY (2012) Targeting the hepcidin-ferroportin axis to develop new treatment strategies for anemia of chronic disease and anemia of inflammation. Am J Hematol 87(4):392–400. doi:10.1002/ajh.23110 Epub 2012 Jan 31. Review
43. Theurl I, Schroll A, Sonnweber T, Nairz M, Theurl M, Willenbacher W, Eller K, Wolf D, Seifert M, Sun CC, Babitt JL, Hong CC, Menhall T, Gearing P, Lin HY, Weiss G (2011) Pharmacologic inhibition of hepcidin expression reverses anemia of chronic inflammation in rats. Blood 118(18):4977–4984. doi:10.1182/blood-2011-03-345066 Epub 2011 Jul 5
44. Kovac S, Böser P, Cui Y, Ferring-Appel D, Casarrubea D, Huang L, Fung E, Popp A, Mueller BK, MW H (2016) Anti-hemojuvelin antibody corrects anemia caused by inappropriately high hepcidin levels. Haematologica 101(5):e173–e176. doi:10.3324/haematol.2015.140772 Epub 2016 Mar 4. No abstract available

BMPs in Inflammation

Lovorka Grgurevic, Ivo Dumic-Cule, and Slobodan Vukicevic

Abstract Bone morphogenetic proteins are regulators of embryonic development with multiple functions in adult organs and tissues. Here we summarize effects of BMPs outside the musculoskeletal system, focused on their role in inflammatory disorders, e.g., fibrosis, inflammatory bowel disease, anchylosing spondylitis, and rheumatoid arthritis. Additionally, we discuss the interplay between BMPs and vascular disorders leading to atherosclerosis and decipher the key role of BMP in iron metabolism.

Keywords Bone morphogenetic protein • Inflammatory bowel disease • Iron metabolism • Rheumatoid arthritis • Atherosclerosis

Bone morphogenetic proteins (BMPs), originally identified by a unique capability to induce ectopic bone formation, are classified in TGFβ superfamily. BMPs were described to act as important regulators of differentiation and patterning of organs and tissues originating from all three developmental layers. They also exert multiple actions in various inflammatory conditions such as inflammatory bowel disease, chronic liver disease, iron deficiency anemia, rheumatoid arthritis, ankylosing spondylitis, vascular disease, and atherosclerosis.

BMPs are secreted as active dimeric complexes. Their communication with neighboring cells is primarily exerted in paracrine and autocrine manner [1]. Local concentration level of BMPs is thus important for embryogenesis and organogenesis. However, the presence of several BMPs in blood has been demonstrated recently, including BMP6, BMP9, and BMP10, suggesting their endocrine role [2, 3].

L. Grgurevic (✉) • I. Dumic-Cule • S. Vukicevic
Center for Translational and Clinical Research, Laboratory for Mineralized Tissues, University of Zagreb School of Medicine, Salata 11, Zagreb 10000, Croatia
e-mail: lgrgurev@mef.hr; ivodc1@gmail.com; vukicev@mef.hr

S. Vukicevic, K.T. Sampath (eds.), *Bone Morphogenetic Proteins: Systems Biology Regulators*, Progress in Inflammation Research,
DOI 10.1007/978-3-319-47507-3_16

Utilizing heparin affinity chromatography purification and proteomic techniques, we have recently discovered that BMP1 isoforms have been found to circulate in the blood of patients with different fibrotic diseases [4]. In this chapter current insights into BMPs' role in inflammation will be presented, and their potential underlying mechanism of action will be discussed.

1 BMPs in Inflammatory Bowel Disease and Associated Iron Deficiency Anemia

The importance of BMP signaling in the gastrointestinal tract development has been already determined by the detection of BMPs and their components in all three developmental germ layers [5]. Inflammatory bowel disease (IBD) and IBD-related iron deficiency anemia will be described in this paragraph.

IBD is the entity comprised of Crohn's disease and ulcerative colitis (UC) with both genetic and multifactorial environmental etiology, which is not completely elucidated yet. These patients are genetically prone to disturbed interaction between the intestinal microflora and inflammatory cells that will lead to typical destruction of intestinal tissue with accompanied active inflammation. Genome-wide expression analysis of mucosal biopsies from patients with UC has been recently performed [6]. Interestingly, BMP/retinoic acid-inducible neural-specific protein 3 (BRINP3) was revealed to be significantly downregulated in patients with UC, thereby serving as a marker of severe mucosal inflammation. Animal model of trinitrobenzene sulfonic acid (TNBS)-induced colitis enables an assessment of both early and late course of the disease. In contrast, early phase in humans is mostly asymptomatic, while late stages are manifested with signs and symptoms of severely damaged intestinal wall. BMP7 has been shown to have beneficial effects on the course of TNBS-induced colitis in rats when administered both prophylactically and therapeutically [7]. The rationale for targeting BMP7 was cognition of its abundant expression in the developing intestine. Immunohistochemical and RT-PCR analyses have shown an elevated expression of pro-inflammatory cytokines, especially interleukin-6 (IL-6), which was significantly reduced upon BMP7 treatment and correlated with a less severe inflammation and improved UC lesions detected by macroscopic and histological observation. In the recent study, an adeno-associated virus vector for delivering BMP7 was designed and ameliorated severity of the disease in rats with induced UC, by decreasing the disease activity index and reducing the rate of oxygen damage [8, 12].

TGFβ1 is known as a protective agent in UC, confirming the key role of TGFβ/BMP signaling cascade [9]. Nevertheless, TGFβ1 was found to be increased in patients with IBD [10]. Further elucidation of the mechanism of action revealed that increased expression of Smad7 made inflammatory cells less prone to TGFβ1 stimulation, thereby diminishing its defensive role, and might lead to an increased inflammation in the gut. Therefore, preserving normal Smad7 signaling seems mandatory for maintenance of the intestinal homeostasis. In line with this, BMP7 was

shown to downregulate the expression of inhibitory Smads. In the active form of the disease, SMAD7 protein binds to a TGFβ1 receptor and blocks TGFβ anti-inflammatory signaling. Based on this mechanism, an oral antisense SMAD7 oligonucleotide called mongersen was produced and tested in patients with IBD. The data from the phase II multicenter RCT showed a significant clinical benefit in patients with active Crohn's disease, significantly supporting and extending the remission time following therapy [11].

A prolonged period of an active IBD is accompanied with an iron deficiency anemia, due to the intestinal blood loss and deficient erythropoiesis caused by iron restriction following an increased hepcidin level. Hepcidin is the key hormone controlling the iron homeostasis via numerous proteins including hemojuvelin, BMP6, hereditary hemochromatosis protein, and others [13]. Hepcidin upregulation leads to an iron deficiency due to inhibition of iron recycling from erythrocytes and restricted absorption from the diet as a result of a decreased expression of ferroportin on macrophages and duodenal enterocytes. Consequence of the intestinal blockage of iron intake anemia in such patients is resistant to oral iron supplements. Hepcidin could be regulated via at least two interconnected pathways – inflammatory response mediated by IL-6 and "iron-sensing" pathway via BMP6/SMAD. IL-6 was shown to be the key driver in development of anemia of chronic disease, mostly by hepcidin-dependent signaling increasing its expression both *in vivo* and *in vitro* [14, 15]. Inflammatory cytokines, mainly IL-6, upregulate hepcidin expression by stimulating BMP signaling in the liver. IL-1β was shown to possess an ability to increase the level of both hepcidin and BMP signaling in *in vitro* conditions and in the mouse liver [16]. BMP exerts its action via the BMP receptor I and the co-receptor hemojuvelin, thereby enabling phosphorylation and nuclear translocation of SMAD 1/5/8 transcription factors [17]. Presently, BMP6 seems to have the highest potential in hepcidin regulation, since BMP6 knockout mice revealed a significant iron overload [18]. However, BMP6 mRNA was not upregulated in intestinal inflammation, while IL-6 was significantly increased. The IL-6 rapid increase was obviously sufficient for the induction of hepcidin transcription. Collectively, tight regulation of both pathways is mandatory, specifically intact BMP/SMAD signaling cascade, because misregulation of either one will cause a profound anemia [19]. Molecules with power to inhibit hepcidin expression could be valuable therapeutic options for patients with refractory anemia due to IBD [20].

2 BMP in Liver Diseases of Different Etiology

The liver can be affected with systemic and local diseases of various etiologies and is characterized by a unique regenerative capacity after acute damage and formation of fibrotic tissue upon chronic injuries as a result of excessive accumulation of extracellular matrix (ECM) proteins [21]. Fibrogenesis, often provoked by inflammation, represents a common pathophysiological pathway of many chronic liver conditions including hereditary diseases (hemochromatosis, Wilson's disease, deficiency of α-1

antitrypsin), alcoholic liver disease, drug toxicity, viral hepatitis, autoimmune hepatitis, and cholestatic diseases. Hepatic tissue demonstrates a tight interplay between epithelial cells, inflammatory cells, myofibroblasts, and ECM components activated as a response to injury. Besides hepatic stellate cells (HSC), myofibroblasts and cells of the bone marrow origin have been shown to exhibit fibrogenic properties [22]. Inflammation promotes activation of HSC, which undergoes phenotypic change in the aspect of acquisition of fibrogenic features and subsequent abundant collagen production. So far, a variety of signaling pathways and cytokines have been shown to regulate the initiation and progression of liver fibrosis, including TGFβ1, connective tissue growth factor (CTGF), BMPs, and others [23]. With the aggravation of hepatic fibrosis, CTGF and TGFβ1 expression in the liver increased, suggesting that those molecules and liver fibrosis are closely related [24]. TGFβ1 was classified as one of the most profibrotic cytokines due to its ability to enhance the transition of HSC toward a myofibroblast-like phenotype and additional inhibition of ECM degradation by HSC through the expression of tissue inhibitor of metalloproteinases (TIMPs). More accurately, TGFβ1 will not directly affect contractile myofibroblasts, but will predominantly exert its actions on HSC stimulation [25].

BMPs are known as important regulators of liver development and regeneration [26]. Administration of rhBMP7 significantly improved liver regeneration and function after partial hepatectomy in mice. Moreover, neutralization of endogenous BMP7 resulted in improper regeneration of the liver. Liver regeneration was mediated by ALK3, which increased nuclear translocation of phosphorylated Smad1, thereby suggesting that the endogenous BMP7 is involved in the liver regeneration. However, surprisingly BMP7 expression was not detected in the healthy and injured liver tissue, while the presence of corresponding receptors was found [27]. Therefore, it was suggested that circulating BMP7 serves as an endogenous regulator of hepatocyte health and function. Expression of BMP9 as the precursor protein that undergoes cleavage by serine endoprotease was confirmed in the liver [28]. The main BMP9 receptor in hepatocytes, ALK1, activates the target gene inhibitor of differentiation 1 (Id1) via the Smad1 pathway, which then stimulates HSC-mediated ECM overproduction that contributes to the development of fibrosis [29]. Additionally, BMP9 induces Snail expression, known as an upregulator of different profibrotic cytokines like CTGF and TGFβ1 [30, 31].

3 BMPs in Skeletal and Joint Disorders

BMPs were originally discovered in the bone, followed by their localization in the cartilage. However, few bone and cartilage diseases are not well understood and are probably, according to available clinical data, partly based on the modulation of BMP signaling in inflammatory conditions: rheumatoid arthritis (RA) and ankylosing spondylitis (AS). Proteomic analysis of plasma samples from patients with RA and noninflammatory rheumatic conditions revealed differently expressed proteins [32]. Serum concentration of BMP2 and BMP7 was higher in patients with both RA

and AS when compared to healthy controls [33]. Expression of BMP4 mRNA was found to be significantly reduced in the synovial tissue of RA patients in comparison with healthy donors [34, 35]. BMP signaling pathway activation upon inflammation is recognized as the key event in bone loss in RA and bone gain in AS. The pro-inflammatory cytokines like tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6 significantly increase BMP2 and BMP6 expression in the arthritic synovium, which upon activation exert their actions mainly via modulating fibroblast-like synoviocytes [36]. Quantitative PCR was utilized to determine the expression of different components of BMP pathway in RA synoviocytes prior to and upon the treatment with pro-inflammatory cytokines TNF-α and IL-17 [37]. BMP signaling complex has basal functional activity in human RA synoviocytes, while its inhibition by dorsomorphin homologue 1 in turn augments the pro-inflammatory phenotype induced by TNF-α and IL-17. This finding confirmed the beneficial role of BMP in the severity of RA. However, antirheumatic treatment diminished the synovial inflammation but did not significantly influence the BMP expression level [38]. Lactoferrin was reported as a marker of Bmp7 gene activation through the mitogen-activated protein kinase ERK pathway in joint chondrocytes. In contrast, FGF-8 was shown to suppress BMP-induced osteoblast differentiation via the ERK pathway and was additionally downregulated by TNF-α stimulation [39]. This and earlier findings should elucidate multiple effects of pro-inflammatory cytokines on the BMP signaling cascade. It seems that positive effects in RA mediated by BMPs will be exerted only upon their activation via noninflammatory pathways or following exogenous administration.

Structural damage in AS is characterized by the new cartilage and bone formation, which led to progressive ankylosis of the spine and sacroiliac joints resulting in the fusion of various vertebral segments and subsequent disability. Inflammation in the early phase of AS was shown to significantly impact the later function impairment [40]. The disease is characterized by bone loss in the trabecular area and new bone formation in the cortex, which will form a typical ankylosis. Trabecular bone loss is a consequence of inflammation, while new bone formation can be explained with a profound stimulation of particular BMPs [41]. Local production of BMP2, BMP4, and BMP7 is stimulated by the peripheral blood mononuclear cells upon strong signaling from TNF-α and IL-1β [42]. In AS patients IgG autoantibodies against noggin were found to be increased when compared to healthy individuals, which can additionally contribute to the ossification potential [43]. Recently, a large cohort of AS patients to define the possible correlation between radiographically determined disease severity and genes associated with bone formation has been analyzed [44]. The presence of syndesmophytes and lumbar or cervical fusion were considered as severe AS, while lack of that finding was classified as mild AS. Two single nucleotide polymorphisms in BMP6 were for the first time identified as a marker of radiologic severity of AS.

Possible explanation of the diverse pathophysiological course of RA and AS is a different level of inflammation between two diseases. In AS patients inflammation is less pronounced and could allow BMP signaling to stimulate production of new bone.

4 Interplay Between Vascular Inflammation and BMPs

Additionally to its contribution in early heart development and establishment of vessel network, BMP endocrine-like role in adult cardiovascular homeostasis is recognized and explained in both clinical and experimental observations [45].

Atherosclerosis and plaque rupture due to its progression is a common underlying pathological event prior to regional ischemia and its consequences such as stroke and myocardial infarction. The initiation and progression of atherosclerosis are complicated, multifaceted pathologic events which are not completely elucidated. Key processes include endothelial cell dysfunction, infiltration of inflammatory cells, lipid dysregulation, and vascular smooth muscle cell differentiation that lead to the chronic inflammatory state.

Studies demonstrated that vascular endothelial and smooth muscle cells are a significant source of BMPs [46]. BMPs, specifically BMP2, could act as modulators of endothelial cell inflammation and differentiation via NF-κB activation after exposure to mechanical stress and/or pro-inflammatory cytokines linked to an increased level of reactive oxygen species [47]. TNF-α induces an overexpression of BMP2 mRNA in endothelial cells. BMP2 level remained normal upon pharmacological inhibition of NF-κB signaling utilizing pyrrolidine dithiocarbamate and SN-50. This study revealed that TNF-α substantially increased the NADPH oxidase-derived H_2O_2 production in endothelial cells, which is the key step for BMP2 induction. Both exogenous administration of H_2O_2 and endogenous induction by high intraluminal pressure significantly augment the BMP2 release. Association between increased vascular expression of BMP2 and hyperhomocysteinemia, vascular inflammation, and upregulated TNF-α has been demonstrated in coronary arteries of male rats [48]. BMP4 was found to be upregulated upon exposure to oscillatory shear stress, which was not the case with laminar shear [49]. It is already known that atherosclerosis more frequently occurs on arterial regions with turbulent flow, while vessels exposed to laminar shear are less prone to endothelial injury. Additionally, BMP4 was found to be notably expressed only in particular parts of human coronary arteries which contain foam cells. Both BMP2 and BMP4, with similar amino acid sequence, exert their pro-inflammatory effects by overexpression of adhesion molecules, mainly ICAM-1, on endothelial surface and profound monocyte recruitment and accumulation. Moreover, chronic infusion of recombinant BMP4 activated NADPH oxidases, thereby increasing the concentration of reactive oxygen species and decreased NO production, which subsequently led to endothelium dysfunction and hypertension [50]. Reactive oxygen species also stimulate expression of ICAM-1 and monocyte binding, which are prerequisites for foam cell formation and atherosclerosis progression. BMP2 has a potential to incorporate inside endothelial microparticles released by endothelial cells stimulated by inflammatory cytokines and may cause osteogenic differentiation of vascular smooth muscle cells [51]. Numerous experimental *in vitro* and gene expression studies suggested a close resemblance between a pathologic vascular calcification and bone remodeling [52, 53]. BMP2 and BMP4 were shown to be

upregulated in atherosclerotic plaques in the human abdominal aorta. The presence of osteoprotegerin (OPG) and OPG ligand, important modulators of osteoclastogenesis, was confirmed in non-diseased aortas. In contrast, increased expression of OPG was found in calcified lesions, suggesting a regulatory role of this pathway in atherosclerosis [54]. Possible explanation of a calcified plaque formation is timed and plaque-restricted activation of proteins like BMP2 and BMP4 that overrules inhibitors of calcification such as matrix Gla protein, osteocalcin, and bone sialoprotein.

BMP4 adverse effects on the process of endothelial dysfunction and atherosclerosis progression have been shown in mice with a knockout for BMPRII that exhibited pronounced vascular inflammation and a marked atherosclerosis through an elevated monocyte adhesion via increased expression of ICAM-1 and VCAM-1, independently from BMP4 [55]. Knockdown of BMPRII also increased the reactive oxygen species in endothelial cells.

Matrix Gla protein (MGP), the well-known antagonist of BMP signaling, reduced the vascular BMP concentration, the size of atherosclerotic lesion, and the vascular wall calcification in apolipoprotein E -/- mice [56]. In addition, the activin-like kinase receptor 1 and vascular endothelial growth factor, members of the BMP-activated pathway that regulate angiogenesis and potentially enhance lesion formation and calcification, were also reduced. Collectively, different studies suggested that the loss of MGP and consequently magnified BMP signaling induced the calcification in arterial medial cells, basically by reprogramming of smooth muscle cells toward an osteochondrogenic lineage [57].

Many studies suggested an important role of the BMP signaling, especially intact BMPIIR and homeostasis between BMP and their antagonists in the early and late pathophysiology of atherosclerosis.

References

1. Martinovic S, Borovecki F, Miljavac V, Kisic V, Maticic D, Francetic I, Vukicevic S (2006) Requirement of a bone morphogenetic protein for the maintenance and stimulation of osteoblast differentiation. Arch Histol Cytol 69:23–36
2. David L, Mallet C, Keramidas M, Lamandé N, Gasc JM, Dupuis-Girod S, Plauchu H, Feige JJ, Bailly S (2008) Bone morphogenetic protein-9 is a circulating vascular quiescence factor. Circ Res 102:914–922
3. Jiang H, Salmon RM, Upton PD, Wei Z, Lawera A, Davenport AP, Morrell NW, Li W (2016) The prodomain-bound form of bone morphogenetic protein 10 is biologically active on endothelial cells. J Biol Chem 291:2954–2966
4. Grgurevic L, Macek B, Healy DR, Brault AL, Erjavec I, Cipcic A, Grgurevic I, Rogic D, Galesic K, Brkljacic J, Stern-Padovan R, Paralkar VM, Vukicevic S (2011) Circulating bone morphogenetic protein 1-3 isoform increases renal fibrosis. J Am Soc Nephrol 22:681–692
5. Batts LE, Polk DB, Dubois RN, Kulessa H (2006) Bmp signaling is required for intestinal growth and morphogenesis. Dev Dyn 235:1563–1570
6. Smith PJ, Levine AP, Dunne J, Guilhamon P, Turmaine M, Sewell GW, O'Shea NR, Vega R, Paterson JC, Oukrif D, Beck S, Bloom SL, Novelli M, Rodriguez-Justo M, Smith AM, Segal

AW (2014) Mucosal transcriptomics implicates under expression of BRINP3 in the pathogenesis of ulcerative colitis. Inflamm Bowel Dis 20:1802–1812
7. Maric I, Poljak L, Zoricic S, Bobinac D, Bosukonda D, Sampath KT, Vukicevic S (2003) Bone morphogenetic protein-7 reduces the severity of colon tissue damage and accelerates the healing of inflammatory bowel disease in rats. J Cell Physiol 196:258–264
8. Hao Z, Yang X, Lv Y, Li S, Purbey BK, Su H (2012) Intracolonically administered adeno-associated virus-bone morphogenetic protein-7 ameliorates dextran sulphate sodium-induced acute colitis in rats. J Gene Med 14:482–490
9. Monteleone G, Pallone F, MacDonald TT (2004) Smad7 in TGF-beta-mediated negative regulation of gut inflammation. Trends Immunol 25:513–517
10. MacDonald TT, Bell I, Monteleone G (2011) The opposing roles of IL-21 and TGFβ1 in chronic inflammatory bowel disease. Biochem Soc Trans 39:1061–1066
11. Monteleone G, Neurath MF, Ardizzone S, Di Sabatino A, Fantini MC, Castiglione F, Scribano ML, Armuzzi A, Caprioli F, Sturniolo GC, Rogai F, Vecchi M, Atreya R, Bossa F, Onali S, Fichera M, Corazza GR, Biancone L, Savarino V, Pica R, Orlando A, Pallone F (2015) Mongersen, an oral SMAD7 antisense oligonucleotide, and Crohn's disease. N Engl J Med 372:1104–1113
12. Theiss AL, Idell RD, Srinivasan S, Klapproth JM, Jones DP, Merlin D, Sitaraman SV (2007) Prohibitin protects against oxidative stress in intestinal epithelial cells. FASEB J 21:197–206
13. Zhao N, Zhang AS, Enns CA (2013) Iron regulation by hepcidin. J Clin Invest 123:2337–2343
14. Wang CY, Babitt JL (2016) Hepcidin regulation in the anemia of inflammation. Curr Opin Hematol 23:189–197
15. Grgurevic L, Christensen GL, Schulz TJ, Vukicevic S (2016) Bone morphogenetic proteins in inflammation, glucose homeostasis and adipose tissue energy metabolism. Cytokine Growth Factor Rev 27:105–118
16. Shanmugam NK, Chen K, Cherayil BJ (2015) Commensal bacteria-induced interleukin 1β (IL-1β) secreted by macrophages up-regulates hepcidin expression in hepatocytes by activating the bone morphogenetic protein signaling pathway. J Biol Chem 290:30637–30647
17. Andriopoulos B Jr, Corradini E, Xia Y, Faasse SA, Chen S, Grgurevic L, Knutson MD, Pietrangelo A, Vukicevic S, Lin HY, Babitt JL (2009) BMP6 is a key endogenous regulator of hepcidin expression and iron metabolism. Nat Genet 41:482–487
18. Pauk M, Grgurevic L, Brkljacic J, Kufner V, Bordukalo-Niksic T, Grabusic K, Razdorov G, Rogic D, Zuvic M, Oppermann H, Babitt JL, Lin HY, Volarevic S, Vukicevic S (2015) Exogenous BMP7 corrects plasma iron overload and bone loss in Bmp6-/- mice. Int Orthop 39:161–172
19. Casanovas G, Banerji A, d'Alessio F, Muckenthaler MU, Legewie S (2014) A multi-scale model of hepcidin promoter regulation reveals factors controlling systemic iron homeostasis. PLoS Comput Biol 10:e1003421
20. Wang L, Trebicka E, Fu Y, Ellenbogen S, Hong CC, Babitt JL, Lin HY, Cherayil BJ (2012) The bone morphogenetic protein-hepcidin axis as a therapeutic target in inflammatory bowel disease. Inflamm Bowel Dis 18:112–119
21. Bataller R, Brenner DA (2005) Liver fibrosis. J Clin Invest 115:209–218
22. Forbes SJ, Russo FP, Rey V, Burra P, Rugge M, Wright NA, Alison MR (2004) A significant proportion of myofibroblasts are of bone marrow origin in human liver fibrosis. Gastroenterology 126:955–963
23. Gressner OA, Rizk MS, Kovalenko E, Weiskirchen R, Gressner AM (2008) Changing the pathogenetic roadmap of liver fibrosis? Where did it start; where will it go? J Gastroenterol Hepatol 23:1024–1035
24. Li FB, Zhao H, Peng KR, Gao ZG, Huang SJ, Tou JF, Shu XL, Gu WZ (2016) Expression of transforming growth factor-β1 and connective tissue growth factor in congenital biliary atresia and neonatal hepatitis liver tissue. Genet Mol Res 15

25. Bauer M, Schuppan D (2001) TGFbeta1 in liver fibrosis: time to change paradigms? FEBS Lett 502:1–3
26. Vukicevic S, Latin V, Chen P, Batorsky R, Reddi AH, Sampath TK (1994) Localization of osteogenic protein-1 (bone morphogenetic protein-7) during human embryonic development: high affinity binding to basement membranes. Biochem Biophys Res Commun 198: 693–700
27. Sugimoto H, Yang C, LeBleu VS, Soubasakos MA, Giraldo M, Zeisberg M, Kalluri R (2007) BMP-7 functions as a novel hormone to facilitate liver regeneration. FASEB J 21:256–264
28. Song JJ, Celeste AJ, Kong FM, Jirtle RL, Rosen V, Thies RS (1995) Bone morphogenetic protein-9 binds to liver cells and stimulates proliferation. Endocrinology 136:4293–4297
29. Muñoz-Félix JM, González-Núñez M, López-Novoa JM (2013) ALK1-Smad1/5 signaling pathway in fibrosis development: friend or foe? Cytokine Growth Factor Rev 24:523–537
30. Li Q, Gu X, Weng H, Ghafoory S, Liu Y, Feng T, Dzieran J, Li L, Ilkavets I, Kruithof-de Julio M, Munker S, Marx A, Piiper A, Augusto Alonso E, Gretz N, Gao C, Wölfl S, Dooley S, Breitkopf-Heinlein K (2013) Bone morphogenetic protein-9 induces epithelial to mesenchymal transition in hepatocellular carcinoma cells. Cancer Sci 104:398–408
31. Bi J, Ge S (2014) Potential roles of BMP9 in liver fibrosis. Int J Mol Sci 15:20656–20667
32. Grazio S, Razdorov G, Erjavec I, Grubisic F, Kusic Z, Punda M, Anticevic D, Vukicevic S, Grgurevic L (2013) Differential expression of proteins with heparin affinity in patients with rheumatoid and psoriatic arthritis: a preliminary study. Clin Exp Rheumatol 31:665–671
33. Park MC, Chung SJ, Park YB, Lee SK (2008) Relationship of angiogenic factors to disease activity and radiographic damage in rheumatoid arthritis. Clin Exp Rheumatol 26:881–886
34. Grcevic D, Jajic Z, Kovacic N, Lukic IK, Velagic V, Grubisic F, Ivcevic S, Marusic A (2010) Peripheral blood expression profiles of bone morphogenetic proteins, tumor necrosis factor-superfamily molecules, and transcription factor Runx2 could be used as markers of the form of arthritis, disease activity, and therapeutic responsiveness. J Rheumatol 37:246–256
35. Bramlage CP, Häupl T, Kaps C, Ungethüm U, Krenn V, Pruss A, Müller GA, Strutz F, Burmester GR (2006) Decrease in expression of bone morphogenetic proteins 4 and 5 in synovial tissue of patients with osteoarthritis and rheumatoid arthritis. Arthritis Res Ther 8:R58
36. Lories RJ, Derese I, Ceuppens JL, Luyten FP (2003) Bone morphogenetic proteins 2 and 6, expressed in arthritic synovium, are regulated by proinflammatory cytokines and differentially modulate fibroblast-like synoviocyte apoptosis. Arthritis Rheum 48:2807–2818
37. Varas A, Valencia J, Lavocat F, Martínez VG, Thiam NN, Hidalgo L, Fernández-Sevilla LM, Sacedón R, Vicente A, Miossec P (2015) Blockade of bone morphogenetic protein signaling potentiates the pro-inflammatory phenotype induced by interleukin-17 and tumor necrosis factor-α combination in rheumatoid synoviocytes. Arthritis Res Ther 17:192
38. Verschueren PC, Lories RJ, Daans M, Théate I, Durez P, Westhovens R, Luyten FP (2009) Detection, identification and in vivo treatment responsiveness of bone morphogenetic protein (BMP)-activated cell populations in the synovium of patients with rheumatoid arthritis. Ann Rheum Dis 68:117–123
39. Katsuyama T, Otsuka F, Terasaka T, Inagaki K, Takano-Narazaki M, Matsumoto Y, Sada KE, Makino H (2015) Regulatory effects of fibroblast growth factor-8 and tumor necrosis factor-α on osteoblast marker expression induced by bone morphogenetic protein-2. Peptides 73:88–94
40. Machado P, Landewé R, Braun J, Hermann KG, Baker D, van der Heijde D (2010) Both structural damage and inflammation of the spine contribute to impairment of spinal mobility in patients with ankylosing spondylitis. Ann Rheum Dis 69:1465–1470
41. Carter S, Lories RJ (2011) Osteoporosis: a paradox in ankylosing spondylitis. Curr Osteoporos Rep 9:112–115
42. Chen MH, Chen HA, Chen WS, Chen MH, Tsai CY, Chou CT (2015) Upregulation of BMP-2 expression in peripheral blood mononuclear cells by proinflammatory cytokines and radiographic progression in ankylosing spondylitis. Mod Rheumatol 25:913–918

43. Tsui FW, Tsui HW, Las Heras F, Pritzker KP, Inman RD (2014) Serum levels of novel noggin and sclerostin-immune complexes are elevated in ankylosing spondylitis. Ann Rheum Dis 73:1873–1879
44. Joo YB, Bang SY, Kim TH, Shim SC, Lee S, Joo KB, Kim JH, Min HJ, Rahman P, Inman RD (2014) Bone morphogenetic protein 6 polymorphisms are associated with radiographic progression in ankylosing spondylitis. PLoS One 9:e104966
45. Morrell NW, Bloch DB, Ten Dijke P, Goumans MJ, Hata A, Smith J, Yu PB, Bloch KD (2016) Targeting BMP signalling in cardiovascular disease and anaemia. Nat Rev Cardiol 13:106–120
46. Willette RN, Gu JL, Lysko PG, Anderson KM, Minehart H, Yue T (1999) BMP-2 gene expression and effects on human vascular smooth muscle cells. J Vasc Res 36:120–125
47. Csiszar A, Smith KE, Koller A, Kaley G, Edwards JG, Ungvari Z (2005) Regulation of bone morphogenetic protein-2 expression in endothelial cells: role of nuclear factor-kappaB activation by tumor necrosis factor-alpha, H2O2, and high intravascular pressure. Circulation 111:2364–2372
48. Ungvari Z, Csiszar A, Edwards JG, Kaminski PM, Wolin MS, Kaley G, Koller A (2003) Increased superoxide production in coronary arteries in hyperhomocysteinemia: role of tumor necrosis factor-alpha, NAD(P)H oxidase, and inducible nitric oxide synthase. Arterioscler Thromb Vasc Biol 23:418–424
49. Sorescu GP, Sykes M, Weiss D, Platt MO, Saha A, Hwang J, Boyd N, Boo YC, Vega JD, Taylor WR, Jo H (2003) Bone morphogenic protein 4 produced in endothelial cells by oscillatory shear stress stimulates an inflammatory response. J Biol Chem 278:31128–31135
50. Miriyala S, Gongora Nieto MC, Mingone C, Smith D, Dikalov S, Harrison DG, Jo H (2006) Bone morphogenic protein-4 induces hypertension in mice: role of noggin, vascular NADPH oxidases, and impaired vasorelaxation. Circulation 113:2818–2825
51. Buendía P, Montes de Oca A, Madueño JA, Merino A, Martín-Malo A, Aljama P, Ramírez R, Rodríguez M, Carracedo J (2015) Endothelial microparticles mediate inflammation-induced vascular calcification. FASEB J 29:173–181
52. Tintut Y, Patel J, Parhami F, Demer LL (2000) Tumor necrosis factor-alpha promotes in vitro calcification of vascular cells via the cAMP pathway. Circulation 102:2636–2642
53. Boström K, Watson KE, Stanford WP, Demer LL (1995) Atherosclerotic calcification: relation to developmental osteogenesis. Am J Cardiol 75:88B–91B
54. Dhore CR, Cleutjens JP, Lutgens E, Cleutjens KB, Geusens PP, Kitslaar PJ, Tordoir JH, Spronk HM, Vermeer C, Daemen MJ (2001) Differential expression of bone matrix regulatory proteins in human atherosclerotic plaques. Arterioscler Thromb Vasc Biol 21:1998–2003
55. Kim CW, Song H, Kumar S, Nam D, Kwon HS, Chang KH, Son DJ, Kang DW, Brodie SA, Weiss D, Vega JD, Alberts-Grill N, Griendling K, Taylor WR, Jo H (2013) Anti-inflammatory and antiatherogenic role of BMP receptor II in endothelial cells. Arterioscler Thromb Vasc Biol 33:1350–1359
56. Yao Y, Bennett BJ, Wang X, Rosenfeld ME, Giachelli C, Lusis AJ, Boström KI (2010) Inhibition of bone morphogenetic proteins protects against atherosclerosis and vascular calcification. Circ Res 107:485–494
57. Luo G, Ducy P, McKee MD, Pinero GJ, Loyer E, Behringer RR, Karsenty G (1997) Spontaneous calcification of arteries and cartilage in mice lacking matrix GLA protein. Nature 386:78–81

Physiological and Pathological Consequences of Vascular BMP Signaling

Andreas Benn, Julia Haupt, Susanne Hildebrandt, Christian Kaehler, and Petra Knaus

Abstract BMPs regulate multiple essential processes contributing to the formation and homeostasis of the vascular system. Here we describe the impact of BMP signaling in mesoderm formation, vasculogenesis, arteriovenous differentiation, sprouting angiogenesis, endothelial-to-mesenchymal transition (EndMT), and the barrier function of the endothelium.

Aberrant signaling during vascular morphogenesis as well as morphogenic processes, such as endothelial-to-mesenchymal transition (EndMT), have been implicated in several pathological conditions, including tumor neovascularization, hereditary hemorrhagic telangiectasia (HHT), cerebral cavernous malformation (CCM), and fibrodysplasia ossificans progressiva (FOP). We emphasize the molecular mechanisms underlying BMP-dependent regulations of endothelial cell functions and highlight possible applications in the treatment of vascular diseases.

Keywords Bone morphogenetic proteins (BMPs) in vasculogenesis • Mesoderm formation • Epithelial to mesenchymal transition • Tumor neovascularization • Hereditary hemorrhagic telangiectasia (HHT) • Cerebral cavernous malformation (CCM) • Fibrodysplasia ossificans progressiva (FOP)

A. Benn • P. Knaus (✉)
Institute for Chemistry and Biochemistry, Freie Universität Berlin, 14195 Berlin, Germany

DFG Graduate School 1093 Berlin School of Integrative Oncology, 13353 Berlin, Germany

DFG Graduate School 203 Berlin-Brandenburg School for Regenerative Therapies, 13353 Berlin, Germany
knaus@zedat.fu-berlin.de

J. Haupt • C. Kaehler
Institute for Chemistry and Biochemistry, Freie Universität Berlin, 14195 Berlin, Germany

S. Hildebrandt
Institute for Chemistry and Biochemistry, Freie Universität Berlin, 14195 Berlin, Germany

DFG Graduate School 203 Berlin-Brandenburg School for Regenerative Therapies, 13353 Berlin, Germany

S. Vukicevic, K.T. Sampath (eds.), *Bone Morphogenetic Proteins: Systems Biology Regulators*, Progress in Inflammation Research,
DOI 10.1007/978-3-319-47507-3_17

1 Introduction

The vascular system is a complex hierarchical network of arteries, arterioles, capillaries, venules, and veins that ensures oxygen and nutrient supply as well as metabolic waste disposal between tissues and organs. Thus, the development of blood vessels and function of the vascular system is critical during embryogenesis and tissue homeostasis in adult organisms. In the early phase of development, blood vessels form de novo via vasculogenesis involving the coalescence of progenitor cells into a primitive vasculature. At later stages of development and in adults, new blood vessels are generated from preexisting vasculature via angiogenesis [121]. Angiogenesis is orchestrated by numerous signaling pathways, including vascular endothelial growth factor (VEGF), Notch/Delta-like ligand 4 (DLL4), and bone morphogenetic protein (BMP) pathways, and requires a dynamic balance between pro- and antiangiogenic cues [48, 96]. Aberrant signaling during vascular morphogenesis as well as recapitulation of morphogenic processes, such as an endothelial-to-mesenchymal transition (EndMT), has been implicated in several pathological conditions, including tumor neovascularization, hereditary hemorrhagic telangiectasia (HHT), cerebral cavernous malformation (CCM), and fibrodysplasia ossificans progressiva (FOP) [86, 87, 261, 302].

The mature vascular system consists of endothelial cells (ECs) lining the luminal surface of blood vessels, which are covered by mural cells (vascular smooth muscle cells on arteries and veins, pericytes supporting capillaries). ECs form a semipermeable barrier to control blood-tissue exchange of fluids, solutes, and cells [149]. Vascular permeability is regulated by several signaling pathways, including VEGF and BMP, and is essential for tissue homeostasis and adaptation to numerous environmental cues [26, 102]. Intriguingly, impaired permeability has been associated with a multitude of blood vessel-associated pathologies, including inflammation, atherosclerosis, and pulmonary arterial hypertension (PAH) [101, 204].

In this chapter, we will discuss the role of vascular BMP signaling during vessel morphogenesis and vascular permeability in physiological and pathological conditions. We will emphasize the molecular mechanisms underlying BMP-dependent regulations of EC functions and highlight possible applications in the treatment of vascular diseases.

2 BMP Signaling

BMPs represent the largest subgroup within the transforming growth factor β (TGF-β) family and can be subdivided into four groups: (I) BMP2 and BMP4; (II) BMP5, BMP6 (also known as vegetal-related-1, Vgr-1), BMP7 (osteogenic protein-1, OP-1), and BMP8 (OP-2); (III) BMP9 (growth and differentiation factor 2, GDF2) and BMP10; and (IV) BMP12 (GDF7), BMP13 (GDF6), and BMP14 (GDF5) [38]. BMPs are secreted dimeric ligands that bind to heteromeric complexes of transmembrane serine/threonine kinase receptors, subdivided into type I receptors,

including activin receptor-like kinase 1 (ALK1; activin A receptor type II-like 1, ACVRL1), ALK2 (activin A receptor type I, ACVR1), ALK3 (BMP receptor type IA, BMPRIA), and ALK6 (BMPRIB), and type II receptors, including BMP receptor type II (BMPRII), activin A receptor type IIA (ActRIIA), and ActRIIB [210]. Ligand binding induces a transphosphorylation of the type I receptor by the constitutive active type II receptor and results in the activation of the type I receptor [330]. In the canonical SMAD pathway, the activated type I receptor phosphorylates receptor-regulated SMADs (R-SMADs; SMAD1, 5, and 8) that form a complex with the common-mediator SMAD (co-SMAD; SMAD4), translocate to the nucleus, and bind to SMAD-binding elements (SBEs) in the promoter region of target genes. Together with coactivator or corepressor DNA-binding partners, SMADs regulate BMP target gene expression [188]. Among the best described target genes of BMP-SMAD signaling are members of the inhibitor of differentiation (ID) protein family as their gene promoters contain a specific sequence element, the BMP-responsive element (BRE), which facilitates SMAD-DNA binding and concomitantly gene transcription [143, 150, 213]. Besides SMAD signaling, BMP ligands can also activate numerous other signaling pathways, including mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K)/AKT, and Rho GTPase pathways [209]. These are collectively referred to as noncanonical or non-SMAD pathways and promote transcriptional as well as non-transcriptional responses, including cytoskeletal rearrangements and cell migration [278].

BMP signaling is regulated and modulated on multiple levels by secreted antagonists, such as chordin, noggin, gremlin1 (GREM1), or BMP-binding endothelial cell precursor-derived regulator (BMPER) [40]; co-receptors, such as endoglin [207]; receptor endocytosis [80]; and intracellular effectors, including inhibitory SMADs (I-SMADs; SMAD6 and 7) [112, 132] or the E3 ubiquitin ligase SMAD ubiquitin regulatory factor 1 (SMURF1) [262]. Furthermore, cross talk between SMAD, non-SMAD, and other signaling pathways fine-tunes signals from BMP ligands and creates a large diversity of cellular outcomes. This reflects the pleiotropicity of BMP signaling in regulating various cellular processes, such as cell proliferation, differentiation, migration, and apoptosis, in different tissues and organs in physiological as well as pathological conditions [148, 278]. Thus, it has been suggested to regard BMP ligands as body rather than bone morphogenetic proteins [122, 244, 311].

3 BMP Signaling in Vascular Morphogenesis

3.1 BMP Signaling and Vasculogenesis

Shortly after gastrulation, the first blood vessels arise de novo in embryonic and extraembryonic tissues via vasculogenesis. Vasculogenesis is initiated as endothelial progenitors from the lateral plate mesoderm start to differentiate, migrate, and coalesce to generate a primitive vasculature [121, 224]. In the yolk sac,

vasculogenesis results in the formation of the primitive plexus, while in the embryo proper, vascular progenitors arrange in a bidirectional fashion and form the major embryonic vessels, the dorsal aorta and the cardinal vein [14].

Several studies highlighted that activation of the VEGF pathway is crucial for vasculogenesis; consequently mouse embryos lacking either VEGF ligands or the VEGF receptor kinase insert domain receptor (KDR; VEGF receptor type II, VEGFR2; fetal liver kinase 1, FLK1) die shortly after gastrulation displaying major vasculogenic defects [74, 84, 269]. Interestingly, BMP signaling acts upstream of the vasculogenic cascade as targeted gene disruption approaches in mice demonstrated that *Bmp2* and *Bmp4* are required for the initial mesoderm formation and patterning [326, 344] (Fig. 1). BMP4 induces the expression of KDR in the lateral plate mesoderm, thereby initiating differentiation of vascular progenitors in avian embryos and cultured human embryoid bodies [37, 218]. Furthermore, the zebrafish *vegf* promoter contains SBEs, and Smad1 stimulates *vegf* promoter activity. This correlates with the observation that ectopic expression of *BMP4* in zebrafish results in elevated *vegf* and *kdr* expression in vascular progenitors [114]. However, considering that genetic ablation of *Smad1* or *Smad5* as well as endothelial-specific *Smad1/5* knockout results in the formation of blood vessels in transgenic mice [51, 165, 208], this suggests that BMP-SMAD signaling is dispensable for vasculogenesis. Nevertheless, *Smad1/5*-deficient mutant mice die in utero due to severe vascular remodeling defects, indicating that BMP signaling is temporally regulated during mesoderm formation, endothelial progenitor differentiation, and vascular remodeling to control blood vessel development. Intriguingly, *Bmper* is expressed by *Kdr*-positive cells of mouse embryoid bodies and antagonizes BMP2 and BMP4 in vitro [207], thus highlighting one mode of temporal regulation of BMP signaling during vascular development. Nevertheless, given that BMP4 is required for vasculogenesis, yet SMAD1/5 signaling is dispensable, further studies focusing on the balance of BMP-induced SMAD and non-SMAD signaling are needed to elucidate the role of BMP signaling during vasculogenesis.

3.2 BMP Signaling During Arteriovenous Differentiation

Immediately after the first blood vessels are generated via vasculogenesis, arterial and venous identity of ECs is established via arteriovenous differentiation. A series of findings have demonstrated that arteriovenous differentiation is mainly driven by genetic factors, rather than hemodynamic forces during embryonic development [14]. Notochord-derived sonic hedgehog (shh) induces *vegf* expression in the somites, and subsequently VEGF-A promotes Notch signaling to initiate arterial differentiation in the developing zebrafish embryo [162, 163, 241]. Notch signaling components, including the Notch receptors NOTCH1 and NOTCH4 as well as their ligands jagged 1 (JAG1) and DLL4, are mainly expressed in mouse arteries [310], and activated Notch signaling in ECs results in the expression of Ephrin-B2 (*EFNB2*) in cultured human umbilical vein ECs (HUVECs) [133]. In contrast, the orphan nuclear receptor chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII; nuclear

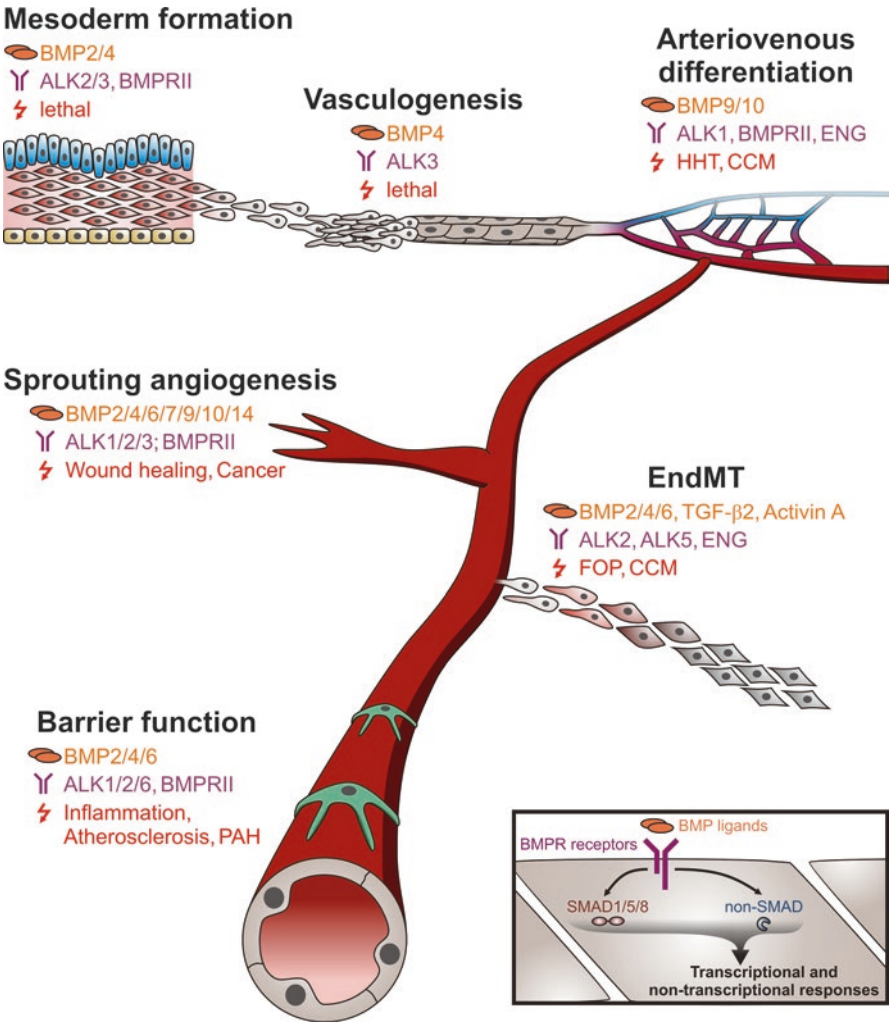


Fig. 1 Vascular BMP signaling. BMP signaling regulates a multitude of processes, including mesoderm formation, vasculogenesis, arteriovenous differentiation, sprouting angiogenesis, endothelial-to-mesenchymal transition (EndMT), and the barrier function of the endothelium, to contribute to proper vascular morphogenesis. Signaling is initiated by numerous BMP ligands (orange ellipses) and transduced via type I and type II serine/threonine kinase receptors (purple sticks) activating SMAD1/5/8 and non-SMAD pathways. These in turn induce transcriptional and non-transcriptional responses. Aberrant BMP signaling, caused, for example, by mutation or deregulated expression of ligands or receptors, impairs a BMP-dependent control of endothelial (beige) or mural cell (green) functions in physiological and pathological conditions (red lightning). Blood vessel-associated pathologies include human hereditary telangiectasia (HHT), cerebral cavernous malformation (CCM), fibrodysplasia ossificans progressiva (FOP), inflammation, atherosclerosis, and pulmonary arterial hypertension (PAH)

receptor subfamily 2 group F member 2, NR2F2) promotes Ephrin receptor B4 (*EPHB4*) expression by repressing Notch signaling, thereby establishing venous identity in mouse vascular networks [340]. The complementary expression of Ephrin-B2 and EphB4 in arterial and venous ECs generates a bidirectional repulsion and mediates arteriovenous segregation in zebrafish [120]. Furthermore, this crucial complementary expression can also be observed in the developing murine vasculature, as homozygous null mutants (*Efnb2*$^{-/-}$ and *Ephb4*$^{-/-}$) die in utero around E9.5, displaying arteriovenous malformations (AVMs) [152, 313].

Intriguingly, BMP signaling induces venous differentiation by promoting expression of the COUP-TFII gene *nr2f2* in zebrafish. This requires a Bmp-dependent upregulation of the angiogenic factor with G patch and FHA domains 1 (*aggf1*), which subsequently enhances β-catenin-dependent gene transcription to promote *nr2f2* expression [142]. This observation is in line with several recent reports demonstrating that BMP signaling is required for the morphogenesis of zebrafish venous vascular beds [119, 145, 312, 325]. It has been suggested that the venous specificity is conferred by a selective enrichment of BMP signaling components, including *bmpr2a* and *bmpr2b*, the zebrafish orthologues of *BMPR2* [199], and Disabled 2 (Dab2), a cargo-specific adaptor protein for clathrin that regulates receptor endocytosis and enhances BMP-SMAD1/5 signaling [145].

However, these findings in zebrafish contrast other in vitro and in vivo studies. In transgenic mice expressing green fluorescent protein (GFP) under a BMP-SMAD-dependent promoter (BRE:GFP reporter), GFP expression is observed in arterial and venous ECs [200, 208]. Furthermore, the BMP type I receptor ALK1 is mainly expressed in arteries and regulates arterial identity during murine development [268, 304]. In cultured human umbilical artery ECs (HUAECs), BMP9-ALK1 signaling promotes EphrinB2 expression via an ID1/ID3-dependent mechanism [146] (Fig. 1). In mice, it was reported that the BMP-ALK1-dependent arterial differentiation is mediated by the intracellular transmembrane protein 100 (TMEM100) and targeted gene disruption of *Tmem100* results in severe AVMs and embryonic lethality around E11.0 [283], resembling *Alk1* null mutant mice [222, 305]. These findings provide a first mechanistic insight into the BMP-dependent regulation of arterial differentiation. Considering that BMP signaling synergizes with Notch pathways during murine vascular morphogenesis [160, 208] and Notch activity is required for arterial differentiation in zebrafish and mice [75, 93, 151, 162, 163], these results indicate a context-dependent regulation of vascular BMP signaling during arteriovenous differentiation in mice and zebrafish that requires further clarification.

3.3 BMP Signaling in Sprouting Angiogenesis

Once a primitive vascular system is generated, new blood vessels form from preexisting ones via angiogenesis. This process is crucial to vasculature expansion during embryogenesis as well as during physiological and pathological situations, including the female menstrual cycle, wound healing, inflammation, and tumor

neovascularization [338]. Sprouting angiogenesis is orchestrated by several signaling pathways and depends on a series of distinct events: (I) vessel destabilization and mural cell detachment, (II) selection of a leading tip cell migrating in the direction of an angiogenic cue, (III) proliferation of trailing stalk cells and lumen formation to ensure proper sprout elongation, (IV) vessel fusion via anastomosis, and (V) vessel maturation by reversion of activated ECs to a quiescent phenotype and mural cell recruitment [48, 121]. The most potent angiogenic cue is VEGF-A, a member of the VEGF family of secreted growth factors. In hypoxic tissues, activation of the hypoxia-inducible factor-1 (HIF-1) pathway results in VEGF-A expression [89]. This generates a VEGF-A gradient from highly hypoxic avascular to normoxic vascularized tissues, thereby providing a spatially confined stimulatory signal [257], which promotes tip cell selection and migration [99]. Subsequently, VEGF-dependent signal transduction results in expression of DLL4, thereby activating Notch signaling in neighboring cells [118]. DLL4/Notch signaling represses the tip cell phenotype and establishes stalk cell identity [169, 178, 279, 292]. Negative feedback loops, cross talk to other pathways, differential expression of crucial signaling components, and differential adhesion during sprout elongation contribute to the dynamic process of sprouting angiogenesis as ECs constantly compete for the tip cell position in a mechanism resembling a tug-of-war [27, 136, 230, 231].

Several in vitro and in vivo studies have shown that BMPs regulate EC functions, including proliferation, migration, and network formation, and substantially contribute to vascular morphogenesis by controlling activation and maturation phases during sprouting angiogenesis. In vitro, BMP2 promotes proliferation of human pulmonary artery ECs (HPAECs) via a β-catenin-dependent mechanism [66]. Interestingly, while BMP2 stimulates proliferation of human aortic ECs (HAECs), it has no effect on the growth of HUVECs or human dermal microvascular ECs (HDMECs) [20, 85, 157, 242], suggesting a cell type-specific regulation. Furthermore, BMP2 induces migration and tube formation of human microvascular ECs (HMECs) [254], HDMECs [242], HAECs [157], and HUVECs [85]. The pro-angiogenic properties of BMP2 have also been demonstrated using Matrigel plugs with A549 cells and BMP2 supplementation that increases tumor neovascularization upon injection into nude mice [157]. Similar results were obtained using the mouse sponge assay as well as ectopic expression of BMP2 in MCF7 breast cancer cell-containing xenografts [242], thereby supporting the notion that BMP2 activates the endothelium.

Besides inducing blood vessel formation in avian embryos [218], BMP4 promotes proliferation and migration of mouse embryonic stem cell-derived ECs (MESECs) and HMECs [254, 295]. Mechanistically, it has been suggested that these functions require a BMP4-dependent activation of VEGF-A/KDR and angiopoietin 1 (ANG1)/TIE2 pathways [295], although a more detailed understanding of this cross talk mechanism is still lacking. Interestingly, BMP4-induced sprouting from HUVEC spheroids is abolished in the presence of pharmacological inhibitors targeting the extracellular signal-regulated kinases 1 and 2 (ERK1/2), while siRNA-mediated knockdown of *SMAD4* has no effect [348]. This provides insights into the role of non-SMAD signaling in controlling EC functions and sprouting angiogenesis.

In bovine aortic ECs (BAECs), BMP6 stimulation increases proliferation, migration, and tube formation via an ID1-dependent mechanism [306], thus suggesting that BMP6-induced SMAD1/5 signaling is required to activate BAECs. Furthermore, BMP6-induced migration and tube formation of mouse embryonic ECs (MEECs) require myosin-X (MYO10), an unconventional myosin that is essential for filopodia formation [233]. Another study provided evidence that BMP6-induced activation of MEECs requires a SMAD1-dependent upregulation of the cyclooxygenase 2 gene (*Cox2*) and pharmacological inhibition of COX2 blocks the pro-angiogenic activity of BMP6 on MEECs and mouse aortic rings [245]. Similar to BMP6, BMP7 has been described to exert pro-angiogenic functions and promotes proliferation and tube formation of HUVECs [5] as well as network formation in the chicken chorioallantoic membrane (CAM) assay [243].

The CAM assay also demonstrated that BMP14 (GDF5) has pro-angiogenic properties and further in vitro analyses showed that BMP14 promotes migration, but not proliferation of BAECs [333]. Collectively, these results highlight that BMP2, BMP4, BMP6, BMP7, and BMP14 activate ECs and positively regulate angiogenic processes (Fig. 1). However, current data suggests that these effects are strongly context and cell type dependent and may be differentially regulated by SMAD and non-SMAD signaling.

In contrast, BMP9 and BMP10 have been reported to promote the maturation of blood vessels by acting as quiescence factors [63]. While members of the BMP2/4 and BMP5/6/7/8 subgroup mainly signal via ALK2, ALK3, and ALK6 [115, 161, 263, 299], BMP9 and BMP10 utilize ALK1 as their high-affinity type I receptor [64] and require endoglin as a co-receptor [219]. BMP9-ALK1 signaling blocks basic fibroblast growth factor (bFGF)-dependent proliferation of BAECs and VEGF-induced network formation of mouse fetal bone explants [265]. Furthermore, BMP9 inhibits blood vessel formation in the CAM assay and bFGF-induced vascularization in the mouse sponge assay [63]. Inhibition of ALK1 with an anti-ALK1 antibody abolishes VEGF-induced sprouting from HUVEC spheroids [309]. Similarly, BMP9-dependent inhibition of HUVEC and HUAEC spheroid sprouting is mediated by ALK1 as demonstrated by siRNA-mediated knockdown [146]. Interestingly, ectopic expression of a constitutive active ALK1 mutant in combination with expression of the inhibitory SMAD6 or pharmacological inhibition of MAPKs demonstrated that the inhibitory effect of ALK1 signaling on HMEC migration is SMAD independent and possibly requires JNK and ERK1/2 pathways [65]. Furthermore, while the aforementioned studies report that BMP9/10-ALK1 signaling promotes EC quiescence, other results seem to contradict this notion. BMP9 stimulates proliferation of MESECs as well as network formation in mouse allantoic explants, in the Matrigel plug assay and in a xenograft model using human pancreatic cancer cells [296]. Intriguingly, BMP9 induces tube formation of HPAECs and requires SMAD1 and p38 MAPK activity yet is independent of SMAD4 [225]. Collectively, these findings demonstrate that BMP9/10-ALK1 signaling exerts context- and cell type-specific effects. The importance of this signaling axis has been extensively studied using transgenic mice and revealed that BMP9, BMP10, ALK1, and endoglin are essential regulators of blood vessel development [96] (Fig. 1).

Furthermore, numerous recent in vivo studies elegantly demonstrated that BMP-dependent signaling is required for proper angiogenesis and depends on the activity of several signaling components. In zebrafish, BMP signaling induces venous sprouting independently of VEGF and requires Smad1/5/8 and Erk1/2 activity [325]. Venous-specific BMP signaling requires the clathrin adaptor Dab2 [145] and mediates vessel formation via β-catenin-dependent upregulation of *nr2f2* [142] as well as Cdc42-mediated activation of formin-like 3 (Fmnl3)-dependent filopodia formation [312]. In mice, it was reported that BMP-SMAD signaling is required to establish stalk cell identity. In endothelial-specific SMAD1/5 knockout mice, a hypersprouting phenotype can be observed, and embryos die around E10.5 displaying severe vascular defects [208]. A similar phenotype was reported for postnatal retinal angiogenesis in the presence of an ALK1-neutralizing antibody [160]. Further investigations highlighted that a synergism between BMP-SMAD and Notch signaling controls stalk cell competence. While the co-stimulation of HUVECs with BMP9 and the soluble Notch ligand DLL4 (sDLL4) results in strong upregulation of the stalk cell-associated genes *HEY1*, *JAG1*, and *FLT1* [160], their transcript levels are significantly diminished upon siRNA-mediated knockdown of *SMAD1* and *SMAD5* [208]. In vitro, *HES1*, *HEY1*, and *JAG1* were shown to be direct target genes of SMAD1/5 [202], and HEY1 gene transcription is cooperatively induced by BMP6-SMAD1 and Notch signaling [134]. These results provide strong evidence that HES1 and HEY1 expressions, which are critical for the stalk cell phenotype, depend on the integration of BMP-SMAD1/5 and Notch pathways. Besides, it was reported that members of the ID protein family act as stalk cell competence factors by forming heteromers with HES1 [208], thereby alleviating the negative autoregulation of HES1 without impairing HES1-dependent repression of target genes with class C site-containing promoter regions [19], such as *KDR* [298]. Intriguingly, a recent study revealed that BMP9-dependent SMAD2/3 signaling is inhibited in the presence of the transmembrane protein neuropilin 1 (NRP1) to repress the stalk cell phenotype in tip cells [13]. On the other hand, Notch signaling represses *NRP1* expression, thereby relieving the NRP1-dependent repression of SMAD2/3 signaling to establish stalk cell identity [13]. This work demonstrates that BMP-SMAD signaling is differentially balanced between tip and stalk cells to ensure proper sprout formation. BMP-SMAD1/5-dependent transcriptional activity is scattered throughout the developing murine vasculature [208] and supports the hypothesis that besides the differential expression of the VEGF receptors KDR and FLT1 [136], BMP-SMAD signaling prepatterns the endothelium conferring a spatiotemporal regulation of tip and stalk competence [24, 208].

Taken together, vascular BMP signaling is essential for proper blood vessel development and requires many signaling components, which seem to be regulated in a ligand- and context-dependent manner and reflect EC heterogeneity. Unfortunately, most studies focus on the function of BMP-induced SMAD1/5 signaling; thus the role of non-SMAD signaling in (sprouting) angiogenesis is still to be defined. Interestingly, in vivo studies revealed that several non-SMAD signaling components, including MAPKs and PI3K [209], are critical regulators of murine

blood vessel development [107, 203], thus highlighting their importance. Accordingly, we are still in need of further mechanistic insights that will enable a more detailed understanding of the function of BMP signaling in several blood vessel-associated pathologies.

4 Aberrant BMP Signaling in Pathologies of Vascular Morphogenesis

4.1 Hereditary Hemorrhagic Telangiectasia (HHT)

4.1.1 Pathophysiology and Genetics of HHT

HHT or Rendu-Osler-Weber syndrome is a heterozygous, autosomal dominant disorder of the vasculature [211, 282]. A large heterogeneity is found in HHT patients, but hallmark clinical signs encompass recurrent epistaxis, chronically dilated capillaries, and other small blood vessels, called telangiectasia, in the nose, fingers, and lips as well as gastrointestinal telangiectasia and AVMs particularly at pulmonary, hepatic, and/or cerebral sites [77, 275]. AVMs are prone to rupture and cause hemorrhages as walls of affected blood vessels appear thin and fragile [106]. A correlation exists between age and number of lesions as well as for increased prevalence in woman to develop pulmonary and hepatic AVMs [170]. Major clinical complications arise from AVMs due to hemorrhages that may lead to anemia, development of shunts followed by hypoxemia, liver disease, pulmonary hypertension, and embolism leading to life-threatening conditions in patients [106, 302].

The etiology of HHT lies in a deregulation of the TGF-β/BMP signaling pathway and can be classified into different subtypes based on affected genes [94, 139, 189, 317]. Mutations in the endoglin (*ENG*; CD105) or ALK1 gene cause HHT1 (OMIM #187300) or HHT2 (OMIM #600376), respectively. Mutations result in haploinsufficiency by either underproduction, inactivation, or retention of the protein [2, 22, 94, 139, 276]. Recently, missense mutations in the BMP9 gene were found in patients with a HHT overlap phenotype presenting with epistaxis and dermal telangiectasia [327]. Identified mutations, located in the pro- and the mature domain, were shown to affect protein processing or reduced bioactivity, respectively. One patient with overlapping syndromes of HHT and PAH was reported with a nonsense mutation in the BMPRII gene [248]. Multiple families have been diagnosed with both pathologies highlighting the importance of the BMP pathway in vascular homeostasis [1] (Fig. 1).

HHT1 and HHT2 can be discriminated based on the incident of pulmonary AVMs (PAMVs) that appear significantly more frequent in HHT1 [168, 258]. HHT1 patients show earlier onset of epistaxis and telangiectasis, while HHT2 patients present with an overall milder phenotype, high variability of onset and location of vascular lesions, and increased hepatic dysfunction [23, 28, 277]. The

majority (80–85 %) of HHT patients carry either *ALK1* or *ENG* heterozygous mutations [302]. An overlapping syndrome of HHT with juvenile polyposis (JP/HHT, OMIM #175050) accounts for 2–3 % of all HHT cases and is caused by mutations in co-SMAD4. Occurrence of hamartomatous polyps throughout the gastrointestinal tract in combination with increased risk for the development of gastrointestinal cancer is characteristic for juvenile polyposis syndrome (JPS; OMIM #174900), which is also linked to *SMAD4* mutations [46, 126, 342]. JPS-associated mutations were found throughout the entire *SMAD4* gene and are predicted to cause protein truncation [42, 45, 90, 125–128, 158, 252, 264, 328]. Immunohistochemical analysis of polyps from JPS patients showed loss of epithelial SMAD4 expression in almost 50 % of investigated samples [158]. Mutations in the overlap syndrome JP/HHT cluster at the carboxy terminus of the MAD homology 2 (MH2) domain of *SMAD4*, a domain responsible for complex formation with R-SMADs and for binding of transcriptional cofactors [94, 240, 324]. However, identical mutations have been reported for JPS and JP/HHT patients leading to the hypothesis that JPS patients might have undiagnosed HHT symptoms and are at risk to develop vascular dysplasia [11, 45, 94, 240].

Two additional loci associated with HHT3 (on chromosome 5q; OMIM # 601101) and HHT4 (on chromosome 7p; OMIM # 610655) exist, but the affected genes for both syndromes are currently unknown [23, 57].

4.1.2 Animal Models of HHT

Key features of human HHT (epistaxis, telangiectasia, AVMs in the lung, brain, and gastrointestinal tract) are recapitulated in vivo by either heterozygous loss-of-function mutations of *Eng* or *Alk1* or conditional deletion in mice [12, 35, 36, 172, 227, 228, 289, 303]. Consistently with a proposed in utero lethality in homozygous humans [82, 141], global knockout of either *Alk1* or *Eng* results in prenatal lethality in mice due to severe vessel abnormalities and heart development defects [12, 205, 222, 289, 305]. Mouse models of HHT1 revealed that initial stages of vasculogenesis is normal but fails to mature further. This arrest of endothelial remodeling is in agreement with normal vasculogenesis and age-dependent dysplasia of the vasculature in HHT patients [12, 35, 172]. Conditional deletion of *Smad4* in ECs also results in embryonic lethality due to severe cardiovascular defects, compromised vascular integrity, and impaired development of vascular smooth muscle cells underlining that BMP/TGF-β signaling components are essential for angiogenesis [156].

4.1.3 BMP Signaling Components Involved in HHT and Their Function in the Vasculature

Endoglin is a transmembrane receptor with an extracellular ligand binding domain for members of the TGF-β family (BMPs, TGF-βs, and activin) dependent on the presence of the respective ligand bound serine/threonine kinase receptors. The cytosolic

domain is very short and lacks a kinase domain [7, 21, 25, 52, 104]. Endoglin thereby acts as co-receptor by forming heteromeric complexes with TGF-β type I and II receptors and modulates signal transmission in response to ligands in a cell context-dependent manner [7, 49, 332, 345]. The cytoplasmic tail of endoglin contains a PDZ-binding motif for binding of PDZ-containing proteins, which can exert a modulating function on signaling pathways. Association of the PDZ-domain protein GAIP-interacting protein C-terminus (GIPC) with endoglin promotes TGF-β-mediated inhibition of migration in MEECs and HMECs [167]. Importantly, endoglin was shown to increase signaling through the ALK1 receptor by stabilizing binding of ligands to the receptor complex [195].

ALK1 expression is primarily found in ECs of arterial vessels [268], while endoglin is expressed on all vascular ECs, activated monocytes, and mesenchymal cells including fibroblasts and vascular smooth muscle cells (VSMCs) [3, 103, 290, 291]. Thus, mutations affecting functionality of either receptor will interfere with normal vascular function as confirmed by various animal models. Expression of endoglin is elevated in pathophysiological processes, for example, in angiogenic vasculature of solid tumors, and monoclonal antibodies that block functionality of either endoglin or ALK1 are deployed as therapeutic strategy to counteract neovascularization in cancer [301, 315, 316], as reviewed in [140].

Earlier studies investigating the underlying pathophysiology of HHT centered on the inhibitory function of endoglin on TGF-β signal transmission in ECs. TGF-β exerts a dual function on ECs by stimulating and inhibiting proliferation and migration in a dose- and context-dependent manner. ECs express the TGF-β type I receptor ALK5, which complexes with the TGF-β type II receptor (TGFßRII) to activate signal propagation via the SMAD2/3 branch resulting in inhibition of EC proliferation and migration in the quiescent endothelium, while ALK1-SMAD1/5/8 signaling stimulates these processes and concomitantly promotes angiogenesis [105, 164]. Endoglin is implicated in modulating the response to TGF-β ligands by inhibiting ALK5-mediated signal transduction, therefore balancing pro- and anti-angiogenic properties [30, 164, 229]. Endoglin is required in ECs to allow SMAD1/5 signal propagation via ALK1 as demonstrated by inhibition of EC proliferation in cells with reduced levels of endoglin, while simultaneously ALK5 signal transduction is stimulated [164]. Application of an anti-endoglin antibody suppresses EC proliferation and angiogenesis in vitro and in vivo and has tumor-suppressive property [270]. Consistently, elevated endoglin levels correlate with increased proliferation of ECs by overcoming TGF-β-induced growth arrest [88, 164, 171]. This pro-angiogenic property of endoglin was recapitulated in a mouse model of retinal angiogenesis where haploinsufficiency impairs angiogenesis [226]. It was postulated that ALK1/endoglin competes with ALK5 for TGF-β ligand binding, yet the biological meaning of this interaction has been questioned as ALK1 and ALK5 show distinct expression patterns in the vasculature in vivo [267]. Furthermore this notion was consolidated by the finding that only conditional deletion of *Alk1*, but not *Alk5* or *Tgfbr2*, induces a HHT vascular phenotype in transgenic mice [227].

Based on the large heterogeneity that exists among HHT patients with respect to severity, age of onset, site, and number of vascular lesions, it is hypothesized that additional factors of either environmental, physical, or genetic nature are required to induce vascular lesions [55, 180, 181, 228]. This is further supported by the notion that patients with HHT show normal vasculogenesis and age-dependent progression of vessel malformation. Moreover, there is a paradox that vascular lesions only develop within certain organs rather than systemically throughout the body. Endoglin also participates in facilitating extravasation of immune cells during vascular repair/remodeling process, a mechanism that might be altered in HHT [135, 308]. In conclusion, the molecular mechanisms of how loss-of-function mutations cause vascular dysplasia in HHT need further investigations to answer remaining questions.

4.2 Endothelial-to-Mesenchymal Transition (EndMT)

Similar to the more intensively studied epithelial-to-mesenchymal transition (EMT), EndMT has also been described as an important biological process in development and disease progression. Initially, EndMT was described in embryonic heart development [81, 147]. Early embryonic chick heart studies have reported that endocardial cushion tissue originates from ECs transdifferentiating into mesenchymal cells [147]. Although, in the past, EndMT was often restricted to embryonic development, its occurrence in the adult vasculature and its participation in pathological processes have been described with increasing frequency in recent years.

The transdifferentiation from ECs to mesenchymal cells is a complex and dynamic process causing disruption of cell-cell junctions from dense, organized layers of resident ECs accompanied by the loss of characteristic endothelial markers (VE-cadherin, VEGFR, CD31/PECAM, etc.). Mesenchymal cells arising through EndMT lose EC characteristics and acquire a mesenchymal spindle-shaped phenotype along with invasive migratory properties. Furthermore, they express mesenchymal-specific markers, including fibroblast-specific protein-1 (FSP-1), alpha-smooth muscle actin (α-SMA), fibronectin, and N-cadherin, and have the potential to differentiate into multiple cell types, such as chondrocytes, osteoblasts, fibroblasts, and adipocytes, as reviewed in [176].

4.2.1 BMP Signaling in EndMT

Phenotypic changes require elementary molecular changes and architectural rearrangements and are controlled by several signaling pathways, including TGF-β [309], BMP [214], WNT [175], bFGF [166], and Notch signaling [50]. These pathways target similar downstream transcription factors, such as Snail [47], Slug [31], and Twist [336], which repress epithelial (E-)cadherin. Currently, EndMT is most

frequently associated with TGF-β/BMP signaling [261]. SMAD4, mediator of both TGF-β and BMP signals, is crucial for EndMT, and *Smad4* deficiency prevents EndMT of murine endocardial cells [67, 339].

TGF-β2 and BMP4 were identified to stimulate EndMT in HUVECs [194], and knockout mouse models display impaired EndMT during heart cushion development [18, 191]. Furthermore, several reports have shown that BMP2 released from the myocardium acts as an inductive signal initiating the onset of EndMT [249, 293] (Fig. 1). This myocardial signal stimulates TGF-β synthesis in endocardial cells inducing EndMT in an autocrine manner [214, 339].

Several studies have investigated the requirement of the BMP type I receptor ALK2 in EndMT. Endothelial-specific *Alk2* knockout mice display defects in atrioventricular septa and valves resulting from failure of endocardial cells to appropriately undergo mesenchymal transdifferentiation during heart development [314]. Consistently, siRNA-mediated knockdown or pharmacological inhibition of ALK2 is sufficient to prevent EndMT in HUVECs and human cutaneous microvascular ECs (HCMECs), thus indicating that ALK2 is a crucial regulator of EndMT [194]. Based on the observation that TGF-β2 and BMP4 also induce phosphorylation of the TGF-β-specific SMAD2, it was hypothesized that EndMT requires the activation of ALK2 and the TGF-β type I receptor ALK5 to activate both SMAD1/5/8 and SMAD2/3 signaling pathways [4, 194]. Interestingly, a hypersensitive ALK2 mutant (ALK2-R206H), which favors EndMT during FOP progression, specifically interacts with ALK5 even in the absence of a ligand [194]. The assumption that induction of EndMT requires the activation of ALK2 and ALK5 receptors is further supported by studies reporting that siRNA-mediated knockdown and pharmacological inhibition of ALK5 effectively abolish EndMT of HUVECs and umbilical cord blood-derived endothelial colony forming cells (UC-ECFCs) [194, 201] and genetic ablation of *Alk5* inhibits EndMT of endocardial cells during murine heart development [288]. Besides, using *Eng*-deficient embryonic stem cells to generate chimeric mice demonstrated that endoglin is required for EndMT transition during endocardial cushion formation [220], thus indicating that ALK2, ALK5, and endoglin exert crucial functions during EndMT (Fig. 1).

Interestingly, however, systemic administration of the ALK2 ligand BMP7 in mice inhibits EndMT and the progression of cardiac fibrosis [343]. Consistently with these in vivo observations, treatment of HUVECs with BMP7 does not promote ALK2-ALK5 complex formation and maintains endothelial marker expression [194]. However, the exact mechanism whereby BMP7 inhibits EndMT is still unknown, yet current data suggests that BMP7 antagonizes TGF-β2 [92] and activates ALK2-SMAD1/5/8 signaling [194], thereby inhibiting EndMT [192] (Fig. 1).

4.2.2 EndMT in Human Diseases

Although EndMT is normally restricted to embryonic development and inactive in adult tissues, pathological conditions in disease and tissue repair can activate this process. The acquisition of mesenchymal properties and loss of endothelial

characteristics is a complex, multistep biological phenomenon involved in the initiation and progression of several blood vessel-associated pathologies, including FOP [194], CCM [187], atherosclerosis [53], and PAH [10].

Heterotopic Ossification (HO) and Fibrodysplasia Ossificans Progressiva (FOP)

HO is a severe pathological condition in which bone forms in soft tissue in response to injury, inflammation, or genetic disease [190]. Among the most severe and disabling pathologies associated with HO is the rare genetic bone disorder FOP (OMIM #135100), an autosomal dominant disease caused by a sporadic or hereditable gain-of-function mutation in ALK2. The most prevalent FOP-associated ALK2 mutation, ALK2-R206H, causes an amino acid exchange in the regulatory glycine-serine-rich GS-Box of ALK2, leading to a hypersensitive signal transduction [271, 274] (Fig. 1). Formation of ectopic bone in soft tissues requires a promotive tissue microenvironment and a trigger to initiate the cellular and molecular events that lead to bone formation. Ectopic bone that forms in FOP is qualitatively normal and requires precursor cells that have the potential to differentiate into bone through endochondral ossification. Thus, HO parallels events that occur in normal embryonic bone development or bone regeneration during fracture healing [273]. Several studies have been performed to reveal the identity of progenitor cells of ectopic bone, yet only a fraction have been characterized so far.

Surprisingly, skeletal muscle precursors contributed minimally to HO [182], while mesenchymal cells residing in the interstitium surrounding skeletal muscle tissue appear to be a source of bone progenitor cells [329]. Cre/loxP lineage tracing approaches using the endothelial markers TIE2 or vascular endothelial (VE)-cadherin demonstrated that the majority of cells in heterotopic bones are of endothelial origin [182, 194, 329]. Interestingly, ectopic expression of the ALK2-R206H mutant enhances EndMT and mutant ECs display multipotency as well as the ability to differentiate into osteoblasts, chondrocytes, and adipocytes in vitro and in vivo [194]. Taken together, these studies suggest that ECs contribute to heterotopic bone formation by undergoing EndMT to rather dedifferentiate into multipotent mesenchymal progenitor cells, which subsequently differentiate into multiple cell types [194], than a direct transformation [236].

With respect to FOP, ALK2-R206H mutant cells are more susceptible to BMP ligands and even gain responsiveness to the otherwise antagonistic activin A, thereby causing enhanced signaling [113] (Fig. 1). Thus, hypersensitizing mutations of ALK2 may result in increased EndMT and eventually osteogenic differentiation, presumably triggered by intermittent episodes of inflammation [261]. Recently, it was shown that human-induced pluripotent stem cells (hiPSCs) from FOP patient-derived urine cells carrying the ALK2-R206H mutation exhibit a reduced potential for endothelial differentiation, but EndMT is unaffected [44]. Hence, the low EC yield likely results from increased EndMT of ALK2-R206H mutant ECs and corresponds with an observed enhanced BMP signaling [44]. However, apart from ECs, it is still unclear from which cell type the remaining cell

population in heterotopic bone tissues originates. Due to their chondrogenic and osteogenic differentiation capacity, pericytes have been suggested as potential candidates [182, 193]. Interestingly, FOP pericytes show increased mineralization ability, which is abolished in the presence of the pharmacological ALK2 kinase inhibitor LDN-212854 [44].

Taken together, in a certain microenvironment ECs have the potential to undergo EndMT to eventually differentiate into bone or cartilage and contribute as the major cell type to the formation of heterotopic bone in soft tissues. The genetic FOP mutation in the ALK2-R206H receptor causes aberrant BMP signaling and leads to the most disabling form of HO, thus suggesting that the FOP mutation in the endothelium rather plays a role in favoring EndMT than directly impairing the vasculature.

Cerebral Cavernous Malformation (CCM)

Another blood vessel-associated pathology associated with increased EndMT is CCM. CCMs are vascular malformations that can occur as a sporadic (80 % of cases) or familial (20 % of cases) autosomal dominant disorder affecting up to 0.5 % of the human population [86, 153]. CCM lesions are formed by enlarged irregular venous blood vessels with impaired inter-EC adhesion that often results in cerebral hemorrhages [86]. Treatment options are currently limited to risky neurosurgery [173, 247]. So far, the three genes *CCM1* (*KRIT1*; OMIM #116860), *CCM2* (*OSM*; OMIM #603284), and *CCM3* (*PDCD10*; OMIM #603285) have been identified as leading causes of their eponymous disease [29, 153, 154, 246]. CCM genes are crucial during vascular morphogenesis, yet current data suggests a context-dependent regulation. Homozygous null *Ccm1* mice die in utero with defects in the arterial vasculature [323], while postnatal endothelial-specific deletion of single CCM genes leads to severe venous vascular malformations resembling the human disease [34]. When *Ccm2* is mutated or absent in immortalized murine brain ECs (bEnd.3), CCM2 can no longer sequester the E3 ubiquitin-protein ligase SMURF1 leading to an accumulation of RhoA [59]. This results in increased stress fiber formation, remodeling of endothelial cell-cell contacts, and elevated vascular permeability. These studies suggest that impaired RhoA signaling substantially contributes to pathology of CCM.

Interestingly, EC-specific disruption of *Ccm1* favors EndMT in vitro and in vivo [187]. *Ccm1*-null ECs show a specific upregulation of *Bmp6*, and recombinant BMP6 induces phosphorylation of SMAD1 in cultured murine wild-type lung ECs. Furthermore, pharmacological BMP receptor kinase inhibitors as well as siRNA-mediated knockdown of *Bmp6* inhibit EndMT [187] (Fig. 1). Moreover, CCM1 is required for Notch signaling, and loss of *Ccm1* results in Notch inhibition. This in turn promotes *Bmp6* expression resulting in autocrine BMP signaling and increased EndMT [187]. Intriguingly, while this study indicates that BMP6 expression is negatively regulated by Notch signaling, it was recently reported that Kruppel-like factor 4 (KLF4) promotes *Bmp6* expression and EndMT in *Ccm1*-knockout ECs [62]. Ablation of *Ccm1* results in enhanced MEKK3-MEK5-dependent activation of

ERK5, which induces the upregulation of *Klf4* in cultured brain ECs. Subsequently, KLF4 binds to the promoter region of *Bmp6* as well as of *Fsp1* and *Sca1*, marker genes associated with EndMT, to stimulate gene expression. Thus, KLF4 induces EndMT in CCM1 mutants by stimulating BMP6-dependent signaling and expression of EndMT-associated genes. Accordingly, EndMT and lesion formation is strongly reduced in endothelial-specific *Ccm1*/*Klf4* knockout mice [62]. However, the prevalence of KLF4 expression and lesion formation in the brain of CCM patients remain unexplained at present, yet context-dependent regulation of KLF4 function in arteries and veins might provide a first insight into the development of venous-derived CCM lesions [34, 187].

In sum, there is increasing evidence that EndMT contributes to the initiation or progression of several blood vessel-associated diseases, thus indicating that EndMT might be a potential therapeutic target in clinical applications. In line with the current data, targeting BMP/TGF-β signaling as a potent inducer of EndMT has naturally been considered. Recently, EndMT in a vein graft mouse model as well as murine vascular malformations and hemorrhages were shown to be susceptible to TGF-β signaling blockade by using a TGF-β-neutralizing antibody or a pharmacological ALK5 kinase inhibitor, respectively [58, 187]. Furthermore, the BMP type I receptor kinase inhibitor LDN-193189 has been shown to block HO in an FOP mouse model [197], and further potential strategies aiming to normalize aberrant BMP signaling are currently tested [113, 198, 272]. Thus, targeting the BMP pathway during EndMT represents a novel approach to treat several human diseases associated with impaired vascular morphogenesis.

5 BMPs and Vascular Permeability

5.1 BMP Signaling in the Regulation of Vascular Permeability

Once established, the endothelium provides a semipermeable barrier to control blood-tissue exchange of fluids, solutes, plasma proteins, and cells. Vascular permeability is regulated by transcytosis via specific intracellular vesicles and vacuoles as well as by paracellular pathways through the openings of inter-EC junctions. Transcellular permeability requires endocytosis of distinct vesicles, and several reports demonstrated that caveolae trafficking is an essential component of transcytosis [149]. Accordingly, caveolin 1 (CAV1), the major structural protein of caveolae [253], has been shown to regulate transcytosis of plasma proteins, such as albumin [196]. In contrast, regulation of paracellular permeability is mediated by tight and adherens junction proteins that link the actin cytoskeleton of adjacent ECs, thereby conferring cell-cell adhesion [149]. The most studied regulator of paracellular permeability is VE-cadherin (CDH5; CD144), which mediates cell-cell adhesion via *cis*- and *trans*-homophilic interactions [39]. VE-cadherin links to the actin cytoskeleton by binding to adaptor proteins of the catenin family, including p120-, β-, and γ-catenin, which associate with the actin binding proteins α-catenin

and vinculin [97]. Endocytosis as well as phosphorylation of specific tyrosine or serine residues in the cytoplasmic domain of VE-cadherin disrupt VE-cadherin-catenin interactions resulting in impaired inter-EC adhesion and increased permeability [101]. These processes are activated by many stimuli, including growth factors, such as VEGF [83, 98], shear stress [223], or leukocytes [319].

Interestingly, we recently demonstrated that endocytosis and tyrosine phosphorylation of VE-cadherin is promoted by BMP6 in HUVECs [26] (Fig. 1). We showed that BMP6-ALK2 signaling results in activation of the non-receptor tyrosine kinase cellular (c-) SRC resulting in phosphorylation of VE-cadherin and ultimately facilitating BMP6-induced hyperpermeability of HUVEC monolayers. Furthermore, VE-cadherin promotes BMP receptor complex stability and is required for proper BMP signal transduction [26], thereby highlighting that VE-cadherin itself is critical for efficient growth factor-induced signaling in the endothelium. This is in accordance with VEGF and TGF-β pathways, which are also regulated by VE-cadherin to control EC functions, including proliferation and migration [108, 155, 256]. Moreover, our study provides mechanistic insights into a BMP-dependent regulation of vascular permeability, which has frequently been addressed with respect to hyperpermeability-associated pathological conditions, such as inflammation, atherosclerosis, and PAH [109].

5.2 BMP Signaling in Hyperpermeability-Associated Pathological Conditions

5.2.1 Inflammation and Atherosclerosis

Shear stress induced by blood flow triggers the synthesis of nitric oxide (NO) in the resting endothelium. The basal NO production in turn regulates vasoconstriction and thereby vascular pressure, but also keeps the endothelium quiescent inhibiting pro-inflammatory gene expression and activation of leukocytes [235]. Inflammation is characterized by increased blood flow associated with warmth (calor) and red color (rubor), swelling of tissue (tumor), and pain (dolor) due to leukocyte infiltration [235]. Upon acute inflammation, ECs are activated. During fast type I activation [234], induction of G-protein-coupled receptor (GPCR) signaling by binding of agonists, including thrombin and histamine, triggers phospholipase C isoform β (PLCβ) activation and release of Ca^{2+} from the endoplasmic reticulum resulting in increased production of NO by activated nitric oxide synthase (NOS) and secretion of prostaglandin PGI_2 [294]. NO and PGI_2 are vasodilators and cause elevated blood flow. Furthermore, calcium-dependent phosphorylation of myosin light chain (MLC) leads to contraction of actin filaments opening the attached tight and adherens junctions, thereby resulting in vascular hyperpermeability. Moreover, intracellular signaling results in exocytosis of Weibel-Palade bodies (WPB), which subsequently targets P-selectin to the cell surface to attract leukocytes [251].

During sustained inflammation, leukocytes secrete tumor necrosis factor-α (TNFα) and interleukin-1 (IL-1) triggering type II activation of ECs [234]. This involves the activation of activation protein 1 (AP1) and nuclear factor-κB (NFκB)-dependent transcription and expression of pro-inflammatory proteins, such as chemokines and adhesion molecules [216].

Atherosclerosis is a vascular disease accompanied by chronic inflammation of the arterial wall and atheromatous lesions, and it represents one of the leading causes of mortality worldwide [297]. One prominent event of atherosclerosis is vascular calcification, which is characterized by deposition of calcium phosphate salts and partially resembles bone mineralization [129].

Expression of BMP2 and BMP4 is increased at sites of vascular calcifications in mouse and human and promotes monocyte adhesion, thus indicating a role of BMP signaling during atherosclerotic development [33, 72, 280] (Fig. 1). Mice treated with the pharmacological BMP receptor kinase inhibitor dorsomorphin show reduced endothelial inflammation indicating a crucial role of BMP signaling in EC homeostasis [117]. While BMP2 is upregulated during vascular inflammation [61, 117], expression of the negative regulator SMAD6 is downregulated [174]. The secreted BMP antagonist BMPER, exerting a protective function, was also downregulated during inflammation [116, 117], and heterozygous null *Bmper* mice exhibit accelerated atherosclerotic development [232]. Furthermore, ectopic expression of matrix Gla protein (MGP), an inhibitor of BMP signaling, blocks BMP2-induced osteogenic differentiation of human sarcoma cells [32] and reduces inflammation, calcification, and atherosclerotic lesion formation in an apolipoprotein E-deficient ($ApoE^{-/-}$) atherosclerosis mouse model [337]. In contrast, BMP7 enhances macrophage differentiation leading to decreased pro-inflammatory cytokine secretion and plaque formation in $ApoE^{-/-}$ mice suggesting an atheroprotective role of BMP7 [250, 281]. BMP6 induces osteogenic differentiation of BAECs, synergized by oxidized low-density lipoprotein (oxLDL) indicating a role of oxidative stress in supporting vascular calcification [341] (Fig. 1). Interestingly, it has been suggested that damaged HUVECs release endothelial microparticles (EMPs) containing BMP2 and calcium to enhance calcification of VSMCs [41]. Besides, dedifferentiated VSMCs are able to induce osteogenic differentiation and atherosclerotic calcification by paracrine BMP2 release [212].

ECs express specific mechanotransducers to convert blood flow as a mechanical stress into biochemical signals. Besides cell adhesion complexes, cytoskeletal elements, caveolae, and membrane receptors, the glycocalyx, the nucleus, and the primary cilium are involved in sensation and transduction of mechanical stimuli [9, 68, 110]. Intriguingly, atherosclerotic plaques are more frequently located at specific atheroprone sites, such as arterial branches and bifurcations, which are characterized by either low laminar or disturbed (oscillatory or turbulent) blood flow, while steady and moderate endothelial shear-stress due to atheroprotective flow prevents atherosclerosis [297, 318]. Atherogenic oscillatory flow induces BMP4 expression in mouse aortic ECs (MAECs) to stimulate inflammatory processes [137, 284, 285], whereas protective laminar flow inhibits BMP4 [60]. Diminished

expression of BMPRII was observed in human atherosclerotic lesions as well as after pro-atherogenic disturbed flow or pro-inflammatory stimuli in HUVECs, whereas *BMPR2* expression is upregulated in response to anti-atherogenic laminar flow or statin treatment [144]. Finally, pro-atherogenic oscillatory shear stress induces sustained association of ALK6 with β3 integrin leading to focal adhesion kinase (FAK)-dependent SMAD1/5 activation and thus increased EC proliferation [346, 347] (Fig. 1).

Interestingly, mouse Tg737$^{orpk/orpk}$ *(Orpk*$^{-/-}$*)* ECs lacking the primary cilium, which is assembled in disturbed flow conditions in vivo [79, 307], are prone to EndMT [78] and calcification via BMP-dependent transdifferentiation into osteogenic cells [260]. In this light, a recent study demonstrated that an endothelial-specific conditional *Tg737/Ift88* knockout in *ApoE*$^{-/-}$ mice abolishes ciliogenesis and leads to increased atherogenesis indicating that primary cilia inhibit atherosclerosis [73].

Considering that LDN-193189 inhibits the development of atheroma in LDL receptor-deficient (*Ldlr*$^{-/-}$) or *ApoE*$^{-/-}$ mice, these findings collectively highlight that targeting the BMP pathway might be a promising way for treating atherosclerosis [71, 205, 259].

5.2.2 Pulmonary Arterial Hypertension (PAH)

PAH is a rare disease characterized by vasoconstriction of pulmonary arteries, resulting in increased pulmonary arterial pressure and ultimately heart failure [204]. PAH is further associated with an increased vascular resistance due to altered balance between proliferation and apoptosis and disturbed cross talk between ECs and VSMCs in the vascular wall [204].

Linkage analysis to map the PAH associated locus and sequencing identified the *BMPR2* as the causative gene [69, 70, 131, 206, 217]. Mutations in the *BMPR2* gene account for 70 % of cases of heritable or familial PAH (FPAH; OMIM #178600) and 10–40 % of cases of sporadic or idiopathic PAH (IPAH) [184, 300] (Fig. 1). Missense mutations can cause nonsense-mediated mRNA decay (NMD) of the respective mutant transcript, a reduced receptor trafficking to the cell surface with retention in the ER, or correct trafficking of kinase-inactive receptor forms [91, 138, 255]. All mutations seem to arise independently but ultimately lead to diminished BMP signaling [186], and BMPRII expression is strongly reduced in lung tissue of patients with FPAH and IPAH [15]. Accordingly, conditional deletion of *Bmpr2* in PAECs or expression of a dominant-negative *Bmpr2* mutant in VSMCs recapitulates the PAH disease phenotype in transgenic mouse models [124, 322]. Moreover, heterozygous null *Bmpr2* mutant PAECs display an increased SRC-dependent vesicle trafficking accompanied by a hyperpermeability phenotype [237]. Besides, PAH is also characterized by reduced NO synthesis, leading to vasoconstriction. The reduction in NO is due to increased arginase activity [331] resulting in a substrate depletion and inhibition of the endothelial NO synthase (eNOS) [239]. Interestingly, BMPRII is able to activate eNOS in response to BMP2 and BMP4 in healthy but not PAH patient-derived PAECs carrying *BMPR2* mutations [95]. We

reported that the cGMP-dependent kinase I (cGKI), a key mediator of vasodilation [123], enhances BMP signaling via association both with BMPRII at the plasma membrane and with SMAD1 in the nucleus [266]. Importantly, cGKI can compensate for the PAH-related defects in BMP signaling and aberrant proliferation of human VSMCs [266].

The penetrance of *BMPR2* mutations is incomplete since only 20 % of mutations lead to a disease phenotype [159, 177]. *BMPR2* is expressed in two alternative splice variants, a full-length long form (LF) and a short form (SF), the latter missing the terminal exon 12 resulting in a shortened C-terminal tail which is known to mediate binding of many signaling proteins [278]. Both isoforms exhibit different translation and internalization rates leading to higher expression of BMPRII-SF at the plasma membrane [8]. Interestingly, PAH patients are more likely to have higher ratios of the short isoform relative to the long isoform [56]. Lower expression of estrogen metabolizing gene *CYP1B1* was found in female PAH patients [321], and *BMPR2* expression was shown to be negatively regulated by the estrogen receptor α [16], which might explain the about 2.5-fold increased frequency of PAH in female mutations carriers [159, 183]. Additional mutations within other genes or environmental factors as second hits are thought to trigger disease progression [184]. Such modifiers include mutations of the *SMAD8* gene identified in PAH patients [6]. Moreover, directed sequencing of *SMAD* genes identified variants in *SMAD1*, *SMAD4*, and *SMAD9* (OMIM #615342) [215]. Moreover, mutated *ALK6* was described in IPAH patients [54], indicating that deficiency of diverse parts of the BMP signaling pathway can contribute to PAH. Moreover, conditional *Smad1* knockout in PAECs or pulmonary artery SMCs (PASMCs) predispose transgenic mice for pulmonary hypertension [111]. Homozygous *Smad8* knockout mice exhibit defective pulmonary vascular remodeling and a PAH phenotype [130]. Rare cases of PAH have been associated also with mutations in the genes encoding CAV1 (OMIM #615343) or the KCNK3 potassium channel (OMIM #615344) [17, 185]. A genome-wide association study (GWAS) has recently identified the cerebellin 2 (*CBLN2*) locus to confer susceptibility for PAH in patients without *BMPR2* mutations [100].

Different options are available for PAH treatment [205, 320]. Prostacyclin analogs are used to interfere with abnormal TGF-β1-induced SMAD-dependent and SMAD-independent signaling [221] while restoring deficient BMP signaling [334]. Leukocyte recruitment can be inhibited by a CXCR1/2 antagonist which reverses disease phenotype in PAH mice with endothelial-specific loss of BMPRII [43]. Anti-inflammatory dexamethasone treatment reduces aberrant proliferation and can prevent and reverse monocrotaline-induced PAH in an experimental rat model [238]. In a similar model, the phosphodiesterase PDE-5 inhibitor sildenafil was shown to partly restore deficient BMP signaling and prevent PAH pathogenesis via cyclic GMP and cGKI [335]. A drug screen for compounds inducing BMPRII signaling identified the immunosuppressant FK506 (tacrolimus), which is now in clinical trials [286, 287]. By releasing FKBP12 from BMP type I receptors, FK506 proved to reverse dysfunctional BMPRII signaling in patient-derived PAECs and a monocrotaline rat model of PAH. The antimalarial drug chloroquine restores com-

promised cell surface expression of BMPRII in PAECs and BMP9-mediated signaling [76]. Finally, administration of BMP9 was recently demonstrated to reverse PAH disease phenotype in a transgenic knockin mouse model carrying a human *BMPR2* mutation [179]. Taken together, besides mutations in *BMPR2*, defects in other pathway components also contribute to BMP signaling deficiency in PAH, making it a promising target for therapy.

6 Conclusions

BMP signaling in the vascular system is essential for physiological development and tissue homeostasis yet also contributes to the initiation or progression of several blood vessel-associated pathologies. Most importantly, vascular BMP signaling is strictly ligand, cell type, and context specific. BMP ligands can either induce activation or promote quiescence of ECs, depending on the ligand, the vascular bed, the model system, and the developmental stage, thereby emphasizing the pleiotropic effects of BMP signaling in the endothelium. This notion is even more strengthened by the observations that the highly similar ALK2 ligands BMP6 and BMP7 both lead to an activation of ECs, yet, while BMP6 promotes EndMT and vascular calcification, BMP7 inhibits these processes. These apparent differences hamper the development of suitable drugs targeting the BMP pathway in numerous diseases, including HHT, FOP, CCM, inflammation, and PAH, albeit there has been a substantial progress in the recent years demonstrating that vascular BMP signaling is druggable in pathological conditions. Nevertheless, underlying mechanisms in different vascular beds and developmental stages are still only poorly understood and should be addressed by future studies as they are essential for the development of novel and innovative therapeutic strategies targeting the BMP pathway in the correct endothelial cell type.

Acknowledgments We would like to acknowledge funding from the BMBF (PrevOP/OVERLOAD) to PK. AB was supported by a fellowship from the Berlin School of Integrative Oncology (DFG graduate school 1093), and SH by a fellowship from the SFB958.

References

1. Abdalla SA, Gallione CJ, Barst RJ, Horn EM, Knowles JA, Marchuk DA, Letarte M, Morse JH (2004) Primary pulmonary hypertension in families with hereditary haemorrhagic telangiectasia. Eur Respir J 23:373–377
2. Abdalla SA, Letarte M (2006) Hereditary haemorrhagic telangiectasia: current views on genetics and mechanisms of disease. J Med Genet 43:97–110
3. Adam PJ, Clesham GJ, Weissberg PL (1998) Expression of endoglin mRNA and protein in human vascular smooth muscle cells. Biochem Biophys Res Commun 247:33–37
4. Akhurst RJ, Derynck R (2001) TGF-beta signaling in cancer--a double-edged sword. Trends Cell Biol 11:S44–S51

5. Akiyama I, Yoshino O, Osuga Y, Shi J, Harada M, Koga K, Hirota Y, Hirata T, Fujii T, Saito S, Kozuma S (2014) Bone morphogenetic protein 7 increased vascular endothelial growth factor (VEGF)-a expression in human granulosa cells and VEGF receptor expression in endothelial cells. Reprod Sci 21:477–482
6. Aldred MA, Comhair SA, Varella-Garcia M, Asosingh K, Xu W, Noon GP, Thistlethwaite PA, Tuder RM, Erzurum SC, Geraci MW, Coldren CD (2010) Somatic chromosome abnormalities in the lungs of patients with pulmonary arterial hypertension. Am J Respir Crit Care Med 182:1153–1160
7. Alt A, Miguel-Romero L, Donderis J, Aristorena M, Blanco FJ, Round A, Rubio V, Bernabeu C, Marina A (2012) Structural and functional insights into endoglin ligand recognition and binding. PLoS One 7:e29948
8. Amsalem AR, Marom B, Shapira KE, Hirschhorn T, Preisler L, Paarmann P, Knaus P, Henis YI, Ehrlich M (2016) Differential regulation of translation and endocytosis of alternatively spliced forms of the type II bone morphogenetic protein (BMP) receptor. Mol Biol Cell 27:716–730
9. Ando J, Yamamoto K (2013) Flow detection and calcium signalling in vascular endothelial cells. Cardiovasc Res 99:260–268
10. Arciniegas E, Frid MG, Douglas IS, Stenmark KR (2007) Perspectives on endothelial-to-mesenchymal transition: potential contribution to vascular remodeling in chronic pulmonary hypertension. Am J Physiol Lung Cell Mol Physiol 293:L1–L8
11. Aretz S, Stienen D, Uhlhaas S, Stolte M, Entius MM, Loff S, Back W, Kaufmann A, Keller KM, Blaas SH, Siebert R, Vogt S, Spranger S, Holinski-Feder E, Sunde L, Propping P, Friedl W (2007) High proportion of large genomic deletions and a genotype phenotype update in 80 unrelated families with juvenile polyposis syndrome. J Med Genet 44:702–709
12. Arthur HM, Ure J, Smith AJ, Renforth G, Wilson DI, Torsney E, Charlton R, Parums DV, Jowett T, Marchuk DA, Burn J, Diamond AG (2000) Endoglin, an ancillary TGFbeta receptor, is required for extraembryonic angiogenesis and plays a key role in heart development. Dev Biol 217:42–53
13. Aspalter IM, Gordon E, Dubrac A, Ragab A, Narloch J, Vizan P, Geudens I, Collins RT, Franco CA, Abrahams CL, Thurston G, Fruttiger M, Rosewell I, Eichmann A, Gerhardt H (2015) Alk1 and Alk5 inhibition by Nrp1 controls vascular sprouting downstream of Notch. Nat Commun 6:7264
14. Atkins GB, Jain MK, Hamik A (2011) Endothelial differentiation: molecular mechanisms of specification and heterogeneity. Arterioscler Thromb Vasc Biol 31:1476–1484
15. Atkinson C, Stewart S, Upton PD, Machado R, Thomson JR, Trembath RC, Morrell NW (2002) Primary pulmonary hypertension is associated with reduced pulmonary vascular expression of type II bone morphogenetic protein receptor. Circulation 105:1672–1678
16. Austin ED, Hamid R, Hemnes AR, Loyd JE, Blackwell T, Yu C, Phillips Iii JA, Gaddipati R, Gladson S, Gu E, West J, Lane KB (2012) BMPR2 expression is suppressed by signaling through the estrogen receptor. Biol Sex Diff 3:6
17. Austin ED, Ma L, LeDuc C, Berman Rosenzweig E, Borczuk A, Phillips JA 3rd, Palomero T, Sumazin P, Kim HR, Talati MH, West J, Loyd JE, Chung WK (2012) Whole exome sequencing to identify a novel gene (caveolin-1) associated with human pulmonary arterial hypertension. Circ Cardiovasc Genet 5:336–343
18. Azhar M, Runyan RB, Gard C, Sanford LP, Miller ML, Andringa A, Pawlowski S, Rajan S, Doetschman T (2009) Ligand-Specific Function of Transforming Growth Factor Beta in Epithelial-Mesenchymal Transition in Heart Development. Dev Dyn 238:431–442
19. Bai G, Sheng N, Xie Z, Bian W, Yokota Y, Benezra R, Kageyama R, Guillemot F, Jing N (2007) Id sustains Hes1 expression to inhibit precocious neurogenesis by releasing negative autoregulation of Hes1. Dev Cell 13:283–297
20. Bai Y, Leng Y, Yin G, Pu X, Huang Z, Liao X, Chen X, Yao Y (2014) Effects of combinations of BMP-2 with FGF-2 and/or VEGF on HUVECs angiogenesis in vitro and CAM angiogenesis in vivo. Cell Tissue Res 356:109–121

21. Barbara NP, Wrana JL, Letarte M (1999) Endoglin is an accessory protein that interacts with the signaling receptor complex of multiple members of the transforming growth factor-beta superfamily. J Biol Chem 274:584–594
22. Bayrak-Toydemir P, Mao R, Lewin S, McDonald J (2004) Hereditary hemorrhagic telangiectasia: an overview of diagnosis and management in the molecular era for clinicians. Genet Med 6:175–191
23. Bayrak-Toydemir P, McDonald J, Akarsu N, Toydemir RM, Calderon F, Tuncali T, Tang W, Miller F, Mao R (2006) A fourth locus for hereditary hemorrhagic telangiectasia maps to chromosome 7. Am J Med Genet A 140:2155–2162
24. Beets K, Huylebroeck D, Moya IM, Umans L, Zwijsen A (2013) Robustness in angiogenesis: notch and BMP shaping waves. Trends Genet 29:140–149
25. Bellon T, Corbi A, Lastres P, Cales C, Cebrian M, Vera S, Cheifetz S, Massague J, Letarte M, Bernabeu C (1993) Identification and expression of two forms of the human transforming growth factor-beta-binding protein endoglin with distinct cytoplasmic regions. Eur J Immunol 23:2340–2345
26. Benn A, Bredow C, Casanova I, Vukicevic S, Knaus P (2016) VE-cadherin facilitates BMP-induced endothelial cell permeability and signaling. J Cell Sci 129:206–218
27. Bentley K, Franco CA, Philippides A, Blanco R, Dierkes M, Gebala V, Stanchi F, Jones M, Aspalter IM, Cagna G, Westrom S, Claesson-Welsh L, Vestweber D, Gerhardt H (2014) The role of differential VE-cadherin dynamics in cell rearrangement during angiogenesis. Nat Cell Biol 16:309–321
28. Berg J, Porteous M, Reinhardt D, Gallione C, Holloway S, Umasunthar T, Lux A, McKinnon W, Marchuk D, Guttmacher A (2003) Hereditary haemorrhagic telangiectasia: a questionnaire based study to delineate the different phenotypes caused by endoglin and ALK1 mutations. J Med Genet 40:585–590
29. Bergametti F, Denier C, Labauge P, Arnoult M, Boetto S, Clanet M, Coubes P, Echenne B, Ibrahim R, Irthum B, Jacquet G, Lonjon M, Moreau JJ, Neau JP, Parker F, Tremoulet M, Tournier-Lasserve E, Société Française de N (2005) Mutations within the programmed cell death 10 gene cause cerebral cavernous malformations. Am J Hum Genet 76:42–51
30. Blanco FJ, Santibanez JF, Guerrero-Esteo M, Langa C, Vary CP, Bernabeu C (2005) Interaction and functional interplay between endoglin and ALK-1, two components of the endothelial transforming growth factor-beta receptor complex. J Cell Physiol 204:574–584
31. Bolós V, Peinado H, Pérez-Moreno MA, Fraga MF, Esteller M, Cano A (2003) The transcription factor Slug represses E-cadherin expression and induces epithelial to mesenchymal transitions: a comparison with Snail and E47 repressors. J Cell Sci 116:499–511
32. Bostrom K, Tsao D, Shen S, Wang Y, Demer LL (2001) Matrix GLA protein modulates differentiation induced by bone morphogenetic protein-2 in C3H10T1/2 cells. J Biol Chem 276:14044–14052
33. Bostrom K, Watson KE, Horn S, Wortham C, Herman IM, Demer LL (1993) Bone morphogenetic protein expression in human atherosclerotic lesions. J Clin Investig 91:1800–1809
34. Boulday G, Rudini N, Maddaluno L, Blécon A, Arnould M, Gaudric A, Chapon F, Adams RH, Dejana E, Tournier-Lasserve E (2011) Developmental timing of CCM2 loss influences cerebral cavernous malformations in mice. J Exp Med 208:1835–1847
35. Bourdeau A, Dumont DJ, Letarte M (1999) A murine model of hereditary hemorrhagic telangiectasia. J Clin Invest 104:1343–1351
36. Bourdeau A, Faughnan ME, Letarte M (2000) Endoglin-deficient mice, a unique model to study hereditary hemorrhagic telangiectasia. Trends Cardiovasc Med 10:279–285
37. Boyd NL, Dhara SK, Rekaya R, Godbey EA, Hasneen K, Rao RR, West FD 3rd, Gerwe BA, Stice SL (2007) BMP4 promotes formation of primitive vascular networks in human embryonic stem cell-derived embryoid bodies. Exp Biol Med (Maywood) 232:833–843
38. Bragdon B, Moseychuk O, Saldanha S, King D, Julian J, Nohe A (2011) Bone morphogenetic proteins: a critical review. Cell Signal 23:609–620
39. Bravi L, Dejana E, Lampugnani MG (2014) VE-cadherin at a glance. Cell Tissue Res 355:515–522

40. Brazil DP, Church RH, Surae S, Godson C, Martin F (2015) BMP signalling: agony and antagony in the family. Trends Cell Biol 25:249–264
41. Buendia P, Montes de Oca A, Madueno JA, Merino A, Martin-Malo A, Aljama P, Ramirez R, Rodriguez M, Carracedo J (2015) Endothelial microparticles mediate inflammation-induced vascular calcification. FASEB J 29:173–181
42. Burger B, Uhlhaas S, Mangold E, Propping P, Friedl W, Jenne D, Dockter G, Back W (2002) Novel de novo mutation of MADH4/SMAD4 in a patient with juvenile polyposis. Am J Med Genet 110:289–291
43. Burton VJ, Holmes AM, Ciuclan LI, Robinson A, Roger JS, Jarai G, Pearce AC, Budd DC (2011) Attenuation of leukocyte recruitment via CXCR1/2 inhibition stops the progression of PAH in mice with genetic ablation of endothelial BMPR-II. Blood 118:4750–4758
44. Cai J, Orlova VV, Cai X, Eekhoff EMW, Zhang K, Pei D, Pan G, Mummery CL, Ten Dijke P (2015) Induced Pluripotent Stem Cells to Model Human Fibrodysplasia Ossificans Progressiva. Stem Cell Rep 5:963–970
45. Calva-Cerqueira D, Chinnathambi S, Pechman B, Bair J, Larsen-Haidle J, Howe JR (2009) The rate of germline mutations and large deletions of SMAD4 and BMPR1A in juvenile polyposis. Clin Genet 75:79–85
46. Calva D, Howe JR (2008) Hamartomatous polyposis syndromes. Surg Clin North Am 88(779–817):vii
47. Cano A, Pérez-Moreno MA, Rodrigo I, Locascio A, Blanco MJ, del Barrio MG, Portillo F, Nieto MA (2000) The transcription factor Snail controls epithelial–mesenchymal transitions by repressing E-cadherin expression. Nat Cell Biol 2:76–83
48. Carmeliet P, Jain RK (2011) Molecular mechanisms and clinical applications of angiogenesis. Nature 473:298–307
49. Castonguay R, Werner ED, Matthews RG, Presman E, Mulivor AW, Solban N, Sako D, Pearsall RS, Underwood KW, Seehra J, Kumar R, Grinberg AV (2011) Soluble endoglin specifically binds bone morphogenetic proteins 9 and 10 via its orphan domain, inhibits blood vessel formation, and suppresses tumor growth. J Biol Chem 286:30034–30046
50. Chang ACY, Fu Y, Garside VC, Niessen K, Chang L, Fuller M, Setiadi A, Smrz J, Kyle A, Minchinton A, Marra M, Hoodless PA, Karsan A (2011) Notch initiates the endothelial-to-mesenchymal transition in the atrioventricular canal through autocrine activation of soluble guanylyl cyclase. Dev Cell 21:288–300
51. Chang H, Huylebroeck D, Verschueren K, Guo Q, Matzuk MM, Zwijsen A (1999) Smad5 knockout mice die at mid-gestation due to multiple embryonic and extraembryonic defects. Development 126:1631–1642
52. Cheifetz S, Bellon T, Cales C, Vera S, Bernabeu C, Massague J, Letarte M (1992) Endoglin is a component of the transforming growth factor-beta receptor system in human endothelial cells. J Biol Chem 267:19027–19030
53. Chen P-Y, Qin L, Baeyens N, Li G, Afolabi T, Budatha M, Tellides G, Schwartz MA, Simons M (2015) Endothelial-to-mesenchymal transition drives atherosclerosis progression. J Clin Invest 125:4514–4528
54. Chida A, Shintani M, Nakayama T, Furutani Y, Hayama E, Inai K, Saji T, Nonoyama S, Nakanishi T (2012) Missense mutations of the BMPR1B (ALK6) gene in childhood idiopathic pulmonary arterial hypertension. Circ J 76:1501–1508
55. Choi EJ, Kim YH, Choe SW, Tak YG, Garrido-Martin EM, Chang M, Lee YJ, Oh SP (2013) Enhanced responses to angiogenic cues underlie the pathogenesis of hereditary hemorrhagic telangiectasia 2. PLoS One 8:e63138
56. Cogan J, Austin E, Hedges L, Womack B, West J, Loyd J, Hamid R (2012) Role of BMPR2 alternative splicing in heritable pulmonary arterial hypertension penetrance. Circulation 126:1907–1916
57. Cole SG, Begbie ME, Wallace GM, Shovlin CL (2005) A new locus for hereditary haemorrhagic telangiectasia (HHT3) maps to chromosome 5. J Med Genet 42:577–582
58. Cooley BC, Nevado J, Mellad J, Yang D, Hilaire CS, Negro A, Fang F, Chen G, San H, Walts AD, Schwartzbeck RL, Taylor B, Lanzer JD, Wragg A, Elagha A, Beltran LE, Berry C, Feil R,

Virmani R, Ladich E, Kovacic JC, Boehm M (2014) TGF-β signaling mediates endothelial to mesenchymal transition (EndMT) during vein graft remodeling. Sci Transl Med 6:227ra234
59. Crose LES, Hilder TL, Sciaky N, Johnson GL (2009) Cerebral Cavernous Malformation 2 Protein Promotes Smad Ubiquitin Regulatory Factor 1-mediated RhoA Degradation in Endothelial Cells. J Biol Chem 284:13301–13305
60. Csiszar A, Labinskyy N, Smith KE, Rivera A, Bakker EN, Jo H, Gardner J, Orosz Z, Ungvari Z (2007) Downregulation of bone morphogenetic protein 4 expression in coronary arterial endothelial cells: role of shear stress and the cAMP/protein kinase A pathway. Arterioscler Thromb Vasc Biol 27:776–782
61. Csiszar A, Smith KE, Koller A, Kaley G, Edwards JG, Ungvari Z (2005) Regulation of bone morphogenetic protein-2 expression in endothelial cells: role of nuclear factor-kappaB activation by tumor necrosis factor-alpha, H2O2, and high intravascular pressure. Circulation 111:2364–2372
62. Cuttano R, Rudini N, Bravi L, Corada M, Giampietro C, Papa E, Morini MF, Maddaluno L, Baeyens N, Adams RH, Jain MK, Owens GK, Schwartz M, Lampugnani MG, Dejana E (2015) KLF4 is a key determinant in the development and progression of cerebral cavernous malformations. EMBO Mol Med 8:6–24
63. David L, Mallet C, Keramidas M, Lamande N, Gasc JM, Dupuis-Girod S, Plauchu H, Feige JJ, Bailly S (2008) Bone morphogenetic protein-9 is a circulating vascular quiescence factor. Circ Res 102:914–922
64. David L, Mallet C, Mazerbourg S, Feige JJ, Bailly S (2007) Identification of BMP9 and BMP10 as functional activators of the orphan activin receptor-like kinase 1 (ALK1) in endothelial cells. Blood 109:1953–1961
65. David L, Mallet C, Vailhe B, Lamouille S, Feige JJ, Bailly S (2007) Activin receptor-like kinase 1 inhibits human microvascular endothelial cell migration: potential roles for JNK and ERK. J Cell Physiol 213:484–489
66. de Jesus Perez VA, Alastalo TP, Wu JC, Axelrod JD, Cooke JP, Amieva M, Rabinovitch M (2009) Bone morphogenetic protein 2 induces pulmonary angiogenesis via Wnt-beta-catenin and Wnt-RhoA-Rac1 pathways. J Cell Biol 184:83–99
67. Deckers M, van Dinther M, Buijs J, Que I, Löwik C, van der Pluijm G, ten Dijke P (2006) The tumor suppressor Smad4 is required for transforming growth factor beta-induced epithelial to mesenchymal transition and bone metastasis of breast cancer cells. Cancer Res 66:2202–2209
68. Deng Q, Huo Y, Luo J (2014) Endothelial mechanosensors: the gatekeepers of vascular homeostasis and adaptation under mechanical stress. Sci China Life Sci 57:755–762
69. Deng Z, Haghighi F, Helleby L, Vanterpool K, Horn EM, Barst RJ, Hodge SE, Morse JH, Knowles JA (2000) Fine mapping of PPH1, a gene for familial primary pulmonary hypertension, to a 3-cM region on chromosome 2q33. Am J Respir Crit Care Med 161:1055–1059
70. Deng Z, Morse JH, Slager SL, Cuervo N, Moore KJ, Venetos G, Kalachikov S, Cayanis E, Fischer SG, Barst RJ, Hodge SE, Knowles JA (2000) Familial primary pulmonary hypertension (gene PPH1) is caused by mutations in the bone morphogenetic protein receptor-II gene. Am J Hum Genet 67:737–744
71. Derwall M, Malhotra R, Lai CS, Beppu Y, Aikawa E, Seehra JS, Zapol WM, Bloch KD, Yu PB (2012) Inhibition of bone morphogenetic protein signaling reduces vascular calcification and atherosclerosis. Arterioscler Thromb Vasc Biol 32:613–622
72. Dhore CR, Cleutjens JP, Lutgens E, Cleutjens KB, Geusens PP, Kitslaar PJ, Tordoir JH, Spronk HM, Vermeer C, Daemen MJ (2001) Differential expression of bone matrix regulatory proteins in human atherosclerotic plaques. Arterioscler Thromb Vasc Biol 21:1998–2003
73. Dinsmore C, Reiter JF (2016) Endothelial primary cilia inhibit atherosclerosis. EMBO Rep
74. Drake CJ, LaRue A, Ferrara N, Little CD (2000) VEGF regulates cell behavior during vasculogenesis. Dev Biol 224:178–188
75. Duarte A, Hirashima M, Benedito R, Trindade A, Diniz P, Bekman E, Costa L, Henrique D, Rossant J (2004) Dosage-sensitive requirement for mouse Dll4 in artery development. Genes Dev 18:2474–2478

76. Dunmore BJ, Drake KM, Upton PD, Toshner MR, Aldred MA, Morrell NW (2013) The lysosomal inhibitor, chloroquine, increases cell surface BMPR-II levels and restores BMP9 signalling in endothelial cells harbouring BMPR-II mutations. Hum Mol Genet 22:3667–3679
77. Dupuis-Girod S, Bailly S, Plauchu H (2010) Hereditary hemorrhagic telangiectasia: from molecular biology to patient care. J Thromb Haemost 8:1447–1456
78. Egorova AD, Khedoe PP, Goumans MJ, Yoder BK, Nauli SM, ten Dijke P, Poelmann RE, Hierck BP (2011) Lack of primary cilia primes shear-induced endothelial-to-mesenchymal transition. Circ Res 108:1093–1101
79. Egorova AD, van der Heiden K, Poelmann RE, Hierck BP (2012) Primary cilia as biomechanical sensors in regulating endothelial function. Differentiation 83:S56–S61
80. Ehrlich M (2015) Endocytosis and trafficking of BMP receptors: Regulatory mechanisms for fine-tuning the signaling response in different cellular contexts. Cytokine Growth Factor Rev 27:35–42
81. Eisenberg LM, Markwald RR (1995) Molecular regulation of atrioventricular valvuloseptal morphogenesis. Circ Res 77:1–6
82. El-Harith el HA, Kuhnau W, Schmidtke J, Gadzicki D, Ahmed M, Krawczak M, Stuhrmann M (2006) Hereditary hemorrhagic telangiectasia is caused by the Q490X mutation of the ACVRL1 gene in a large Arab family: support of homozygous lethality. Eur J Med Genet 49:323–330
83. Esser S, Lampugnani MG, Corada M, Dejana E, Risau W (1998) Vascular endothelial growth factor induces VE-cadherin tyrosine phosphorylation in endothelial cells. J Cell Sci 111(Pt 13):1853–1865
84. Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell-Braxton L, Hillan KJ, Moore MW (1996) Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature 380:439–442
85. Finkenzeller G, Hager S, Stark GB (2012) Effects of bone morphogenetic protein 2 on human umbilical vein endothelial cells. Microvasc Res 84:81–85
86. Fischer A, Zalvide J, Faurobert E, Albiges-Rizo C, Tournier-Lasserve E (2013) Cerebral cavernous malformations: from CCM genes to endothelial cell homeostasis. Trends Mol Med 19:302–308
87. Folkman J (2007) Angiogenesis: an organizing principle for drug discovery? Nat Rev Drug Discov 6:273–286
88. Fonsatti E, Del Vecchio L, Altomonte M, Sigalotti L, Nicotra MR, Coral S, Natali PG, Maio M (2001) Endoglin: An accessory component of the TGF-beta-binding receptor-complex with diagnostic, prognostic, and bioimmunotherapeutic potential in human malignancies. J Cell Physiol 188:1–7
89. Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD, Semenza GL (1996) Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol 16:4604–4613
90. Friedl W, Kruse R, Uhlhaas S, Stolte M, Schartmann B, Keller KM, Jungck M, Stern M, Loff S, Back W, Propping P, Jenne DE (1999) Frequent 4-bp deletion in exon 9 of the SMAD4/MADH4 gene in familial juvenile polyposis patients. Genes Chromosomes Cancer 25:403–406
91. Frump AL, Lowery JW, Hamid R, Austin ED, de Caestecker M (2013) Abnormal trafficking of endogenously expressed BMPR2 mutant allelic products in patients with heritable pulmonary arterial hypertension. PLoS One 8:e80319
92. Fuchshofer R, Yu AHL, Welge-Lu¨ssen U, Tamm ER (2007) Bone morphogenetic protein-7 is an antagonist of transforming growth factor-β2 in human trabecular meshwork cells. Investig Opthalmol Visual Sci 48:715
93. Gale NW, Dominguez MG, Noguera I, Pan L, Hughes V, Valenzuela DM, Murphy AJ, Adams NC, Lin HC, Holash J, Thurston G, Yancopoulos GD (2004) Haploinsufficiency of delta-like 4 ligand results in embryonic lethality due to major defects in arterial and vascular development. Proc Natl Acad Sci U S A 101:15949–15954
94. Gallione CJ, Repetto GM, Legius E, Rustgi AK, Schelley SL, Tejpar S, Mitchell G, Drouin E, Westermann CJ, Marchuk DA (2004) A combined syndrome of juvenile polyposis and heredi-

tary haemorrhagic telangiectasia associated with mutations in MADH4 (SMAD4). Lancet 363:852–859
95. Gangopahyay A, Oran M, Bauer EM, Wertz JW, Comhair SA, Erzurum SC, Bauer PM (2011) Bone morphogenetic protein receptor II is a novel mediator of endothelial nitric-oxide synthase activation. J Biol Chem 286:33134–33140
96. Garcia de Vinuesa A, Abdelilah-Seyfried S, Knaus P, Zwijsen A, Bailly S (2016) BMP signaling in vascular biology and dysfunction. Cytokine Growth Factor Rev 27:65–79
97. Gavard J (2014) Endothelial permeability and VE-cadherin: a wacky comradeship. Cell Adh Migr 8:158–164
98. Gavard J, Gutkind JS (2006) VEGF controls endothelial-cell permeability by promoting the beta-arrestin-dependent endocytosis of VE-cadherin. Nat Cell Biol 8:1223–1234
99. Gerhardt H, Golding M, Fruttiger M, Ruhrberg C, Lundkvist A, Abramsson A, Jeltsch M, Mitchell C, Alitalo K, Shima D, Betsholtz C (2003) VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. J Cell Biol 161:1163–1177
100. Germain M, Eyries M, Montani D, Poirier O, Girerd B, Dorfmuller P, Coulet F, Nadaud S, Maugenre S, Guignabert C, Carpentier W, Vonk-Noordegraaf A, Levy M, Chaouat A, Lambert JC, Bertrand M, Dupuy AM, Letenneur L, Lathrop M, Amouyel P, de Ravel TJ, Delcroix M, Austin ED, Robbins IM, Hemnes AR, Loyd JE, Berman-Rosenzweig E, Barst RJ, Chung WK, Simonneau G, Tregouet DA, Humbert M, Soubrier F (2013) Genome-wide association analysis identifies a susceptibility locus for pulmonary arterial hypertension. Nat Genet 45:518–521
101. Giannotta M, Trani M, Dejana E (2013) VE-cadherin and endothelial adherens junctions: active guardians of vascular integrity. Dev Cell 26:441–454
102. Goddard LM, Iruela-Arispe ML (2013) Cellular and molecular regulation of vascular permeability. Thromb Haemost 109:407–415
103. Gougos A, Letarte M (1988) Identification of a human endothelial cell antigen with monoclonal antibody 44G4 produced against a pre-B leukemic cell line. J Immunol 141: 1925–1933
104. Gougos A, Letarte M (1990) Primary structure of endoglin, an RGD-containing glycoprotein of human endothelial cells. J Biol Chem 265:8361–8364
105. Goumans MJ, Valdimarsdottir G, Itoh S, Rosendahl A, Sideras P, ten Dijke P (2002) Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors. EMBO J 21:1743–1753
106. Govani FS, Shovlin CL (2009) Hereditary haemorrhagic telangiectasia: a clinical and scientific review. Eur J Hum Genet 17:860–871
107. Graupera M, Guillermet-Guibert J, Foukas LC, Phng LK, Cain RJ, Salpekar A, Pearce W, Meek S, Millan J, Cutillas PR, Smith AJ, Ridley AJ, Ruhrberg C, Gerhardt H, Vanhaesebroeck B (2008) Angiogenesis selectively requires the p110alpha isoform of PI3K to control endothelial cell migration. Nature 453:662–666
108. Grazia Lampugnani M, Zanetti A, Corada M, Takahashi T, Balconi G, Breviario F, Orsenigo F, Cattelino A, Kemler R, Daniel TO, Dejana E (2003) Contact inhibition of VEGF-induced proliferation requires vascular endothelial cadherin, beta-catenin, and the phosphatase DEP-1/CD148. J Cell Biol 161:793–804
109. Grgurevic L, Christensen GL, Schulz TJ, Vukicevic S (2015) Bone morphogenetic proteins in inflammation, glucose homeostasis and adipose tissue energy metabolism. Cytokine Growth Factor Rev 27:105–18
110. Hahn C, Schwartz MA (2009) Mechanotransduction in vascular physiology and atherogenesis. Nat Rev Mol Cell Biol 10:53–62
111. Han C, Hong KH, Kim YH, Kim MJ, Song C, Kim MJ, Kim SJ, Raizada MK, Oh SP (2013) SMAD1 deficiency in either endothelial or smooth muscle cells can predispose mice to pulmonary hypertension. Hypertension 61:1044–1052
112. Hanyu A, Ishidou Y, Ebisawa T, Shimanuki T, Imamura T, Miyazono K (2001) The N domain of Smad7 is essential for specific inhibition of transforming growth factor-beta signaling. J Cell Biol 155:1017–1027

113. Hatsell SJ, Idone V, Wolken DMA, Huang L, Kim HJ, Wang L, Wen X, Nannuru KC, Jimenez J, Xie L, Das N, Makhoul G, Chernomorsky R, D'Ambrosio D, Corpina RA, Schoenherr CJ, Feeley K, Yu PB, Yancopoulos GD, Murphy AJ, Economides AN (2015) ACVR1R206H receptor mutation causes fibrodysplasia ossificans progressiva by imparting responsiveness to activin A. Sci Transl Med 7:303ra137
114. He C, Chen X (2005) Transcription regulation of the vegf gene by the BMP/Smad pathway in the angioblast of zebrafish embryos. Biochem Biophys Res Commun 329:324–330
115. Heinecke K, Seher A, Schmitz W, Mueller TD, Sebald W, Nickel J (2009) Receptor oligomerization and beyond: a case study in bone morphogenetic proteins. BMC Biol 7:59
116. Helbing T, Rothweiler R, Heinke J, Goetz L, Diehl P, Zirlik A, Patterson C, Bode C, Moser M (2010) BMPER is upregulated by statins and modulates endothelial inflammation by intercellular adhesion molecule-1. Arterioscler Thromb Vasc Biol 30:554–560
117. Helbing T, Rothweiler R, Ketterer E, Goetz L, Heinke J, Grundmann S, Duerschmied D, Patterson C, Bode C, Moser M (2011) BMP activity controlled by BMPER regulates the proinflammatory phenotype of endothelium. Blood 118:5040–5049
118. Hellstrom M, Phng LK, Hofmann JJ, Wallgard E, Coultas L, Lindblom P, Alva J, Nilsson AK, Karlsson L, Gaiano N, Yoon K, Rossant J, Iruela-Arispe ML, Kalen M, Gerhardt H, Betsholtz C (2007) Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. Nature 445:776–780
119. Hen G, Nicenboim J, Mayseless O, Asaf L, Shin M, Busolin G, Hofi R, Almog G, Tiso N, Lawson ND, Yaniv K (2015) Venous-derived angioblasts generate organ-specific vessels during zebrafish embryonic development. Development 142:4266–4278
120. Herbert SP, Huisken J, Kim TN, Feldman ME, Houseman BT, Wang RA, Shokat KM, Stainier DY (2009) Arterial-venous segregation by selective cell sprouting: an alternative mode of blood vessel formation. Science 326:294–298
121. Herbert SP, Stainier DY (2011) Molecular control of endothelial cell behaviour during blood vessel morphogenesis. Nat Rev Mol Cell Biol 12:551–564
122. Hiepen C, Yadin D, Rikeit P, Dorpholz G, Knaus P (2016) Actions from head to toe: an update on bone/body morphogenetic proteins in health and disease. Cytokine Growth Factor Rev 27:1–11
123. Hofmann F, Feil R, Kleppisch T, Schlossmann J (2006) Function of cGMP-dependent protein kinases as revealed by gene deletion. Physiol Rev 86:1–23
124. Hong KH, Lee YJ, Lee E, Park SO, Han C, Beppu H, Li E, Raizada MK, Bloch KD, Oh SP (2008) Genetic ablation of the BMPR2 gene in pulmonary endothelium is sufficient to predispose to pulmonary arterial hypertension. Circulation 118:722–730
125. Houlston R, Bevan S, Williams A, Young J, Dunlop M, Rozen P, Eng C, Markie D, Woodford-Richens K, Rodriguez-Bigas MA, Leggett B, Neale K, Phillips R, Sheridan E, Hodgson S, Iwama T, Eccles D, Bodmer W, Tomlinson I (1998) Mutations in DPC4 (SMAD4) cause juvenile polyposis syndrome, but only account for a minority of cases. Hum Mol Genet 7:1907–1912
126. Howe JR, Roth S, Ringold JC, Summers RW, Jarvinen HJ, Sistonen P, Tomlinson IP, Houlston RS, Bevan S, Mitros FA, Stone EM, Aaltonen LA (1998) Mutations in the SMAD4/DPC4 gene in juvenile polyposis. Science 280:1086–1088
127. Howe JR, Sayed MG, Ahmed AF, Ringold J, Larsen-Haidle J, Merg A, Mitros FA, Vaccaro CA, Petersen GM, Giardiello FM, Tinley ST, Aaltonen LA, Lynch HT (2004) The prevalence of MADH4 and BMPR1A mutations in juvenile polyposis and absence of BMPR2, BMPR1B, and ACVR1 mutations. J Med Genet 41:484–491
128. Howe JR, Shellnut J, Wagner B, Ringold JC, Sayed MG, Ahmed AF, Lynch PM, Amos CI, Sistonen P, Aaltonen LA (2002) Common deletion of SMAD4 in juvenile polyposis is a mutational hotspot. Am J Hum Genet 70:1357–1362
129. Hruska KA, Mathew S, Saab G (2005) Bone morphogenetic proteins in vascular calcification. Circ Res 97:105–114
130. Huang Z, Wang D, Ihida-Stansbury K, Jones PL, Martin JF (2009) Defective pulmonary vascular remodeling in Smad8 mutant mice. Hum Mol Genet 18:2791–2801

131. International PPHC, Lane KB, Machado RD, Pauciulo MW, Thomson JR, Phillips JA, 3rd, Loyd JE, Nichols WC, Trembath RC (2000) Heterozygous germline mutations in BMPR2, encoding a TGF-beta receptor, cause familial primary pulmonary hypertension. Nat Genet 26:81–84
132. Ishida W, Hamamoto T, Kusanagi K, Yagi K, Kawabata M, Takehara K, Sampath TK, Kato M, Miyazono K (2000) Smad6 is a Smad1/5-induced smad inhibitor. Characterization of bone morphogenetic protein-responsive element in the mouse Smad6 promoter. J Biol Chem 275:6075–6079
133. Iso T, Maeno T, Oike Y, Yamazaki M, Doi H, Arai M, Kurabayashi M (2006) Dll4-selective Notch signaling induces ephrinB2 gene expression in endothelial cells. Biochem Biophys Res Commun 341:708–714
134. Itoh F, Itoh S, Goumans MJ, Valdimarsdottir G, Iso T, Dotto GP, Hamamori Y, Kedes L, Kato M, ten Dijke PP (2004) Synergy and antagonism between Notch and BMP receptor signaling pathways in endothelial cells. EMBO J 23:541–551
135. Jaipersad AS, Lip GY, Silverman S, Shantsila E (2014) The role of monocytes in angiogenesis and atherosclerosis. J Am Coll Cardiol 63:1–11
136. Jakobsson L, Franco CA, Bentley K, Collins RT, Ponsioen B, Aspalter IM, Rosewell I, Busse M, Thurston G, Medvinsky A, Schulte-Merker S, Gerhardt H (2010) Endothelial cells dynamically compete for the tip cell position during angiogenic sprouting. Nat Cell Biol 12:943–953
137. Jo H, Song H, Mowbray A (2006) Role of NADPH oxidases in disturbed flow- and BMP4-induced inflammation and atherosclerosis. Antioxid Redox Signal 8:1609–1619
138. John A, Kizhakkedath P, Al-Gazali L, Ali BR (2015) Defective cellular trafficking of the bone morphogenetic protein receptor type II by mutations underlying familial pulmonary arterial hypertension. Gene 561:148–156
139. Johnson DW, Berg JN, Baldwin MA, Gallione CJ, Marondel I, Yoon SJ, Stenzel TT, Speer M, Pericak-Vance MA, Diamond A, Guttmacher AE, Jackson CE, Attisano L, Kucherlapati R, Porteous ME, Marchuk DA (1996) Mutations in the activin receptor-like kinase 1 gene in hereditary haemorrhagic telangiectasia type 2. Nat Genet 13:189–195
140. Jonker L (2014) TGF-beta & BMP receptors endoglin and ALK1: overview of their functional role and status as antiangiogenic targets. Microcirculation 21:93–103
141. Karabegovic A, Shinawi M, Cymerman U, Letarte M (2004) No live individual homozygous for a novel endoglin mutation was found in a consanguineous Arab family with hereditary haemorrhagic telangiectasia. J Med Genet 41:e119
142. Kashiwada T, Fukuhara S, Terai K, Tanaka T, Wakayama Y, Ando K, Nakajima H, Fukui H, Yuge S, Saito Y, Gemma A, Mochizuki N (2015) beta-Catenin-dependent transcription is central to Bmp-mediated formation of venous vessels. Development 142:497–509
143. Katagiri T, Imada M, Yanai T, Suda T, Takahashi N, Kamijo R (2002) Identification of a BMP-responsive element in Id1, the gene for inhibition of myogenesis. Genes Cells 7: 949–960
144. Kim CW, Song H, Kumar S, Nam D, Kwon HS, Chang KH, Son DJ, Kang DW, Brodie SA, Weiss D, Vega JD, Alberts-Grill N, Griendling K, Taylor WR, Jo H (2013) Anti-inflammatory and antiatherogenic role of BMP receptor II in endothelial cells. Arterioscler Thromb Vasc Biol 33:1350–1359
145. Kim JD, Kang H, Larrivee B, Lee MY, Mettlen M, Schmid SL, Roman BL, Qyang Y, Eichmann A, Jin SW (2012) Context-dependent proangiogenic function of bone morphogenetic protein signaling is mediated by disabled homolog 2. Dev Cell 23:441–448
146. Kim JH, Peacock MR, George SC, Hughes CC (2012) BMP9 induces EphrinB2 expression in endothelial cells through an Alk1-BMPRII/ActRII-ID1/ID3-dependent pathway: implications for hereditary hemorrhagic telangiectasia type II. Angiogenesis 15:497–509
147. Kinsella MG, Fitzharris TP (1980) Origin of cushion tissue in the developing chick heart: cinematographic recordings of in situ formation. Science (New York, NY) 207:1359–1360
148. Kishigami S, Mishina Y (2005) BMP signaling and early embryonic patterning. Cytokine Growth Factor Rev 16:265–278

149. Komarova Y, Malik AB (2010) Regulation of endothelial permeability via paracellular and transcellular transport pathways. Annu Rev Physiol 72:463–493
150. Korchynskyi O, ten Dijke P (2002) Identification and functional characterization of distinct critically important bone morphogenetic protein-specific response elements in the Id1 promoter. J Biol Chem 277:4883–4891
151. Krebs LT, Shutter JR, Tanigaki K, Honjo T, Stark KL, Gridley T (2004) Haploinsufficient lethality and formation of arteriovenous malformations in Notch pathway mutants. Genes Dev 18:2469–2473
152. Krebs LT, Starling C, Chervonsky AV, Gridley T (2010) Notch1 activation in mice causes arteriovenous malformations phenocopied by ephrinB2 and EphB4 mutants. Genesis 48:146–150
153. Labauge P, Denier C, Bergametti F, Tournier-Lasserve E (2007) Genetics of cavernous angiomas. Lancet Neurol 6:237–244
154. Laberge-le Couteulx S, Jung HH, Labauge P, Houtteville JP, Lescoat C, Cecillon M, Marechal E, Joutel A, Bach JF, Tournier-Lasserve E (1999) Truncating mutations in CCM1, encoding KRIT1, cause hereditary cavernous angiomas. Nat Genet 23:189–193
155. Lampugnani MG, Orsenigo F, Gagliani MC, Tacchetti C, Dejana E (2006) Vascular endothelial cadherin controls VEGFR-2 internalization and signaling from intracellular compartments. J Cell Biol 174:593–604
156. Lan Y, Liu B, Yao H, Li F, Weng T, Yang G, Li W, Cheng X, Mao N, Yang X (2007) Essential role of endothelial Smad4 in vascular remodeling and integrity. Mol Cell Biol 27: 7683–7692
157. Langenfeld EM, Langenfeld J (2004) Bone morphogenetic protein-2 stimulates angiogenesis in developing tumors. Mol Cancer Res 2:141–149
158. Langeveld D, van Hattem WA, de Leng WW, Morsink FH, Ten Kate FJ, Giardiello FM, Offerhaus GJ, Brosens LA (2010) SMAD4 immunohistochemistry reflects genetic status in juvenile polyposis syndrome. Clin Cancer Res 16:4126–4134
159. Larkin EK, Newman JH, Austin ED, Hemnes AR, Wheeler L, Robbins IM, West JD, Phillips JA 3rd, Hamid R, Loyd JE (2012) Longitudinal analysis casts doubt on the presence of genetic anticipation in heritable pulmonary arterial hypertension. Am J Respir Crit Care Med 186:892–896
160. Larrivee B, Prahst C, Gordon E, del Toro R, Mathivet T, Duarte A, Simons M, Eichmann A (2012) ALK1 signaling inhibits angiogenesis by cooperating with the Notch pathway. Dev Cell 22:489–500
161. Lavery K, Swain P, Falb D, Alaoui-Ismaili MH (2008) BMP-2/4 and BMP-6/7 differentially utilize cell surface receptors to induce osteoblastic differentiation of human bone marrow-derived mesenchymal stem cells. J Biol Chem 283:20948–20958
162. Lawson ND, Scheer N, Pham VN, Kim CH, Chitnis AB, Campos-Ortega JA, Weinstein BM (2001) Notch signaling is required for arterial-venous differentiation during embryonic vascular development. Development 128:3675–3683
163. Lawson ND, Vogel AM, Weinstein BM (2002) sonic hedgehog and vascular endothelial growth factor act upstream of the Notch pathway during arterial endothelial differentiation. Dev Cell 3:127–136
164. Lebrin F, Goumans MJ, Jonker L, Carvalho RL, Valdimarsdottir G, Thorikay M, Mummery C, Arthur HM, ten Dijke P (2004) Endoglin promotes endothelial cell proliferation and TGF-beta/ALK1 signal transduction. EMBO J 23:4018–4028
165. Lechleider RJ, Ryan JL, Garrett L, Eng C, Deng C, Wynshaw-Boris A, Roberts AB (2001) Targeted mutagenesis of Smad1 reveals an essential role in chorioallantoic fusion. Dev Biol 240:157–167
166. Lee JG, Kay EP (2006) FGF-2-mediated signal transduction during endothelial mesenchymal transformation in corneal endothelial cells. Exp Eye Res 83:1309–1316
167. Lee NY, Ray B, How T, Blobe GC (2008) Endoglin promotes transforming growth factor beta-mediated Smad 1/5/8 signaling and inhibits endothelial cell migration through its association with GIPC. J Biol Chem 283:32527–32533

168. Lesca G, Olivieri C, Burnichon N, Pagella F, Carette MF, Gilbert-Dussardier B, Goizet C, Roume J, Rabilloud M, Saurin JC, Cottin V, Honnorat J, Coulet F, Giraud S, Calender A, Danesino C, Buscarini E, Plauchu H (2007) Genotype-phenotype correlations in hereditary hemorrhagic telangiectasia: data from the French-Italian HHT network. Genet Med 9:14–22
169. Leslie JD, Ariza-McNaughton L, Bermange AL, McAdow R, Johnson SL, Lewis J (2007) Endothelial signalling by the Notch ligand Delta-like 4 restricts angiogenesis. Development 134:839–844
170. Letteboer TG, Mager JJ, Snijder RJ, Koeleman BP, Lindhout D, Ploos van Amstel JK, Westermann CJ (2006) Genotype-phenotype relationship in hereditary haemorrhagic telangiectasia. J Med Genet 43:371–377
171. Li C, Hampson IN, Hampson L, Kumar P, Bernabeu C, Kumar S (2000) CD105 antagonizes the inhibitory signaling of transforming growth factor beta1 on human vascular endothelial cells. FASEB J 14:55–64
172. Li DY, Sorensen LK, Brooke BS, Urness LD, Davis EC, Taylor DG, Boak BB, Wendel DP (1999) Defective angiogenesis in mice lacking endoglin. Science 284:1534–1537
173. Li DY, Whitehead KJ (2010) Evaluating strategies for the treatment of cerebral cavernous malformations. Stroke 41:S92–S94
174. Li X, Lim J, Lu J, Pedego TM, Demer L, Tintut Y (2015) Protective Role of Smad6 in Inflammation-Induced Valvular Cell Calcification. J Cell Biochem 116:2354–2364
175. Liebner S, Cattelino A, Gallini R, Rudini N, Iurlaro M, Piccolo S, Dejana E (2004) Beta-catenin is required for endothelial-mesenchymal transformation during heart cushion development in the mouse. J Cell Biol 166:359–367
176. Lin F, Wang N, Zhang T-C (2012) The role of endothelial–mesenchymal transition in development and pathological process. IUBMB Life 64:717–723
177. Liu D, Morrell NW (2013) Genetics and the molecular pathogenesis of pulmonary arterial hypertension. Curr Hypertens Rep 15:632–637
178. Lobov IB, Renard RA, Papadopoulos N, Gale NW, Thurston G, Yancopoulos GD, Wiegand SJ (2007) Delta-like ligand 4 (Dll4) is induced by VEGF as a negative regulator of angiogenic sprouting. Proc Natl Acad Sci U S A 104:3219–3224
179. Long L, Ormiston ML, Yang X, Southwood M, Graf S, Machado RD, Mueller M, Kinzel B, Yung LM, Wilkinson JM, Moore SD, Drake KM, Aldred MA, Yu PB, Upton PD, Morrell NW (2015) Selective enhancement of endothelial BMPR-II with BMP9 reverses pulmonary arterial hypertension. Nat Med 21:777–785
180. Lopez-Novoa JM (2012) Angiogenic stimuli and endoglin absence induces brain arteriovenous malformations: are local endoglin deletion and angiogenesis the ‘second hit’ that is necessary for arteriovenous malformations formation in HHT-1? Cerebrovasc Dis 33:548
181. Lopez-Novoa JM, Bernabeu C (2010) The physiological role of endoglin in the cardiovascular system. Am J Physiol Heart Circ Physiol 299:H959–H974
182. Lounev VY, Ramachandran R, Wosczyna MN, Yamamoto M, Maidment ADA, Shore EM, Glaser DL, Goldhamer DJ, Kaplan FS (2009) Identification of progenitor cells that contribute to heterotopic skeletogenesis. J Bone Joint Surg Am 91:652–663
183. Loyd JE, Butler MG, Foroud TM, Conneally PM, Phillips JA 3rd, Newman JH (1995) Genetic anticipation and abnormal gender ratio at birth in familial primary pulmonary hypertension. Am J Respir Crit Care Med 152:93–97
184. Ma L, Chung WK (2014) The genetic basis of pulmonary arterial hypertension. Hum Genet 133:471–479
185. Ma L, Roman-Campos D, Austin ED, Eyries M, Sampson KS, Soubrier F, Germain M, Tregouet DA, Borczuk A, Rosenzweig EB, Girerd B, Montani D, Humbert M, Loyd JE, Kass RS, Chung WK (2013) A novel channelopathy in pulmonary arterial hypertension. N Engl J Med 369:351–361
186. Machado RD, Aldred MA, James V, Harrison RE, Patel B, Schwalbe EC, Gruenig E, Janssen B, Koehler R, Seeger W, Eickelberg O, Olschewski H, Elliott CG, Glissmeyer E, Carlquist J, Kim M, Torbicki A, Fijalkowska A, Szewczyk G, Parma J, Abramowicz MJ, Galie N, Morisaki H, Kyotani S, Nakanishi N, Morisaki T, Humbert M, Simonneau G, Sitbon O,

Soubrier F, Coulet F, Morrell NW, Trembath RC (2006) Mutations of the TGF-beta type II receptor BMPR2 in pulmonary arterial hypertension. Hum Mutat 27:121–132
187. Maddaluno L, Rudini N, Cuttano R, Bravi L, Giampietro C, Corada M, Ferrarini L, Orsenigo F, Papa E, Boulday G, Tournier-Lasserve E, Chapon F, Richichi C, Retta SF, Lampugnani MG, Dejana E (2013) EndMT contributes to the onset and progression of cerebral cavernous malformations. Nature 498:492–496
188. Massague J, Seoane J, Wotton D (2005) Smad transcription factors. Genes Dev 19:2783–2810
189. McAllister KA, Grogg KM, Johnson DW, Gallione CJ, Baldwin MA, Jackson CE, Helmbold EA, Markel DS, McKinnon WC, Murrell J et al (1994) Endoglin, a TGF-beta binding protein of endothelial cells, is the gene for hereditary haemorrhagic telangiectasia type 1. Nat Genet 8:345–351
190. McCarthy EF, Sundaram M (2005) Heterotopic ossification: a review. Skeletal Radiol 34:609–619
191. McCulley DJ, Kang J-O, Martin JF, Black BL (2008) BMP4 is required in the anterior heart field and its derivatives for endocardial cushion remodeling, outflow tract septation, and semilunar valve development. Dev Dyn 237:3200–3209
192. Medici D, Kalluri R (2012) Endothelial-mesenchymal transition and its contribution to the emergence of stem cell phenotype. Semin Cancer Biol 22:379–384
193. Medici D, Olsen BR (2012) The role of endothelial-mesenchymal transition in heterotopic ossification. J Bone Miner Res 27: 1619–22
194. Medici D, Shore EM, Lounev VY, Kaplan FS, Kalluri R, Olsen BR (2010) Conversion of vascular endothelial cells into multipotent stem-like cells. Nat Med 16:1400–1406
195. Meurer SK, Alsamman M, Scholten D, Weiskirchen R (2014) Endoglin in liver fibrogenesis: Bridging basic science and clinical practice. World J Biol Chem 5:180–203
196. Minshall RD, Tiruppathi C, Vogel SM, Malik AB (2002) Vesicle formation and trafficking in endothelial cells and regulation of endothelial barrier function. Histochem Cell Biol 117:105–112
197. Mohedas AH (2014) Development of BMP type I receptor kinase inhibitors for the treatment of fibrodysplasia ossificans progressiva and the study of the BMP signaling pathway. PhD thesis, Massachusetts Institute of Technology (http://hdl.handle.net/1721.1/90173)
198. Mohedas AH, Xing X, Armstrong KA, Bullock AN, Cuny GD, Yu PB (2013) Development of an ALK2-biased BMP type I receptor kinase inhibitor. ACS Chem Biol 8:1291–1302
199. Monteiro R, van Dinther M, Bakkers J, Wilkinson R, Patient R, ten Dijke P, Mummery C (2008) Two novel type II receptors mediate BMP signalling and are required to establish left-right asymmetry in zebrafish. Dev Biol 315:55–71
200. Monteiro RM, de Sousa Lopes SM, Bialecka M, de Boer S, Zwijsen A, Mummery CL (2008) Real time monitoring of BMP Smads transcriptional activity during mouse development. Genesis 46:335–346
201. Moonen J-RAJ, Krenning G, Brinker MGL, Koerts JA, van Luyn MJA, Harmsen MC (2010) Endothelial progenitor cells give rise to pro-angiogenic smooth muscle-like progeny. Cardiovasc Res 86:506–515
202. Morikawa M, Koinuma D, Tsutsumi S, Vasilaki E, Kanki Y, Heldin CH, Aburatani H, Miyazono K (2011) ChIP-seq reveals cell type-specific binding patterns of BMP-specific Smads and a novel binding motif. Nucleic Acids Res 39:8712–8727
203. Morioka S, Inagaki M, Komatsu Y, Mishina Y, Matsumoto K, Ninomiya-Tsuji J (2012) TAK1 kinase signaling regulates embryonic angiogenesis by modulating endothelial cell survival and migration. Blood 120:3846–3857
204. Morrell NW, Adnot S, Archer SL, Dupuis J, Jones PL, MacLean MR, McMurtry IF, Stenmark KR, Thistlethwaite PA, Weissmann N, Yuan JX, Weir EK (2009) Cellular and molecular basis of pulmonary arterial hypertension. J Am Coll Cardiol 54:S20–S31
205. Morrell NW, Bloch DB, Ten Dijke P, Goumans MJ, Hata A, Smith J, Yu PB, Bloch KD (2016) Targeting BMP signalling in cardiovascular disease and anaemia. Nat Rev Cardiol 13:106–120

206. Morse JH, Jones AC, Barst RJ, Hodge SE, Wilhelmsen KC, Nygaard TG (1997) Mapping of familial primary pulmonary hypertension locus (PPH1) to chromosome 2q31-q32. Circulation 95:2603–2606
207. Moser M, Binder O, Wu Y, Aitsebaomo J, Ren R, Bode C, Bautch VL, Conlon FL, Patterson C (2003) BMPER, a novel endothelial cell precursor-derived protein, antagonizes bone morphogenetic protein signaling and endothelial cell differentiation. Mol Cell Biol 23:5664–5679
208. Moya IM, Umans L, Maas E, Pereira PN, Beets K, Francis A, Sents W, Robertson EJ, Mummery CL, Huylebroeck D, Zwijsen A (2012) Stalk cell phenotype depends on integration of Notch and Smad1/5 signaling cascades. Dev Cell 22:501–514
209. Mu Y, Gudey SK, Landstrom M (2012) Non-Smad signaling pathways. Cell Tissue Res 347:11–20
210. Mueller TD, Nickel J (2012) Promiscuity and specificity in BMP receptor activation. FEBS Lett 586:1846–1859
211. Muller JY, Michailov T, Izrael V, Bernard J (1978) Hereditary haemorrhagic telanglectasia in a large Saharan familly. 87 cases in the same family (author's transl). Nouv Presse Med 7:1723–1725
212. Nakagawa Y, Ikeda K, Akakabe Y, Koide M, Uraoka M, Yutaka KT, Kurimoto-Nakano R, Takahashi T, Matoba S, Yamada H, Okigaki M, Matsubara H (2010) Paracrine osteogenic signals via bone morphogenetic protein-2 accelerate the atherosclerotic intimal calcification in vivo. Arterioscler Thromb Vasc Biol 30:1908–1915
213. Nakahiro T, Kurooka H, Mori K, Sano K, Yokota Y (2010) Identification of BMP-responsive elements in the mouse Id2 gene. Biochem Biophys Res Commun 399:416–421
214. Nakajima Y, Yamagishi T, Hokari S, Nakamura H (2000) Mechanisms involved in valvuloseptal endocardial cushion formation in early cardiogenesis: roles of transforming growth factor (TGF)-beta and bone morphogenetic protein (BMP). Anat Rec 258:119–127
215. Nasim MT, Ogo T, Ahmed M, Randall R, Chowdhury HM, Snape KM, Bradshaw TY, Southgate L, Lee GJ, Jackson I, Lord GM, Gibbs JS, Wilkins MR, Ohta-Ogo K, Nakamura K, Girerd B, Coulet F, Soubrier F, Humbert M, Morrell NW, Trembath RC, Machado RD (2011) Molecular genetic characterization of SMAD signaling molecules in pulmonary arterial hypertension. Hum Mutat 32:1385–1389
216. Natoli G, Ghisletti S, Barozzi I (2011) The genomic landscapes of inflammation. Genes Dev 25:101–106
217. Nichols WC, Koller DL, Slovis B, Foroud T, Terry VH, Arnold ND, Siemieniak DR, Wheeler L, Phillips JA 3rd, Newman JH, Conneally PM, Ginsburg D, Loyd JE (1997) Localization of the gene for familial primary pulmonary hypertension to chromosome 2q31-32. Nat Genet 15:277–280
218. Nimmagadda S, Geetha Loganathan P, Huang R, Scaal M, Schmidt C, Christ B (2005) BMP4 and noggin control embryonic blood vessel formation by antagonistic regulation of VEGFR-2 (Quek1) expression. Dev Biol 280:100–110
219. Nolan-Stevaux O, Zhong W, Culp S, Shaffer K, Hoover J, Wickramasinghe D, Ruefli-Brasse A (2012) Endoglin requirement for BMP9 signaling in endothelial cells reveals new mechanism of action for selective anti-endoglin antibodies. PLoS One 7:e50920
220. Nomura-Kitabayashi A, Anderson GA, Sleep G, Mena J, Karabegovic A, Karamath S, Letarte M, Puri MC (2009) Endoglin is dispensable for angiogenesis, but required for endocardial cushion formation in the midgestation mouse embryo. Dev Biol 335:66–77
221. Ogo T, Chowdhury HM, Yang J, Long L, Li X, Torres Cleuren YN, Morrell NW, Schermuly RT, Trembath RC, Nasim MT (2013) Inhibition of overactive transforming growth factor-beta signaling by prostacyclin analogs in pulmonary arterial hypertension. Am J Respir Cell Mol Biol 48:733–741
222. Oh SP, Seki T, Goss KA, Imamura T, Yi Y, Donahoe PK, Li L, Miyazono K, ten Dijke P, Kim S, Li E (2000) Activin receptor-like kinase 1 modulates transforming growth factor-beta 1 signaling in the regulation of angiogenesis. Proc Natl Acad Sci U S A 97:2626–2631
223. Orsenigo F, Giampietro C, Ferrari A, Corada M, Galaup A, Sigismund S, Ristagno G, Maddaluno L, Koh GY, Franco D, Kurtcuoglu V, Poulikakos D, Baluk P, McDonald D,

Grazia Lampugnani M, Dejana E (2012) Phosphorylation of VE-cadherin is modulated by haemodynamic forces and contributes to the regulation of vascular permeability in vivo. Nat Commun 3:1208
224. Park C, Kim TM, Malik AB (2013) Transcriptional regulation of endothelial cell and vascular development. Circ Res 112:1380–1400
225. Park JE, Shao D, Upton PD, Desouza P, Adcock IM, Davies RJ, Morrell NW, Griffiths MJ, Wort SJ (2012) BMP-9 induced endothelial cell tubule formation and inhibition of migration involves Smad1 driven endothelin-1 production. PLoS One 7:e30075
226. Park S, Dimaio TA, Liu W, Wang S, Sorenson CM, Sheibani N (2013) Endoglin regulates the activation and quiescence of endothelium by participating in canonical and non-canonical TGF-beta signaling pathways. J Cell Sci 126:1392–1405
227. Park SO, Lee YJ, Seki T, Hong KH, Fliess N, Jiang Z, Park A, Wu X, Kaartinen V, Roman BL, Oh SP (2008) ALK5- and TGFBR2-independent role of ALK1 in the pathogenesis of hereditary hemorrhagic telangiectasia type 2. Blood 111:633–642
228. Park SO, Wankhede M, Lee YJ, Choi EJ, Fliess N, Choe SW, Oh SH, Walter G, Raizada MK, Sorg BS, Oh SP (2009) Real-time imaging of de novo arteriovenous malformation in a mouse model of hereditary hemorrhagic telangiectasia. J Clin Invest 119:3487–3496
229. Pepper MS (1997) Transforming growth factor-beta: vasculogenesis, angiogenesis, and vessel wall integrity. Cytokine Growth Factor Rev 8:21–43
230. Phng LK, Gerhardt H (2009) Angiogenesis: a team effort coordinated by notch. Dev Cell 16:196–208
231. Phng LK, Potente M, Leslie JD, Babbage J, Nyqvist D, Lobov I, Ondr JK, Rao S, Lang RA, Thurston G, Gerhardt H (2009) Nrarp coordinates endothelial Notch and Wnt signaling to control vessel density in angiogenesis. Dev Cell 16:70–82
232. Pi X, Lockyer P, Dyer LA, Schisler JC, Russell B, Carey S, Sweet DT, Chen Z, Tzima E, Willis MS, Homeister JW, Moser M, Patterson C (2012) Bmper inhibits endothelial expression of inflammatory adhesion molecules and protects against atherosclerosis. Arterioscler Thromb Vasc Biol 32:2214–2222
233. Pi X, Ren R, Kelley R, Zhang C, Moser M, Bohil AB, Divito M, Cheney RE, Patterson C (2007) Sequential roles for myosin-X in BMP6-dependent filopodial extension, migration, and activation of BMP receptors. J Cell Biol 179:1569–1582
234. Pober JS, Cotran RS (1990) The role of endothelial cells in inflammation. Transplantation 50:537–544
235. Pober JS, Sessa WC (2007) Evolving functions of endothelial cells in inflammation. Nat Rev Immunol 7:803–815
236. Potenta S, Zeisberg E, Kalluri R (2008) The role of endothelial-to-mesenchymal transition in cancer progression. Br J Cancer 99:1375–1379
237. Prewitt AR, Ghose S, Frump AL, Datta A, Austin ED, Kenworthy AK, de Caestecker MP (2015) Heterozygous null bone morphogenetic protein receptor type 2 mutations promote SRC kinase-dependent caveolar trafficking defects and endothelial dysfunction in pulmonary arterial hypertension. J Biol Chem 290:960–971
238. Price LC, Montani D, Tcherakian C, Dorfmuller P, Souza R, Gambaryan N, Chaumais MC, Shao DM, Simonneau G, Howard LS, Adcock IM, Wort SJ, Humbert M, Perros F (2011) Dexamethasone reverses monocrotaline-induced pulmonary arterial hypertension in rats. Eur Respir J 37:813–822
239. Pullamsetti S, Kiss L, Ghofrani HA, Voswinckel R, Haredza P, Klepetko W, Aigner C, Fink L, Muyal JP, Weissmann N, Grimminger F, Seeger W, Schermuly RT (2005) Increased levels and reduced catabolism of asymmetric and symmetric dimethylarginines in pulmonary hypertension. FASEB J 19:1175–1177
240. Pyatt RE, Pilarski R, Prior TW (2006) Mutation screening in juvenile polyposis syndrome. J Mol Diagn 8:84–88
241. Quillien A, Moore JC, Shin M, Siekmann AF, Smith T, Pan L, Moens CB, Parsons MJ, Lawson ND (2014) Distinct Notch signaling outputs pattern the developing arterial system. Development 141:1544–1552

242. Raida M, Clement JH, Leek RD, Ameri K, Bicknell R, Niederwieser D, Harris AL (2005) Bone morphogenetic protein 2 (BMP-2) and induction of tumor angiogenesis. J Cancer Res Clin Oncol 131:741–750
243. Ramoshebi LN, Ripamonti U (2000) Osteogenic protein-1, a bone morphogenetic protein, induces angiogenesis in the chick chorioallantoic membrane and synergizes with basic fibroblast growth factor and transforming growth factor-beta1. Anat Rec 259:97–107
244. Reddi AH (2005) BMPs: from bone morphogenetic proteins to body morphogenetic proteins. Cytokine Growth Factor Rev 16:249–250
245. Ren R, Charles PC, Zhang C, Wu Y, Wang H, Patterson C (2007) Gene expression profiles identify a role for cyclooxygenase 2-dependent prostanoid generation in BMP6-induced angiogenic responses. Blood 109:2847–2853
246. Riant F, Cecillon M, Saugier-Veber P, Tournier-Lasserve E (2013) CCM molecular screening in a diagnosis context: novel unclassified variants leading to abnormal splicing and importance of large deletions. Neurogenetics 14:133–141
247. Rigamonti D, Hadley MN, Drayer BP, Johnson PC, Hoenig-Rigamonti K, Knight JT, Spetzler RF (1988) Cerebral Cavernous Malformations. N Engl J Med 319:343–347
248. Rigelsky CM, Jennings C, Lehtonen R, Minai OA, Eng C, Aldred MA (2008) BMPR2 mutation in a patient with pulmonary arterial hypertension and suspected hereditary hemorrhagic telangiectasia. Am J Med Genet A 146a:2551–2556
249. Rivera-Feliciano J, Tabin CJ (2006) Bmp2 instructs cardiac progenitors to form the heart-valve-inducing field. Dev Biol 295:580–588
250. Rocher C, Singla DK (2013) SMAD-PI3K-Akt-mTOR pathway mediates BMP-7 polarization of monocytes into M2 macrophages. PLoS One 8:e84009
251. Rondaij MG, Bierings R, Kragt A, van Mourik JA, Voorberg J (2006) Dynamics and plasticity of Weibel-Palade bodies in endothelial cells. Arterioscler Thromb Vasc Biol 26:1002–1007
252. Roth S, Sistonen P, Salovaara R, Hemminki A, Loukola A, Johansson M, Avizienyte E, Cleary KA, Lynch P, Amos CI, Kristo P, Mecklin JP, Kellokumpu I, Jarvinen H, Aaltonen LA (1999) SMAD genes in juvenile polyposis. Genes Chromosomes Cancer 26:54–61
253. Rothberg KG, Heuser JE, Donzell WC, Ying YS, Glenney JR, Anderson RG (1992) Caveolin, a protein component of caveolae membrane coats. Cell 68:673–682
254. Rothhammer T, Bataille F, Spruss T, Eissner G, Bosserhoff AK (2007) Functional implication of BMP4 expression on angiogenesis in malignant melanoma. Oncogene 26:4158–4170
255. Rudarakanchana N, Flanagan JA, Chen H, Upton PD, Machado R, Patel D, Trembath RC, Morrell NW (2002) Functional analysis of bone morphogenetic protein type II receptor mutations underlying primary pulmonary hypertension. Hum Mol Genet 11:1517–1525
256. Rudini N, Felici A, Giampietro C, Lampugnani M, Corada M, Swirsding K, Garre M, Liebner S, Letarte M, ten Dijke P, Dejana E (2008) VE-cadherin is a critical endothelial regulator of TGF-beta signalling. EMBO J 27:993–1004
257. Ruhrberg C, Gerhardt H, Golding M, Watson R, Ioannidou S, Fujisawa H, Betsholtz C, Shima DT (2002) Spatially restricted patterning cues provided by heparin-binding VEGF-A control blood vessel branching morphogenesis. Genes Dev 16:2684–2698
258. Sabba C, Pasculli G, Lenato GM, Suppressa P, Lastella P, Memeo M, Dicuonzo F, Guant G (2007) Hereditary hemorrhagic telangiectasia: clinical features in ENG and ALK1 mutation carriers. J Thromb Haemost 5:1149–1157
259. Saeed O, Otsuka F, Polavarapu R, Karmali V, Weiss D, Davis T, Rostad B, Pachura K, Adams L, Elliott J, Taylor WR, Narula J, Kolodgie F, Virmani R, Hong CC, Finn AV (2012) Pharmacological suppression of hepcidin increases macrophage cholesterol efflux and reduces foam cell formation and atherosclerosis. Arterioscler Thromb Vasc Biol 32:299–307
260. Sanchez-Duffhues G, de Vinuesa AG, Lindeman JH, Mulder-Stapel A, DeRuiter MC, Van Munsteren C, Goumans MJ, Hierck BP, Ten Dijke P (2015) SLUG is expressed in endothelial cells lacking primary cilia to promote cellular calcification. Arterioscler Thromb Vasc Biol 35:616–627
261. Sanchez-Duffhues G, Orlova V, Ten Dijke P (2016) In Brief: Endothelial-to-mesenchymal transition. J Pathol 238:378–380

262. Sapkota G, Alarcon C, Spagnoli FM, Brivanlou AH, Massague J (2007) Balancing BMP signaling through integrated inputs into the Smad1 linker. Mol Cell 25:441–454
263. Saremba S, Nickel J, Seher A, Kotzsch A, Sebald W, Mueller TD (2008) Type I receptor binding of bone morphogenetic protein 6 is dependent on N-glycosylation of the ligand. FEBS J 275:172–183
264. Sayed MG, Ahmed AF, Ringold JR, Anderson ME, Bair JL, Mitros FA, Lynch HT, Tinley ST, Petersen GM, Giardiello FM, Vogelstein B, Howe JR (2002) Germline SMAD4 or BMPR1A mutations and phenotype of juvenile polyposis. Ann Surg Oncol 9:901–906
265. Scharpfenecker M, van Dinther M, Liu Z, van Bezooijen RL, Zhao Q, Pukac L, Lowik CW, ten Dijke P (2007) BMP-9 signals via ALK1 and inhibits bFGF-induced endothelial cell proliferation and VEGF-stimulated angiogenesis. J Cell Sci 120:964–972
266. Schwappacher R, Weiske J, Heining E, Ezerski V, Marom B, Henis YI, Huber O, Knaus P (2009) Novel crosstalk to BMP signalling: cGMP-dependent kinase I modulates BMP receptor and Smad activity. EMBO J 28:1537–1550
267. Seki T, Hong KH, Oh SP (2006) Nonoverlapping expression patterns of ALK1 and ALK5 reveal distinct roles of each receptor in vascular development. Lab Invest 86:116–129
268. Seki T, Yun J, Oh SP (2003) Arterial endothelium-specific activin receptor-like kinase 1 expression suggests its role in arterialization and vascular remodeling. Circ Res 93:682–689
269. Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, Schuh AC (1995) Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature 376:62–66
270. She X, Matsuno F, Harada N, Tsai H, Seon BK (2004) Synergy between anti-endoglin (CD105) monoclonal antibodies and TGF-beta in suppression of growth of human endothelial cells. Int J Cancer 108:251–257
271. Shen Q, Little SC, Xu M, Haupt J, Ast C, Katagiri T, Mundlos S, Seemann P, Kaplan FS, Mullins MC, Shore EM (2009) The fibrodysplasia ossificans progressiva R206H ACVR1 mutation activates BMP-independent chondrogenesis and zebrafish embryo ventralization. J Clin Invest 119:3462–3472
272. Shimono K, W-e T, Macolino C, Chi AH-T, Didizian JJ, Mundy C, Chandraratna RA, Mishina Y, Iwamoto ME, Pacifici M, Iwamoto M (2011) Potent Inhibition of Heterotopic Ossification by Nuclear Retinoic Acid Receptor γ Agonists. Nat Med 17:454–460
273. Shore EM, Kaplan FS (2010) Inherited human diseases of heterotopic bone formation. Nat Rev Rheumatol 6:518–527
274. Shore EM, Xu M, Feldman GJ, Fenstermacher DA, Cho T-J, Choi IH, Connor JM, Delai P, Glaser DL, LeMerrer M, Morhart R, Rogers JG, Smith R, Triffitt JT, Urtizberea JA, Zasloff M, Brown MA, Kaplan FS (2006) A recurrent mutation in the BMP type I receptor ACVR1 causes inherited and sporadic fibrodysplasia ossificans progressiva. Nat Genet 38:525–527
275. Shovlin CL, Guttmacher AE, Buscarini E, Faughnan ME, Hyland RH, Westermann CJ, Kjeldsen AD, Plauchu H (2000) Diagnostic criteria for hereditary hemorrhagic telangiectasia (Rendu-Osler-Weber syndrome). Am J Med Genet 91:66–67
276. Shovlin CL, Hughes JM, Tuddenham EG, Temperley I, Perembelon YF, Scott J, Seidman CE, Seidman JG (1994) A gene for hereditary haemorrhagic telangiectasia maps to chromosome 9q3. Nat Genet 6:205–209
277. Shovlin CL, Letarte M (1999) Hereditary haemorrhagic telangiectasia and pulmonary arteriovenous malformations: issues in clinical management and review of pathogenic mechanisms. Thorax 54:714–729
278. Sieber C, Kopf J, Hiepen C, Knaus P (2009) Recent advances in BMP receptor signaling. Cytokine Growth Factor Rev 20:343–355
279. Siekmann AF, Lawson ND (2007) Notch signalling limits angiogenic cell behaviour in developing zebrafish arteries. Nature 445:781–784
280. Simoes Sato AY, Bub GL, Campos AH (2014) BMP-2 and −4 produced by vascular smooth muscle cells from atherosclerotic lesions induce monocyte chemotaxis through direct BMPRII activation. Atherosclerosis 235:45–55

281. Singla DK, Singla R, Wang J (2016) BMP-7 Treatment Increases M2 Macrophage Differentiation and Reduces Inflammation and Plaque Formation in Apo E−/− Mice. PLoS One 11:e0147897
282. Snyder LH, Doan, C. A. (1944) Clinical and experimental studies in human inheritance: is the homozygous form of multiple telangiectasia lethal? J Lab Clin Med 29:1211–1216
283. Somekawa S, Imagawa K, Hayashi H, Sakabe M, Ioka T, Sato GE, Inada K, Iwamoto T, Mori T, Uemura S, Nakagawa O, Saito Y (2012) Tmem100, an ALK1 receptor signaling-dependent gene essential for arterial endothelium differentiation and vascular morphogenesis. Proc Natl Acad Sci U S A 109:12064–12069
284. Sorescu GP, Song H, Tressel SL, Hwang J, Dikalov S, Smith DA, Boyd NL, Platt MO, Lassegue B, Griendling KK, Jo H (2004) Bone morphogenic protein 4 produced in endothelial cells by oscillatory shear stress induces monocyte adhesion by stimulating reactive oxygen species production from a nox1-based NADPH oxidase. Circ Res 95:773–779
285. Sorescu GP, Sykes M, Weiss D, Platt MO, Saha A, Hwang J, Boyd N, Boo YC, Vega JD, Taylor WR, Jo H (2003) Bone morphogenic protein 4 produced in endothelial cells by oscillatory shear stress stimulates an inflammatory response. J Biol Chem 278:31128–31135
286. Spiekerkoetter E, Sung YK, Sudheendra D, Bill M, Aldred MA, van de Veerdonk MC, Vonk Noordegraaf A, Long-Boyle J, Dash R, Yang PC, Lawrie A, Swift AJ, Rabinovitch M, Zamanian RT (2015) Low-Dose FK506 (Tacrolimus) in End-Stage Pulmonary Arterial Hypertension. Am J Respir Crit Care Med 192:254–257
287. Spiekerkoetter E, Tian X, Cai J, Hopper RK, Sudheendra D, Li CG, El-Bizri N, Sawada H, Haghighat R, Chan R, Haghighat L, de Jesus PV, Wang L, Reddy S, Zhao M, Bernstein D, Solow-Cordero DE, Beachy PA, Wandless TJ, Ten Dijke P, Rabinovitch M (2013) FK506 activates BMPR2, rescues endothelial dysfunction, and reverses pulmonary hypertension. J Clin Invest 123:3600–3613
288. Sridurongrit S, Larsson J, Schwartz R, Ruiz-Lozano P, Kaartinen V (2008) Signaling via the Tgf-beta type I receptor Alk5 in heart development. Dev Biol 322:208–218
289. Srinivasan S, Hanes MA, Dickens T, Porteous ME, Oh SP, Hale LP, Marchuk DA (2003) A mouse model for hereditary hemorrhagic telangiectasia (HHT) type 2. Hum Mol Genet 12:473–482
290. St-Jacques S, Cymerman U, Pece N, Letarte M (1994) Molecular characterization and in situ localization of murine endoglin reveal that it is a transforming growth factor-beta binding protein of endothelial and stromal cells. Endocrinology 134:2645–2657
291. St-Jacques S, Forte M, Lye SJ, Letarte M (1994) Localization of endoglin, a transforming growth factor-beta binding protein, and of CD44 and integrins in placenta during the first trimester of pregnancy. Biol Reprod 51:405–413
292. Suchting S, Freitas C, le Noble F, Benedito R, Breant C, Duarte A, Eichmann A (2007) The Notch ligand Delta-like 4 negatively regulates endothelial tip cell formation and vessel branching. Proc Natl Acad Sci U S A 104:3225–3230
293. Sugi Y, Yamamura H, Okagawa H, Markwald RR (2004) Bone morphogenetic protein-2 can mediate myocardial regulation of atrioventricular cushion mesenchymal cell formation in mice. Dev Biol 269:505–518
294. Sun L, Ye RD (2012) Role of G protein-coupled receptors in inflammation. Acta Pharmacol Sin 33:342–350
295. Suzuki Y, Montagne K, Nishihara A, Watabe T, Miyazono K (2008) BMPs promote proliferation and migration of endothelial cells via stimulation of VEGF-A/VEGFR2 and angiopoietin-1/Tie2 signalling. J Biochem 143:199–206
296. Suzuki Y, Ohga N, Morishita Y, Hida K, Miyazono K, Watabe T (2010) BMP-9 induces proliferation of multiple types of endothelial cells in vitro and in vivo. J Cell Sci 123:1684–1692
297. Tabas I, Garcia-Cardena G, Owens GK (2015) Recent insights into the cellular biology of atherosclerosis. J Cell Biol 209:13–22
298. Taylor KL, Henderson AM, Hughes CC (2002) Notch activation during endothelial cell network formation in vitro targets the basic HLH transcription factor HESR-1 and downregulates VEGFR-2/KDR expression. Microvasc Res 64:372–383

299. ten Dijke P, Yamashita H, Sampath TK, Reddi AH, Estevez M, Riddle DL, Ichijo H, Heldin CH, Miyazono K (1994) Identification of type I receptors for osteogenic protein-1 and bone morphogenetic protein-4. J Biol Chem 269:16985–16988
300. Thomson JR, Machado RD, Pauciulo MW, Morgan NV, Humbert M, Elliott GC, Ward K, Yacoub M, Mikhail G, Rogers P, Newman J, Wheeler L, Higenbottam T, Gibbs JS, Egan J, Crozier A, Peacock A, Allcock R, Corris P, Loyd JE, Trembath RC, Nichols WC (2000) Sporadic primary pulmonary hypertension is associated with germline mutations of the gene encoding BMPR-II, a receptor member of the TGF-beta family. J Med Genet 37: 741–745
301. Thorpe PE, Burrows FJ (1995) Antibody-directed targeting of the vasculature of solid tumors. Breast Cancer Res Treat 36:237–251
302. Tillet E, Bailly S (2014) Emerging roles of BMP9 and BMP10 in hereditary hemorrhagic telangiectasia. Front Genet 5:456
303. Torsney E, Charlton R, Diamond AG, Burn J, Soames JV, Arthur HM (2003) Mouse model for hereditary hemorrhagic telangiectasia has a generalized vascular abnormality. Circulation 107:1653–1657
304. Tual-Chalot S, Mahmoud M, Allinson KR, Redgrave RE, Zhai Z, Oh SP, Fruttiger M, Arthur HM (2014) Endothelial depletion of Acvrl1 in mice leads to arteriovenous malformations associated with reduced endoglin expression. PLoS One 9:e98646
305. Urness LD, Sorensen LK, Li DY (2000) Arteriovenous malformations in mice lacking activin receptor-like kinase-1. Nat Genet 26:328–331
306. Valdimarsdottir G, Goumans MJ, Rosendahl A, Brugman M, Itoh S, Lebrin F, Sideras P, ten Dijke P (2002) Stimulation of Id1 expression by bone morphogenetic protein is sufficient and necessary for bone morphogenetic protein-induced activation of endothelial cells. Circulation 106:2263–2270
307. Van der Heiden K, Hierck BP, Krams R, de Crom R, Cheng C, Baiker M, Pourquie MJ, Alkemade FE, DeRuiter MC, Gittenberger-de Groot AC, Poelmann RE (2008) Endothelial primary cilia in areas of disturbed flow are at the base of atherosclerosis. Atherosclerosis 196:542–550
308. van Laake LW, van den Driesche S, Post S, Feijen A, Jansen MA, Driessens MH, Mager JJ, Snijder RJ, Westermann CJ, Doevendans PA, van Echteld CJ, ten Dijke P, Arthur HM, Goumans MJ, Lebrin F, Mummery CL (2006) Endoglin has a crucial role in blood cell-mediated vascular repair. Circulation 114:2288–2297
309. van Meeteren LA, Thorikay M, Bergqvist S, Pardali E, Stampino CG, Hu-Lowe D, Goumans MJ, ten Dijke P (2012) Anti-human activin receptor-like kinase 1 (ALK1) antibody attenuates bone morphogenetic protein 9 (BMP9)-induced ALK1 signaling and interferes with endothelial cell sprouting. J Biol Chem 287:18551–18561
310. Villa N, Walker L, Lindsell CE, Gasson J, Iruela-Arispe ML, Weinmaster G (2001) Vascular expression of Notch pathway receptors and ligands is restricted to arterial vessels. Mech Dev 108:161–164
311. Wagner DO, Sieber C, Bhushan R, Borgermann JH, Graf D, Knaus P (2010) BMPs: from bone to body morphogenetic proteins. Sci Signal 3:mr1
312. Wakayama Y, Fukuhara S, Ando K, Matsuda M, Mochizuki N (2015) Cdc42 mediates Bmp-induced sprouting angiogenesis through Fmnl3-driven assembly of endothelial filopodia in zebrafish. Dev Cell 32:109–122
313. Wang HU, Chen ZF, Anderson DJ (1998) Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. Cell 93:741–753
314. Wang J, Sridurongrit S, Dudas M, Thomas P, Nagy A, Schneider MD, Epstein JA, Kaartinen V (2005) Atrioventricular cushion transformation is mediated by ALK2 in the developing mouse heart. Dev Biol 286:299–310
315. Wang JM, Kumar S, Pye D, van Agthoven AJ, Krupinski J, Hunter RD (1993) A monoclonal antibody detects heterogeneity in vascular endothelium of tumours and normal tissues. Int J Cancer 54:363–370

316. Wang JM, Kumar S, van Agthoven A, Kumar P, Pye D, Hunter RD (1995) Irradiation induces up-regulation of E9 protein (CD105) in human vascular endothelial cells. Int J Cancer 62:791–796
317. Wehner LE, Folz BJ, Argyriou L, Twelkemeyer S, Teske U, Geisthoff UW, Werner JA, Engel W, Nayernia K (2006) Mutation analysis in hereditary haemorrhagic telangiectasia in Germany reveals 11 novel ENG and 12 novel ACVRL1/ALK1 mutations. Clin Genet 69:239–245
318. Wentzel JJ, Chatzizisis YS, Gijsen FJ, Giannoglou GD, Feldman CL, Stone PH (2012) Endothelial shear stress in the evolution of coronary atherosclerotic plaque and vascular remodelling: current understanding and remaining questions. Cardiovasc Res 96:234–243
319. Wessel F, Winderlich M, Holm M, Frye M, Rivera-Galdos R, Vockel M, Linnepe R, Ipe U, Stadtmann A, Zarbock A, Nottebaum AF, Vestweber D (2014) Leukocyte extravasation and vascular permeability are each controlled in vivo by different tyrosine residues of VE-cadherin. Nat Immunol 15:223–230
320. West J, Austin E, Fessel JP, Loyd J, Hamid R (2014) Rescuing the BMPR2 signaling axis in pulmonary arterial hypertension. Drug Discov Today 19:1241–1245
321. West J, Cogan J, Geraci M, Robinson L, Newman J, Phillips JA, Lane K, Meyrick B, Loyd J (2008) Gene expression in BMPR2 mutation carriers with and without evidence of pulmonary arterial hypertension suggests pathways relevant to disease penetrance. BMC Med Genomics 1:45
322. West J, Fagan K, Steudel W, Fouty B, Lane K, Harral J, Hoedt-Miller M, Tada Y, Ozimek J, Tuder R, Rodman DM (2004) Pulmonary hypertension in transgenic mice expressing a dominant-negative BMPRII gene in smooth muscle. Circ Res 94:1109–1114
323. Whitehead KJ, Plummer NW, Adams JA, Marchuk DA, Li DY (2004) Ccm1 is required for arterial morphogenesis: implications for the etiology of human cavernous malformations. Development (Cambridge, England) 131:1437–1448
324. Whitman M (1998) Smads and early developmental signaling by the TGFbeta superfamily. Genes Dev 12:2445–2462
325. Wiley DM, Kim JD, Hao J, Hong CC, Bautch VL, Jin SW (2011) Distinct signalling pathways regulate sprouting angiogenesis from the dorsal aorta and the axial vein. Nat Cell Biol 13:686–692
326. Winnier G, Blessing M, Labosky PA, Hogan BL (1995) Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse. Genes Dev 9:2105–2116
327. Wooderchak-Donahue WL, McDonald J, O'Fallon B, Upton PD, Li W, Roman BL, Young S, Plant P, Fulop GT, Langa C, Morrell NW, Botella LM, Bernabeu C, Stevenson DA, Runo JR, Bayrak-Toydemir P (2013) BMP9 mutations cause a vascular-anomaly syndrome with phenotypic overlap with hereditary hemorrhagic telangiectasia. Am J Hum Genet 93:530–7
328. Woodford-Richens KL, Rowan AJ, Poulsom R, Bevan S, Salovaara R, Aaltonen LA, Houlston RS, Wright NA, Tomlinson IP (2001) Comprehensive analysis of SMAD4 mutations and protein expression in juvenile polyposis: evidence for a distinct genetic pathway and polyp morphology in SMAD4 mutation carriers. Am J Pathol 159:1293–1300
329. Wosczyna MN, Biswas AA, Cogswell CA, Goldhamer DJ (2012) Multipotent progenitors resident in the skeletal muscle interstitium exhibit robust BMP-dependent osteogenic activity and mediate heterotopic ossification. J Bone Miner Res 27:1004–1017
330. Wrana JL, Attisano L, Wieser R, Ventura F, Massague J (1994) Mechanism of activation of the TGF-beta receptor. Nature 370:341–347
331. Xu W, Kaneko FT, Zheng S, Comhair SA, Janocha AJ, Goggans T, Thunnissen FB, Farver C, Hazen SL, Jennings C, Dweik RA, Arroliga AC, Erzurum SC (2004) Increased arginase II and decreased NO synthesis in endothelial cells of patients with pulmonary arterial hypertension. FASEB J 18:1746–1748
332. Yamashita H, Ichijo H, Grimsby S, Moren A, ten Dijke P, Miyazono K (1994) Endoglin forms a heteromeric complex with the signaling receptors for transforming growth factor-beta. J Biol Chem 269:1995–2001

333. Yamashita H, Shimizu A, Kato M, Nishitoh H, Ichijo H, Hanyu A, Morita I, Kimura M, Makishima F, Miyazono K (1997) Growth/differentiation factor-5 induces angiogenesis in vivo. Exp Cell Res 235:218–226
334. Yang J, Li X, Al-Lamki RS, Southwood M, Zhao J, Lever AM, Grimminger F, Schermuly RT, Morrell NW (2010) Smad-dependent and smad-independent induction of id1 by prostacyclin analogues inhibits proliferation of pulmonary artery smooth muscle cells in vitro and in vivo. Circ Res 107:252–262
335. Yang J, Li X, Al-Lamki RS, Wu C, Weiss A, Berk J, Schermuly RT, Morrell NW (2013) Sildenafil potentiates bone morphogenetic protein signaling in pulmonary arterial smooth muscle cells and in experimental pulmonary hypertension. Arterioscler Thromb Vasc Biol 33:34–42
336. Yang J, Mani SA, Donaher JL, Ramaswamy S, Itzykson RA, Come C, Savagner P, Gitelman I, Richardson A, Weinberg RA (2004) Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. Cell 117:927–939
337. Yao Y, Bennett BJ, Wang X, Rosenfeld ME, Giachelli C, Lusis AJ, Bostrom KI (2010) Inhibition of bone morphogenetic proteins protects against atherosclerosis and vascular calcification. Circ Res 107:485–494
338. Yoo SY, Kwon SM (2013) Angiogenesis and its therapeutic opportunities. Mediators Inflamm 2013:127170
339. Yoshimatsu Y, Watabe T (2011) Roles of TGF-β signals in endothelial-mesenchymal transition during cardiac fibrosis. Int J Inflam 2011:724080
340. You LR, Lin FJ, Lee CT, DeMayo FJ, Tsai MJ, Tsai SY (2005) Suppression of Notch signalling by the COUP-TFII transcription factor regulates vein identity. Nature 435:98–104
341. Yung LM, Sanchez-Duffhues G, Ten Dijke P, Yu PB (2015) Bone morphogenetic protein 6 and oxidized low-density lipoprotein synergistically recruit osteogenic differentiation in endothelial cells. Cardiovasc Res 108:278–287
342. Zbuk KM, Eng C (2007) Hamartomatous polyposis syndromes. Nat Clin Pract Gastroenterol Hepatol 4:492–502
343. Zeisberg EM, Tarnavski O, Zeisberg M, Dorfman AL, McMullen JR, Gustafsson E, Chandraker A, Yuan X, Pu WT, Roberts AB, Neilson EG, Sayegh MH, Izumo S, Kalluri R (2007) Endothelial-to-mesenchymal transition contributes to cardiac fibrosis. Nat Med 13:952–961
344. Zhang H, Bradley A (1996) Mice deficient for BMP2 are nonviable and have defects in amnion/chorion and cardiac development. Development 122:2977–2986
345. Zhang H, Shaw AR, Mak A, Letarte M (1996) Endoglin is a component of the transforming growth factor (TGF)-beta receptor complex of human pre-B leukemic cells. J Immunol 156:564–573
346. Zhou G, Hamik A, Nayak L, Tian H, Shi H, Lu Y, Sharma N, Liao X, Hale A, Boerboom L, Feaver RE, Gao H, Desai A, Schmaier A, Gerson SL, Wang Y, Atkins GB, Blackman BR, Simon DI, Jain MK (2012) Endothelial Kruppel-like factor 4 protects against atherothrombosis in mice. J Clin Invest 122:4727–4731
347. Zhou J, Lee PL, Lee CI, Wei SY, Lim SH, Lin TE, Chien S, Chiu JJ (2013) BMP receptor-integrin interaction mediates responses of vascular endothelial Smad1/5 and proliferation to disturbed flow. J Thromb Haemost 11:741–755
348. Zhou Q, Heinke J, Vargas A, Winnik S, Krauss T, Bode C, Patterson C, Moser M (2007) ERK signaling is a central regulator for BMP-4 dependent capillary sprouting. Cardiovasc Res 76:390–399

Bone Morphogenetic Proteins in the Initiation and Progression of Breast Cancer

Jiang Ren and Peter ten Dijke

Abstract Due to their vast roles in human development, differentiation, homeostasis, and disease, bone morphogenetic proteins (BMP) have evolved along with numerous potentiating and inhibitory mechanisms to fine-tune signaling outcomes. As such, this chapter focuses on some of the best-studied and utilized extracellular mechanisms of BMP signal regulation. Due to their inherent binding characteristics, BMP ligands are often found engaged with at least of one of these many interacting partners. From a structural and functional perspective, we discuss our current understanding of how BMP ligands interact with these numerous binding partners, including secreted extracellular antagonists, BMP prodomains, and various co-receptors and noncanonical binding partners. Interestingly, while the BMP ligands themselves exhibit very redundant structural features, the composition and structure of their interacting proteins is quite diverse, lending to different ligand-binding modes and mechanisms, which lead to very different biological outcomes. Collectively, biochemical and structural characterization of these important interactions has provided valuable insight into BMP signal regulation.

Keywords BMP • Breast cancer • Metastasis • Proliferation • DAN family • Follistatin • Chordin • Noggin • Antagonism

J. Ren
Department of Molecular Cell Biology and Cancer Genomics Centre Netherlands, Leiden University Medical Center, Leiden, The Netherlands

P. ten Dijke (✉)
Department of Molecular Cell Biology and Cancer Genomics Centre Netherlands, Leiden University Medical Center, Leiden, The Netherlands

Ludwig Institute for Cancer Research, Science for Life Laboratory, Uppsala University, Box 595, 75124 Uppsala, Sweden
e-mail: p.ten_dijke@lumc.nl

S. Vukicevic, K.T. Sampath (eds.), *Bone Morphogenetic Proteins: Systems Biology Regulators*, Progress in Inflammation Research,
DOI 10.1007/978-3-319-47507-3_18

1 Introduction

Bone morphogenetic proteins (BMPs) were originally identified as osteogenic factors with the ability to induce cartilage and bone formation at ectopic sites [1]. Accumulating evidence thereafter showed that BMPs (of which about 20 members have been identified in mammals) can perform versatile functions in embryonic development and in maintenance of adult tissue homeostasis. BMPs were found to regulate proliferation, survival, migration, differentiation, and lineage commitment of many different cell types [2, 3]. Perturbation in BMP signal transduction processes may lead to disease states, including tumorigenesis [3]. BMPs belong to the transforming growth factor β (TGFβ) superfamily, which are dimeric ligands that signal via specific transmembrane type I and type II serine/threonine kinase receptors and intracellular SMAD transduction factors. Each step of the BMP signaling pathway is carefully regulated, e.g., through ligand-binding proteins that sequester ligand from binding to receptors and coreceptors that present ligand to these receptors [4]. Recent years have seen an increasing interest in the role of BMP signaling in the development and progression of several cancers [5]. Similar as found for TGFβ, BMPs may act as tumor suppressor and/or promoter in a highly contextual manner [5].

BMPs play an important role in the development of embryonic mammary gland [6]. Of interest is also that breast cancer is frequently accompanied by osteolytic metastasis, which accounts for significant morbidity [7]. BMPs are present with high abundance in bone and have the ability to stimulate bone formation [8]. In this review, we aim to overview the recent studies on the relationship between BMPs and breast cancer pathology. After a brief introduction to the key components of BMP signaling pathways and their regulation, we discuss the aberrant expression of canonical BMP/SMAD signaling components and the underlying prognostic value in breast cancer. We then focus on the functions of BMPs in breast cancer initiation, proliferation, apoptosis, tumor microenvironment, as well as the processes of metastasis. The possibilities utilizing these controlling mechanisms of BMPs for therapeutic intervention against breast cancer are also discussed.

2 BMP Signaling and Its Regulation

BMPs are produced as larger dimeric precursor proteins, which are proteolytically processed thereby generating a carboxy-terminal bioactive domain with highly conserved cysteine residues. This mature dimer may undergo further posttranslational modification such as glycosylation [4, 9]. The BMP signaling cascade is initiated by binding of BMPs to two types of transmembrane serine/threonine kinase receptors, i.e., BMP type I and type II receptors (BMPRIs and BMPRIIs, respectively) [10]. Generally, initial binding occurs to BMPRIs, i.e., activin receptor-like kinase (ALK)1, ALK2 (or ACVR1A), ALK3 (or BMPRIA), and ALK6 (or BMPRIB), to which BMPs interact with higher affinity as compared to BMPRIIs. Thereafter,

BMPs recruit BMPRII, which is specific for BMPs, or activin type II A receptor (ACVR2A) and activin type IIB receptor (ACVR2B), which are shared type II receptors with the activins (Table 1) [4].

As described in Fig. 1, upon BMP-induced formation of a heteromeric receptor complex, the constitutively active BMPRIIs kinase can phosphorylate BMPRI in the highly conserved glycine-serine-rich (GS) juxtamembrane domain. Then, the activated BMP type I receptor in turn can incur intracellular signaling by phosphorylating specific SMADs (R-SMADs), SMAD1/5/8 [9]. These BMP R-SMADs are distinct from TGFβ and activin receptor-induced R-SMADs, i.e., SMAD2 and SMAD3. Phosphorylated R-SMADs form heteromeric complexes with common-partner SMAD (Co-SMAD), i.e., SMAD4 [11]. Subsequently, these SMAD complexes can translocate into the nucleus where they serve as transcription factors and recognize specific BMP response elements (BRE) (also termed SMAD-binding elements (SBE)) located within the promoters or enhancers of target genes. In collaboration with other transcription factors and transcriptional coactivators/corepressors, they mediate the transcription of BMP target genes, such as inhibitor of differentiation (ID) 1–3, inhibitory SMAD6, and runt-related transcription factor 2 (RUNX2) [12–14]. Besides the canonical SMAD-dependent pathway, BMPs have also been reported to activate non-SMAD pathways, including stress-activated protein kinase/c-Jun NH2-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38 mitogen-activated protein kinase (MAPK) pathways, as well as phosphoinositide 3-kinase (PI3K)-AKT, protein kinase C (PKC), TGFβ-activated kinase 1 (TAK1), and small Rho-GTPases pathways [9, 15].

The BMP signaling cascade is subject to intricate regulation at multiple levels. Extracellular antagonists prevent binding of BMPs to receptors either by sequestering the BMP ligands or by binding to the BMP receptors themselves [2]. Like the BMP ligands, the BMP antagonists have a cysteine knot structure, which can be divided into several subclasses: twisted gastrulation, Noggin and Chordin family, and differential screening-selected gene aberrative in neuroblastoma (DAN) family (including DAN, Cerberus, Gremlin 1, protein related to Dan or Cerberus (PRDC), Sclerostin, uterine sensitization-associated gene 1 (USAG1), Caronte, and Coco) [9, 16]. Another type of inhibitors involves soluble receptors in the extracellular environment, which also can sequester BMPs from binding to their transmembrane receptors [17]. Regulation at the cell membrane level is mediated by various membrane proteins. The BMP and activin membrane-bound inhibitor (BAMBI) inhibit BMP signaling by interfering with receptor complex formation [18]. In addition, BMP signaling can be potentiated by some membrane proteins, such as members of

Table 1 BMP subclasses and receptor-binding preference

Ligands	Type I receptors	Type II receptors
BMP2/4	ALK3, 6	BMPRII, ACVR2A, ACVR2B
BMP5/6/7/8	ALK2, 3, 6	BMPRII, ACVR2A, ACVR2B
GDF5/6/7	ALK3, 6	BMPRII, ACVR2A, ACVR2B
BMP9	ALK1, 2	BMPRII, ACVR2A

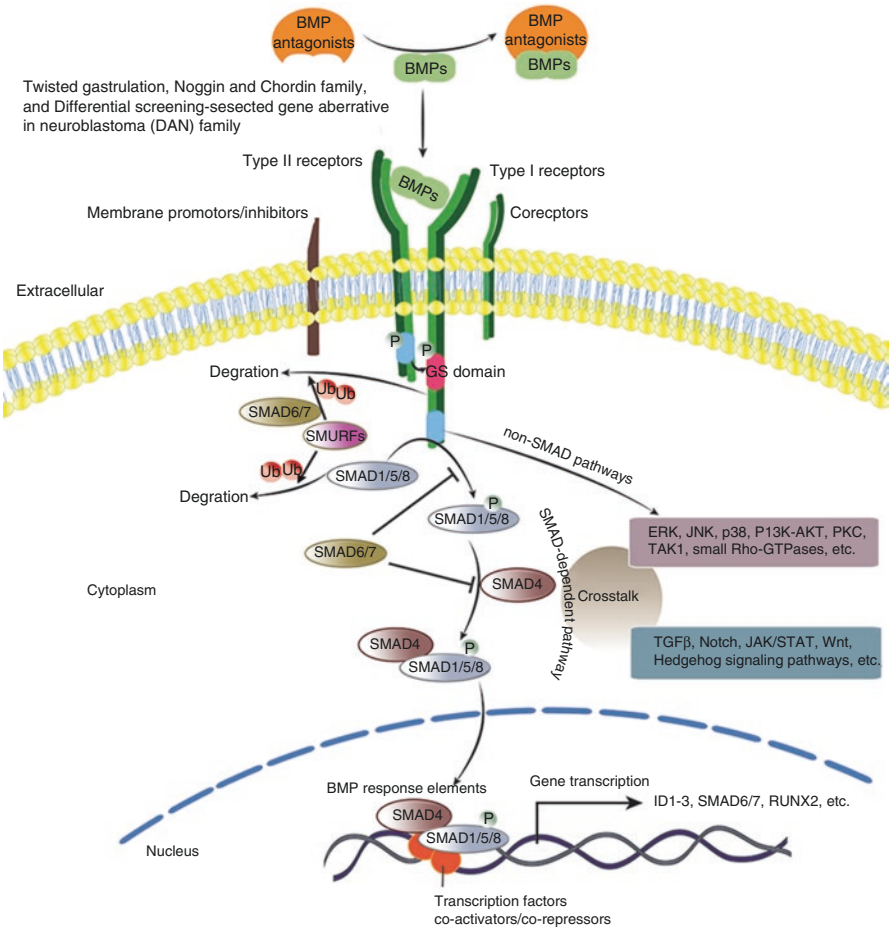


Fig. 1 Schematic presentation of the BMP signaling pathway. BMP binds and induces heterotetrameric complex formation of specific single transmembrane-spanning BMP type I and type II receptors. Upon heteromeric complex formation, the extracellular BMP signal is transduced across the membrane by the phosphorylation of BMP type I receptors in the glycine-serine-rich (GS) juxtamembrane domain by the constitutively active type II receptors kinase. The intracellular signal is initiated by the phosphorylation of SMAD1/5/8. These activated R-SMADs can then form heteromeric complexes with SMAD4, which translocate into the nucleus where they collaborate with other DNA-binding transcription factors and transcriptional coactivators/corepressors to regulate the transcription of BMP target genes (SMAD-dependent pathway). The BMP signal can also be transduced via non-SMAD pathways. BMP signaling is subject to multiple regulations, such as extracellular antagonists, coreceptors, membrane promoters/inhibitors, and inhibitory SMAD6/7. There also exists extensive cross talk between BMP signaling pathways and other signaling pathways

the repulsive guidance molecule (RGM) family [19], and coreceptors betaglycan [20] and endoglin (CD105) [21, 22].

Within the cell, Endofin acts as an anchor between SMAD1 and activated BMPRIs to facilitate SMAD1 phosphorylation. Meanwhile, Endofin can mediate the dephosphorylation and inactivation of BMPRIs by its motif for protein phosphatase binding [23]. FK506 binding protein 12 (FKBP12) can bind to the GS domain of BMPRIs, thereby shielding the serine and threonine residues from being phosphorylated by BMPRIIs and stabilizing the inactive conformation [24, 25]. The drug FK506 (tacrolimus) that binds FKBP12 was shown to relieve this inhibition and to potentiate BMP signaling [24, 25]. BMP signaling is also restricted intracellularly by the inhibitory SMADs (I-SMADs), i.e., SMAD6 and SMAD7, which compete with SMAD1/5/8 for interaction with BMPRIs and with SMAD4 for complex formation with SMAD1 [26, 27]. Both SMAD1 and SMAD5 can be targeted for proteasomal degradation via addition of ubiquitin chains by SMAD ubiquitin regulatory factors (SMURFs). Additionally, by interacting with I-SMADs that can be recruited to activated BMPRI, SMURFs are also capable of decreasing the stability of BMPRI [28].

Importantly, many of the (negative) regulators of BMP signaling themselves are BMP target genes, creating auto-feedback loops that ensure increased fine-tuning of signaling [2, 28, 29]. Additional facets of BMP signaling include cross talk with other signaling pathways, such as TGFβ, Notch, Janus kinase/signal transducers and activators of transcription (JAK/STAT), Wnt, and Hedgehog, which further broaden the cellular responses to BMP signaling [30]. Thus, the actual outcome of BMP signaling results from levels and activities of all those cellular context-dependent components mentioned above, explaining the diversity of observed effects.

3 Aberrant Expression of BMP Signaling Components in Breast Cancer

In the normal breast, all the necessary components of the canonical BMP signaling pathway (i.e., BMP ligands, BMP receptors, and SMADs) are expressed [31]. Aberrant expression of these components has been observed for breast cancer cell lines with different characteristics and/or has been detected in breast cancer cell lines compared to normal cell lines, in primary tumor tissues compared to normal tissues, and in recurrent tumor tissues compared to primary tumor tissues, however, often with inconsistent and frequent contradictory results. In part, this may be caused by cell lines that were cultured under different conditions and tumors that were not characterized and, for example, not subdivided based upon their genetic alterations and stroma content.

In the forthcoming section, we have listed some examples. Significant lower levels of BMP2 transcript and protein were detected in both noninvasive and invasive

breast cancer cell lines and/or cancer cells in breast cancer tissues [31–33]. There were no significant differences in the percentage of BMP2-positive tumors found with respect to cancer cell subtype [31] and grades [33]. What is intriguing, BMP2 protein levels were found to be increased significantly in luminal tumor tissues compared to normal tissues [31]. Immunohistochemical (IHC) staining revealed that BMP2 protein was mainly produced by endothelial cells, fibroblasts, and other stromal cells in luminal tumor microenvironment, not by tumor cells themselves [31]. BMP2 is also highly enriched in bone marrow microenvironment during the process of breast cancer bone metastases [34]. These results indicate that breast tumor cells are the target of BMP2, rather than the source of overexpression.

BMP4 is expressed with wide variation in levels among breast cancer cell lines and/or primary cancer tissues [32, 33, 35–39]. While low levels of BMP4 protein were observed only in normal mammary gland tissue, it was strongly stained in 25 % of patients and more frequent in lobular carcinoma compared to the ductal carcinoma, suggesting that strong expression is cancer specific [39]. Breast cancer patients with strong BMP4 staining suffered from increased frequency of local and distant tumor recurrence [39]. Another study showed that a four-marker panel with low methylation in breast cancer (paired-like homeodomain 2 (PITX2), BMP4, fibroblast growth factor (FGF) 4, and family with sequence similarity 110, member A (FAM110A)) is associated with a longer duration to distant metastasis [36]. However, opposite results were reported in a study by Kretschmer and coworkers indicating that BMP4 mRNA and protein are clearly reduced in ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC) compared to nonmalignant human and murine mammary tissues [40]. A negative correlation between BMP4 mRNA level and tumor grade was reported by Ketolainen et al. [37]. Accordingly, lower BMP4 mRNA expression correlated with poor disease-free survival in breast cancer patients [41].

BMP6 mRNA and/or protein expression was consistently found to be significantly downregulated in breast cancer cell lines or primary cancer tissues [33, 42–46]. Downregulation of BMP6 mRNA correlated with the increase in breast tumor histologic grade [46]. Interestingly, compared to estrogen receptor-positive (ER^+) breast cancers, BMP6 mRNA level is significantly higher in estrogen receptor-negative (ER^-) breast cancers [43, 45, 46].

BMP7 has been described as being amplified at the gene levels [47, 48] and overexpressed at the mRNA and/or protein levels [33, 47, 49–51] frequently in breast cancer cell lines and/or tissues. BMP7 protein expression was also found to be tumor subtype dependent; 57 % of the lobular carcinomas but only 37 % of the ductal carcinomas are BMP7 positive [50]. Increased *BMP7* DNA copy number was reported to show significant correlation with a high Ki67 proliferation index and high histological tumor grade [47]. In addition, BMP7 overexpression was regarded as an independent prognostic marker for early bone metastasis development by multivariate analysis, especially in ductal carcinomas [50]. But contradicting results for BMP7 expression in breast cancer to those just mentioned have also been reported. For example, extreme low levels of BMP7 mRNA were detected in aggressive cells [52, 53]. Moreover, BMP7 mRNA levels in primary breast cancers involving bone

metastases were found lower when compared with those involving visceral (lung and liver) metastases [52]. In addition, lower BMP7 levels in patients show a moderate and poor clinical outcome [33].

Relatively few studies have appeared on the expression of other BMP ligands in breast cancer. No difference in BMP3 mRNA levels between breast tumors and normal tissues was detected, but lower BMP3 transcript levels correlated with a poorer prognosis [33]. Lower BMP5 mRNA levels were observed in breast tumors compared to normal breast tissues [54] and correlated with cancer recurrence, particularly in patients with ERα-negative cancers [54]. In contrast, another study showed that patients with higher levels of BMP5 transcript were associated with moderate and poor prognosis [33]. Moreover, decreased expression of BMP9 [55], BMP10 [56], growth and differentiation factor (GDF) 9a [57], GDF-9b/BMP15 [57], and BMP12 [58] along with poor prognosis was observed in breast cancer compared with matched normal tissues.

Investigations into the expression profiles of BMP receptors and downstream SMAD signaling components have been conducted rather infrequently for breast cancer. BMPRIs, BMPRIIs, and SMAD4 and inhibitory SMAD6 and 7 were found expressed rather uniformly in breast cancer cells or tissues [35, 38, 59]. DNA homozygous deletion and mRNA downregulation of BMP receptors are rare in breast cancer according to the provisional breast in The Cancer Genome Atlas (TCGA, Provisional) database [60]. BMPRIA [31, 35, 61] and BMPRIB [31, 35, 62] expressions were found overall increased in tumors compared to normal breast tissues. BMPRIB and BMPRII expression is significantly increased in highly metastatic breast cancer cells [51]. Tissue microarrays demonstrated that high expression of BMPRIA [48, 63] and BMPRII [48] correlated with poor relapse-free survival (RFS) or survival. Strong expression of BMPRIB is associated with high proliferation, cytogenetic instability, high grade, and poor prognosis in ER^+ breast cancer [62]. However, the results from Bokobza et al. [64] showed that a decreased level of BMPRIB in breast cancer is associated with poor prognosis.

Only a small portion of breast cancer cell lines and clinical samples were identified as homozygous deletion and reduced mRNA and/or protein expression of SMAD4 [48, 65]. But SMAD4 mutations, which are usually found in pancreatic [66] and colorectal [67] cancer, are rare in breast cancer [65]. Secreted BMP antagonists, such as Gremlin 1 [40, 48, 68, 69], Noggin [31, 48], and Chordin [48], are amplified and/or expressed at higher levels in breast cancer tissues compared to nonmalignant tissues. Of which, Gremlin 1 expression was below detection in breast cancer cells [70] but frequently found expressed in stromal cells within the microenvironment of human breast cancers [68]. In addition, a study conducted by Tarragona et al. indicated that higher levels of Noggin were found in breast cancer bone metastatic tissues compared to lung, brain, and liver metastatic tissues [71].

Taken together, the results of the studies above on the expression of BMP signaling components suggest a highly context-dependent and multifunctional role of BMPs in breast cancer.

4 Status of BMP/SMAD Signaling in Breast Cancer

Even though the expression frequencies and levels of BMPs and other BMP signaling components varied considerably among different studies, human breast cancers and their metastases retain BMP/SMAD signaling [48, 61, 72], as well as several mouse models of breast cancer [61].

Strong phospho-SMAD1/5/8 staining, indicative for active BMP receptor signaling, was demonstrated in human breast cancer tissues [48, 61, 72] and not confined to specific cancer cell types within the tumor tissue [48, 61]. This is consistent with the already mentioned finding that the core BMP canonical signaling components were found to be expressed in breast cancer cells. Metastatic breast cancer to the brain, bone, liver, lymph node, and lung was also found to be positive for phospho-SMAD1/5/8 [48, 72]. Lymph node metastasis tissues were demonstrated to be weaker in phospho-SMAD1/5/8 levels than bone metastasis tissues [72]. Moreover, BMP/SMAD signaling is specifically absent in the stroma of human ductal and lobular carcinoma in situ (DCIS and LCIS). Yet after progression to invasion, breast cancers of many distinct subtypes contained a stroma active for BMP signaling [73].

5 Regulation of the Expression of BMP Signaling Components by Other Factors in Breast Cancer

The expression of BMPs and other pathway components has been shown to be regulated by several other factors, such as estrogen [43, 45, 46, 49], epidermal growth factor (EGF) [49], and p53 [74]. Estrogen represents the primary stimulant in the development and progression of breast cancers. ER status is a determinant for selecting endocrine therapies to block estrogen signaling [75]. A possible relationship between BMP signaling and ER is therefore an interesting area of research. Estrogen has been shown to alter BMP signaling by downregulating specific BMPs and their receptors in ER^+ MCF-7 cells, including BMP7, BMPRIA, BMPRIB, ACVR2A, and ACVR2B, but no effect was detected on ACVR1 and BMPRII [59, 76]. In addition, estrogen can suppress BMP2-induced activation of the SMAD pathway and BMP-mediated gene expression [77]. This effect probably depends on the direct physical interaction of SMAD4 with ERα/ERβ [78]. The antiestrogen modulator raloxifene can increase the promoter activity of BMP4 in U2OS osteoblast-like cells in the presence of ERα [79]. In contrast, promoter hypermethylation was found to lead to BMP6 downregulation in ER^- breast cancer tissues, while lower methylation frequency was detected in ER^+ cases [43, 45, 46, 80]. Moreover, BMP6 gene expression can be upregulated by estrogen-mediated demethylation of the BMP6 promoter in ER^+ MCF-7 cells in a dose-dependent manner [81].

Apart from upregulation of BMP2 and BMP6, a derivative of vitamin D can reduce inhibitory SMAD6 expression and enhance SMAD1/5 phosphorylation [82, 83]. EGF treatment can also lead to elevated levels of BMP6 mRNA in a dose-dependent

manner [42]. FGF8 was found to inhibit BMP receptor-mediated SMAD1/5/8 phosphorylation and mitigate BMP target gene ID1 promoter activity by suppressing BMPRII expression and by increasing I-SMAD expression [84]. Parathyroid hormone-related protein (PTHrP) can function as the upstream regulator of BMP6 through the protein kinase A (PKA) pathway and exert its anti-mitogenic effect through downregulating BMP6 mRNA expression [85]. Furthermore, BMP7 is a target gene of the p53 family [61, 74] and LIM domain only protein 4 (LMO-4) [86], which activate BMP signaling by inducing the expression of BMP7 in breast cancer.

In short, many different signaling pathways regulate BMP signaling; these findings explain in part the contextual functions of BMPs.

6 BMP Signaling in Stem Cell Self-Renewal and Initiation of Breast Cancer

In human breast cancer, a subpopulation of cancer cells with an $ALDH^{high}/CD44^{high}/CD24^{low}$ phenotype is highly enriched for cancer stem cells (CSCs), also termed tumor-initiating cells (TICs), which are capable of initiating and sustaining tumorigenesis [87]. CSCs may be generated from the adult somatic stem cell by disturbing the processes of normal self-renewal or from more differentiated cells through certain processes to reacquire stem cell-like characteristics, such as epithelial to mesenchymal transition (EMT) [87, 88]. BMPs are indispensable for tissue homeostasis in adults, regulating somatic stem cells and controlling differentiation. Aberrant regulation of the BMP signaling pathway could therefore be a target in early phases of tumorigenesis [5].

The evidence points activation of BMP signaling as an early event during primary breast cancer initiation from malignant transformation [31, 48, 61]. Clinically defined samples demonstrate increased BMP signaling in premalignant luminal epithelial cells within the area of DCIS lesions [61]. BMP signaling is also hyperactivated in both epithelium and surrounding stroma in the premalignant mammary gland of transgenic mice model with mouse mammary tumor virus (MMTV)-derived oncogene expression [48, 61]. Chapellier et al. [31] showed that stimulation with BMP2 rapidly induced sustained upregulation of a well-known luminal differentiation regulator, GATA3, and progressive switch of the forkhead box (FOX) A1/FOXC1 balance in favor of FOXA1 through BMPRIB-dependent signaling, thereby leading to differentiation of normal mammary epithelial cell to luminal and expansion of luminal immature progenitors. In addition, abnormal high levels of BMP2 are produced in the mammary microenvironment upon exposure to common carcinogens. Chronic exposure of MCF10A breast epithelial cells to high levels of BMP2 thus initiates transformation of luminal immature progenitor cells toward a luminal tumorlike phenotype in vitro [31].

The small-molecule BMPRIs kinase inhibitor Dorsomorphin and its more selective analogs LDN193189 and DMH1 provide the chance to evaluate the effects of BMP type I receptor signaling on tumorigenesis. In vitro analysis revealed that sup-

pressing BMP signaling in premalignant murine mammary cells or immortalized mammary epithelial cells (IMECs) repressed mammosphere formation [89] and clonogenic capacity and diminishes the CSC-enriched ALDH1^{high} population [61]. Accordingly, the expression of stem markers, spinocerebellar ataxia type 1(SCA1) and NOTCH1, are markedly reduced [89]. Consistently, BMP4 stimulation increased the number and size of primary mammospheres [89]. Thus, BMP signaling is essential for maintenance of CSCs in breast cancer. Importantly, the BMP receptor kinase inhibitor blocks the ability of ALDH1^{high} fraction to resubstitute the mixed ALDH1^{high}/ALDH1^{low} parental culture, implicating that BMP signaling may control the aspects of cellular plasticity within tumor hierarchies [61]. Furthermore, LDN193189 restricts the tumorigenic capacity of allografts and increases tumor latency in vivo [61]. Therefore, these data implicate that BMP signaling is central to regulating mammary epithelial cell stemness, plasticity, and potentially supports maintenance and progression of tumorigenesis.

Interestingly, BMPs also seem to pose a substantial barrier to tumor stemness, when it comes to aggressive and metastatic breast cancers, or rather metastasis-initiating cells. Besides reduced BMP7 expression, an aggressive clone from MCF-7 cell line shows CD44 upregulation and CD24 downregulation, indicative of a CSC phenotype [90]. BMP4 inhibits mammosphere-forming and tumor-initiating ability in IMEC-transformed derivatives with high motility and high percentage of CD44^{high}/CD24^{low} subpopulation [91]. Multiple BMPs (BMP2, BMP7, BMP2/7) decrease the size of ALDH^{high}/CD44^{high}/CD24^{low} stem/progenitor subpopulation in MDA-MB-231 [92]. Elevated expression of BMP6 in MDA-MB-231 cells results in decreased tumorigenesis in vivo [93]. Furthermore, colonization of metastatic cancer cells in the target organs is thought of as another type of tumor initiation, while CSCs are commonly considered as the culprits [94]. High-metastatic cells expressing high levels of the BMP antagonist Noggin [71] or Coco [95] are associated with CSCs traits, with the ability to form more tumor spheres and a higher CD44^{high}/CD24^{low} population that display a higher capacity for metastatic colonization. Mechanistically, Coco induces CSC traits of metastatic cells by sustaining the expression of stem cell transcription factors, NANOG, SRY-related HMG-box (SOX) 2, octamer-binding transcription factor (OCT) 4, and transcriptional coactivator TAFAZZIN (TAZ). BMP4 suppresses their expression [95].

Taken together, with respect to CSCs development and tumorigenesis, it can be concluded that BMP signaling can act as promoter of premalignant mammary cells and as suppressor of aggressive mammary cancer cells.

7 Effects of BMPs on Breast Cancer Proliferation and Apoptosis

BMPs have been reported to regulate breast cancer cell growth with context pleiotropy. For the same BMP ligand, the responses can vary within different tumor types. For example, BMP7 was reported to promote cell proliferation of BT-474 and MDA-MB-231 breast cancer cells but to decrease cell proliferation of other breast

cancer cell lines (including MDA-MB-361, HCC1954, ZR-75-30, and T-47D) [53]. Even for the same BMP ligand and cell line, different conditions may cause a different response. BMP4 does not have any inhibitory effects on the proliferation of MDA-MB-231 cells in two-dimensional (2D) cell culture but inhibits proliferation in 3D [96]. BMP2 was found to inhibit the hormone-independent growth of MCF-7 in vitro [97–99], but the contrary was reported in vivo [100]. BMP4 and BMP7 have also been shown to promote anchorage-independent MCF-7 cell proliferation [51, 89].

In most of the studies, BMP2 [31, 97–103], BMP4 [31, 37, 96], BMP6 [46, 93, 104], BMP9 [105, 106], and BMP10 [56] were found to trigger cytostatic effects on multiple breast cancer cells. The underlying mechanism could be that BMP signaling has evident effects on the expression of mitotic checkpoint proteins. Chemical inhibition of BMP signaling by BMPRIs kinase inhibitor Dorsomorphin abrogates Nocodazole-mediated mitotic arrest [107]. Simultaneously, levels of mitotic checkpoint proteins, budding uninhibited by benzimidazoles 3 (BUB3), highly expressed protein in cancer (HEC1), monopolar spindle 1 (MPS1), and mitotic arrest deficient 2 (MAD2), which ensures proper chromosome segregation during mitosis, were dramatically downregulated. Overexpressing these proteins significantly recovers the defect in mitotic arrest caused by BMP inhibition [107]. Some of BMPs are demonstrated to delay cell cycle reentry in breast cancer cells. BMP2 [99, 102, 108, 109], BMP4 [37, 96], and BMP6 [46, 93, 104] induce G1 cell cycle arrest caused by increased expression of the cell cycle inhibitor p21 [96, 99, 102, 108, 109]. p21 promoter activity in turn inactivates cyclin D1 and cyclin E and results in retinoblastoma protein (pRb) hypophosphorylation [101]. The process of cell cycle arrest requires active BMPRIs, and the cytoplasmic signal transducers SMAD1/5 and SMAD4 are indispensable [102]. Upregulation of protein tyrosine phosphatases (PTPs), such as protein tyrosine phosphatase gamma (PTPRG), MAPK phosphatase (MKP), and phosphatase and tensin homolog (PTEN), may also contribute to increased levels of p21 in cells where BMP induced antiproliferative effects [110, 111]. In addition, BMP7 [84] and BMP9 [105] can lead to an accumulation of the G2/M phase in breast cancer cells.

BMPs can also influence the effect of other factors on breast cancer cell proliferation. BMP4 itself cannot significantly stimulate the proliferation but potently enhances the mitogenic activity of EGF, FGF, and hepatocyte growth factor (HGF) on murine mammary epithelial cells [112]. BMP2, in contrast to BMP4, prevents EGF-induced proliferation of MDA-MB-231 cells [108]. The estrogen-induced mitotic effects can be suppressed by BMP2 [59, 101], BMP4 [59], BMP6 [59], and BMP7 [59, 84], with the effects of BMP6 and BMP7 being more potent than those of BMP2 and BMP4 [59]. AB215, an activin A/BMP2 chimera, has increased BMP2-like signaling potency via the SMAD1/5/8 pathway and exerts stronger inhibitory effects on estradiol-induced proliferation in ER^+ breast cancer cells than BMP2 [113]. Estradiol rapidly activates MAPK phosphorylation including ERK1/2, p38, and JNK pathways [59, 84]. BMP6 and 7 can preferentially inhibit estradiol-induced p38 phosphorylation [59]. BMP6 is also believed to decrease the chemoresistance of MCF-7 breast cancer cells to doxorubicin through inactivation of ERK signaling and upregulation of P-glycoprotein (P-GP) [46]. Furthermore, BMP9 can

inhibit expression of HER2, phosphorylation of ERK1/2 (without effect on p38 and JNK), and PI3K/AKT in SK-BR-3 cells, thereby suppressing the growth of HER2-positive SK-BR-3 cells in vitro and in vivo [106].

Obviously, the distinct BMP receptors present also explain the diversity of effects of BMP signaling on breast cancer proliferation. BMPRIA was identified as a positive regulator of breast cancer at primary and secondary sites through activation of the SMAD pathway [72]. In contrast, another type I receptor, BMPRIB, plays a negative role in the proliferation of breast cancer cells. Downregulation of BMPRIB in MDA-MB-231 cells leads to promotion of cell growth in vitro [64]. Overexpression of a BMPRII-dominant negative (DN) mutant interferes with the phosphorylation of SMAD1, resulting in G1 phase cell cycle arrest of T-47D cells [109]. However, in the MMTV polyoma middle T antigen mice model of spontaneous mammary tumor formation, BMPRII-DN-expressing tumor cells have higher proliferation rates [114].

A few studies have pointed out pro-apoptotic roles for BMPs in breast cancer cells [86, 99, 105, 115]. BMP2 regulates the expression of apoptosis-related genes, especially protein kinase R (PKR) and activates its substrate α-subunit of eukaryotic initiation factor 2, thereby showing a pro-apoptotic effect in MCF-7 cells under normal culture conditions [115]. However, when these cells are deprived of serum, BMPs display a contrasting function by exerting an anti-apoptotic effect. BMP2 increases the resistance to hypoxia-induced apoptosis in MCF-7 cells via activation of the MAPK and ID1 pathways and suppression of caspase-3 [116, 117]. In parallel, BMP6, which can inhibit the proliferation of MDA-MB-231 cells, inhibits serum starvation-induced apoptosis through SMAD-dependent upregulation of Survivin and non-SMAD-dependent activation of p38 MAPK [104].

8 BMPs and the Tumor Microenvironment

Accumulating evidence indicates that the tumor microenvironment is a pathologically active niche that shapes tumor evolution. Hypoxia, low pH, immune evasion, chronic inflammation, and neovasculature can be considered as enabling characteristics [118]. Disruption of BMP signaling brings about alterations in the breast tumor microenvironment and accelerates tumor progression [41, 114, 119]. Deletion of BMPRII in mammary tumors [114] or in fibroblasts within the tumor stroma [119] can result in increased expression of chemokines, such as chemokine (C-C motif) ligand 5 and 9 (CCL5, 9), interferon gamma-induced protein 10 (IP-10), and granulocyte colony-stimulating factor (G-CSF), which facilitate inflammation by a sustained increase of myeloid cells infiltration, especially myeloid-derived suppressor cells (MDSCs) [114, 119]. Accordingly, the T-cell population is reduced due to a main function of MDSCs in the inhibition of T-cell proliferation [114]. As a classical stress response pathway, nuclear factor-κB (NF-κB) activation can be detected in a majority of cancers [120]. BMP4 has been shown to attenuate NF-κB activity in breast cancer [41]. Thereby lower levels of chemokines result from the attenuation of its known regulator NF-κB, leading to reduced numbers and immunosuppressive activity of MDSCs [41, 114].

Meanwhile, increased T-cell populations are observed within stromal tissues, and many immune-related genes are significantly upregulated by BMP4, indicating BMP4 triggers an enhanced antitumor immune response [41]. Therefore, it can be concluded that BMP signaling could inhibit inflammatory infiltrates and tumor progression through suppressing an inflammatory chemokine profile in tumor microenvironment.

Intriguingly, BMP signaling could also induce a series of cytokines which trigger CAF-mediated pro-tumorigenic stimulation on epithelial cells directly. BMP4 treatment of normal mammary fibroblasts or carcinoma-associated mammary fibroblasts (CAFs) induces an increase in secreted matrix metalloproteases (MMPs) and pro-inflammatory cytokines, which enhance mammary carcinoma cell invasion [73, 121]. Furthermore, inhibition of BMP signaling alters fibroblasts, macrophages, and lymphatic vessels to be less tumor promoting in vivo [48].

It has been reported that BMPs can promote endothelial cell (EC) proliferation and migration [122]. Consistent with this notion, BMP signaling is required for appropriate angiogenesis [123]. BMP2 promotes vascularization by stimulating the ID1 and p38 MAPK pathways. Overexpression of BMP2 in MCF-7 cells induces vascularized tumors eventually upon injection in vivo [124]. The signaling mediated by BMP type I receptor ALK1 has a critical role in regulation of both developmental and pathologic blood vessel formation [125]. ALK1 is mainly expressed at the sites of angiogenesis during embryogenesis and is expressed at lower levels in adult vasculature. Yet its expression increases in neoangiogenic vessels of wounds and cancer [125]. BMP9 binds to ALK1 in ECs with high affinities [126]. There have been divergent results with respect to the effects of BMP9/ALK1 signaling on ECs. Some reports demonstrate that high-dose BMP9/ALK1 signaling exhibits antiangiogenic effects, by inhibiting FGF-induced angiogenesis [127, 128], while other reports have shown induction of proliferation by low dose of BMP9 in several types of ECs and proangiogenic effects of BMP9 in Matrigel plug assays [129, 130]. The apparent discrepancy between these reports might reflect the contextual function of BMPs, in which the concentration plays an important role. In addition, common proangiogenic factors (VEGF-A and bFGF) can stimulate ALK1-mediated BMP/SMAD-like signaling, leading to cell spreading, and tubulogenesis of ECs [131]. Inhibition of ALK1 signaling by gene silencing, ligand traps, or antibodies can significantly suppress the growth and progression of tumors, including breast cancer, with substantial reduction of angiogenesis, supporting the notion that ALK1 is an important target for antiangiogenic treatment [131, 132].

9 Roles of BMPs in the Migration, Invasion, and Metastasis of Breast Cancer

It is clear that BMPs and their receptors modulate key pathways mediating breast cancer cell invasion and migration, critical parameters of metastatic dissemination. But the conclusions also seem paradoxical, indicating dependence on particular cell types and contexts.

9.1 BMPs and EMT

The development of metastasis involves the replacement with new phenotypes in cancer cells to facilitate detachment from the primary site [133]. Many epithelial cancer cells can acquire sufficient phenotypic plasticity by EMT, which implies the conversion of a proliferative epithelial state into nonproliferative mesenchymal state with the ability to migrate and invade adjacent tissue [134]. Restriction in BMP signaling level is frequently needed for efficient EMT [54, 91, 135]. Significant downregulation of some BMPs and upregulation of two secreted BMP antagonists, Chordin-like (CHRDL) 2 and Gremlin, were observed when human mammary epithelial cells pass through an EMT [91]. A subsequent study showed that the transcription factor zinc finger E-box-binding homeobox 1 (ZEB1) which mediates EMT can directly upregulate the expression of the BMPs antagonists Noggin, Follistatin, and CHRDL1 [135]. Likewise, a newly identified EMT pathway mediated by the transcriptional repressor Blimp-1 (PRDM1) leads to SNAIL induction via repression of BMP5 [54]. Of note, during acquisition of metastatic ability, EMT in mammary cells is strongly correlated with a $CD44^{high}/CD24^{low}$ stem cell phenotype [90, 91, 136]. These studies thus support a mechanistic link between BMP downregulation, EMT, and stem cell signature in cancer.

In addition, some BMPs are capable of reversing EMT or EMT markers in breast cancer cells [52, 80, 137]. E-cadherin-mediated cell-to-cell adhesion can be restored through inhibition of ZEB1 by BMP6 in breast cancer cells [44, 137, 138]. Stimulation with exogenous BMP7, which can decrease vimentin and increase cytokeratin expression in vitro and in vivo, gives rise to an epithelial-like phenotype [52]. BMPs can also oppose EMT inducers, e.g., TGFβ, in normal mammary epithelial cells or IMECs [54, 91, 139–142] and in breast cancer cells [52, 92, 140]. For example, the loss of E-cadherin expression on the surface of NMuMG cells in response to TGFβ1 is largely overridden by BMP5, and the fibroblastoid phenotype is also substantially reversed [54]. BMP7 has also been shown to reverse TGFβ-induced EMT [139–141], which increases E-cadherin expression through upregulation of ID2 and ID3. Interestingly, when knocking down ID2 or ID3, BMP7 actually induces the expression of α-smooth muscle actin (αSMA) and stimulates EMT [140, 141]. Thus, BMP signaling impedes the progression of breast cancer to an invasive state and prevents metastasis in the aforementioned studies. However, the BMP pathway was found to maintain a mesenchymal stem cell phenotype of breast cancer cells and render cells more migratory, invasive in other in vitro [89, 143, 144] and in vivo [61, 143] studies. BMP2 transforms MCF-7 cells from a round-like shape into a spindle-like shape with some specialized structures, such as filopodia, lamellipodia, and membrane protrusions, which are essential for cell migration and spreading [100, 144]. BMP4 blocks the capacity of mammary epithelial cells to form polarized lumen-containing structures and renders them invasive properties [145]. Of note, in 4T1.2 cells expressing BMP4,

genes associated with EMT are upregulated but no change was observed in their migratory capacity [41].

9.2 BMPs and Components of the Extracellular Matrix (ECM)

EMT is not an "all-or-nothing" event; it's highly dynamic. Studies have shown that BMPs induce MMP-dependent migration and invasion of breast cancer [48, 96, 121]. MMPs are known for degrading surrounding ECM components during cancer invasion and metastasis [146]. Treatment of primary tumors with BMPRI kinase inhibitor DMH1 reduced MMP2 and CCL9 in CAFs [48]. BMP4 induces the expression of multiple MMPs in mouse mammary fibroblasts and in cancer-associated human mammary fibroblasts [121] and dramatically increases MMP3 and MMP4 expression in 3D-cultured MDA-MB-231 cells [96]. However, another study showed that BMP4 suppresses the activity of MMP9 in 2D culture, rather than MMP1 and MMP3 [147]. Moreover, BMP6 was found to inhibit MMP9 activation via SMAD-dependent induction of heme oxygenase 1 (HO1) in MCF-7 cells [148]. BMP9 can inhibit MMP9 by inhibiting the AKT signaling pathway [106, 149].

ECM-associated protein Wnt1-inducible secreted protein 3 (WISP-3/CCN6) binds directly to BMP4 to antagonize BMP4-induced SMAD-independent activation of TAK1/p38 kinases, decreases the invasiveness of breast cancer cells in 3D, and also reduces distant metastasis in xenografts [143]. In contrast, the expression of ECM proteins tenascin-W, which can promote the motility of breast cancer cells expressing α8 integrin, is induced by BMP2-mediated p38 MAPK and JNK signaling pathways [150].

9.3 Interplay Between BMPs and TGFβ

Apart from EMT as previously mentioned, other features of cancer cells such as migration and invasion are also affected by a mutual antagonism between BMPs and TGFβ. Overexpression of type III TGFβ receptor inhibited BMP-mediated SMAD1/5/8 phosphorylation and BMP-induced migration [151]. BMP7 treatment significantly increases migration and invasion in MDA-MB-231 cells [53, 152]. This effect is substantially inhibited by costimulation with TGFβ by inducing the formation of complexes involving phosphorylated SMAD1/5 and SMAD3 [152]. Moreover, BMP2-mediated upregulation of ID1 may be a contributing factor in BMP2-related aggressiveness of breast cancer cells. Aberrant activation of SRC kinase resulting in increased SMAD1/5 signaling can change ID1 expression, which is positively controlled via SMAD1/5 by BMP2 and negatively via SMAD2/3 by TGFβ [153]. Conversely, BMP7 inhibits TGFβ-induced expression of αvβ3 integrin and invasion of the metastatic breast cancer cell line MCF-10CA1a in a spheroid model [154].

10 BMPs and Metastasis

Common sites of metastatic dissemination, such as the bone and lung, are the main targets of metastatic breast cancer [7]. In the process of bone metastasis, breast cancer triggers predominantly an osteoclast-mediated osteolytic lesion [155]. BMP signaling is shown to shift the osteoblast/osteoclast differentiation balance in favor of stimulating osteoblast differentiation [70, 71, 156]. By inactivating BMP signaling, BMP antagonists, such as Noggin, Follistatin, and CHRDL1, have been linked to the induction of osteoclast differentiation, as well as the formation of osteolytic bone metastases [71, 135, 156]. Lack of Noggin expression by breast cancer cells is a determinant of osteoblastic activities [70]. In an intracardiac xenograft model, evidence was found that Noggin is expressed in metastatic breast cancer cells during the late events of metastasis. In particular, it facilitates the metastatic capabilities of breast cancer cells to the bone by promoting osteoclast differentiation and bone degradation [71].

In contrast, when MCF-7 or MDA-MB-231 cells are cocultured with osteoblast-like cells, Noggin effectively inhibits migration and invasion of breast cancer cells by downregulating MMP1 and CXCR4 and improves bone remodeling by increasing the ratio of osteoprotegerin (OPG)/nuclear factor kappa B ligand (RANKL) [38]. The BMP target gene and cofactor RUNX2 are required for breast cancer osteolytic metastases [157, 158]. miR-135 impairs the BMP-RUNX2 axis by directly targeting SMAD5 and subsequently reduces the osteolytic properties of breast cancer cells [158]. Likewise, expression of dominant-negative receptors (DN-ALK3) for BMPs reduces interleukin-11 (IL-11) expression and inhibits bone metastasis in xenograft model [72].

As for individual BMP, BMP9, which is one of the most effective BMPs in osteogenesis, can inhibit osteolytic injury and bone metastasis caused by MDA-MB-231 cells by downregulating PTHrP, IL6, RANKL, and connective tissue growth factor (CTGF) [55, 149]. BMP2, 7, and 2/7 heterodimer inhibits bone metastases formation in MDA-MB-231 cells [52, 92]. Contradicting results showed that BMP7 overexpression could lead to accelerated bone metastasis formation of breast cancer cells [50, 51, 53].

BMP signaling can also prevent the colonization of metastatic cells in the lung by repressing key CSCs traits and enforcing cancer cells into dormancy. Overexpression of the BMP antagonist Coco permits a few dormant cancer cells to break through the barrier imposed by BMP signaling and to establish clinically meaningful metastases [95].

11 Conclusions and Perspectives

As discussed above, there are conflicting views regarding the significance of BMPs in breast cancer, based both on in vitro and in vivo studies. This has been attributed to multiple factors, including the (dose- and context-dependent) differential effects

of different BMP ligands and differences in the genetic patterns of breast cancer subtypes, as well as differences in the research models that were used. Most results are obtained using only a few types of cancer cell lines or single and different animal models and are therefore difficult to compare to each other. What is clear is that BMPs are emerging as key factors in many aspects of breast cancer. Aberrant changes in BMP signaling/components have been detected in breast cancer and metastatic recurrence and have deepened our understanding of the pathogenesis of breast cancer. The majority of studies indicate that BMP signaling is a critical negative regulator in multiple breast cancer cell lines both in vitro and in vivo. Restoration or amplification of specific aspects of BMP signaling may be potentially exploited for therapeutic intervention strategies.

To this point, context is critical. For instance, even an agonist or coactivator with precisely delivered BMP signaling input will not make any contribution to overcome the shortages that derive from functional deficiency of BMP receptors or any critical downstream components. It is therefore necessary to identify more potential targets or markers of the specific signaling defect(s). This might be pursued by using the latest types of high-throughput (epi)genetic, proteomic, and metabolomic analysis to systematically investigate the BMP responses to multiple cell types of the different breast cancer subclasses and/or patient-derived (organoid) (co)cultures grown in 3D and investigating the effect of misexpression of BMP receptor components or pharmacological inhibition of BMP receptor signaling in relevant transgenic mouse models and patient-derived xenografts with clear classification of histological pathology. This may provide effective principles to better illuminate the context-dependent roles of BMP family signaling in breast cancer. Via these approaches the opportunities for pharmacological intervention to rectify aberrant BMP family signaling in specific contexts are likely to be increased.

Acknowledgments We are grateful to Philip Owens, Miriam de Boeck, and Hans van Dam for critical reading and comments. Our studies on BMP in cancer and vascular diseases are supported by the Cancer Genomics Centre, Netherlands, and Swedish Cancerfonden (090773).

References

1. Urist MR (1965) Bone: formation by autoinduction. Science 150:893–899
2. Brazil DP, Church RH, Surae S, Godson C, Martin F (2015) BMP signalling: agony and antagony in the family. Trends Cell Biol 25:249–264
3. Wang RN, Green J, Wang Z, Deng Y, Qiao M, Peabody M, Zhang Q, Ye J, Yan Z, Denduluri S (2014) Bone Morphogenetic Protein (BMP) signaling in development and human diseases. Genes Dis 1:87–105
4. Miyazono K, Kamiya Y, Morikawa M (2010) Bone morphogenetic protein receptors and signal transduction. J Biol Chem 147:35–51
5. Ehata S, Yokoyama Y, Takahashi K, Miyazono K (2013) Bi-directional roles of bone morphogenetic proteins in cancer: another molecular Jekyll and Hyde? Pathol Int 63:287–296
6. Robinson GW (2008) Cooperation of signalling pathways in embryonic mammary gland development. Nat Rev Genet 9:566–566

7. Lorusso G, Rüegg C (2012) New insights into the mechanisms of organ-specific breast cancer metastasis. Semin Cancer Biol 22:226–233
8. Long F (2012) Building strong bones: molecular regulation of the osteoblast lineage. Nat Rev Mol Cell Biol 13:27–38
9. Bragdon B, Moseychuk O, Saldanha S, King D, Julian J, Nohe A (2011) Bone morphogenetic proteins: a critical review. Cell Signal 23:609–620
10. Wakefield LM, Hill CS (2013) Beyond TGFβ: roles of other TGFβ superfamily members in cancer. Nat Rev Cancer 13:328–341
11. Heldin CH, Miyazono K, Ten Dijke P (1997) TGF-β signalling from cell membrane to nucleus through SMAD proteins. Nature 390:465–471
12. López-Rovira T, Chalaux E, Massagué J, Rosa JL, Ventura F (2002) Direct binding of Smad1 and Smad4 to two distinct motifs mediates bone morphogenetic protein-specific transcriptional activation of Id1 gene. J Biol Chem 277:3176–3185
13. Ishida W, Hamamoto T, Kusanagi K, Yagi K, Kawabata M, Takehara K, Sampath TK, Kato M, Miyazono K (2000) Smad6 is a Smad1/5-induced Smad inhibitor characterization of bone morphogenetic protein-responsive element in the mouse Smad6 promoter. Trends Cell Biol 20:244–256
14. Lee KS, Kim HJ, Li QL, Chi XZ, Ueta C, Komori T, Wozney JM, Kim EG, Choi JY, Ryoo HM (2000) Runx2 is a common target of transforming growth factor β1 and bone morphogenetic protein 2, and cooperation between Runx2 and Smad5 induces osteoblast-specific gene expression in the pluripotent mesenchymal precursor cell line C2C12. Mol Cell Biol 20: 8783–8792
15. Zhang YE (2009) Non-Smad pathways in TGF-β signaling. Cell Res 19:128–139
16. Walsh DW, Godson C, Brazil DP, Martin F (2010) Extracellular BMP-antagonist regulation in development and disease: tied up in knots. Trends Cell Biol 20(5):244–256
17. Singhatanadgit W, Salih V, Olsen I (2006) Shedding of a soluble form of BMP receptor-IB controls bone cell responses to BMP. Bone 39:1008–1017
18. Onichtchouk D, Chen YG, Dosch R, Gawantka V, Delius H, Massague J, Niehrs C (1999) Silencing of TGF-β signalling by the pseudoreceptor BAMBI. Nature 401:480–485
19. Halbrooks PJ, Ding R, Wozney JM, Bain G (2007) Role of RGM coreceptors in bone morphogenetic protein signaling. J Mol Signal 2:4
20. Kirkbride KC, Townsend TA, Bruinsma MW, Barnett JV, Blobe GC (2008) Bone morphogenetic proteins signal through the transforming growth factor-β type III receptor. J Biol Chem 283:7628–7637
21. Scherner O, Meurer SK, Tihaa L, Gressner AM, Weiskirchen R (2007) Endoglin differentially modulates antagonistic transforming growth factor-β1 and BMP-7 signaling. J Biol Chem 282:13934–13943
22. Alt A, Miguel-Romero L, Donderis J, Aristorena M, Blanco FJ, Round A, Rubio V, Bernabeu C, Marina A (2012) Structural and functional insights into endoglin ligand recognition and binding. PLoS One 7:e29948
23. Shi W, Chang C, Nie S, Xie S, Wan M, Cao X (2007) Endofin acts as a Smad anchor for receptor activation in BMP signaling. J Cell Sci 120:1216–1224
24. Kugimiya F, Yano F, Ohba S, Igawa K, Nakamura K, Kawaguchi H, Chung UI (2005) Mechanism of osteogenic induction by FK506 via BMP/Smad pathways. Biochem Biophys Res Commun 338:872–879
25. Spiekerkoetter E, Tian X, Cai J, Hopper RK, Sudheendra D, Li CG, El-Bizri N, Sawada H, Haghighat R, Chan R (2013) FK506 activates BMPR2, rescues endothelial dysfunction, and reverses pulmonary hypertension. J Clin Invest 123:3600–3613
26. Ten Dijke P, Goumans MJ, Itoh F, Itoh S (2002) Regulation of cell proliferation by Smad proteins. J Cell Physiol 191:1–16
27. Massagué J, Seoane J, Wotton D (2005) Smad transcription factors. Genes Dev 19:2783–2810
28. Itoh S, ten Dijke P (2007) Negative regulation of TGF-β receptor/Smad signal transduction. Curr Opin Cell Biol 19:176–184
29. Massagué J, Chen YG (2000) Controlling TGF-β signaling. Genes Dev 14:627–644

30. Guo X, Wang XF (2009) Signaling cross-talk between TGF-β/BMP and other pathways. Cell Res 19:71–88
31. Chapellier M, Bachelard-Cascales E, Schmidt X, Clément F, Treilleux I, Delay E, Jammot A, Ménétrier-Caux C, Pochon G, Besançon R (2015) Disequilibrium of BMP2 levels in the breast stem cell niche launches epithelial transformation by overamplifying BMPR1B cell response. Stem Cell Rep 4:239–254
32. Reinholz MM, Iturria SJ, Ingle JN, Roche PC (2002) Differential gene expression of TGF-β family members and osteopontin in breast tumor tissue: analysis by real-time quantitative PCR. Breast Cancer Res Treat 74:255–269
33. Davies SR, Watkins G, Douglas-Jones A, Mansel RE, Jiang WG (2007) Bone morphogenetic proteins 1 to 7 in human breast cancer, expression pattern and clinical/prognostic relevance. J Exp Ther Oncol 7:327–338
34. Zhang XHF, Wang Q, Gerald W, Hudis CA, Norton L, Smid M, Foekens JA, Massagué J (2009) Latent bone metastasis in breast cancer tied to Src-dependent survival signals. Cancer Cell 16:67–78
35. Alarmo EL, Kuukasjärvi T, Karhu R, Kallioniemi A (2007) A comprehensive expression survey of bone morphogenetic proteins in breast cancer highlights the importance of BMP4 and BMP7. Breast Cancer Res Treat 103:239–246
36. Hartmann O, Spyratos F, Harbeck N, Dietrich D, Fassbender A, Schmitt M, Eppenberger-Castori S, Vuaroqueaux V, Lerebours F, Welzel K (2009) DNA methylation markers predict outcome in node-positive, estrogen receptor-positive breast cancer with adjuvant anthracycline-based chemotherapy. Clin Cancer Res 15:315–323
37. Ketolainen JM, Alarmo EL, Tuominen VJ, Kallioniemi A (2010) Parallel inhibition of cell growth and induction of cell migration and invasion in breast cancer cells by bone morphogenetic protein 4. Breast Cancer Res Treat 124:377–386
38. Guo D, Huang J, Gong J (2012) Bone morphogenetic protein 4 (BMP4) is required for migration and invasion of breast cancer. Mol Cell Biochem 363:179–190
39. Alarmo EL, Huhtala H, Korhonen T, Pylkkänen L, Holli K, Kuukasjärvi T, Parkkila S, Kallioniemi A (2013) Bone morphogenetic protein 4 expression in multiple normal and tumor tissues reveals its importance beyond development. Mod Pathol 26:10–21
40. Kretschmer C, Conradi A, Kemmner W, Sterner-Kock A (2011) Latent transforming growth factor binding protein 4 (LTBP4) is downregulated in mouse and human DCIS and mammary carcinomas. Cell Oncol 34:419–434
41. Cao Y, Slaney CY, Bidwell BN, Parker BS, Johnstone CN, Rautela J, Eckhardt BL, Anderson RL (2014) BMP4 inhibits breast cancer metastasis by blocking myeloid-derived suppressor cell activity. Cancer Res 74:5091–5102
42. Clement JH, Sänger J, Höffken K (1999) Expression of bone morphogenetic protein 6 in normal mammary tissue and breast cancer cell lines and its regulation by epidermal growth factor. Int J Cancer 80(2):250–256
43. Zhang M, Wang Q, Yuan W, Yang S, Wang X, Yan JD, Du J, Yin J, Gao SY, Sun BC (2007) Epigenetic regulation of bone morphogenetic protein-6 gene expression in breast cancer cells. J Steroid Biochem Mol Biol 105(1):91–97
44. Du J, Yang S, An D, Hu F, Yuan W, Zhai C, Zhu T (2009) BMP-6 inhibits microRNA-21 expression in breast cancer through repressing δEF1 and AP-1. Cell Res 19(4):487–496
45. Barekati Z, Radpour R, Lu Q, Bitzer J, Zheng H, Toniolo P, Lenner P, Zhong XY (2012) Methylation signature of lymph node metastases in breast cancer patients. BMC Cancer 12:244
46. Lian WJ, Liu G, Liu YJ, Zhao ZW, Yi T, Zhou HY (2013) Downregulation of BMP6 enhances cell proliferation and chemoresistance via activation of the ERK signaling pathway in breast cancer. Oncol Rep 30:193–200
47. Alarmo EL, Rauta J, Kauraniemi P, Karhu R, Kuukasjärvi T, Kallioniemi A (2006) Bone morphogenetic protein 7 is widely overexpressed in primary breast cancer. Genes Chromosomes Cancer 45:411–419
48. Owens P, Pickup MW, Novitskiy SV, Giltnane JM, Gorska AE, Hopkins CR, Hong CC, Moses HL (2014) Inhibition of bmp signaling suppresses metastasis in mammary cancer. Oncogene 34:2437–2449

49. Schwalbe M, Sänger J, Eggers R, Naumann A, Schmidt A, Höffken K, Clement JH (2003) Differential expression and regulation of bone morphogenetic protein 7 in breast cancer. Int J Oncol 23:89–95
50. Alarmo EL, Korhonen T, Kuukasjärvi T, Huhtala H, Holli K, Kallioniemi A (2007) Bone morphogenetic protein 7 expression associates with bone metastasis in breast carcinomas. Ann Oncol 19:308–314
51. Sakai H, Furihata M, Matsuda C, Takahashi M, Miyazaki H, Konakahara T, Imamura T, Okada T (2012) Augmented autocrine bone morphogenic protein (BMP) 7 signaling increases the metastatic potential of mouse breast cancer cells. Clin Exp Metastasis 29:327–338
52. Buijs JT, Henriquez NV, Van Overveld PG, Van der Horst G, Que I, Schwaninger R, Rentsch C, Ten Dijke P, Cleton-Jansen AM, Driouch K (2007) Bone morphogenetic protein 7 in the development and treatment of bone metastases from breast cancer. Cancer Res 67:8742–8751
53. Alarmo EL, Pärssinen J, Ketolainen JM, Savinainen K, Karhu R, Kallioniemi A (2009) BMP7 influences proliferation, migration, and invasion of breast cancer cells. Cancer Lett 275:35–43
54. Romagnoli M, Belguise K, Yu Z, Wang X, Landesman-Bollag E, Seldin DC, Chalbos D, Barillé-Nion S, Jézéquel P, Seldin ML (2012) Epithelial-to-mesenchymal transition induced by TGF-β1 is mediated by Blimp-1–dependent repression of BMP-5. Cancer Res 72:6268–6278
55. Ren W, Sun X, Wang K, Feng H, Liu Y, Fei C, Wan S, Wang W, Luo J, Shi Q (2014) BMP9 inhibits the bone metastasis of breast cancer cells by downregulating CCN2 (connective tissue growth factor, CTGF) expression. Mol Biol Rep 41:1373–1383
56. Ye L, Bokobza S, Li J, Moazzam M, Chen J, Mansel RE, Jiang WG (2010) Bone morphogenetic protein-10 (BMP-10) inhibits aggressiveness of breast cancer cells and correlates with poor prognosis in breast cancer. Cancer Sci 101:2137–2144
57. Hanavadi S, Martin T, Watkins G, Mansel R, Jiang W (2007) The role of growth differentiation factor-9 (GDF-9) and its analog, GDF-9b/BMP-15, in human breast cancer. Ann Surg Oncol 14:2159–2166
58. Li J, Ye L, Parr C, Douglas-Jones A, Kyanaston H, Mansel RE, Jiang WG (2009) The aberrant expression of bone morphogenetic protein 12 (BMP-12) in human breast cancer and its potential prognostic value. Gene Ther Mol Biol 13:186–193
59. Takahashi M, Otsuka F, Miyoshi T, Otani H, Goto J, Yamashita M, Ogura T, Makino H, Doihara H (2008) Bone morphogenetic protein 6 (BMP6) and BMP7 inhibit estrogen-induced proliferation of breast cancer cells by suppressing p38 mitogen-activated protein kinase activation. J Endocrinol 199(3):445–455
60. Network CGA (2012) Comprehensive molecular portraits of human breast tumours. Nature 490:61–70
61. Balboni AL, Hutchinson JA, DeCastro AJ, Cherukuri P, Liby K, Sporn MB, Schwartz GN, Wells WA, Sempere LF, Paul BY (2013) ΔNp63α-mediated activation of bone morphogenetic protein signaling governs stem cell activity and plasticity in normal and malignant mammary epithelial cells. Cancer Res 73:1020–1030
62. Helms MW, Packeisen J, August C, Schittek B, Boecker W, Brandt BH, Buerger H (2005) First evidence supporting a potential role for the BMP/SMAD pathway in the progression of oestrogen receptor-positive breast cancer. J Pathol 206:366–376
63. Hover LD, Pickup MW, Gorska AE, Chytil A, Guo Y, Novitskiy SV, Moses HL, Owens P (2015) Deletion of the BMP receptor BMPR1a results in EMT and impairs mammary gland tumor formation and metastasis. Cancer Res 75:4083–4083
64. Bokobza SM, Ye L, Kynaston HE, Mansel RE, Jiang WG (2009) Reduced expression of BMPR-IB correlates with poor prognosis and increased proliferation of breast cancer cells. Cancer Genom Proteom 6:101–108
65. Zhong D, Morikawa A, Guo L, Colpaert C, Xiong L, Nassar A, Chen C, Lamb N, Dong J-T, Zhou W (2006) Homozygous deletion of SMAD4 in breast cancer cell lines and invasive ductal carcinomas. Cancer Biol Ther 5:601–607
66. Valero V III, Saunders TJ, He J, Weiss MJ, Cameron JL, Dholakia A, Wild AT, Shin EJ, Khashab MA, O'Broin-Lennon AM (2015) Reliable detection of somatic mutations in

fine needle aspirates of pancreatic cancer with next-generation sequencing. Ann Surg 263: 153–161
67. Voorneveld PW, Kodach LL, Jacobs RJ, Liv N, Zonnevylle AC, Hoogenboom JP, Biemond I, Verspaget HW, Hommes DW, de Rooij K (2014) Loss of SMAD4 alters BMP signaling to promote colorectal cancer cell metastasis via activation of Rho and ROCK. Gastroenterology 147:196–208
68. Sneddon JB, Zhen HH, Montgomery K, van de Rijn M, Tward AD, West R, Gladstone H, Chang HY, Morganroth GS, Oro AE (2006) Bone morphogenetic protein antagonist gremlin 1 is widely expressed by cancer-associated stromal cells and can promote tumor cell proliferation. Proc Natl Acad Sci 103:14842–14847
69. Ma X-J, Dahiya S, Richardson E, Erlander M, Sgroi DC (2009) Gene expression profiling of the tumor microenvironment during breast cancer progression. Breast Cancer Res 11:R7
70. Schwaninger R, Rentsch CA, Wetterwald A, van der Horst G, van Bezooijen RL, van der Pluijm G, Löwik CW, Ackermann K, Pyerin W, Hamdy FC (2007) Lack of noggin expression by cancer cells is a determinant of the osteoblast response in bone metastases. Am J Pathol 170:160–175
71. Tarragona M, Pavlovic M, Arnal-Estapé A, Urosevic J, Morales M, Guiu M, Planet E, González-Suárez E, Gomis RR (2012) Identification of NOG as a specific breast cancer bone metastasis-supporting gene. J Biol Chem 287:21346–21355
72. Katsuno Y, Hanyu A, Kanda H, Ishikawa Y, Akiyama F, Iwase T, Ogata E, Ehata S, Miyazono K, Imamura T (2008) Bone morphogenetic protein signaling enhances invasion and bone metastasis of breast cancer cells through Smad pathway. Oncogene 27:6322–6333
73. Owens P, Polikowsky H, Pickup MW, Matise LA, Gorska AE, Shaw AK, Novitskiy SV, Aakre ME, Hong CC, Moses HL (2012) Bone morphogenetic proteins stimulate mammary fibroblasts to promote mammary tumorigenesis. Cancer Res 72:1500–1500
74. Yan W, Chen X (2007) Targeted repression of bone morphogenetic protein 7, a novel target of the p53 family, triggers proliferative defect in p53-deficient breast cancer cells. Cancer Res 67:9117–9124
75. Huang B, Warner M (2015) Gustafsson J-Å (2014) Estrogen receptors in breast carcinogenesis and endocrine therapy. Mol Cell Endocrinol 418:240–244
76. Kusumegi T, Tanaka J, Kawano M, Yonemoto J, Tohyama C, Sone H (2004) BMP7/ActRIIB regulates estrogen-dependent apoptosis: New biomarkers for environmental estrogens. J Biochem Mol Toxicol 18:1–11
77. Yamamoto T, Saatcioglu F, Matsuda T (2002) Cross-talk between bone morphogenic proteins and estrogen receptor signaling. Endocrinology 143:2635–2642
78. Páez-Pereda M, Giacomini D, Refojo D, Nagashima AC, Hopfner U, Grübler Y, Chervin A, Goldberg V, Goya R, Hentges ST (2003) Involvement of bone morphogenetic protein 4 (BMP-4) in pituitary prolactinoma pathogenesis through a Smad/estrogen receptor crosstalk Proc Natl Acad Sci 100: 1034–1039
79. Van den Wijngaard A, Mulder W, Dijkema R, Boersma C, Mosselman S, van Zoelen E, Olijve W (2000) Antiestrogens specifically up-regulate bone morphogenetic protein-4 promoter activity in human osteoblastic cells. Mol Endocrinol 14:623–633
80. Liu G, Liu YJ, Lian WJ, Zhao ZW, Yi T, Zhou HY (2014) Reduced BMP6 expression by DNA methylation contributes to EMT and drug resistance in breast cancer cells. Oncol Rep 32:581–588
81. Zhang M, Yan JD, Zhang L, Wang Q, Lü SJ, Zhang J, Zhu TH (2005) Activation of bone morphogenetic protein-6 gene transcription in MCF-7 cells by estrogen. Chin Med J (Engl) 118:1629–1636
82. Lee HJ, Liu H, Goodman C, Ji Y, Maehr H, Uskokovic M, Notterman D, Reiss M, Suh N (2006) Gene expression profiling changes induced by a novel Gemini Vitamin D derivative during the progression of breast cancer. Biochem Pharmacol 72:332–343
83. Lee HJ, Wislocki A, Goodman C, Ji Y, Ge R, Maehr H, Uskokovic M, Reiss M, Suh N (2006) A novel vitamin D derivative activates bone morphogenetic protein signaling in MCF10 breast epithelial cells. Mol Pharmacol 69:1840–1848
84. Masuda H, Otsuka F, Matsumoto Y, Takano M, Miyoshi T, Inagaki K, Shien T, Taira N, Makino H, Doihara H (2011) Functional interaction of fibroblast growth factor-8, bone morphogenetic

protein and estrogen receptor in breast cancer cell proliferation. Mol Cell Endocrinol 343:7–17
85. Mi D, Zhang M, Yan JD, Zhang J, Wang X, Wang Q, Yang S, Zhu TH (2011) PTHrP inhibits BMP-6 expression through the PKA signaling pathway in breast cancer cells. J Cancer Res Clin Oncol 137:295–303
86. Wang N, Lin K, Lu Z, Lam K, Newton R, Xu X, Yu Z, Gill G, Andersen B (2007) The LIM-only factor LMO4 regulates expression of the BMP7 gene through an HDAC2-dependent mechanism, and controls cell proliferation and apoptosis of mammary epithelial cells. Oncogene 26:6431–6441
87. Wei W, Lewis MT (2015) Identifying and targeting tumor-initiating cells in the treatment of breast cancer. Endocr Relat Cancer 22:R135–R155
88. Oshimori N, Fuchs E (2012) The harmonies played by TGF-β in stem cell biology. Cell Stem Cell 11:751–764
89. Garulli C, Kalogris C, Pietrella L, Bartolacci C, Andreani C, Falconi M, Marchini C, Amici A (2014) Dorsomorphin reverses the mesenchymal phenotype of breast cancer initiating cells by inhibition of bone morphogenetic protein signaling. Cell Signal 26:352–362
90. Uchino M, Kojima H, Wada K, Imada M, Onoda F, Satofuka H, Utsugi T, Murakami Y (2010) Nuclear β-catenin and CD44 upregulation characterize invasive cell populations in non-aggressive MCF-7 breast cancer cells. BMC Cancer 10:414
91. Scheel C, Eaton EN, Li SH-J, Chaffer CL, Reinhardt F, Kah K-J, Bell G, Guo W, Rubin J, Richardson AL (2011) Paracrine and autocrine signals induce and maintain mesenchymal and stem cell states in the breast. Cell 145:926–940
92. Buijs J, Van Der Horst G, Van Den Hoogen C, Cheung H, De Rooij B, Kroon J, Petersen M, Van Overveld P, Pelger R, Van Der Pluijm G (2012) The BMP2/7 heterodimer inhibits the human breast cancer stem cell subpopulation and bone metastases formation. Oncogene 31:2164–2174
93. Hu F, Meng X, Tong Q, Liang L, Xiang R, Zhu T, Yang S (2013) BMP-6 inhibits cell proliferation by targeting microRNA-192 in breast cancer. Biochim Biophys Acta 1832:2379–2390
94. Clevers H (2011) The cancer stem cell: premises, promises and challenges. Nat Med 17:313–319
95. Gao H, Chakraborty G, Lee-Lim AP, Mo Q, Decker M, Vonica A, Shen R, Brogi E, Brivanlou AH, Giancotti FG (2012) The BMP inhibitor Coco reactivates breast cancer cells at lung metastatic sites. Cell 150:764–779
96. Ampuja M, Jokimäki R, Juuti-Uusitalo K, Rodriguez-Martinez A, Alarmo EL, Kallioniemi A (2013) BMP4 inhibits the proliferation of breast cancer cells and induces an MMP-dependent migratory phenotype in MDA-MB-231 cells in 3D environment. BMC Cancer 13:429
97. Arnold S, Tims E, McGrath B (1999) Identification of bone morphogenetic proteins and their receptors in human breast cancer cell lines: importance of BMP2. Cytokine 11:1031–1037
98. Wang D, Huang P, Zhu B, Sun L, Huang Q, Wang J (2012) Induction of estrogen receptor α-36 expression by bone morphogenetic protein 2 in breast cancer cell lines. Mol Med Rep 6:591–559
99. Chen A, Wang D, Liu X, He S, Yu Z, Wang J (2012) Inhibitory effect of BMP-2 on the proliferation of breast cancer cells. Mol Med Rep 6:615–620
100. Clement JH, Raida M, Sänger J, Bicknell R, Liu J, Naumann A, Geyer A, Waldau A, Hortschansky P, Schmidt A (2005) Bone morphogenetic protein 2 (BMP-2) induces in vitro invasion and in vivo hormone independent growth of breast carcinoma cells. Int J Oncol 27:401–407
101. Ghosh-Choudhury N, Ghosh-Choudhury G, Celeste A, Ghosh PM, Moyer M, Abboud SL, Kreisberg J (2000) Bone morphogenetic protein-2 induces cyclin kinase inhibitor p21 and hypophosphorylation of retinoblastoma protein in estradiol-treated MCF-7 human breast cancer cells. Biochim Biophys Acta 1497:186–196
102. Pouliot F, Labrie C (2002) Role of Smad1 and Smad4 proteins in the induction of p21WAF1, Cip1 during bone morphogenetic protein-induced growth arrest in human breast cancer cells. J Endocrinol 172:187–198

103. Dumont N, Arteaga CL (2003) A kinase-inactive type II TGFβ receptor impairs BMP signaling in human breast cancer cells. Biochem Biophys Res Commun 301:108–112
104. Du J, Yang S, Wang Z, Zhai C, Yuan W, Lei R, Zhang J, Zhu T (2008) Bone morphogenetic protein 6 inhibit stress-induced breast cancer cells apoptosis via both smad and P38 pathways. J Cell Biochem 103:1584–1597
105. Wang K, Feng H, Ren W, Sun X, Luo J, Tang M, Zhou L, Weng Y, He TC, Zhang Y (2011) BMP9 inhibits the proliferation and invasiveness of breast cancer cells MDA-MB-231. J Cancer Res Clin Oncol 137:1687–1696
106. Ren W, Liu Y, Wan S, Fei C, Wang W, Chen Y, Zhang Z, Wang T, Wang J, Zhou L (2014) BMP9 Inhibits proliferation and metastasis of HER2-positive SK-BR-3 breast cancer cells through ERK1/2 and PI3K/AKT pathways. PLoS One 9:e96816
107. Yan H, Zhu S, Song C, Liu N, Kang J (2012) Bone morphogenetic protein (BMP) signaling regulates mitotic checkpoint protein levels in human breast cancer cells. Cell Signal 24:961–968
108. Ghosh-Choudhury N, Woodruff K, Qi W, Celeste A, Abboud SL, Choudhury GG (2000) Bone morphogenetic protein-2 blocks MDA MB 231 human breast cancer cell proliferation by inhibiting cyclin-dependent kinase-mediated retinoblastoma protein phosphorylation. Biochem Biophys Res Commun 272:705–711
109. Pouliot F, Blais A, Labrie C (2003) Overexpression of a dominant negative type II bone morphogenetic protein receptor inhibits the growth of human breast cancer cells. Cancer Res 63:277–281
110. Waite KA, Eng C (2003) BMP2 exposure results in decreased PTEN protein degradation and increased PTEN levels. Hum Mol Genet 12:679–684
111. Rodriguez-Martinez A, Alarmo E-L, Saarinen L, Ketolainen J, Nousiainen K, Hautaniemi S, Kallioniemi A (2011) Analysis of BMP4 and BMP7 signaling in breast cancer cells unveils time-dependent transcription patterns and highlights a common synexpression group of genes. BMC Med Genomics 4:80
112. Montesano R, Sarközi R, Schramek H (2008) Bone morphogenetic protein-4 strongly potentiates growth factor-induced proliferation of mammary epithelial cells. Biochem Biophys Res Commun 374:164–168
113. Jung JW, Shim SY, Lee DK, Kwiatkowski W, Choe S (2014) An Activin A/BMP2 chimera, AB215, blocks estrogen signaling via induction of ID proteins in breast cancer cells. BMC Cancer 14:549
114. Owens P, Pickup MW, Novitskiy SV, Chytil A, Gorska AE, Aakre ME, West J, Moses HL (2012) Disruption of bone morphogenetic protein receptor 2 (BMPR2) in mammary tumors promotes metastases through cell autonomous and paracrine mediators. Proc Natl Acad Sci 109:2814–2819
115. Steinert S, Kroll TC, Taubert I, Pusch L, Hortschansky P, Höffken K, Wölfl S, Clement JH (2008) Differential expression of cancer-related genes by single and permanent exposure to bone morphogenetic protein 2. J Cancer Res Clin Oncol 134:1237–1245
116. Clement JH, Marr N, Meissner A, Schwalbe M, Sebald W, Kliche K-O, Höffken K, Wölfl S (2000) Bone morphogenetic protein 2 (BMP-2) induces sequential changes of Id gene expression in the breast cancer cell line MCF-7. J Cancer Res Clin Oncol 126:271–279
117. Raida M, Clement JH, Ameri K, Han C, Leek RD, Harris A (2005) Expression of bone morphogenetic protein 2 in breast cancer cells inhibits hypoxic cell death. Int J Oncol 26:1465–1470
118. Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144:646–674
119. Pickup MW, Hover LD, Polikowsky ER, Chytil A, Gorska AE, Novitskiy SV, Moses HL, Owens P (2015) BMPR2 loss in fibroblasts promotes mammary carcinoma metastasis via increased inflammation. Mol Oncol 9:179–191
120. Perkins ND (2012) The diverse and complex roles of NF-κB subunits in cancer. Nat Rev Cancer 12:121–132
121. Owens P, Polikowsky H, Pickup MW, Gorska AE, Jovanovic B, Shaw AK, Novitskiy SV, Hong CC, Moses HL (2013) Bone morphogenetic proteins stimulate mammary fibroblasts to promote mammary carcinoma cell invasion. PLoS One 8:e67533

122. Cai J, Pardali E, Sánchez-Duffhues G, ten Dijke P (2012) BMP signaling in vascular diseases. FEBS Lett 586:1993–2002
123. David L, Feige JJ, Bailly S (2009) Emerging role of bone morphogenetic proteins in angiogenesis. Cytokine Growth Factor Rev 20:203–212
124. Raida M, Clement JH, Leek RD, Ameri K, Bicknell R, Niederwieser D, Harris AL (2005) Bone morphogenetic protein 2 (BMP-2) and induction of tumor angiogenesis. J Cancer Res Clin Oncol 131:741–750
125. Cunha SI, Pietras K (2011) ALK1 as an emerging target for antiangiogenic therapy of cancer. Blood 117:6999–7006
126. Brown MA, Zhao Q, Baker KA, Naik C, Chen C, Pukac L, Singh M, Tsareva T, Parice Y, Mahoney A (2005) Crystal structure of BMP-9 and functional interactions with pro-region and receptors. J Biol Chem 280:25111–25118
127. Scharpfenecker M, van Dinther M, Liu Z, van Bezooijen RL, Zhao Q, Pukac L, Löwik CW, ten Dijke P (2007) BMP-9 signals via ALK1 and inhibits bFGF-induced endothelial cell proliferation and VEGF-stimulated angiogenesis. J Cell Sci 120:964–972
128. David L, Mallet C, Keramidas M, Lamandé N, Gasc JM, Dupuis-Girod S, Plauchu H, Feige JJ, Bailly S (2008) Bone morphogenetic protein-9 is a circulating vascular quiescence factor. Circ Res 102:914–922
129. Suzuki Y, Montagne K, Nishihara A, Watabe T, Miyazono K (2008) BMPs promote proliferation and migration of endothelial cells via stimulation of VEGF-A/VEGFR2 and angiopoietin-1/Tie2 signalling. J Biochem 143:199–206
130. Suzuki Y, Ohga N, Morishita Y, Hida K, Miyazono K, Watabe T (2010) BMP-9 induces proliferation of multiple types of endothelial cells in vitro and in vivo. J Cell Sci 123: 1684–1692
131. Hu-Lowe DD, Chen E, Zhang L, Watson KD, Mancuso P, Lappin P, Wickman G, Chen JH, Wang J, Jiang X (2011) Targeting activin receptor-like kinase 1 inhibits angiogenesis and tumorigenesis through a mechanism of action complementary to anti-VEGF therapies. J Biochem 143:199–206
132. Cunha SI, Pardali E, Thorikay M, Anderberg C, Hawinkels L, Goumans MJ, Seehra J, Heldin CH, ten Dijke P, Pietras K (2010) Genetic and pharmacological targeting of activin receptor-like kinase 1 impairs tumor growth and angiogenesis. J Exp Med 207:85–100
133. Chaffer CL, Weinberg RA (2011) A perspective on cancer cell metastasis. Science 331:1559–1564
134. Polyak K, Weinberg RA (2009) Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. Nat Rev Cancer 9:265–273
135. Mock K, Preca B, Brummer T, Brabletz S, Stemmler M, Brabletz T (2015) The EMT-activator ZEB1 induces bone metastasis associated genes including BMP-inhibitors. Oncotarget 6:14399–14412
136. Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, Brooks M, Reinhard F, Zhang CC, Shipitsin M (2008) The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell 133:704–715
137. Yang S, Du J, Wang Z, Yuan W, Qiao Y, Zhang M, Zhang J, Gao S, Yin J, Sun B (2007) BMP-6 promotes E-cadherin expression through repressing δEF1 in breast cancer cells. BMC Cancer 7:211
138. Yang S, Du J, Wang Z, Yan J, Yuan W, Zhang J, Zhu T (2009) Dual mechanism of δEF1 expression regulated by bone morphogenetic protein-6 in breast cancer. Int J Biochem Cell Biol 41:853–861
139. Zeisberg M, J-i H, Sugimoto H, Mammoto T, Charytan D, Strutz F, Kalluri R (2003) BMP-7 counteracts TGF-β1–induced epithelial-to-mesenchymal transition and reverses chronic renal injury. Nat Med 9:964–968
140. Kowanetz M, Valcourt U, Bergström R, Heldin C-H, Moustakas A (2004) Id2 and Id3 define the potency of cell proliferation and differentiation responses to transforming growth factor β and bone morphogenetic protein. Mol Cell Biol 24:4241–4254

141. Valcourt U, Kowanetz M, Niimi H, Heldin C-H, Moustakas A (2005) TGF-β and the Smad signaling pathway support transcriptomic reprogramming during epithelial-mesenchymal cell transition. Mol Biol Cell 16:1987–2002
142. Buijs JT, Henriquez NV, van Overveld PG, van der Horst G, ten Dijke P, van der Pluijm G (2007) TGF-β and BMP7 interactions in tumour progression and bone metastasis. Clin Exp Metastasis 24:609–617
143. Pal A, Huang W, Li X, Toy KA, Nikolovska-Coleska Z, Kleer CG (2012) CCN6 modulates BMP signaling via the Smad-independent TAK1/p38 pathway, acting to suppress metastasis of breast cancer. Cancer Res 72:4818–4828
144. Jin H, Pi J, Huang X, Huang F, Shao W, Li S, Chen Y, Cai J (2012) BMP2 promotes migration and invasion of breast cancer cells via cytoskeletal reorganization and adhesion decrease: an AFM investigation. Appl Microbiol Biotechnol 93:1715–1723
145. Montesano R (2007) Bone morphogenetic protein-4 abrogates lumen formation by mammary epithelial cells and promotes invasive growth. Biochem Biophys Res Commun 353:817–822
146. Friedl P, Alexander S (2011) Cancer invasion and the microenvironment: plasticity and reciprocity. Cell 147:992–1009
147. Shon SK, Kim A, Kim JY, Kim KI, Yang Y, Lim JS (2009) Bone morphogenetic protein-4 induced by NDRG2 expression inhibits MMP-9 activity in breast cancer cells. Biochem Biophys Res Commun 385:198–203
148. Wang C, Hu F, Guo S, Mi D, Shen W, Zhang J, Qiao Y, Zhu T, Yang S (2011) BMP-6 inhibits MMP-9 expression by regulating heme oxygenase-1 in MCF-7 breast cancer cells. J Cancer Res Clin Oncol 137:985–995
149. Wan S, Liu Y, Weng Y, Wang W, Ren W, Fei C, Chen Y, Zhang Z, Wang T, Wang J (2014) BMP9 regulates cross-talk between breast cancer cells and bone marrow-derived mesenchymal stem cells. Cell Oncol 37:363–375
150. Scherberich A, Tucker RP, Degen M, Brown-Luedi M, Andres A-C, Chiquet-Ehrismann R (2005) Tenascin-W is found in malignant mammary tumors, promotes alpha8 integrin-dependent motility and requires p38MAPK activity for BMP-2 and TNF-alpha induced expression in vitro. Oncogene 24:1525–1532
151. Gatza CE, Elderbroom JL, Oh SY, Starr MD, Nixon AB, Blobe GC (2014) The balance of cell surface and soluble type III TGF-β receptor regulates BMP signaling in normal and cancerous mammary epithelial cells. Neoplasia 16:489–500
152. Grönroos E, Kingston IJ, Ramachandran A, Randall RA, Vizán P, Hill CS (2012) Transforming growth factor β inhibits bone morphogenetic protein-induced transcription through novel phosphorylated Smad1/5-Smad3 complexes. Mol Cell Biol 32:2904–2916
153. Gautschi O, Tepper CG, Purnell PR, Izumiya Y, Evans CP, Green TP, Desprez PY, Lara PN, Gandara DR, Mack PC (2008) Regulation of Id1 expression by SRC: implications for targeting of the bone morphogenetic protein pathway in cancer. Cancer Res 68:2250–2258
154. Naber HP, Wiercinska E, Pardali E, van Laar T, Nirmala E, Sundqvist A, van Dam H, van der Horst G, van der Pluijm G, Heckmann B (2012) BMP-7 inhibits TGF-β-induced invasion of breast cancer cells through inhibition of integrin β3 expression. Cell Oncol 35:19–28
155. Roodman GD (2004) Mechanisms of bone metastasis. N Engl J Med 350(16):1655–1664
156. Bunyaratavej P, Hullinger TG, Somerman MJ (2000) Bone morphogenetic proteins secreted by breast cancer cells upregulate bone sialoprotein expression in preosteoblast cells. Exp Cell Res 260:324–333
157. Javed A, Barnes GL, Pratap J, Antkowiak T, Gerstenfeld LC, Van Wijnen AJ, Stein JL, Lian JB, Stein GS (2005) Impaired intranuclear trafficking of Runx2 (AML3/CBFA1) transcription factors in breast cancer cells inhibits osteolysis in vivo. Proc Natl Acad Sci 102:1454–1459
158. Taipaleenmäki H, Browne G, Akech J, Zustin J, van Wijnen AJ, Stein JL, Hesse E, Stein GS, Lian JB (2015) Targeting of Runx2 by miR-135 and miR-203 impairs progression of breast cancer and metastatic bone disease. Cancer Res 75:1433–1444

Index

S. Vukicevic, K.T. Sampath (eds.), *Bone Morphogenetic Proteins: Systems Biology Regulators*, Progress in Inflammation Research,
DOI 10.1007/978-3-319-47507-3

P

R