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A Tissue Regeneration Approach to Bone and Cartilage Repair

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A Tissue Regeneration Approach to Bone and Cartilage Repair

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Contents

Ligand–Receptor Interactions and Their Implications in Delivering Certain Signaling for Bone Regeneration 1
Takenobu Katagiri, Sho Tsukamoto, Kenji Osawa and Shoichiro Kokabu

BMPs and Wnts in Bone and Cartilage Regeneration 17
Di Chen, Shan Li and Tian-Fang Li

Osteocytes and Bone Regeneration 39
Gerald J. Atkins, Matthew Prideaux and David M. Findlay

Skeletal Stem Cells for Bone and Cartilage Tissue Regeneration 53
Walid Zaher, Adiba Isa and Moustapha Kassem

Manipulation of Macrophages to Enhance Bone Repair and Regeneration 65
Sina Nassiri, Pamela Graney and Kara L. Spiller

Cartilage Regeneration Using Induced Pluripotent Stem Cell Technologies 85
Noriyuki Tsumaki

Alveolar Augmentation: Focus on Growth Factors (BMPs) 99
Ulf M.E. Wikesjö, Cristiano Susin and Jaebum Lee

Bone-Biomimetic Biomaterial and Cell Fate Determination 119
ZuFu Lu, Jiao Jiao Li and Hala Zreiqat

Biomimetic Scaffolds for Craniofacial Bone Tissue Engineering: Understanding the Role of the Periosteum in Regeneration 147
Michael E. Frohbergh and Peter I. Lelkes

Biomaterials Used for Maxillofacial Regeneration 167
Xinquan Jiang and Zhiyuan Zhang

Advances and Applications of Nanomechanical Tools in Cartilage Tissue Engineering 191
Lin Han and Alan J. Grodzinsky

Signalling Pathways in Osteochondral Defect Regeneration 219
Henning Madry and Magali Cucchiarini

Polymer-Assisted Cartilage and Tendon Repair 229
Gundula Schulze-Tanzil

Ligand–Receptor Interactions and Their Implications in Delivering Certain Signaling for Bone Regeneration

Takenobu Katagiri, Sho Tsukamoto, Kenji Osawa and Shoichiro Kokabu

Abstract Cartilage and bone tissue formation is observed not only during embryonic development but also in some pathological conditions occurring after birth, including fracture healing. This process is regulated by many stimuli that are applicable to the reconstitution of skeletal tissues using tissue engineering. In particular, members of the transforming growth factor (TGF)-β family play a unique and important role in skeletal tissue formation, wherein they activate specific intracellular signaling pathways by binding two types of serine–threonine kinase receptors and downstream effectors called Smad proteins. The biological activity of TGF-β family members is positively and negatively regulated at multiple steps by various molecules found in the extracellular space, on the cell membrane, and in the intracellular space. The modification of TGF-β family signaling pathways can be used in tissue engineering approaches for skeletal tissue formation.

1 Introduction

Skeletal tissues mainly consist of cartilage and bone, which are formed during embryonic development in vertebrates. There are two processes at work in bone formation—intramembranous ossification and endochondral ossification (Shapiro 2008; Yang 2009). The former process is observed in flat bones, such as the frontal and parietal bones of the skull, whereas most of the long bones are formed through the latter process. During endochondral ossification, undifferentiated

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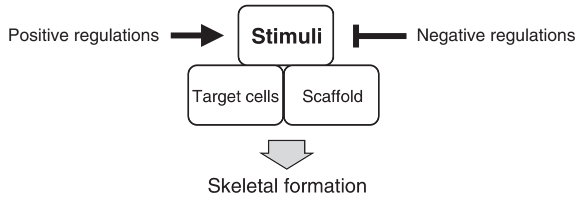

Fig. 1 Both positive and negative regulations of bone formation stimuli can be applied to reconstitute skeletal tissues via tissue engineering approaches, which further require cells and scaffolds

mesenchymal cells form cartilaginous rudiments, and the first bone tissue emerges from terminally differentiated hypertrophic chondrocytes within these rudiments. The processes involved in bone formation are detected not only during embryonic development but also after birth, in pathological conditions such as fracture healing and ectopic bone formation (Katagiri 2010; Shapiro 2008). The molecular mechanisms underlying physiological and pathological bone formation can be applied to reconstitute skeletal tissues via tissue engineering approaches, which require three critical factors—cells, scaffolds, and stimuli (Fig. 1). In this chapter, we focus on the positive and negative regulation of the stimuli component of these critical factors.

2 The Identification of BMPs as Members of the TGF-β Family

Urist (1965) first reported the experimental induction of bone formation in soft tissue through the use of bone matrix. He implanted HCl-treated bone matrix into skeletal muscle tissue and observed the formation of mineralized bone tissue containing newly generated osteoblasts and bone marrow. In addition, he showed that bone induction could be applied to the regeneration of bone tissue with critical defects. Given that the demineralized bone matrix did not contain any living cells, these findings indicated the presence of a novel bone-inducing activity within the demineralized bone matrix. This bone-inducing activity was later named "bone morphogenetic protein (BMP)" (Urist and Strates 1971). BMP could be extracted from demineralized bone matrix using protein denaturation reagents such as urea, suggesting that BMP binds tightly to the organic components of bone (Sampath and Reddi 1981; Urist and Strates 1971). Molecular cloning of the bone-inducing activity of bone matrix revealed that this activity consisted of several related BMPs and that these BMPs belong to the transforming growth factor-β (TGF-β) family, which includes TGF-βs, activin, growth and differentiation factors (GDFs), and others (Celeste et al. 1990; Ozkaynak et al. 1990; Sampath et al. 1990; Wozney et al. 1988) (Fig. 2). The ectopic bone-inducing activity of BMP is observed only with several specific members, such as BMP-2, BMP-4, BMP-5, BMP-6, BMP-7, BMP-9, and GDF5, but not with other

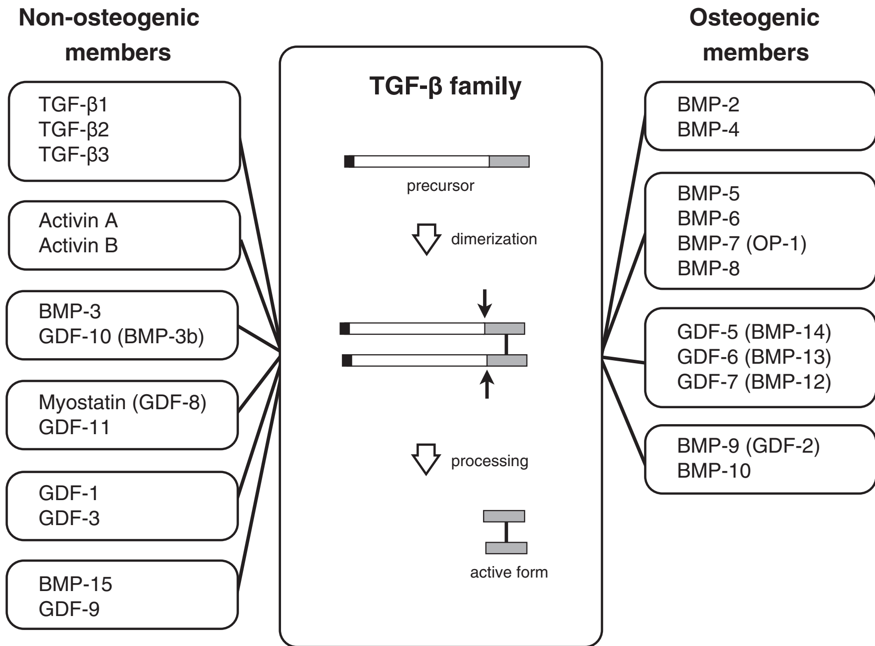

Fig. 2 Typical osteogenic and non-osteogenic members of the TGF-β family. The TGF-β family consists of TGF-βs, BMPs, GDFs, activin, and other members. These proteins form dimers that are processed to release active ligands. Most BMPs are capable of inducing heterotopic bone formation in soft tissues (osteogenic); however, TGF-β proteins, activin, and BMP-3 lack this activity (nonosteogenic)

members of the family or with other growth factors, hormones, or chemical compounds (Kang et al. 2004). The bone-inducing activity of BMPs is both positively and negatively regulated by a variety of molecules that act at multiple steps in the signaling pathway, such as during ligand modification, ligand–receptor interaction, and downstream effector activation. In this chapter, we discuss this multistep regulation of bone-inducing activity and how it might be applied in the reconstruction of skeletal tissues.

3 Experimental Models for Evaluating Bone Formation

Bone-inducing activity can be directly evaluated in vivo by implanting test samples into skeletal muscles or subcutaneous sites (Urist 1965; Wang et al. 1988). Fracture healing is another experimental model that can be used to evaluate the bone-forming capacity of samples in vivo (Urist 1965). In addition, cell cultures and ex vivo cultures function as alternative ways to examine a sample's bone induction capacity in vitro.

Cartilage-inducing activity is studied in vitro using chick limb bud-derived mesenchymal cells. In high-density cultures, these cells express cartilaginous phenotypes in response to appropriate stimuli, which include osteogenic BMPs/GDFs (Seemann et al. 2005). Chondrogenesis can also be induced in a pellet culture system using centrifuge tubes (Kato et al. 1988; Mackay et al. 1998). Alternatively, the mouse ATDC5 cell line can be used to test the chondrogenic activity of samples in vitro (Shukunami et al. 1997).

The murine myoblast C2C12 cell line is widely used to examine the bone-inducing capacity and regulatory mechanisms of various factors, functional receptors, and intracellular signal transducers. C2C12 cells are a subclonal cell line of parental C2 cells that were established from regenerating thigh muscle tissue (Blau et al. 1983). C2C12 cells proliferate as mononuclear cells that express MyoD, a transcription factor that is specific for activated myoblasts, and differentiate into multinucleated myotubes that express contractile proteins including troponin T and myosin heavy chain. However, in the presence of BMP-2, myogenic differentiation is inhibited, and C2C12 cells begin to express genes related to osteoblastic differentiation, such as alkaline phosphatase, osteocalcin, and parathyroid hormone receptor (Katagiri et al. 1994). Although many growth factors, hormones, and small chemical compounds inhibit myogenesis in C2C12 cells, only the osteogenic members of the TGF-β family induce osteoblastic differentiation (Katagiri et al. 1994). Thus, the osteoblastic differentiation of C2C12 cells may at least partially reflect the in vivo osteogenic activity of a test sample, despite being an artificial, in vitro model. Indeed, one of the master regulators of osteoblast differentiation, osterix, was identified using this system (Nakashima et al. 2002). Recently, mesenchymal interstitial cells, but not myogenic cells, were identified as the progenitor cells that differentiate into osteoblasts and chondrocytes during ectopic bone formation induced by BMP-2 in skeletal muscle cells (Wosczyna et al. 2012).

4 Regulation of Ligand Modifications

TGF-β family members have highly conserved amino acid sequences and possess conserved cysteine residues that form both internal and external disulfide bonds (Mueller and Nickel 2012) (Fig. 2). Most TGF-βs form dimers of approximately 30 kDa that consist of two homomeric or heteromeric components linked by a single disulfide bond. The disruption of these disulfide bonds, for example, via treatment with reducing reagents, destroys the biological activities of these proteins. Heterodimers between BMP-7 and BMP-2 or BMP-4 are more potent in inducing ectopic bone formation in vivo and in inducing osteoblastic differentiation in vitro than any of the homodimers (Aono et al. 1995; Israel et al. 1996; Takada et al. 2003; Valera et al. 2010). The domain of BMP-2 that functions in bone induction was identified through the generation of chimeric proteins with the nonosteogenic protein activin (Korupolu et al. 2008).

In cells, the bioactive BMP dimers are made from large precursor proteins (Fig. 2). First, two such precursors form a dimer with a single disulfide bond

linking their C-terminal domains. These precursor complexes are then processed at Arg–X–X–Arg sequences (where X represents any amino acid) by furin family proteinases to release the mature, bioactive dimers, suggesting that this processing step is important for regulating the biological activity of TGF-β family members (Mueller and Nickel 2012). The N-termini of the active dimers of osteogenic BMPs possess stretches of basic amino acids, suggesting a potential role in their bone-inducing activity (Ruppert et al. 1996). In addition, although several BMPs are glycosylated, the deglycosylated proteins can still induce ectopic bone formation in vivo. Indeed, some recombinant osteogenic BMPs can be produced in *Escherichia coli* and refolded in vitro, which allows us to produce large amounts of the active factors at lower costs (Bessa et al. 2009; Yano et al. 2009).

5 Regulation of Receptor Activation

The signals from TGF-β members are transduced via the binding of two types of transmembrane serine (Ser)/threonine (Thr) kinase receptors, namely type I (ALK1, ALK2, ALK3/BMPR-IA, ALK4/ActR-IB, ALK5/TβR-I, ALK6/BMPR-IB, and ALK7) and type II (TβR-II, ActR-IIA, ActR-IIB, and BMPR-II) receptors (Katagiri and Tsukamoto 2013; Mueller and Nickel 2012) (Fig. 3). Type I receptors are distinguished from type II receptors by a conserved "GS domain," which is a glycine and serine-rich juxtamembrane domain located in the intracellular region. In the case of TGF-β and activin, type II receptors are essential for the binding

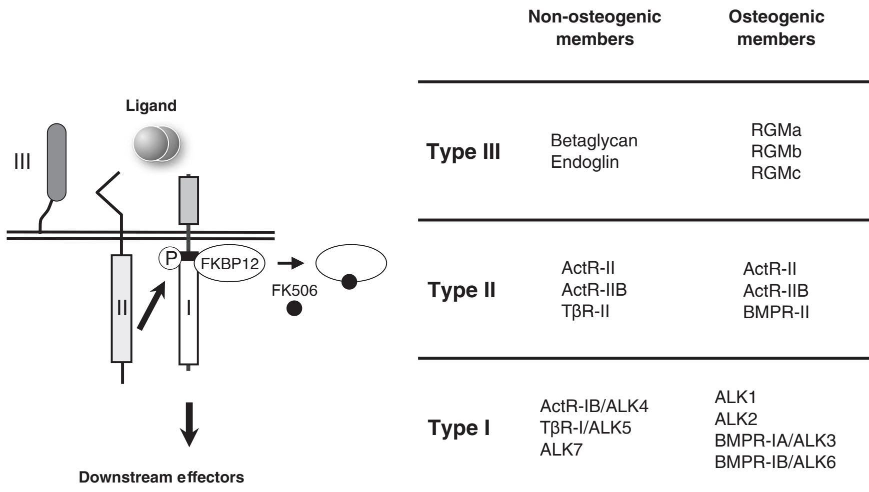

Fig. 3 Regulation of TGF-β receptors. Both type I and type II receptors are transmembrane Ser/Thr kinases. Type III receptors function as co-receptors to enhance ligand binding to type I and type II receptors. Only type I receptors have a GS domain in the juxtamembrane region. FKBP12 binds the unphosphorylated GS domain and inhibits type I receptor kinase activity. FK506 binds to FKBP12 and releases it from the GS domain

of ligands to type I receptors and the subsequent induction of intracellular signal transduction. The three-dimensional structure of a TGF-β-receptor complex further supports the critical role of type II receptors for the ligand-binding capacity of type I receptors (Groppe et al. 2008).

The ligand-binding capacity of the type I and type II receptors can be modified by other ligands and/or by another receptor type called type III receptors (Fig. 3). BMP-3 knockout mice show an unexpected increase in bone mass, suggesting that BMP-3 is a negative regulator of bone formation (Daluiski et al. 2001). The negative effect of BMP-3 seemed to be due to competitive binding by ActR-IIB without the activation of type I receptors, suggesting that inhibitors of BMP-3 will increase bone mass in vivo (Kokabu et al. 2012a). Betaglycan and Endoglin (also known as CD105) act as type III receptors to enhance TGF-β signaling. Repulsive guidance molecules (RGMs), including RGMa, RGMb (also known as DRAGON), and RGMc (also known as hemojuvelin), are GPI-anchored proteins that act as co-receptors for BMPs in iron metabolism (Wu et al. 2012). However, an extracellular domain from DRAGON was reported to suppress BMP-induced osteoblastic differentiation of C2C12 cells; hence, the physiological role of RGMs in bone metabolism remains controversial (Kanomata et al. 2009).

Because both type I and type II receptors possess Ser/Thr kinase domains in their intracellular domains, phosphorylation is a critical event during the induction of intracellular signal transduction by the TGF-β family (Katagiri and Tsukamoto 2013; Mueller and Nickel 2012) (Fig. 3), and directly or indirectly modifying the kinase activity of the receptors changes the biological activity of the ligands. Indeed, ligand binding typically increases receptor kinase activity. Although type II receptors exhibit constitutive kinase activity regardless of ligand binding, type I receptors, which are initially inactive, are activated in response to ligand binding through the phosphorylation of the GS domain by type II receptors. Ligand dimers form a complex on the cell membrane containing two type I and two type II receptors. The phosphorylation of the GS domain in type I receptors releases inhibitors such as FKBP-12, which binds the GS domains of unphosphorylated type I receptors and suppresses their kinase activity; treating cells with FK506, an immunosuppressor and FKBP-12 ligand, releases FKBP-12 from the GS domain (Huse et al. 2001; Nishanian and Waldman 2004). These findings suggest that small chemical compounds that bind to the GS domain can be used to modulate type I receptor kinase activity. In addition, several small molecule inhibitors of type I receptor kinase activity have been developed in order to prevent ectopic bone formation in patients with fibrodysplasia ossificans progressiva, a genetic disorder characterized by ectopic bone formation in soft tissue.

6 Regulation by Antagonists

The bone- and/or cartilage-inducing activities of TGF-β family members are physiologically suppressed by several antagonists present in the extracellular space (Riley et al. 1996; Walsh et al. 2010) (Fig. 4). Noggin was the first BMP

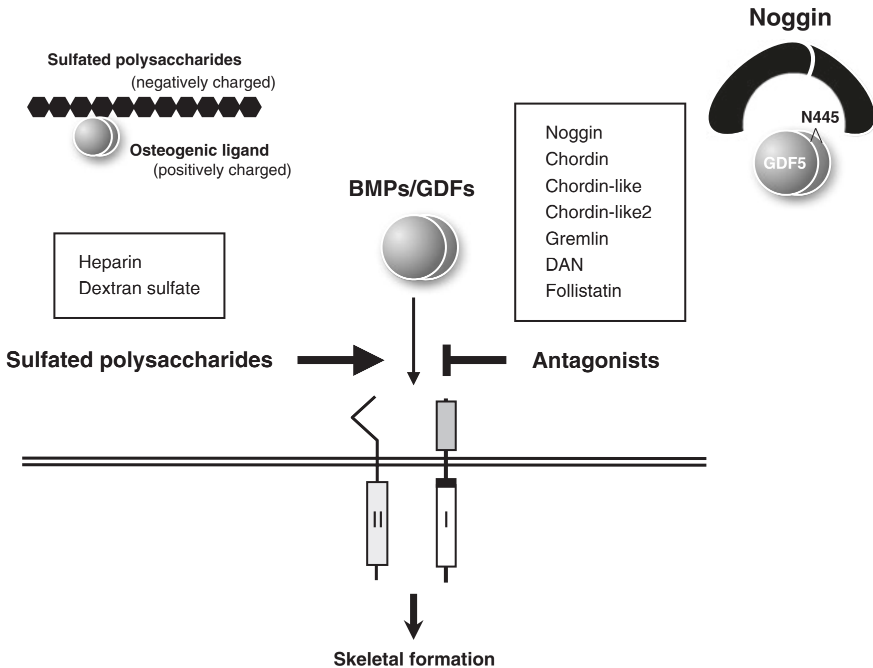

Fig. 4 Regulation of osteogenic BMPs/GDFs by antagonists and sulfated polysaccharides. Several type antagonists have been identified for osteogenic BMPs/GDFs. Antagonists directly bind to the ligands and mask the domains responsible for binding to the functional membrane receptors. In the case of GDF5, substitution mutations that replace asparagine 445 with lysine (N445K) or threonine (N445T) prevent the interaction with noggin and make the mutant proteins resistant to this antagonist. Sulfated polysaccharides, such as heparin and dextran sulfate, enhance BMP-2 activity by protecting the ligands from inhibition by noggin

antagonist to be identified, based on its inhibitory activity against BMP-4 in *Xenopus* embryos. Noggin contains a cystine knot motif, which is a conserved structure found in other BMP antagonists such as Gremlin and DAN (Riley et al. 1996; Walsh et al. 2010). The noggin homodimer binds directly to the active BMP-4 dimer and to other BMPs in the extracellular space, thereby preventing the induction of intracellular signaling. Structural studies indicated that noggin masks a type II receptor-binding domain within BMP-7, suggesting that type II receptors play a critical role in TGF-β signal transduction (Groppe et al. 2002). Noggin mRNA expression increases in response to BMP activity as a part of a negative feedback loop, and loss-of-function mutations in noggin have been identified in patients with skeletal abnormalities, such as multiple synostosis syndrome and proximal symphalangism, indicating that noggin is a physiological inhibitor of BMP activity during skeletal development (Gazzerro et al. 1998; Brunet et al. 1998; Gong et al. 1999; Zimmer et al. 2012). GDF5 is a potential ligand whose biological activity is enhanced by the noggin mutations. Substitution mutations in which asparagine 445 of GDF5 is replaced with lysine (N445K) or threonine

(N445T) were identified in patients with multiple synostosis syndrome (Seemann et al. 2009). Position N445 co-localizes within the type I receptor and noggin interaction sites of GDF5, and the N445K and N445T mutants of GDF5 were insensitive to noggin, compared with wild-type GDF5 or BMP-2 (Seemann et al. 2009). These findings suggest that mutant BMPs/GDFs that are insensitive to antagonists may be effective at inducing bone formation in vivo. Thus, the inhibition of ligand–antagonist interactions is a potential target for stimulating the bone-inducing activity of the TGF-β family.

Sulfated polysaccharides such as native heparin and synthetic dextran sulfate enhance the osteogenic activity of BMPs (Ruppert et al. 1996; Takada et al. 2003; Zhao et al. 2006). In the presence of heparin, BMP-2 induced higher levels of receptor kinase activity in vitro and induced the formation of larger ectopic bones in vivo (Zhao et al. 2006). It has been suggested that the N-terminal basic amino acid stretch found in BMPs serves as the binding domain for heparin (Ruppert et al. 1996) (Fig. 4). At the molecular level, heparin may act by suppressing the inhibitory effect of noggin (Zhao et al. 2006) (Fig. 4). In addition, heparin increased the half-life of active BMP-2 by preventing the binding of BMP-2 to the extracellular matrix (Zhao et al. 2006). Both TGF-βs and osteogenic BMPs induce chondrogenesis in vitro. Interestingly, chondrogenesis was inhibited by the addition of noggin, even though it is a specific antagonist of BMPs, suggesting that TGF-β-induced chondrogenesis is a secondary effect that occurs via osteogenic BMPs (Nakayama et al. 2003). These findings highlight the inhibition of these extracellular negative regulators as a target for tissue engineering of skeletal tissues.

7 Regulation of Intracellular Signaling Effectors

Overexpressing constitutively activated type I BMP receptors in C2C12 cells inhibits myogenesis and induces osteoblastic differentiation without the need for BMP stimulation, suggesting that intracellular effectors are being phosphorylated by type I receptors (Katagiri and Tsukamoto 2013). The Mad and Sma proteins were discovered in *Drosophila* and *Caenorhabditis elegans*, respectively, as signal transducers of the corresponding endogenous TGF-β family members. Eight types of Smad proteins (Smad1 through Smad8) have been identified as the vertebrate counterparts of the Mad and Smad proteins (Fig. 5). Smad proteins all share the Mad homology 1 (MH1) and MH2 domains at their N- and C-termini, respectively (Katagiri and Tsukamoto 2013; Kokabu et al. 2012b; Massague et al. 2005; Zi et al. 2012) (Fig. 5). The MH1 domain is required for DNA binding, while the MH2 domain is required for protein–protein interactions. Smad1, Smad2, Smad3, Smad5, and Smad8 (also known as Smad9) are classified as R-Smads (receptor-regulated Smads) because the SV/MS motif found in their C-termini has been identified as a phosphorylation site for type I receptors. Smad4 is classified as a Co-Smad because it functions as a co-activator of R-Smads in the transcriptional regulation of target genes. Smad6 and Smad7 lack MH1 domains and inhibit type I

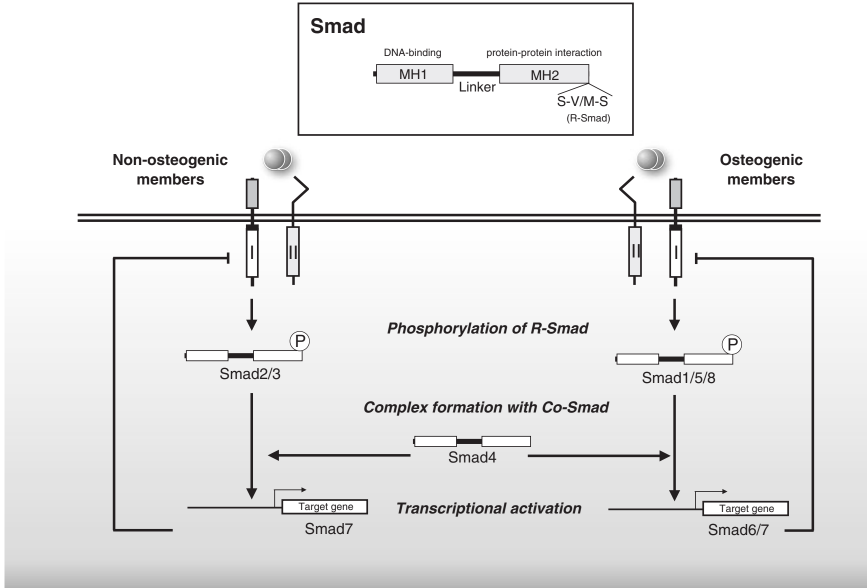

Fig. 5 Smad proteins are critical effectors of intracellular TGF-β signaling. Eight Smad proteins are expressed in vertebrates and are classified as R-Smads (Smad1, Smad2, Smad3, Smad5, and Smad8), Co-Smads (Smad4), and I-Smads (Smad6 and Smad7) based on their structural and functional properties. Osteogenic TGF-β family BMPs/GDFs induce the phosphorylation of Smad1, Smad5, and Smad8, and nonosteogenic BMPs/GDFs induce the phosphorylation of Smad2 and Smad3. I-Smad expression is upregulated by ligand stimulation, resulting in a negative feedback loop

receptors via direct interactions between their MH2 domains and the receptor kinase domains; thus, these Smad proteins are classified as I-Smads (inhibitory Smads). I-Smad mRNA expression is upregulated in response to stimulation by inflammatory cytokines or TGF-β family ligands, suggesting that they form a negative feedback loop during TGF-β signaling (Nakao et al. 1997; Takase et al. 1998) (Fig. 5). It has been suggested that I-Smad inhibitors may enhance the biological activities of TGF-β ligands, although the identification of such molecules has yet to be reported.

Osteogenic BMPs/GDFs induce the phosphorylation of Smad1, Smad5, and Smad8 (also called Smad9), whereas nonosteogenic TGF-βs/activin induce the phosphorylation of Smad2 and Smad3 (Katagiri and Tsukamoto 2013; Kokabu et al. 2012b; Massague et al. 2005; Zi et al. 2012). Thus, the osteogenic and nonosteogenic activities of TGF-βs depend on the differential usage of R-Smad proteins. This hypothesis is further supported by the development of constitutively active R-Smad proteins through the substitution of the SV/MS motif. Overexpression of mutant Smad1 induced ventralization in *Xenopus* embryos

and osteoblastic differentiation in myoblasts in vitro (Nojima et al. 2010). Furthermore, an anti-phospho-Smad1/5/8-specific antibody recognized the mutant Smad1 but not wild-type Smad1, suggesting that conformational changes in the C-terminus induce the transcriptional activity of R-Smads (Nojima et al. 2010). Inhibitors of type I receptor kinase activity block the phosphorylation of R-Smad, and the C-terminus of R-Smad can be dephosphorylated by phosphatases such as protein phosphatase magnesium-dependent 1A (PPM1A) and small C-terminal domain phosphatase 1 (SCP1) (Sanvitale et al. 2013; Bruce and Sapkota 2012; Kokabu et al. 2012b). However, a constitutively active Smad1 mutant, in which the C-terminal motif cannot be dephosphorylated, could still be inhibited by either PPM1A or SCP1, suggesting that these phosphatases may target additional domains and/or molecules to inhibit intracellular signaling by R-Smad Kokabu et al. (2010, 2011). It has also been suggested that chemical inhibitors of these phosphatases may enhance the biological activity of TGF-β ligands.

In addition to inducing the Smad pathway, type I TGF-β receptors also induce Smad-independent signaling pathways (Moustakas and Heldin 2005; Mu et al. 2012; Zhang 2009). The p38 mitogen-activated protein kinase (MAPK) pathway is one such non-Smad pathway that is immediately activated by both osteogenic and nonosteogenic TGF-β family members, although the role of this pathway in the biological activity of TGF-β family members is still unclear. The linker regions in R-Smad proteins contain multiple phosphorylation sites for p38 MAPK, and these sites negatively regulate the transcriptional activity of R-Smads (Kretzschmar et al. 1997). Thus, it seems likely that the biological activity of TGF-β family members is determined by a balance between positive and negative signaling pathways induced by type I receptors.

8 Cross-Talk with Other Signaling Molecules

The Wnt family is one of the most important regulators of bone formation in humans (Baron and Kneissel 2013; Maeda et al. 2013). A loss-of-function mutation in LRP5, which is a membrane receptor for Wnt, was identified in osteoporosis-pseudoglioma syndrome (OPPG) patients with low bone mineral density (Gong et al. 2001). Moreover, a gain-of-function mutation in LRP5 was also found in family members with high bone mineral densities (Boyden et al. 2002; Little et al. 2002). Very recently, WNT1 was identified as a ligand responsible for determining bone mineral density in humans (Laine et al. 2013). Canonical Wnt proteins, such as Wnt1 and Wnt3a, but not noncanonical proteins, such as Wnt5a and Wnt11, stimulated the osteoblastic differentiation of C2C12 cells in vitro in cooperation with BMP-4 (Fukuda et al. 2010). This stimulatory effect could be caused by cross-talk between BMP signaling and GSK3, but not β-catenin, activated by canonical Wnt signaling. The interaction between WNT and BMPs can be observed in sclerosteosis, which is an autosomal recessive disorder characterized by bone overgrowth (Balemans et al. 2001; Brunkow et al. 2001) (Fig. 6). Loss-of-function mutations have been identified

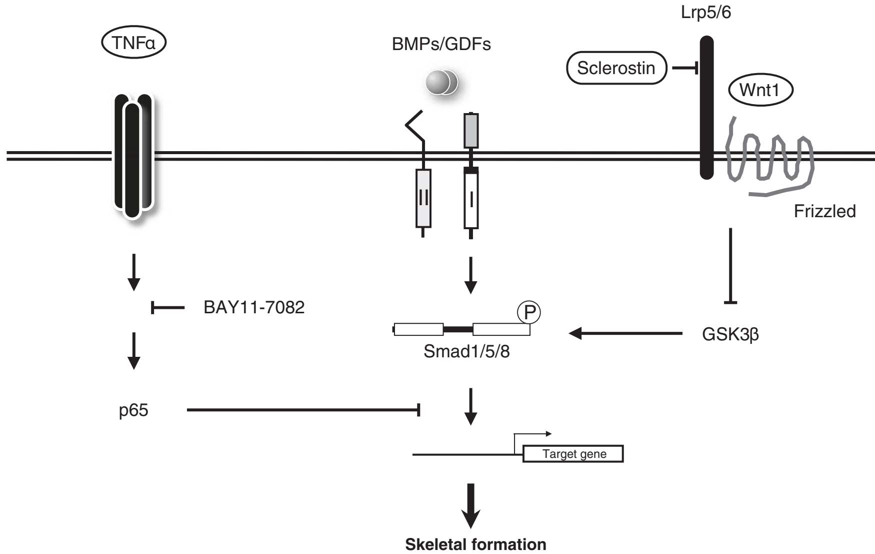

Fig. 6 Cross-talk with other signaling molecules. Wnt proteins that bind to the Lrp5 and Lrp6 receptors, such as Wnt1 and Wnt5a, activate canonical Wnt signaling via the Frizzled receptor-GSK3β pathway. Sclerostin binds to both Lrp5 and Lrp6 and inhibits Wnt signaling. The GSK3β pathway cooperatively increases osteogenic BMP signaling. The inflammatory cytokine TNF-α activates the canonical NF-κB pathway via p65. Activated p65 suppresses BMP signaling by blocking the binding of Smad proteins to their target DNA sequences. BAY11-7082, an inhibitor of the canonical NF-κB pathway, increases BMP activity

in sclerostin, encoded by the SOST gene, which is mainly expressed by osteocytes (Balemans et al. 2001; Brunkow et al. 2001). Although sclerostin has been regarded as an antagonist of osteogenic BMPs, at present, it is classified as WNT antagonist. Sclerostin binds to LRP5 and LRP6 and inhibits WNT signaling, thereby reducing bone mass (Ellies et al. 2006; Li et al. 2005). An anti-sclerostin-neutralizing antibody is being developed as a novel drug for the treatment of osteoporosis, as the systemic administration of this antibody increases bone mineral density in humans (Papapoulos 2011). These findings suggest that similar inhibitors of WNT antagonists may be applicable to the stimulation of bone formation.

The bone-inducing activity of BMPs is suppressed in the presence of inflammatory cytokines, including tumor necrosis factor (TNF)-α. Nuclear factor (NF)-κB signaling is one of the major pathways induced by inflammatory cytokines. In mice lacking p65, a component of the canonical NF-κB pathway, BMP-2 induced the formation of larger ectopic bones in vivo (Yamazaki et al. 2009). It was observed that p65 blocked the DNA-binding capacity of Smads through direct interactions with these proteins (Fig. 6). As expected, treatment with BAY11-7082, a chemical inhibitor of the canonical NF-κB pathway, increased the binding of Smads to their target DNA elements in early responsive genes in vitro and increased ectopic bone formation in mice (Yamazaki et al. 2009).

9 Conclusions

The members of the TGF-β family are critical factors in skeletal formation, and their biological activity is subject to multiple levels of both direct and indirect positive and negative regulation. The inhibition of negative regulators and the stimulation of positive regulators are potential entry points for tissue engineering efforts to reconstruct skeletal tissues.

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BMPs and Wnts in Bone and Cartilage Regeneration

Di Chen, Shan Li and Tian-Fang Li

Abstract Bone morphogenetic proteins (BMPs) play an important role in osteoblast and chondrocyte differentiation and canonical Wnt signaling regulates bone mass. BMP-2 is approved for use in spinal fusions due to degenerative disk disease, and in the treatment of acute open fractures of the tibial shaft. BMP-7 is approved for lumbar spinal fusion and in the treatment of long bone nonunion fractures. Sclerostin monoclonal antibodies are currently under clinical trials for their application in treating patients with osteoporosis and bone fractures. The roles of BMPs and Wnts in bone and cartilage regeneration have been extensively studied in recent years and the progress in this research area is summarized in this chapter.

1 BMP Signaling in Bone and Cartilage Regeneration

Bone morphogenetic proteins (BMPs) are a group of growth factors in the transforming growth factor-β (TGF-β) superfamily (Chen et al. 2004; Cao and Chen 2005). BMPs were originally isolated from bone matrix (Urist 1965; Wozney et al. 1988). However, we now know that BMPs exist in connective tissues of many other organs in the body. For example, BMP-7 is mainly produced in kidney (Ozkaynak et al. 1991; Alper 1994) and BMP-9 is mainly expressed in liver (Song

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et al. 1995). Recombinant BMPs have now been used clinically to treat different types of orthopedic diseases, such as segmental bone defects, nonunion fracture, and for spinal fusion (Gupta and Khan 2005; Garrison et al. 2007).

BMP signaling is a complex process. Smad proteins play a central role in BMP signaling. Smad1/5 transiently and directly interact with activated type I BMP receptors, which phosphorylate the C-terminal SSXS motif of Smad in a ligand-dependent manner (Hoodless et al. 1996; Nishimura et al. 1998). After releasing from the receptor, the phosphorylated Smad proteins form heteromeric complexes with the related protein Smad4, which acts as a shared partner. This complex translocates into the nucleus and participates in gene transcription with other transcription factors (Cao and Chen 2005). Chondrocyte-specific *Smad1/5* double knockout (KO) mice (*Smad1/5^{Col2}*) showed a severe chondrodysplasia phenotype and are embryonic lethal (Retting et al. 2009), suggesting that Smad1/5 signaling is absolutely required for endochondral skeletal development. Since the nuclear translocation of Smad1/5 requires Smad4 binding, the prediction originally was that the chondrocyte-specific deletion of *Smad4* (*Smad4^{Col2}*) will produce similar defects in skeletal development. However, this is not the case. Although *Smad4^{Col2}* mice displayed growth retardation, the skeletal defects of these mice are less severe than those of *Smad1/5^{Col2}* double KO mice and *Smad4^{Col2}* mice survive into adulthood without problems (Zhang et al. 2005a). These findings suggest that, in addition to the Smad4 binding and nuclear translocation, Smad1/5 may be able to use other signaling pathways in chondrocytes.

To better understand bone induction activity among different members of the BMP family, the relative potency of bone formation activity among 14 BMP family members has been compared using an adenovirus gene delivery approach by intramuscular injection of BMP-expressing adenovirus-transduced C2C12 cells into the right quadriceps of nude mice. Radiographic and histological evaluations demonstrated that, in addition to BMP-2 and BMP-7, the well known bone induction agents, BMP-6, and BMP-9 effectively induced ectopic ossification when either AdBMP-transduced osteoblast progenitor cells or the viral vectors were injected into the quadriceps of athymic nude mice (Kang et al. 2004). This study suggests that, in addition to extensively studied BMP-2 and BMP-7, BMP-6, and BMP-9 may also be used clinically for bone and cartilage regeneration approaches.

1.1 Bmp-2

BMP-2 is the most studied BMP family member. BMP-2 is approved for use in spinal fusion due to degenerative disk disease and in treatment of acute open fracture of the tibial shaft (Gupta and Khan 2005; Garrison et al. 2007). The utilization of BMP-2 in segmental bone defects, nonunion fracture, spinal fusion, and other orthopedic diseases has been well documented in recent years (Gautschi et al. 2007; McKay et al. 2007; Khosla et al. 2008; Tumialan et al. 2008; Rosen 2009; Lo et al. 2012; Wei et al. 2012).

Although *Bmp2* has an expression pattern similar to other members of the *Bmp* family, such as *Bmp4*, it seems that *Bmp2* plays a unique role in skeletal

development and fracture healing. The chondrocyte-specific deletion of *Bmp2* (targeted by *Col2-CreER* transgenic mice) showed a severe chondrodysplasia phenotype. In contrast, deletion of *Bmp4* in chondrocytes produced minor changes in skeletal development (Shu et al. 2011). Similarly, deletion of *Bmp2* in limb mesenchymal progenitor cells (targeted by *Prx1-Cre* transgenic mice) led to defects in fracture healing (Tsuji et al. 2006). In contrast, BMP-4 is dispensable for skeletogenesis and fracture healing in the limb tissue, since deletion of *Bmp4* in the mesenchymal progenitor cells using *Prx1-Cre* transgenic mice had minor effects on skeletal development and fracture healing (Tsuji et al. 2008). BMP-2 has been demonstrated to regulate expression of other BMP family members in a paracrine regulation manner (Harris et al. 1994; Ghosh-Choudhury et al. 1994; Chen et al. 1997; Edgar et al. 2007). This may explain why *Bmp2*, but not *Bmp4*, is absolutely required for skeletal development and fracture healing.

Although we know that BMP-2 accelerates fracture healing in different animal models, we do not know on which cell population BMP-2 plays a specific role during the fracture healing process. Using chondrocyte- or osteoblast-specific *Bmp2* conditional KO mice ($Bmp2^{Col2}$ and $Bmp2^{Col1}$), we demonstrated that the fracture healing process was delayed in chondrocyte-specific, but not osteoblast-specific, *Bmp2* conditional KO mice (Mi et al. 2013). This study has provided important information about the time frame for BMP-2 administration when it is used to promote fracture healing.

Bone fracture healing resembles the endochondral skeletal development process and periosteal tissue plays a critical role during fracture healing. The periosteum, which is the membrane that covers the outer surface of long bones, is divided into an outer fibrous layer and inner osteogenic layer. The fibrous layer contains fibroblasts, while the osteogenic layer contains mesenchymal progenitor cells that are able to differentiate into chondrocytes and osteoblasts after a bone fracture (Colnot et al. 2012). Transplantation of a live bone graft harvested from Rosa 26A mice showed that about 70 % of osteogenesis in the graft was attributed to the expansion and differentiation of donor periosteal progenitor cells. Furthermore, engraftment of BMP-2-producing bone marrow stromal cells on non-vital allografts showed marked increases in cortical graft incorporation and neovascularization, suggesting that BMP-2-induced tissue engineered functional periosteum may improve allograft incorporation and repair (Zhang et al. 2005b). This study indicates that periosteal tissue plays a critical role in bone fracture healing and that BMP-2 promotes periosteal progenitor cells to differentiate into chondrocytes and osteoblasts, leading to endochondral bone formation in the fracture callus.

Although BMP-2 has been used successfully to treat different orthopedic diseases, concerns have also been raised. Recent studies suggest that BMP-2 enhances bone resorption in vitro and in vivo. Treatment with BMP-2 in bone grafts might cause a higher nonunion rate compared to nontreatment group, which was attributed to an aggressive bone resorptive phase prior to osteoinduction (Pradhan et al. 2006). In addition, reports also showed that BMP-2-treated bone grafts for spinal fusion lost their original height and structure, probably due to activated bone resorption (Vaidya et al. 2007). It has been reported that treatment

with BMP-2 in a primate bone defect model increased the size of the defect and the number of osteoclasts by inducing bone resorption followed by bone formation (Seeherman et al. 2010). These reports suggest complications in clinical settings where anabolic effects of BMP-2 are expected, but catabolic effects may occur prior to anabolic effects. To prevent catabolic effects of BMPs, several studies of combining BMP therapy with anti-resorptive drugs, such as bisphosphonates, have been conducted. The addition of zoledronic acid to BMP-7 increased a bone volume significantly compared to BMP-7 alone in bone defect and bone graft models in rats (Little et al. 2005; Harding et al. 2008). These reports suggest that combining BMP and bisphosphonate treatments may have synergistic effects on bone regeneration. Randomized controlled clinical trials are required in order to further investigate the efficacy of this combination treatment in patients.

1.2 Bmp-4

1.2.1 Cartilage Repair

The effect of BMP-4 on adult cells is different from those on embryonic stem cells. Muscle derived-stem cells stably expressing *Bmp4* exhibited the chondrocytic phenotype, including *Col2* gene expression. *Bmp4* stably transfected progenitor cells were mixed with fibrin glue and transplanted into cartilage defects in the femoral groves of nude mice. Histological analysis showed that 8 weeks after transplantation, cartilage defects treated with the stem cells overexpressing *Bmp4* were filled with white glossy tissue that was well integrated with the surrounding articular cartilage. The results demonstrated that the transplanted cells became chondrocyte-like cells stained with Safranin O. In contrast, the defects filled with cells stably transfected with LacZ cDNA only contained the fibroblast-like cells (Kuroda et al. 2006).

An important consideration for cartilage repair is possible angiogenesis and osteophyte formation. Muscle-derived stem cells were infected with retroviruses expressing *Bmp4* and soluble *Flt-1* (blocking the VEGF effect). An arthritis model in rats was then established by the intra-articular injection of mono-iodoacetate and the rats were then treated with the cells expressing *Bmp4* and *Flt-1*. The results show that this therapy induced maximal chondrogenesis with undetectable angiogenesis, thus leading to persistent cartilage repair (Matsumoto et al. 2009).

1.2.2 Bone-Tendon-Muscle Interaction

Recent studies suggest that BMP-4 is critical for embryonic development of bone ridges/eminences. Such ridges are the insertion sites of muscles and tendons to bones in embryonic stages and are pivotal for normal biomechanics and the motion

of limbs in adults. Blitz et al. 2009 used the deltoid tuberosity to investigate embryonic bone ridge formation in mice and demonstrated that this process was similar to that of the epiphyseal growth plate. Signals from tendons adjacent to bones initiate the ridge formation and the process was supported and enhanced by the signaling from adjacent muscles. Tendon-specific transcription factor scleraxis (SCX) upregulates BMP-4 expression at the insertion site. The tissue-specific deletion of *Bmp4* in tendons of $Bmp4^{Scx}$ mice resulted in aberrant formation of bone ridges in the axial and appendicular skeletons, indicating that normal *Bmp4* expression in tendons is indispensable for the formation of bone ridges (Blitz et al. 2009). The progenitor cells forming bone ridges are not descendent of chondrocytes; instead, they are the Sox9 and SCX double positive cells regulated by TGF-β in the initial process of bone ridge formation. The subsequent differentiation of such cells is regulated by BMP-4 signaling (Blitz et al. 2013). These observations help us understand the mechanism of the bone-tendon interaction and unravel the pathogenesis of some pediatric orthopedic diseases, such as Osgood-Schlatter syndrome, a disease commonly seen in children about 8 years-old with a major clinical manifestation being pain in the insertion site of the patellar tendon in the tibia (Gholve et al. 2007).

1.3 Bmp-6

BMP-6 null mutant mice show delayed ossification of developing sterna. The observations made by in situ hybridization revealed that *Bmp6* was specifically expressed in the hypertrophic zone of epiphyseal growth plates, implying that BMP-6 can be used as a marker for chondrocyte hypertrophy (Solloway et al. 1998). In *Bmp6* null mutant mice, the diameters of long bones were smaller than their wild-type (WT) littermates, suggesting that BMP-6 may play a role in appositional bone growth. In addition, the longitudinal bone growth was also affected, suggesting that BMP-6 is also important for the normal function of growth plate chondrocytes (Perry et al. 2008). BMP-6 was also expressed in human cartilage and may play a role in maintenance of the homeostasis of articular cartilage (Bobacz et al. 2003).

1.3.1 Cartilage Repair

BMP-6 has been shown to induce the differentiation of adipose tissue-derived stem cells toward chondrocytes with robust expression of *Col2* and *aggrecan* (Estes et al. 2006). In a recent study, adipose tissue-derived stem cells were genetically modified with a baculovirus system for prolonged and sustained production of BMP-6 and TGF-β3. Such cells were cultured in porous scaffolds and transplanted to rabbit knee joints to repair cartilage defects. The induced new cartilage-like tissue exhibited a zonal structure typical of normal articular cartilage. No chondrocyte hypertrophy or joint degeneration was observed.

However, these results were not observed in the rabbits transplanted with the stem cells that transiently expressed BMP-6 and TGF-β3. These findings suggest that prolonged production of these two growth factors and an appropriate scaffold are critical for chondrogenesis and successful cartilage repair (Lu et al. 2014). Consistent with these findings, the injection of adenovirus expressing either BMP-2 or BMP-6 to the knee joint cavity of a pony with large osteochondral defects resulted in the enhanced regeneration of cartilage and subchondral bone, but the long-term effect of such repair was not satisfactory (Menendez et al. 2011).

1.3.2 Bone Regeneration

To investigate the effect of endogenous BMPs, compound deficient mice (*Bmp* $2^{+/-}$*;Bmp6*$^{-/-}$) were generated. Such mice exhibited a reduced bone volume, a phenomenon not seen in single KO mice. Impaired endochondral bone formation, but not intra-membranous growth, was detected in fracture calluses of compound deficient mice, suggesting a synergistic effect of endogenous BMP-2 and BMP-6 in normal bone metabolism and bone repair (Kugimiya et al. 2005). Adenovirus expressing *Bmp6* was injected locally after osteotomy surgery in rabbits. The results demonstrated that BMP-6 is potent for osteoinduction and skeletal repair (Bertone et al. 2004). Non-viral delivery of BMPs holds great promise for skeletal repair. Adipose-derived and bone marrow-derived stem cells were nucleofected with *Bmp2* or *Bmp6* and these cells were mixed with fibrin gel and injected to thigh muscles of mice. Local osteogenesis was monitored by μCT. The results demonstrated that bone marrow-derived cells are superior to the cells from adipose tissue in their potential for osteogenesis and that BMP-6 is a more potent inducer for osteogenesis than BMP-2 (Mizrahi et al. 2013).

1.4 Bmp-7

1.4.1 Cartilage Repair and Arthritis

It has been shown that BMP-7 is expressed in human articular cartilage and BMP-7 increased the synthesis of proteoglyans and collagen type 2 (Col2) in human articular chondrocytes (Huch et al. 1997). The addition of BMP-7 upregulated important molecules for cartilage homeostasis, including hyaluronan and CD44 (Chubinskaya et al. 2000; Nishida et al. 2000). A recent report demonstrated that hyaluronan-CD44 signaling potentiated BMP-7-Smad1 signaling, and loss of CD44 caused partial loss of BMP-7 signaling mediating aggrecan production (Luo et al. 2014).

A model for impact injury in articular cartilage was established in sheep by applying contusive forces to the medial femoral condyles, causing injury to the superficial and middle zones of articular cartilage. The sheep were treated with BMP-7 for different time periods. The results showed that treatment with BMP-7

effectively prevented the progression of joint destruction caused by injury, and that BMP-7 may have a chondro-protective effect on patients with articular injury (Hurtig et al. 2009). Similarly, BMP-7 injection into rat knee joints delayed the cartilage degradation caused by excessive running (Sekiya et al. 2009).

Consistent with these findings, BMP-7 enhanced proteoglycan synthesis in the chondrocytes isolated from donors with osteoarthritis. BMP-7 has a synergistic effect with IGF-1. In normal and osteoarthritic chondrocytes, BMP-7 enhanced proteoglycan synthesis, especially when BMP-7 was added with IGF-1 (Loeser et al. 2003; Chubinskaya et al. 2000). Aging is a significant contributor to OA development and BMP-7 and IGF-1 increased proteoglycan synthesis in chondrocytes derived from either young or aged donors. Aging causes partial inhibition of the chondrogenetic response to IGF-1, or BMP-7 plus IGF-1 in proteoglycan synthesis. Aging-related oxidative stress suppressed the effect of BMP-7 through a p38-Smad1 non-canonical pathway (Loeser et al. 2014).

1.4.2 Meniscus Repair

In a recent study, the effect of BMP-7 on in vivo induction of fibrocartilage was investigated. BMP-7 at different doses was injected directly into the Achilles tendon of adult Lewis rats and the tendon samples were examined at different time points after injection. The results showed that 4-weeks after surgery, fibrocartilage-like tissue were successfully induced from the tendon following BMP-7 injection. The transformed tendon was sutured to repair meniscus defects. Histological and immunohistochemical analysis of the 'tendon-meniscus' samples showed that BMP-7 induced tendon cell transformation to fibrocartilage with enhanced expression of Col2, leading to the regeneration of meniscus and alleviation of articular cartilage degeneration (Ozeki et al. 2013).

1.4.3 Fracture and Spinal Fusion

rhBMP-7 was approved by the FDA in 2001 for the treatment of fracture patients, especially nonunion fractures. BMP-7 has a satisfactory efficacy and an excellent safety profile. Trails have been conducted using BMP-7 with a collagen carrier for revision surgery due to fracture nonunions in different bones, including the tibia and femur. Over 80 % of patients so treated achieved clinical healing. rhBMP-7 and collagen putty have been developed and used for fusion of the cervical and lumbar spine. The outcomes of this treatment are promising despite the common complications, such as soft tissue swelling. Comparative studies of the relative potencies of rhBMP-2 and rh-BMP-7 have been contradictory; one plausible explanation for the discrepancies being the difference in scaffolds. Other factors include the rate of tissue clearance and the numbers of the responding cells near the fracture sites. An important factor that may limit the widespread clinical use of BMP-7 is the cost of the treatment (Lo et al. 2012; Ronga et al. 2013).

1.5 Bmp-9

BMP-9 strongly promoted osteoblast differentiation from mesenchymal stem cells (MSCs) both in vitro and in vivo (Kang et al. 2004; Cheng et al. 2003; Luo et al. 2004; Luu et al. 2007; Peng et al. 2003, 2004). Studies from He's laboratory demonstrated that BMP-9 regulated a distinct set of downstream targets that probably play a role in osteoinduction. Unlike other TGF-β superfamily members, the mature BMP-9 protein retains the N-terminal pro-region that is generally cleaved in other BMPs prior to secretion. Retention of the pro-region did not result in functional inhibition of BMP-9 and may in fact stabilize the mature protein after secretion (Brown et al. 2005). Also, unlike other BMPs, BMP-9 has poor affinity for ALK3 (BMPR-IA), a receptor that generally transduces BMP signaling (Brown et al. 2005). Using dominant-negative mutants of the seven type I receptors, Luo et al. demonstrated that only ALK1 and ALK2 mutants effectively inhibited BMP-9-induced osteogenic differentiation in vitro and in ectopic bone formation assays (Luo et al. 2010). These findings suggest that the mechanisms governing BMP-9-mediated osteoinduction of MSCs may differ from other BMPs (Lamplot et al. 2013).

1.6 Cross-Talk Between BMP and Wnt Signaling

The role of BMPs in skeletal development and pattern formation are well documented, however, the role and mechanism of BMPs in bone formation remain unclear. To investigate the interaction between BMP and Wnt signaling, several in vitro studies using mesenchymal progenitor cell lines or primary osteoblasts have been conducted. Differing results have been found.

Several recent studies show that BMP-2 has a synergistic effect with Wnt ligands and β-catenin. β-catenin was required for BMP-2-induced osteoblast differentiation (Mbalaviele et al. 2005; Chen et al. 2007; Zhang et al., 2009). In vivo studies also demonstrated that BMP-2 induced expression of several Wnt ligands and their receptors, and activated β-catenin-mediated T cell factor (TCF)-dependent transcriptional activity. Mice expressing conditional β-catenin null alleles displayed inhibition of BMP-induced chondrogenesis and osteogenesis (Chen et al. 2007). These findings suggest that BMP-2-induced bone formation may be mediated by canonical Wnt/β-catenin signaling.

In contrast, other reports showed that BMPs induced *Sost* expression in Saos-2 osteosarcoma cells (Yu et al. 2011). Similarly, treatment of cultured calvarial bone with BMP antagonist Noggin increased canonical Wnt signaling (Kamiya et al. 2008). In vivo studies demonstrated that osteoblast-specific conditional KO of BMP receptor type IA (*Bmpr1a*^{Col1}) had increased bone mass during weanling stages. *Bmpr1a*^{Col1} mice show diminished expression of *Sost* and increased Wnt/β-catenin signaling as assessed by Wnt reporter TOPGAL mice and TOP-flash luciferase reporter. Consistent with the negative regulation of the Wnt pathway by

BMPRIA signaling, treatment of osteoblasts with dorsomorphin, an inhibitor of the Smad-dependent BMP pathway, enhanced Wnt signaling. In addition to *Sost*, *Dkk1* was also down-regulated in bone tissue of *Bmpr1a*^{Col1} mice. Expression levels of *Dkk1* and *Sost* were up-regulated by the treatment with BMP-2 and down-regulated by Noggin. Moreover, mice expressing a constitutively active *Bmpr1a* transgene show up-regulation of both *Dkk1* and *Sost* and partially restored the high bone mass phenotype when crossed with *Bmpr1a*^{Col1} KO mice (Kamiya et al. 2010). These results suggest that BMPRIA in osteoblasts negatively regulates bone mass and Wnt/β-catenin signaling. BMPRIA-mediated negative regulation of bone mass may be through promoting *Sost* and *Dkk1* expression in osteoblasts. The discrepancy observed in these studies may be due to stage differences of the target cells.

2 Wnt/β-Catenin Signaling in Bone and Cartilage Regeneration

After more than 10 years research, we now understand that canonical Wnt/β-catenin signaling controls bone mass. Disruption of any molecule in this signaling pathway in genetic mouse models caused significant changes in bone mass (Gong et al. 2001; Babij et al. 2003; Day et al. 2005; Glass et al. 2005; Hill et al. 2005). Human genetic studies also demonstrated that High Bone Mass (HBM) diseases were observed in patients with *Lrp5* gain-of-function mutations or *Sost* loss-of-function mutations (Gong et al. 2001; Boyden et al. 2002; Little et al. 2002; Van Wesenbeeck et al. 2003; Beighton 1976; Beighton et al. 1976; Balemans et al. 2001; Brunkow et al. 2001; Wergedal et al. 2003). LRP5 is a co-receptor of Wnt/β-catenin signaling and sclerostin is a negative regulator of LRP5 signaling (Ke et al. 2012). A recombinant form of parathyroid hormone (PTH), designated Teriparatide or Forteo, is an FDA approved anabolic agent which promotes bone formation in patients with osteoporosis (Tsai et al. 2013). Recent studies suggest that the molecular mechanism of PTH action in bone formation may be through inhibition of *Sost* and *Dkk1* expression in osteocytes and osteoblasts (Keller and Kneissel 2005; Bellido et al. 2005; Silvestrini et al. 2007; Leupin et al. 2007; Guo et al. 2010). Therapeutic PTH is given as a daily subcutaneous injection, and its use is limited to 2 years duration due to observations of induction of osteosarcoma and chondrosarcoma in long-term rodent studies. To better manage osteoporosis and other bone loss-associated diseases, additional bone anabolic agents are needed. Two humanized monoclonal antibodies targeting the Wnt/β-catenin signaling pathway, sclerostin, and Dkk1 antibodies (Scl-Ab and Dkk1-Ab), have been developed in recent years. Preclinical and clinical studies found that these agents have potent anabolic effects on bone formation and fracture healing (Rossini et al. 2013; Weivoda and Oursler 2014).

Sclerostin (Scl) and Dkk1 bind Wnt co-receptors LRP5/6 to inhibit Wnt binding and signaling, leading to a reduction in bone formation. Sclerostin and Dkk1 bind the first β-propeller of LRP5 and LRP6 to inhibit Wnt1 class Wnt signaling (Ettenberg et al. 2010; Bourhis et al. 2010). Dkk1 also binds the third β-propeller to

inhibit Wnt3a class Wnt signaling (Ke et al. 2012). Dkk1 and sclerostin also utilize co-receptors to enhance their inhibitory activity. Dkk1 forms a ternary complex with LRP5 or LRP6 and Kremen receptors 1 or 2, which results in internalization of the complex (Ellwanger et al. 2008; Ke et al. 2012). Scl-Ab and Dkk1-Ab prevent the interaction of these molecules with LRP5 and LRP6, allowing Wnt ligands to bind the LRP5 or LRP6 co-receptor and activate β-catenin signaling.

2.1 Scl-Ab

2.1.1 Scl-Ab in Ovariectomy-Induced Bone Loss

Osteoporosis is a metabolic bone disease characterized by low bone mass and micro-architectural deterioration of bone tissue leading to increased bone fragility. In the United States, approximately 10 million Americans older than 50 years have osteoporosis, and about 1.5 million fragility fractures occur each year. It is estimated that one in two women and one in five men aged 50 years will have an osteoporotic fracture in their remaining lifetime (Harvey et al. 2008).

Sclerostin antibodies (Scl-Abs) have been reported to have significant bone anabolic activity in various animal models. Treatment with Scl-Ab increased bone mineral density and improved cortical and trabecular architecture at the lumbar vertebrae and femur in aged male rats (Li et al. 2010). Treatment with Scl-Ab was associated with marked increases in bone mass at cortical and trabecular sites in gonad-intact primates (Ominsky et al. 2010). Scl-Ab was also found to increase trabecular thickness and bone strength of lumbar vertebrae and the proximal femur (Ominsky et al. 2011). Moreover, increasing bone formation on remodeling surfaces and along quiescent surfaces (modeling surfaces) was found in Scl-Ab treated animals (Ominsky et al. 2014). This implies that treatment with Scl-Ab might exert a modeling effect. The ovariectomized (OVX) rat model is a widely used animal model for hypogonadal estrogen deficiency induced bone loss. Li et al. reported the effect of Scl-Ab on OVX rats (Li et al. 2009). In OVX rats treated with Scl-Ab, trabecular thickness, trabecular BMD and bone volume in distal femur were restored to levels similar to sham controls. In addition, bone formation at the proximal tibia and lumbar vertebrae was significantly increased in Scl-Ab treated rats. Furthermore, treatment with Scl-Ab resulted in increased osteoblast surface and decreased osteoclast surface. Therefore, treatment with Scl-Ab has robust anabolic effects with marked increases in bone formation, and reverses OVX-induced bone loss.

2.1.2 Scl-Ab in Bone Mechanical Strength

In addition to its efficacy in promoting bone formation and increasing bone mass, Scl-Ab also increased mechanical strength of rat bone. Bone strength

parameters, such as peak load, stiffness, and energy to failure were increased in lumbar vertebrae and femoral diaphysis after treatment with Scl-Ab in OVX animals and aged male rats (Li et al. 2010; Ominsky et al. 2010; Li et al. 2009). Scl-Ab also increased bone strength at the femoral neck, the principal site for osteoporotic fracture in humans (Li et al. 2010). These preclinical studies demonstrate that treatment with Scl-Ab promotes bone formation, increases bone mass and bone strength, and reduces the risk of a secondary osteoporotic fracture.

2.1.3 Scl-Ab in Bone Fracture Healing

Skeletal fractures may occur as a consequence of trauma as well as fragility and represent a significant public health problem. Biological therapies, such as local application of BMPs, were developed to accelerate fracture healing and reduce fracture-associated complications. However, to date there are no approved systemic therapies to accelerate fracture healing and reduce fracture-associated complications. It has been shown that Scl-Ab is a potent agent for enhancing fracture healing (Ominsky et al. 2011).

Fracture healing is a complex biologic process, which involves granulation, callus formation, and bone modeling and remodeling. Application of Scl-Ab to enhance fracture healing is an anabolic approach in several bone fracture models. Scl-Ab significantly increased bone mass and bone strength at the site of fracture in a fibular osteotomy model (Ominsky et al. 2011). The fractures in the Scl-Ab group had less callus cartilage with smaller fracture gaps containing more bone and less fibrovascular tissue than the control group. The most recent study has investigated effects on the healing of defects in proximal tibiae of OVX rats (McDonald et al. 2012). Scl-Ab significantly improved repair outcomes, augmenting both intramembranous and endochondral bone formation and enhancing bone formation and bone volume. Diabetes mellitus is recognized as a high-risk factor for fracture incidence and fracture healing delay. ZDF fa/fa rats are an established model of type 2 diabetes mellitus with low bone mass and delayed bone fracture healing. Scl-Ab reversed diabetes-associated low bone density and impaired osteoblast function, improved bone mass and strength, and improved bone defect regeneration in diabetic ZDF rats (Hamann et al. 2013).

2.1.4 Scl-Ab in Osteogenesis Imperfecta

Osteogenesis Imperfecta (OI) is a genetic disorder with the skeletal fragility as the hallmark feature (Cundy 2012). Most patients with OI have mutations in genes encoding type I collagen, *Col1a1* and *Col1a2*, or in genes encoding proteins that participate in the assembly, modification, and/or secretion of type I collagen (Byers and Pyott 2012). LRP5 is a Wnt co-receptor and regulates

bone mass and bone strength in human. Specific missense mutations in *Lrp5* cause an autosomal dominant phenotype characterized by HBM and increased bone strength (Boyden et al. 2002; Little et al. 2002). The HBM-causing missense mutations make LRP5 resistant to its endogenous inhibitors Dkk1 and sclerostin (Boyden et al. 2002; Semenov and He 2006; Balemans et al. 2008; Ellies et al. 2006). To determine if Scl-Ab has potential for use in treatment of OI disease, Jacobsen et al. have performed two proof-of-principle experiments. They showed that increasing bone anabolism via the LRP5 pathway significantly improved bone mass and bone strength in the *Col1a2*$^{+/p.G610C}$ mouse model of OI. *Col1a2*$^{+/p.G610C}$ mice have a missense mutation in the α2 chain of type I collagen, which is identical to that found in a large kindred affected with a moderate form of OI (Daley et al. 2010). The *Col1a2*$^{+/p.G610C}$ mice have lower bone density and bone strength than their WT littermates (Daley et al. 2010). In the first experiment, the authors crossed *Lrp5*$^{+/p.A214V}$ mice with *Col1a2*$^{+/p.G610C}$ mice and determined the effect of the LRP5 HBM allele on bone properties in the offspring. In the second experiment, they administered Scl-Ab (Li et al. 2009) or vehicle alone to WT and to *Col1a2*$^{+/p.G610C}$ mice. They found that *Col1a2*$^{+/p.G610C}$; *Lrp5*$^{+/p.A214V}$ offspring had significantly increased bone mass and strength compared to *Col1a2*$^{+/p.G610C}$; *Lrp5*$^{+/+}$ controls. The improved bone properties were not due to altered mRNA expression of type I collagen or its chaperones, nor were they due to changes in mutant type I collagen secretion. In the second experiment they treated *Col1a2*$^{+/p.G610C}$ mice with Scl-Ab. They found that antibody treated mice had significantly increased bone mass and strength compared to vehicle treated control mice (Jacobsen et al. 2014). These findings indicate increasing bone formation, even without altering bone collagen composition, may benefit patients with OI and that Scl-Ab is a potential treatment for OI disease.

2.1.5 Potential Side Effect

Sclerostin KO (*Sost*$^{-/-}$) mice have HBM with small bone marrow cavities. Hematopoietic cell fate decisions are dependent on the local microenvironment. Osteoblasts and stromal cells support hematopoietic stem cell quiescence as well as facilitate B-cell development. Recent studies demonstrated that the bone marrow of *Sost*$^{-/-}$ mice is specifically depleted of B cells because of elevated apoptosis at all B-cell developmental stages. In contrast, B-cell function in the spleen was normal. Further analysis confirmed that *Sost* is mainly expressed in osteocytes but not in hematopoietic lineage cells, suggesting that the B-cell defects in *Sost*$^{-/-}$ mice are noncell autonomous. This finding was further confirmed by transplantation of WT bone marrow into lethally irradiated *Sost*$^{-/-}$ recipients. WT → *Sost*$^{-/-}$ chimeras displayed a reduction in B cells, whereas reciprocal *Sost*$^{-/-}$ → WT chimeras did not, supporting the idea that the *Sost*$^{-/-}$ bone environment cannot fully support normal B-cell development (Cain et al. 2012). These

results demonstrate a novel role for *Sost* in the regulation of bone marrow environments and B cell development and also suggest that another potential side effect for Scl-Ab is affecting bone marrow B-cell survival.

2.2 Dkk1-Ab

Based on the same principles applied in the development of Scl-Ab, the scientists at the company, Amgen, further developed Dkk1-Ab as an alternative anabolic agent for the treatment of osteoporosis and fracture healing. As predicted, the administration of Dkk1-Ab indeed increased bone formation, reversed ovariectomy-induced bone loss and accelerated fracture healing in animal studies (Li et al. 2011; Agholme et al. 2011). To determine if Dkk1-Ab promotes bone fracture healing through activation of β-catenin signaling, we treated β-catenin conditional KO mice ($\beta\text{-}catenin^{Prx1ER}$) with Dkk1-Ab and found that the Dkk1-Ab-induced fracture healing was significantly delayed in $\beta\text{-}catenin^{Prx1ER}$ mice (Jin et al. 2015). It will be interesting to learn if Scl-Ab and Dkk1-Ab activate β-catenin signaling in different populations of cells during fracture healing. Since sclerostin and Dkk1 have very different expression patterns (Atkins et al. 2011; Moustafa et al. 2012; Guo et al. 2010; Hardy et al. 2012), the prediction is that these two antibodies will act on different populations of cells in periosteum tissue during bone callus formation. Mechanisms of actions of Scl-Ab and Dkk1-Ab on bone require further investigation.

Although Scl-Ab and Dkk1-Ab show promising activities in the treatment of osteoporosis and promoting fracture healing, several issues must be considered, such as the potential role of long-term usage of these antibodies in promoting tumorigenesis, development of osteoarthritis, and other side effects. Although patients with osteoporosis are often elderly and no cancer incidence has been reported in patients with *Lrp5* gain-of-function mutations or *Sost* loss-of-function mutations, long-term monitoring for patients prescribed with these humanized antibodies is necessary. Activation of β-catenin signaling could lead to an osteoarthritis-like phenotype and defects in disk degeneration in mice (Zhu et al. 2009; Wang et al. 2012). Potential side effects, such as osteoarthritis and disk degeneration, require consideration. Recent data suggest that sclerostin is expressed in articular cartilage tissue; however, animals with *Sost* deletion or receiving Scl-Ab do not develop osteoarthritis during aging or following mechanical injury (Roudier et al. 2013). In fact, recent findings demonstrated that systemic bone loss in the spine and periarticular bone loss in the proximal tibia were completely blocked and partially reversed by administration of Scl-Ab, but not by inhibition of tumor necrosis factor (TNF) in hTNF-tg mice. Moreover, Scl-Ab completely arrested the progression of bone erosion in hTNF-tg mice and led to significant regression of cortical bone erosions when Scl-Ab was used in combination with TNF inhibitors (Chen et al. 2013).

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Osteocytes and Bone Regeneration

Gerald J. Atkins, Matthew Prideaux and David M. Findlay

Abstract Bone integrity is essential to maintain its load-bearing capacity and to resist fractures. However, the skeleton can be subject to multiple insults during life, from subtle matrix damage in otherwise intact bone, to frank fracture. Fortunately, bone has a remarkable capacity to repair but because this does not always occur spontaneously, particularly in older individuals, a greater knowledge of the mechanisms of repair is required to enable intelligent intervention. To date, a great deal has been learnt about the roles of osteoblasts and osteoclasts in bone repair, while potential roles of the matrix embedded osteocytes has been much less investigated. Here, we review the evidence for osteocyte involvement in the repair of defects within the bone matrix, such as matrix microdamage, and their potential role in maintenance of a healthy and strong matrix by remodelling the bone from within. We also speculate as to whether osteocytes might be involved in the repair of macro-fractures, by serving as progenitors for the cells that contribute to fracture repair.

1 Introduction

Bone can sustain damage in multiple ways; examples include traumatic fracture in healthy bone or minimal trauma fracture in the frail elderly, orthopaedic procedures, such as drilling or reaming, stress fracture due to overuse, disease processes

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such as Paget's Disease of Bone or subchondral bone damage in osteoarthritis, bone necrosis due to ischemia or chemotherapy and bone defects caused by bone tumours and/or their surgical removal. Fortunately, bone tissue has a remarkable capacity to repair after structural damage and to regenerate after tissue loss. For example, given a favourable environment, both tissue-level matrix damage and macro-scale fractures usually heal, with the bone returning to its pre-injury size and shape. Likewise, bone lost during lactation or periods of weightlessness or bed rest can be recovered. Although bone repair and regeneration have been largely considered with respect to the functions of bone forming osteoblasts and bone resorbing osteoclasts, there is increasing evidence supporting a significant role for osteocytes in the maintenance and repair of bone. Here, we review the evidence for both the direct and indirect involvement of osteocytes in the different modes of bone regeneration.

Osteocytes, which reside in the mineralised matrix of bone, are the dominant cell type in bone, and have diverse roles, both in the skeleton itself and in extra-osseous tissues (Atkins and Findlay 2012; Bonewald 2007). These roles include mechanotransduction (Dallas et al. 2013), regulation of calcium homeostasis (Atkins and Findlay 2012), hormonal regulation of serum phosphate levels (Ito et al. 2014), regulation of haemopoiesis (Fulzele et al. 2013), and regulation of energy storage and use (Brennan-Speranza et al. 2012). Importantly for bone repair, osteocytes are also well placed to detect damage within the bone matrix, and to initiate repair responses. Because of their essential roles, it has become clear that maintenance of healthy osteocytes is central to bone health and it is likely that the loss of osteocytes during ageing is an important driver of bone fragility. Significantly, it has been shown that patients who have suffered a fragility fracture have a lower number of osteocytes per area of bone than age-matched individuals without fracture (Qiu et al. 2003). In addition, osteocytes appear to play an essential role in microfracture callus formation as patients with low osteocyte viability fail to form normal fracture calluses (Dunstan et al. 1993).

2 Bone Matrix Repair by Osteocytes

The best evidence for a role for osteocytes in bone repair is their involvement in the maintenance of bone matrix in an otherwise intact bone. As reviewed below, there are several possible ways that osteocytes do this, with the best understood being an indirect action via induction of osteoclast-initiated bone remodelling. An important mode of bone regeneration is that which is initiated by osteocyte apoptosis. This mechanism of osteocyte-induced repair was first appreciated in the context of the detection, removal and renewal of bone microdamage, but appears to apply also to a variety of, perhaps all, insults that result in osteocyte apoptosis. These include oestrogen deficiency, mechanical unloading of bone, chemotherapy and ischaemia.

2.1 Osteocyte Apoptosis and Bone Matrix Microdamage

Bone microdamage has been demonstrated to initiate signals for the removal and renewal of damaged bone matrix. Osteocytes are ideally situated to detect microdamage within the bone matrix because damage disrupts the dense osteocyte cellular network, comprising cell bodies in lacunae and highly branched and interconnected cell extensions, which run though canaliculi in the bone matrix. It has been shown experimentally that microdamage results in a transient burst of osteocyte apoptosis, which is followed several days later by spatially related osteoclast recruitment (Noble et al. 2003). When microdamage was induced by fatigue loading of rat ulnae, osteocyte apoptosis and the subsequent intra-cortical bone remodelling were found to associate more with linear cracks in the bone matrix than the diffuse type of microdamage, consistent with the latter having little effect on osteocyte viability (Herman et al. 2010). Osteocyte microdamage-induced apoptosis occurs in highly specific spatial association with the subsequent osteoclastic remodelling (Mori and Burr 1993). Verborgt et al. (2002) showed that apoptosing osteocytes close to the site of microdamage express the pro-apoptotic protein Bax, while the cell-survival protein Bcl-2 is expressed by adjacent osteocytes further from the damaged zone. The authors proposed that these mechanisms serve to confine osteocyte apoptosis to sites of microdamage and provide spatial guidance for the resorption processes that occur after microdamage in bone. This provides a potential mechanism for what Parfitt described as the ‘Area Code’, by which pre-osteoclasts find their way out of the circulation to precisely where they are needed in the skeleton (Parfitt 1998). To investigate whether osteocyte apoptosis might be causal of microdamage-induced bone remodelling, Cardoso et al. (2009) performed experiments, in which induction of microdamage in rats was preceded by administration of a pan-caspase inhibitor and showed that the inhibitor blocked both osteocyte apoptosis and the activation of bone resorption. The association between osteocyte death and increased osteoclast formation was also supported by in vitro studies, which demonstrated increased RANKL expression in MLO-Y4 osteocyte-like cells when the cells were damaged by mechanical means (Kurata et al. 2006) or induced to undergo apoptosis by serum starvation (Al-Dujaili et al. 2011). Subsequent investigations in vivo have suggested that, rather than the injured cells themselves producing RANKL, apoptosing osteocytes signal to adjacent viable osteocytes, and that these neighbouring cells produce catabolic signals, including RANKL to initiate osteoclastogenesis and vascular endothelial growth factor (VEGF) to stimulate local angiogenesis (Kennedy et al. 2012). These observations provide an example in the bone context of the general observation in biology that apoptosis is a prerequisite for tissue regeneration (Zimmerman et al. 2013). Thus, for example, skin wounds were shown to heal much more slowly in caspase-3 gene deletion mice, in which apoptosis was deficient (Zimmerman et al. 2013). Moreover, it has also been shown that apoptosing cells signal in a range of important ways to neighbouring cells (Boland et al. 2013), which seems to be phenocopied in a uniquely bone manner to accomplish targeted bone repair.

2.2 Mechanisms of Osteocyte Mediated Matrix Repair

2.2.1 RANKL Mediated Osteoclastogenesis

Osteocytes may orchestrate both osteoclast-mediated repair of bone matrix and also participate directly in the repair process. As reviewed above, osteocyte apoptosis appears to be associated with increased expression of RANKL by populations of osteocytes but RANKL may be part of the comprehensive molecular machinery that osteocytes can call upon to remodel their extracellular space. It is well recognised that RANKL is essential for the differentiation and activation of myeloid osteoclast precursors (reviewed in Findlay and Atkins 2011), in which it binds to its cognate receptor, RANK, thereby promoting osteoclast differentiation and bone resorbing activity (Yasuda et al. 1998). RANKL is a product of both osteoblasts and osteocytes, as well as other cell types, (Findlay and Atkins 2011), but it was recently reported that osteocytes are the predominant source of RANKL for osteoclastogenesis in adult bone (Nakashima et al. 2011; Xiong et al. 2011), at least in mice. Increased RANKL expression in association with osteocyte apoptosis is consistent with the extensive bone resorption, which was observed several days after induction of osteocyte ablation in mice, in association with dramatically increased RANKL expression in the bone (Tatsumi et al. 2007). We have also shown that exposure of osteocytes to exogenous sclerostin, a protein whose expression is largely confined to osteocytes (Poole et al. 2005), increases the expression of a number of genes, including a sufficient increase in RANKL production by the cells to support osteoclast formation and activity (Wijenayaka et al. 2011). In these experiments, an increased rate of osteocyte apoptosis was not observed, suggesting that viable osteocytes may also initiate osteoclastogenesis and progress osteoclastic bone resorption. It is noteworthy that as an osteoclast tunnels through the bone matrix, it would encounter an osteocyte cell process every 2–3 μm in three dimensions, providing ample opportunity for intercellular contact (Atkins and Findlay 2012). Jones and Boyde presented electron micrographic evidence that at least some osteocytes in the path of resorbing osteoclasts survived and could be 'liberated' by the osteoclast (Jones and Boyde 1977), supported by others (Suzuki et al. 2003), while other evidence suggests that osteoclasts engulf (dying) osteocytes (Suzuki et al. 2003; Elmardi et al. 1990; Boabaid et al. 2001; Kogianni et al. 2008), both of which scenarios could provide the opportunity for direct osteocyte-osteoclast communication.

2.2.2 Osteocyte Induction of Angiogenesis

Recent studies have suggested that osteocytes may play an important role in angiogenesis during fracture repair. MLO-Y4 osteocyte-like cells release high levels of VEGF when undergoing apoptosis and conditioned media from these cells induced endothelial cell proliferation, migration and tubule formation in vitro (Cheung et al. 2011). Prasadam et al. (2014) have also shown that the co-culture

of MLO-Y4 cells with human endothelial cells (HUVEC) resulted in upregulation of angiogenic gene expression and capillary formation by these cells. These effects were attenuated by inhibition of VEGF. These findings, in addition to the proximity and connectivity of osteocytes to the bone vasculature, suggest that one of the functions of osteocytes in fracture repair is to initiate and regulate angiogenesis.

2.2.3 Osteocytic Osteolysis

Other osteocyte products are likely to exert direct effects on bone surrounding osteocytes. Recent evidence has been obtained to support the older concept of 'osteocyte osteolysis', a term describing the removal by osteocytes of mineral and probably also organic matrix surrounding the cells (Atkins and Findlay 2012; Belanger et al. 1967; Teti and Zallone 2009; Qing and Bonewald 2009). This process is so far understood in the context of release by the bone of mineral stores to contribute to calcium homeostasis, for example in lactation (Qing and Bonewald 2009). Several investigators have demonstrated that osteocytes produce TRAP in association with osteocyte osteolysis (Qing and Bonewald 2009; Tazawa et al. 2004; Nakano et al. 2004). In addition, Qing et al. (2012) reported increased expression by osteocytes in bone of lactating animals of a number of genes, which collectively suggested that osteocytes remove mineralised matrix by utilising molecular mechanisms similar to those in resorbing osteoclasts. These genes included cathepsin K, carbonic anhydrases 1 and 2, subunits of proton pumps, and matrix metalloproteinase (MMP) 13 (Qing et al. 2012). Interestingly, we have observed that treatment of osteocytes in vitro with exogenous sclerostin also causes increased expression of the above genes, as well as others, which collectively suggest the induction of a catabolic phenotype in osteocytes by sclerostin. In particular, we have reported that sclerostin-induced carbonic anhydrase 2 can acidify the extracellular space and solubilise bone mineral (Kogawa et al. 2013). We have also shown that exposure of osteocytes to polyethylene particles in a 3-dimensional matrix induced a catabolic phenotype, characterised by increased expression of RANKL, IL-8, M-CSF (Atkins et al. 2009) and MMP-13 (Atkins et al., unpublished data).

It is not known whether the ability of osteocytes to remove and replace their extracellular matrix is used by these cells to repair damaged bone matrix. However, a clue that this could be the case is provided by a recent study, in which a role for MMP-13 in the remodelling and maintenance of bone matrix was examined in MMP-13 deficient mice (Tang et al. 2012). These mice displayed regions of hypermineralisation, associated with altered osteocyte morphology, in the mid-cortical zone of long bones, which had reduced fracture resistance. The defects localised to the same mid-cortical bone regions where osteocytes normally show MMP-13, TRAP and sclerostin expression. It was additionally shown that MMP-13 is required for lactation-induced osteocyte peri-lacunar remodelling and for the maintenance of bone quality, measured in terms of matrix organisation, bone fracture toughness and post-yield behaviour. These changes could not be accounted for in terms of altered osteoblast or osteoclast parameters and the authors therefore

proposed that osteocyte perilacunar remodelling of mid-cortical bone matrix requires MMP-13, which is therefore essential for the maintenance of bone quality. How this maintenance occurs mechanistically, and how the matrix replacement component takes place, is not known.

It is interesting to speculate as to the drivers of the expression of catabolic genes in osteocytes. In the case of osteocyte osteolysis for the purposes of calcium homeostasis, these are likely to be hormonal. For example, perilacunar remodelling during lactation does not occur in animals deficient in the parathyroid hormone (PTH) receptor specifically in osteocytes (Qing et al. 2012), suggesting a primary role for PTH in this process.

2.2.4 Osteocyte Control of Bone Matrix Mineralisation

Stimuli to target matrix repair or maintenance could include unloading of bone or increased mineralisation of the matrix, since the former is known to increase sclerostin expression in bone (Robling et al. 2008; Lin et al. 2009) and, as mentioned, hypermineralisation was observed in MMP-13 deficient mice. The latter is consistent with experiments showing that calcium is taken up into bone (alive or dead) along a physicochemical gradient (Stevens and Ray 1967), suggesting that cellular activity is required to modulate this uptake in the interests of maintaining correctly mineralised bone with the optimal biomechanical characteristics. Evidence suggests an important overall role for osteocytes also in the regulation of bone mineralisation. As reviewed in detail by Rowe (2012), osteocytes express a number of proteins important for regulating mineral uptake into newly formed bone: matrix extracellular phosphoglycoprotein (MEPE), produced by late osteoblasts and osteocytes, contains an acidic serine aspartate-rich MEPE associated (ASARM) motif. ASARM peptides released by the proteolytic enzyme cathepsin B bind to nascent calcium phosphate crystals and potently inhibit further mineralisation. Osteopontin, which also contains an ASARM motif, has also been shown to act in this way (Addison et al. 2010). The osteocyte-expressed enzyme, phosphate regulating gene with homologies to proteases on the X-chromosome (PHEX), degrades both MEPE and osteopontin-derived ASARM peptides and releases the inhibition of mineralisation (Rowe 2012; Addison et al. 2010). We reported that one of the actions of sclerostin appears to be to promote MEPE-ASARM activity in pre-osteocytes, and inhibit that of PHEX, shifting the balance between these opposing entities towards inhibition of mineralisation (Atkins et al. 2011). We also reported that calcium itself promotes mineralisation, which was regulated by a MEPE-PHEX response, at least by differentiating osteocytes (Welldon et al. 2013). The osteocyticosteolysis machinery, discussed above, could also be an important control mechanism for bone mineralisation. For example, osteocyte-derived carbonic anhydrase-mediated acidification of the perilacunar matrix, another process which is regulated by osteocyte-expressed sclerostin (Kogawa et al. 2013), may have an effect on the overall mineralisation of both newly formed and mature bone tissue.

3 Osteocytes in Fracture

There has been considerable conjecture about the origin of the mesenchymal progenitor cells that contribute to fracture repair. As reviewed by Murao et al. (2013), progenitor cells have been reported to arise from the bone marrow, the endosteum, vessel walls, surrounding muscle and adipose tissues soft tissues, and the circulation. Many questions remain, but recent studies, using cell lineage tracking techniques, have provided strong evidence that the periosteum plays a crucial role in fracture healing (Murao et al. 2013; Colnot 2009; Ushiku et al. 2010) and contributes precursor cells to the chondrocytes and osteoblasts, respectively, which populate the soft and hard callus. It is not clear whether progenitor cells arise from the bone itself, although there are both new and older data that support this possibility. First, established methods of obtaining human osteoblast-like cells from human bone involved mincing trabecular bone into small pieces, followed by extensive collagenase treatment to remove cells adherent to the bone surface (Robey and Termine 1985). Histologic analysis of the surface of the collagenase digested chips showed that they were almost completely devoid of adherent cells, however, after 1–2 weeks cells were seen emerging from the chips, which then rapidly grew to confluence. It is difficult to escape the conclusion that these cells were osteocytes that had escaped from the bone matrix. In our own work, we have found that human or bovine trabecular bone chips in culture also release cells that rapidly lose their osteocyte gene signature and dedifferentiate to an osteoblast phenotype (Atkins et al., unpublished data). The mechanism, by which cells might escape the bone matrix, is not characterised but it is likely that dedifferentiation of an osteocyte involves the adoption of a catabolic phenotype, as described above for osteocytic osteolysis. Given that mature osteocytes contain relatively few organelles, it is also likely that the cells would adopt a more blastic phenotype, manifesting as dedifferentiation. We have observed that human bone chips exhibit increased expression of the collagenases MMP-13 and cathepsin K (Fig. 1), and release calcium and βCTX fragments into the medium, suggesting osteocyte-mediated degradation of the bone matrix within the chips. Moreover, as discussed above, we have reported that when human bone chips are treated with sclerostin, this catabolism is significantly increased, evidenced by an increased expression of the catabolic mediators carbonic anhydrase 2, cathepsin K and TRAP, and a measurable increase in the size of the osteocyte lacunae (Kogawa et al. 2013). A mouse correlate of these human experiments was recently described, showing that collagenase digested mouse compact bone fragments rapidly (by 48 h) released cells, which again were most likely to have been osteocytes in the bone (Zhu et al. 2010). These cells are described by the authors as mesenchymal stem cells, on the basis of their surface markers, and had the demonstrated capacity to (Han et al. 2004) differentiate into osteoblasts, adipocytes and chondroblasts. Most recently, Torreggiani et al. (2013) have used cell lineage marking to positively identify mouse bone outgrowth cells as osteocytic in origin, using time-lapse photography to show the outward migration of cells previously embedded within the bone

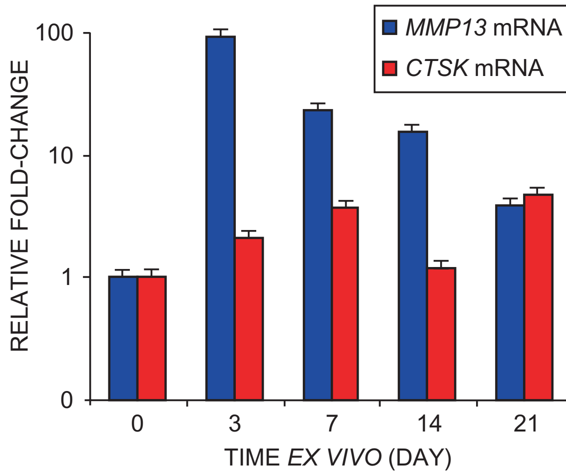

Fig. 1 Human cancellous bone chips cultured ex vivo increase over time their expression of the catabolic enzymes MMP13 and cathepsin K (CTSK), both of which derive from osteocytes. Bone was isolated from the proximal femur of a patient undergoing total hip arthroplasty, dissected into ~4 mm^2 pieces and cultured in αMEM in the presence of 10 % foetal calf serum, as described (Kogawa et al. 2013), for up to 21 days. Relative gene expression was assessed by real-time reverse transcription PCR (Kogawa et al. 2013) for *MMP13* and *CTSK* mRNA

matrix. However, these cells were shown capable of de- or re-differentiation into either osteoblasts or adipocytes, depending on the conditions. These authors found extensive contamination of the released cells with cells expressing haemopoietic markers and it was suggested that the contaminating cells arose from the intracortical vascular elements. The authors speculated that exposure of the bone by fracture might mobilise osteocytes to participate in the repair process. This intriguing possibility remains to be explored.

4 Osteocytes as Regulators of Bone Formation

The maintenance of bone mass and integrity, the repair of fracture and the return of bone shape after fracture, all depend on loading of bone. Moreover, bone repair is only successful to the extent that the bone regains sufficient strength to support the loads that it must bear. Osteocytes have a key role in the regulation of bone formation and the mechanotransduction that underpins it.

The osteocyte network can be considered as a functional syncytium throughout the bone matrix, which is ideally placed to both sense and respond to skeletal loading. There is accumulating evidence that osteocytes are the most important mechanotransducing cells in bone (Bonewald 2007; Han et al. 2004; Santos et al. 2009) and ablation of osteocytes in mice resulted in defective

mechanotransduction and severe bone loss (Tatsumi et al. 2007). It is thought that small physiologic strains imposed on bone are amplified at the level of the osteocyte by the induction of interstitial fluid flow though the lacuno-canalicular network (Han et al. 2004; Fritton and Weinbaum 2009). It was recently demonstrated that cyclic end-compression of the mouse tibia with a moderate loading magnitude (3 N peak load or 400 με surface strain at 0.5 Hz) significantly enhanced solute transport through the lacunar-canalicular system, compared with diffusion alone (Price et al. 2011). Bone loading activates a number of signalling pathways in osteocytes, which may converge to alter Wnt/β-catenin, or canonical Wnt, signalling (Bonewald and Johnson 2008; McBride and Silva 2012). Canonical Wnt signalling, which occurs when Wnts bind frizzled receptors heterodimerised to low density lipoprotein receptors (LRPs) -5 or-6, leads to accumulation of intracellular β-Catenin, which translocates to the nucleus to modulate the expression of multiple genes, including those required for bone formation (Baron and Kneissel 2013). High bone mass conditions due to activating mutations in *LRP5* (Little et al. 2002), and inactivating mutations in the *SOST* gene, which codes for the Wnt antagonist sclerostin (Brunkow et al. 2001), support the key role of Wnt signalling in bone. As discussed above, sclerostin is largely expressed by osteocytes in bone, and acts to inhibit bone formation by osteoblasts (Poole et al. 2005). Sclerostin thus provides a link between loading of bone and bone formation. Robling et al. (2008) showed that *Sost* mRNA and sclerostin protein levels in rodent osteocytes were dramatically reduced by ulnar loading, with the greatest effect in regions of the ulnar cortex receiving the largest strain stimulus. On the other hand, hindlimb unloading resulted in a significant increase in osteocyte expression of *Sost* mRNA in the tibia. The authors concluded that modulation of sclerostin levels appears to be ‘a finely tuned mechanism, by which osteocytes coordinate regional and local osteogenesis in response to increased mechanical stimulation’ (Robling et al. 2008). It thus appears that osteocytes, through their expression of sclerostin, impart a tonic brake on bone formation and that bone formation can occur in specific sites in bone where the concentration of sclerostin is decreased. This essential finding was reported recently by Moustafa et al. (2012).

These data provide a mechanistic explanation for the observed loss of bone due to unloading (Eser et al. 2004; Lang et al. 2004) and suggest that bone repair might require appropriate loading of the bone. They further suggest that antagonising sclerostin might enhance fracture repair, especially of unfixed fractures and fractures for which loading across the fracture site is not feasible. Accordingly, it has been reported that mice lacking the *SOST* gene showed improved healing of femoral fractures, in terms of accelerated bridging, increased callus and bone formation and increased strength of the callus (Li et al. 2011). Similar findings were reported in rat and primate fracture models treated with monoclonal antibodies to sclerostin, in which antibody treatment appeared to enhance fracture repair and increase the biomechanical indices of fracture healing (Ominsky et al. 2011). The potential efficacy of sclerostin antagonism for repair of fractures and bone defects has been reviewed recently (Gamie et al. 2012). The authors also list two clinical trials to investigate the effects of neutralising sclerostin antibody on fresh tibial

diaphyseal fracture healing with an intramedullary nail fixation and unilateral hip fracture following surgical fixation (both trials with scheduled completion late 2012). It remains to be seen, however, whether any effect of neutralising sclerostin on fracture healing is due to inhibition of paracrine signalling from osteocytes adjacent to the fracture site or inhibition of sclerostin expression by cells in the fracture callus itself.

5 Summary

Repair of damage in bone, whether matrix damage within otherwise intact bone or frank fracture likely requires direct and indirect contributions from osteocytes. Further consideration of the role of osteocytes in the complex processes of repair, in addition to those of osteoblasts and osteoclasts, will provide a more complete and ultimately more useful knowledge base, from which to conceptualise new interventions to improve the repair and maintenance of bone in skeletal disease conditions.

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Skeletal Stem Cells for Bone and Cartilage Tissue Regeneration

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Abstract There is an increasing interest in using stem cells in treatment of degenerative diseases such as Parkinson's disease, liver failure, leukemia, diabetes, osteoarthritis (OA), and osteoporosis, for which there is no curative therapy. In this context, skeletal tissue regeneration is being addressed for a number of common clinical conditions including repair of non-healing fractures and bone defects through transplantation of skeletal stem cells (also known as stromal or adult or mesenchymal stem cells, MSCs) either alone or with osteoinductive/osteoconductive scaffolds. In the present Chapter, we will present biological characteristics of MSCs and will give an update regarding their use in skeletal tissue regeneration in preclinical animal models and in clinical trials.

Keywords Skeletal stem cell · Regenerative medicine · Osteogenesis · Bone diseases · Fracture · Scaffold

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1 Bone Marrow Microenvironment and Bone Marrow Stem Cells

Stem cells are traditionally defined as cells that can self-renew and differentiate into a progeny of mature cells (Becker et al. 1963). Stem cells are responsible for tissue homeostasis and repair following damage by generating differentiated progeny. In the postnatal bone marrow; two distinct stem cell systems co-exist: hematopoietic stem cells (HSCs) that give rise to different blood cells, and skeletal (stromal or mesenchymal) stem cells (BMSCs) that give rise to stromal components of the bone marrow and have the ability to differentiation into osteoblastic bone forming cells.

BMSCs are non-hematopoietic, plastic adherent, multipotent, mesodermal germ layer-derived cells that were first identified by Friedenstein et al. (1987) as bone marrow osteogenic stem cells. They are capable of in vitro differentiating into cells of mesodermal lineage including osteoblasts (Kassem et al. 1993), adipocytes (Abdallah et al. 2005) and chondrocytes (Johnstone et al. 1998). When transplanted in vivo (with osteoconductive carrier), they form bone and bone marrow microenvironment and they maintain this capacity during serial transplantations in vivo; providing evidence for their stemness (Sacchetti et al. 2007). In culture, BMSCs are defined by their expression of a number of stromal CD markers, e.g., CD146, CD90, CD73, CD140b, and CD166 while being negative for hematopoietic CD markers: CD31, CD45, CD34, and MHC class II. However, these markers are not specific and they are not able to distinguish true MSCs present within the stromal cell population, from their differentiated progeny (Kuznetsov et al. 1997). Thus, there is a need to identify novel cellular and molecular markers predictive for MSC "stemness". Using DNA microarrays and bioinformatic analysis, our group has identified a number of non-canonical markers that were predictive for the in vivo bone formation ability of BMSCs (Larsen et al. 2009). In addition to their presence in bone marrow, BMSC-like cell populations have been isolated from the stromal compartment of a number of tissues including adipose tissue, umbilical cord, dental pulp, skeletal muscle, synovium, and periodontal ligament (Akiyama et al. 2012; Asakura et al. 2001; De Bari et al. 2001; Seo et al. 2004; Bianco et al. 2001; Zuk et al. 2001; Kermani et al. 2008). Microarray analysis of molecular signatures of these BMSC-like cell populations demonstrated that they are not identical and they exhibit differences in their gene expression pattern and differentiation potential dependent on their tissue of origin (Al-Nbaheen et al. 2013).

Bone marrow stem cells reside in a specialized microenvironment within the bone marrow termed the "Niche" that helps to maintain the identity of stem cells and regulate their functions (Méndez-Ferrer et al. 2010). The in vivo location of BMSCs has been suggested to be in a perivascular niche in close association with pericytes and endothelial cells (Crisan et al. 2008). Interestingly, MSCs form a supportive niche for HSCs in the bone marrow (Mendez-Ferrer et al. 2010) demonstrating the existence of a complex interaction between MSCs and HSCs with clinical relevance to bone and bone marrow regeneration. The BMSC niche is

influenced by a number of chemokines and adhesion molecules. For instance, the stromal cell derived factor-1 alpha (SDF-1α, CXCL12) and its cognate chemokine receptor CXCR4 (chemokine receptor type 4, fusin or CD184) axis play an important role in stem cell homing to bone marrow (Lapidot and Kollet 2002) and injured tissues (Granero-Molto et al. 2009; Wynn et al. 2004). A number of recent studies have shown that osteoblastic cells residing along the endosteal bone surfaces provide a second niche for HSCs that regulate their functions. An increase in osteoblastic cell number and activity leads to an increase in HSC numbers (Calvi et al. 2003) while induced ablation of osteoblastic cells leads to hematopoietic failure (Visnjic et al. 2004). Understanding the biology of stem cell niche may allow developing therapies targeting BMSCs and new approaches to enhance homing of BMSCs to injured skeletal tissues.

2 MSCs: Road to Clinical Use

Bone has a tremendous capacity for self-repair; however, complicated fractures, injuries, or surgically induced defects following resection of tumors or osteomyelitis, are common clinical problems that challenge normal healing processes and conventional surgical procedures. BMSC-based therapy for tissue regeneration has been the focus of a large number of preclinical and clinical studies. Bone marrow derived MSCs are suitable cells for clinical application due to their ease of isolation, stable multi-potency phenotype (Zhao et al. 2010; Nauta and Fibbe 2007), and their excellent safety record (Lepperdinger et al. 2008). MSCs have been employed in an increasing number of clinical studies for enhancing tissue regeneration following injury of both skeletal tissues (Wakitani et al. 2007) as well as non-skeletal tissues, e.g., type I diabetes mellitus (Bhansali et al. 2009; Estrada et al. 2008), Crohn's diseases (Duijvestein et al. 2010; Liang et al. 2012), and following myocardial infarction (Chen et al. 2004b; Hare et al. 2009) and the initial results of several Phase I or Phase I–II trials using stromal stem cells are encouraging (Horwood et al. 2012). The effects of BMSCs on nonskeletal tissue regeneration are based on their ability to secrete regeneration enhancing, inflammation modulating and immune regulatory humoral factors (Aldahmash et al. 2012), the exact identity of these factors are only partially known.

2.1 MSC Homing to Injured Tissues

Most of the clinical studies where BMSCs have been employed were performed using local administration routes (Table 1). However, systemic intravenous infusion of BMSCs is also being tested as a clinically relevant approach. Intravenous infusion is the standard route for HSC therapy in which HSC can home from the circulation to bone marrow and launch hematopoiesis (Magnon and Frenette 2008).

Table 1 Examples of published case reports or clinical studies using adult derived stem cells in patients suffering from bone or cartilage damage

Disease	Study design	Cell type	No. of subjects	Route	Outcome	Reference
Non-union bone fractures	Case series	Autologous bone marrow stem cells	3	Local transplant of loaded hydroxyapatite scaffolds	Good integration of the transplant at the edges of the host bones	Quarto et al. (2001)
	Observational	Autologous bone marrow stem cells	60	Percutaneous	Bone union in 88.3 % of the 53 patients treated	Hernigou et al. (2005)
Achondroplasia and limb hypoplasia	Case series	Autologous bone marrow stem cells (culture-expanded in platelet rich plasma (PRP)	3 (2 + 1)	Local	Acceleration of new bone formation during bone lengthening	Kitoh et al. (2004)
Osteogenesis imperfecta	Case series	Allogeneic bone marrow stem cells	6	Systemic	83 % of the treated children showed improvement in the symptoms and slower progression of the disease	Horwitz et al. (2002).
Osteoarthritis	Double blinded control study	Autologous adipose derived stem cells	40 (20 + 20)	Intra articular	A large and significant reduction in total pain scores	March (2013)
	Observational cohort study	Autologous BM derived stem cells or chondrocytes	72 (36 + 36)	Intra articular	Significant improvement in the patients quality of life in both groups	Nejadnik et al. (2010)

The ability of BMSCs to home to injured tissues is supported by a number of observations where endogenous BMSCs homed to injured tissues of animal models of tissue injury like the brain (Ji et al. 2004) or following acute radiation syndrome (Yang et al. 2012; Lange et al. 2011). Also, the existence of circulating osteoprogenitors or MSC-like cells in the blood suggest their capacity for homing to bone marrow or inflammatory sites (Pignolo and Kassem 2011). Human BMSCs do express variable amounts of many chemokine receptors (Sordi et al. 2005; Wu and Zhao 2012), some of which were identified previously to regulate the homing of leukocytes and HSCs (Mohle et al. 1998; Quesenberry and Becker 1998), but their precise role in BMSC homing is still under investigation. Unfortunately, current studies testing homing of unmodified human BMSCs to tissues (including bone marrow) following intravenous infusion demonstrated their limited homing potential (Bentzon et al. 2005; Karp and Leng Teo 2009). Thus, novel approaches that enhance homing of MSCs to skeletal tissues are needed to further enhance the use of MSCs for clinical applications.

3 Specific Uses of BMSCs in Skeletal Regeneration

Because of their ability to generate bone or cartilage, transplantation of BMSCs has been examined in a number of clinical conditions and is being considered as an attractive alternative approach to bone autograft or allograft techniques.

3.1 Fractures and Bone Defects

Treatment of a number of bone diseases can leave a defect too large for bridging by natural healing and usually requires extensive surgical reconstruction. The use of BMSCs to enhance the repair of complicated fractures has been tried with success (Table 1). Results from preclinical animal models suggest that combining BMSCs with growth factors enhances bone regeneration capacity of the cells and this approach has been tested using genetically modified MSCs (also see below Sect. 3.2). For example, when BMSCs overexpressing VEGF and BMP2 were systemically administered in mice with induced tibial bone defects, they resulted in enhanced bone formation caused by increased osteoblastic cell number as well as by enhanced tissue vascularity at the fracture site (Kumar et al. 2010b). In another study, murine MSCs over-expressing Osterix (an osteoblastic specific transcription factor) were implanted in calvarial bone defects in mice and they resulted in improved healing of bone defects where the total amount of newly formed bone was five times greater in the Osterix treated group compared to controls (Tu et al. 2007).

Human studies tested the ability of a number of heterogeneous cell populations to enhance bone repair. Hernigou et al. (2005) have demonstrated that injection of a autologous bone marrow aspirates (containing a small population of BMSCs)

into the site of bone nonunion fractures in 60 patients resulted in bone union in the 53 (88.3 %) of patients treated. Remarkably, the seven remaining patients in the study that showed failure of bone healing exhibited considerably lower CFU-F (fibroblastic colony forming unit) count, which is a surrogate measure of the number of BMSCs in bone marrow aspirate (Hernigou et al. 2005), providing circumstantial evidence for the importance of BMSCs in bone healing. In a small case series, autologous MSCs were established from bone marrow aspirates and cultured in platelet rich plasma (PRP) followed by transplantation to the site of distraction (femur and tibia) in patients with achondroplasia ($n = 2$) or limb hypoplasia ($n = 1$) undergoing distraction osteogenesis for limb lengthening. Healing was observed in the treated patients with enhancement of new bone formation (Kitoh et al. 2004).

3.2 Osteoporosis

Osteoporosis is a systemic bone disease characterized by low bone mass and bone fragility increasing the risk of fractures of vertebrae, femoral neck, and other peripheral bones. Osteoporosis is caused by impaired bone formation and thus enhancing bone formation through BMSC transplantation is an attractive potential treatment option. Genetically modified MSC overexpressing factors known to enhance their bone forming capacity, e.g., (BMP) (Chen et al. 2004a), α-4 integrin (Yamamoto et al. 1998) or Runx2 (Zhao et al. 2005) have been tested in in osteoporotic animal models. Overexpressing α-4 integrin and BMP2 in murine cells have shown to improve homing to bone marrow and to increase bone mass following systemic infusion (Kumar et al. 2010a). Similarly, a murine bone marrow cell line overexpressing CXCR4 was intravenously administrated in mice with glucocorticoid-induced osteoporosis in and led to enhanced homing of injected cells to bone marrow and consequently an increase in bone mass in the study group (Lien et al. 2009). While it may seem that treatment of osteoporosis with a MSC infusion is a futuristic scenario, one clinical trial has tested this possibility. A trial involving six children with severe osteoporosis due to osteogenesis imperfecta (OI) received systemic infusions of allogeneic BMSCs that resulted in an improvement in their bone mass and halting the progression of the disease in 83.3 % of the children (5 out of 6) and with evidence for engraftment of injected cells in bone (Horwitz et al. 2002).

3.3 Cartilage Repair and Rheumatic Diseases

Rheumatoid arthritis (RA) and osteoarthritis (OA) are among the most common rheumatic diseases, with the latter being most prevalent and a major cause of disability in the aging population worldwide. RA is a complex autoimmune disease

that results in cartilage and bone destruction due to production of inflammatory cytokines, such as TNFα and IL-1β. The use of MSCs in cartilage tissue regeneration is based on their ability to secrete humoral factors with immunomodulatory, anti-inflammatory, and regeneration promoting characteristics (Kristensen et al. 2012). In this regard, the outcome of local delivery of ex vivo cultures of MSCs has been promising in preclinical models (Al Faqeh et al. 2012; Ter Huurne et al. 2012; Desando et al. 2013). For example, surgically induced OA in goats, caused by unilateral excision of the medial meniscus and resection of the anterior cruciate ligament, was treated by intra-articular administration of autologous MSCs in hyaluronan solution and resulted in an enhanced regeneration of the medial meniscus and a decrease in bone and cartilage damage (Murphy et al. 2003). In a recent study, injection of GFP labeled adipose-tissue derived MSCs (ASCs) on day 7 after the onset of collagenase induced OA in rabbits showed localization of ASCs within the synovium and a decreased disease progression (Desando et al. 2013).

Few human studies have tested the effects of BMSC injections on cartilage regeneration. In an observational study, the effects of autologous bone marrow MSCs or cultured chondrocytes on OA progression were tested in 72 patients that were locally injected with either MSCs ($n = 32$) or cultured chondrocytes ($n = 32$). Patients in both groups showed significant improvement in “quality of life” but no differences could be observed between both groups. Results from the osteoarthritis stem cell advanced research study (OSCARS) were recently announced. This was the first randomized clinical trial utilizing ASC in OA treatment. Forty patients were randomized to receive either a single intra-articular injection of autologous adipose-derived cells (average: 50 million cells/dose) or placebo into their knee joint space. A large and significant reduction in total pain scores, serum level of macrophage migration inhibitory factor (MIF) were observed in the treatment group months after cell treatment with no side effects observed (March 2013).

4 Concluding Remarks

BMSCs provide a useful stem cell type for skeletal tissue regeneration. The coming years will witness an increasing number of patients participating in clinical trials receiving unmodified or genetically modified MSCs either alone or within osteoconductive or osteoinductive scaffolds. The cells will be administered through a number of routes either locally or systemically. Results from these trials will establish the exact role of BMSC-based therapies in skeletal regeneration.

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Manipulation of Macrophages to Enhance Bone Repair and Regeneration

Sina Nassiri, Pamela Graney and Kara L. Spiller

Abstract Bone is a unique tissue in that it has the ability to heal itself perfectly, without scarring, under certain conditions. Therapeutic strategies that harness its powerful repair processes have the potential to successfully regenerate bone tissue in large defects, which remain a significant clinical challenge. However, despite the demonstrated importance of the inflammatory response in dictating the success or failure of implanted biomaterials, it is not often considered as an important criterion in the design of tissue engineering scaffolds. This chapter first highlights the role of macrophages in orchestrating the delicate balance between bone formation and resorption. Then, the main strategies that have been explored to actively control the inflammatory response are discussed, including delivery of mesenchymal stem cells, controlled release of immunomodulatory cytokines, and topographical modification of biomaterial scaffolds. Increased understanding of macrophage phenotypes (M1, various M2's, osteoclasts, etc.) will allow us to design therapeutic strategies that tip the balance toward healthy bone regeneration and away from pathologic bone loss.

1 Introduction

Bone is a unique tissue in that small fractures heal perfectly, without scarring (Carano and Filvaroff 2003). Despite this natural ability for bone repair and regeneration, healing of large bone defects remains a significant challenge in orthopedic medicine (Hausman and Rinker 2004). Currently, the standard treatment involves harvesting autologous grafts from other locations in the body and transplantation into the defect, or the transplantation of allografts, which have many drawbacks such as limited tissue supply, donor site morbidity, infection, and poor integration

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(Betz 2002). This intrinsic capacity of bone for self-repair suggests that its natural repair processes can be harnessed to promote regeneration of large bone defects, which remain a significant challenge in orthopedic medicine (Hausman and Rinker 2004).

At the heart of bone repair lies the inflammatory response, orchestrated primarily by macrophages, including osteoclasts, macrophages derived from circulating mononuclear cells in response to injury, and the recently described OsteoMacs (Chang et al. 2008). This chapter will first highlight the roles of macrophages in bone repair and resorption, which hang in the balance of inflammation, and then describe some promising strategies to manipulate the behavior of macrophages to enhance bone regeneration.

2 Role of Inflammation in Bone Healing

In adults, bone fracture disrupts the circulation, triggering a coagulation cascade and inflammatory response (Schindeler et al. 2008). A fibrin mesh forms around the fracture site, and inflammatory cells—first neutrophils and then macrophages—migrate through the fibrin matrix, degrading it and promoting remodeling and healing. Pro-inflammatory signals, especially tumor necrosis factor-α (TNFα) and interleukin-1β (IL1β) secreted mainly from infiltrating macrophages, recruit and stimulate differentiation of MSCs into osteoblasts necessary for bone formation (Glass et al. 2011; Rifas et al. 2003; Bocker et al. 2008). These inflammatory cytokines also stimulate the activation of osteoclasts, a type of bone-resident macrophage that resorbs bone, an important part of remodeling (Kobayashi et al. 2000). Invading capillaries from the bone marrow bring new osteoclasts and osteoprogenitor cells (Andersen et al. 2009). Osteoblast–osteoclast interactions, together with new blood vessel infiltration, drive the coupling of new bone formation and bone resorption (Martin and Sims 2005; Matsuo and Irie 2008) (Fig. 1). This complex process is so successful that most fractures are completely repaired with no evidence of scarring or lasting damage (Carano and Filvaroff 2003).

The importance of inflammation in the initiation of bone healing has been demonstrated experimentally. For example, removal of the fracture hematoma at early times after injury (30 min to 4 days) impaired fracture healing (Grundnes and Reikeras 1993; Park et al. 2002). Moreover, infusion of TNFα to mouse bone fractures at early times (<24 h) after injury significantly accelerated healing (Glass et al. 2011). The addition of the inflammatory cytokines TNFα and IL1β to cell culture media caused increased osteogenesis of mesenchymal stem cells (Lu et al. 2013a).

It should be no surprise that inflammation is especially critical for bone healing, considering its highly vascularized nature (more access to inflammatory cells) and proximity to the bone marrow (the body's reservoir of inflammatory cells). Moreover, bone repair cannot occur without angiogenesis, which relies

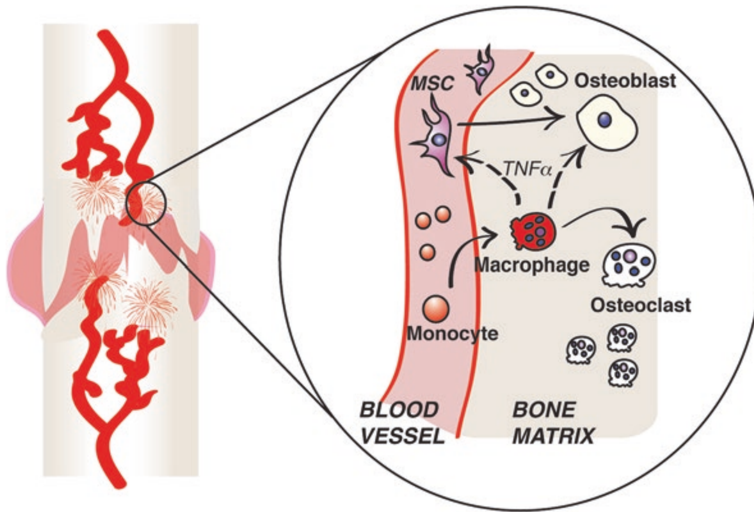

Fig. 1 Role of inflammation in bone repair. Damage to the circulation triggers the inflammatory response. Monocytes are recruited to the site of injury and differentiate into macrophages, which secrete inflammatory cytokines that activate osteoblasts, osteoclasts, and MSCs to generate new bone tissue

on inflammation for initiation and regulation. At the center of it all is the macrophage, the primary cell of the inflammatory response that has long been recognized as a crucial regulator of healing (Murray and Wynn 2011). In response to injury, monocytes are recruited from the circulation and differentiate into macrophages, which exert downstream effects on osteoblasts, osteoclasts, and endothelial cells in the fracture environment. Both mature monocytes and macrophages have the potential to differentiate into bone-specific macrophages, or osteoclasts, in part through release of RANKL by osteoblasts (Martin and Sims 2005; Udagawa et al. 1990). Bone also contains a population of bone-resident macrophages that are distinct from osteoclasts, which have been termed OsteoMacs (Wythe et al. 2014). These OsteoMacs were shown to be part of a canopy covering sites of new bone formation in bone homeostasis and fracture repair (Chang et al. 2008; Alexander et al. 2011). They were required for efficient mineralization of osteoblast cultures in vitro, and their depletion in vivo drastically inhibited new bone formation. Macrophage colony-stimulating factor (MCSF), the main cytokine responsible for the differentiation of macrophages from monocytes, is elevated during fracture healing (Sarahrudi et al. 2010). Macrophages have also been shown to stimulate osteogenic differentiation of MSCs in vitro (Champagne et al. 2002; Nicolaidou et al. 2012; Guihard et al. 2012). Thus, macrophages represent an extremely important cell in bone formation and repair.

3 Impact of Chronic Inflammation on Bone Healing

Although the process of inflammation is critical for the initiation of bone healing, prolonged inflammation beyond the initial phase (~4 days) leads to impaired healing in bone (Schmidt-Bleek et al. 2012) and numerous other tissues (Krishnamoorthy 2006; Khallou-Laschet et al. 2010; Kigerl et al. 2009). Risk of fracture is increased and fracture healing is impaired in patients with chronic inflammatory diseases, such as Crohn's disease (Wythe et al. 2014; Loftus et al. 2002). In one study, increased systemic inflammation, achieved by injecting the inflammatory stimulus lipopolysaccharide into the circulation of rats, caused decreased mechanical properties in bone hematoma after fracture (Reikeras et al. 2005). Macrophages themselves have been pinpointed as important players in pathological bone destruction (Kaneko et al. 2001) and osteoporosis (Cenci et al. 2000). Accordingly, selective depletion of bone macrophages by delivery of bisphosphonates is the most common treatment of osteoporosis (Eslami et al. 2011).

The negative consequences of improper activation of inflammatory macrophages are most comprehensively studied in the context of aseptic loosening in total joint replacements (TJR). More than one million TJR surgeries are performed annually worldwide, in which arthritic or degenerated knee and hip joints are replaced with artificial prostheses (Gallo et al. 2013; Ingham and Fisher 2005; Rao et al. 2012). Although these TJRs are considered successful, they only last for about 10–20 years, resulting in difficult decisions about whether or not to have the procedure for younger patients who do not want to undergo revision surgery while in their 70s, 80s, or 90s.

Due to the heterogeneity of patient needs, prostheses for TJR have been designed using a variety of materials in different combinations, such as metal-on-metal, ceramic-on-ceramic, metal-on-polyethylene, and ceramic-on-polyethylene. The most frequent mode of failure in TJR is the generation of submicron wear debris from articulating implant surfaces (Ingham and Fisher 2005; Nich et al. 2013; Sundfeldt et al. 2006), which activate macrophages and osteoclasts to secrete pro-inflammatory cytokines and proteases that damage the periprosthetic tissue and result in implant loosening (Vanos et al. 2013). In order to overcome TJR failure and minimize the need for revision surgery, recent research efforts have been dedicated to understanding the interactions between macrophages and implant-derived wear debris. These studies are outlined in Table 1.

In vitro studies of macrophages incubated with submicron polyethylene (Green et al. 2000; Matthews et al. 2000a) and titanium (Taira et al. 2010; Valles et al. 2008) particles have clearly demonstrated the relevance of particle size and composition on the phagocytosis activity and response of macrophages. Green et al. (2000) showed an inverse correlation between particle size and biological reactivity, which was also dependent on volumetric particle concentration. In a murine air pouch model, titanium alloy (Ti-6Al-4V) particles elicited more pronounced inflammatory reactions histologically compared to particles of ultra-high molecular weight polyethylene (UHMWPE), poly(methyl methacrylate)

Table 1 Investigations on the response of macrophages to wear particles

Material	Particle size (μm)	Particle volume (μm^3) to cell ratio	Cell line or species	Outcome	References
In vitro					
Polyethylene	0.24, 0.45, 1.71, 7.62, 88	0.1, 10, 100, 1,000	C3H murine cells	Increased bone resorption activity and TNFα secretion by 0.24 (10, 100 μm^3), 0.45, 1.71 μm (100 μm^3) particles	Green et al. (2000)
	0.21, 0.49, 4.4, 7.2, 88	10, 100	HPBMCs	TNFα, IL-6, GM-CSF secretion stimulated by 0.21, 0.49 μm particles	Matthews et al. (2000a) Matthews et al. (2000b)
Alumina ceramic	0.5, 1.5	100[a]	HPBMCs, U937	Enhanced cytokine secretion by 0.5 μm particles	Yagil-Kelmer et al. (2004)
	0.005–0.02, 0.2–10, 0.5	1, 10, 100, 500	HPBMCs	TNFα secretion enhanced at 100, 500 μm^3 particle volumes; 0.5 μm more stimulatory	Hatton et al. (2003)
PMMA	0.1–1, 0.1–10, 1–10, >10	1, 10, 100	HPBMCs	Enhanced cytokine secretion by particles <10 μm containing radio opaque additives	Mitchell et al. (2003)
In vivo					
UHMWPE	3.6	5 % (w/v) particles in 500 μL suspension (SC)	Murine air pouches	Marked increase in IL-1β and IL-6 in response to all particulate biomaterials; UHMWPE and PMMA elicit synergistic increase	Wooley et al. (2002)
PMMA	5.7				
Co–Cr	2.3				
Ti-6Al-4 V	4.0				
Titanium	0.38	12.5 mg (IP)	BALB/c mice	Solid Ti particles induce potent Th2-type inflammatory responses	Mishra et al. (2011)

[a]Reported as particles/cell. Particle sizes shown represent the mean particle size reported

PMMA poly(methyl methacrylate); *UHMWPE* ultrahigh molecular weight polyethylene; *Co–Cr* cobalt–chrome; *Ti-6Al-4* V titanium alloy; *SC*, subcutaneous injection; *IP* intraperitoneal injection; *C3H* murine peritoneal macrophages; *HPBMCs* human peripheral blood mononuclear cells; *U937* human monocytic cell line

(PMMA), or cobalt–chrome (Co–Cr), while UHMWPE particles elicited the thickest fibrous capsule (Wooley et al. 2002). These results suggest that the composition of the particle stimulus plays a role in tissue response. It has also been proposed that the ceramic-on-ceramic prostheses have limited osteolytic potential due to the low wear rates and high volumetric concentration of wear debris needed to generate an osteolytic response (Hatton et al. 2003). Overall, these findings confirm that the size, composition, and volumetric concentration of wear debris are critical factors in macrophage activation. As a result, it is conceivable that modulation of the immune response to minimize inflammation and promote bone healing and repair can overcome periprosthetic osteolysis and aseptic loosening and maximize the longevity of artificial joints.

4 Macrophage Polarization as a Potential Therapeutic Strategy

The explanation behind the apparently contradictory roles of macrophages in bone regeneration and resorption may be related to macrophage polarization states. Despite relatively few studies in the context of bone healing, it is well known from healing of other tissues that macrophages exist on a broad spectrum of phenotypes, ranging from pro-inflammatory to anti-inflammatory, with wildly varying functions (Mosser and Edwards 2008). Manipulation of macrophages from the pro- to the anti-inflammatory states has been used to promote healing in a variety of tissues (see Sect. 5), although its potential for bone regeneration is yet to be fully realized.

In general, pro-inflammatory macrophages, also called "classically activated" or "M1", dominate at early times (1–3 days) after injury, while anti-inflammatory macrophages, also called "alternatively activated" or M2, take over at later stages (4–10 days) (Fig. 2). M1 macrophages secrete inflammatory cytokines that recruit

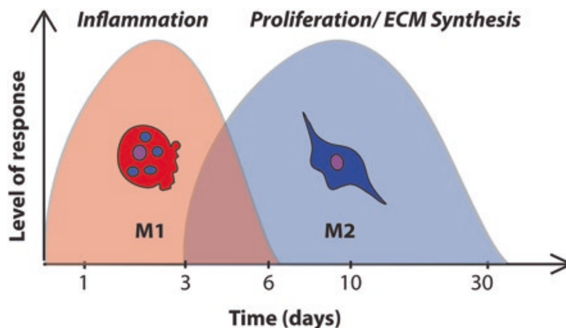

Fig. 2 Macrophage populations in the normal response to injury. M1 macrophages are present at early stages and initiate the process of angiogenesis, and M2 macrophages dominate at later stages, promoting blood vessel stabilization and synthesis of extracellular matrix (ECM) components

other inflammatory cells, initiate angiogenesis, and proteolytic enzymes that clear the area of debris and bacteria (Mosser and Edwards 2008). M2 macrophages promote extracellular matrix (ECM) synthesis, matrix remodeling, and anastomosis of blood vessels (Mosser and Edwards 2008; Fantin et al. 2010; Outtz et al. 2011; Spiller et al. 2013, 2014). If the M1-to-M2 transition is disrupted, depicted by persistent numbers of M1 macrophages, the injury is chronically inflamed and healing is impaired (Kigerl et al. 2009; Brown et al. 2012, 2009). These concepts have also been applied to biomaterial implantation. Increased numbers of M2-activated macrophages in the vicinity of implanted biomaterials at later time points (i.e., not within the first 3 days following implantation) are associated with integration and healing, whereas persistent numbers of M1 macrophages coincide with chronic inflammation (Brown et al. 2009; Madden et al. 2010; Hamlet and Ivanovski 2011). The relative numbers of M1 and M2 macrophages, often determined from immunohistochemical analysis, is approximately proportional to biomaterial integration (Krishnamoorthy 2006; Kigerl et al. 2009; Wang et al. 2007; Mokarram et al. 2012). Thus, macrophages represent an attractive target to reverse damaging inflammation surrounding TJR and to enhance bone regeneration strategies.

To complicate matters further, macrophages of diverse functional phenotypes have been described after culturing them in vitro with various stimuli (Fig. 3). Many of these phenotypes have been designated as a subtype of M2, despite

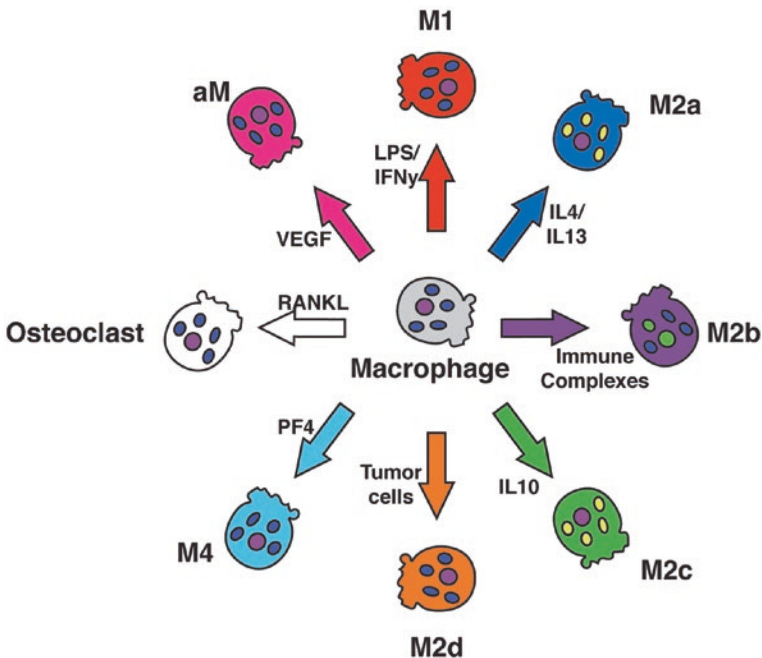

Fig. 3 Diverse macrophage phenotypes and nomenclature. *IL4* interleukin-4; *IL13* interleukin-13; *IL10* interleukin-10; *PF4* platelet factor 4; *RANKL* receptor activator of nuclear factor kappa-B ligand; *VEGF* vascular endothelial growth factor

sharing very little similarities with the originally described "alternatively activated" macrophages stimulated with the T-helper 2 cytokine interleukin-4 (IL4) (Stein et al. 1992). These macrophages are now called M2a, while those stimulated with immune complexes are called M2b (Edwards et al. 2006), those stimulated with IL10 are called M2c (Lolmede et al. 2009), and those stimulated via coculture with tumor cells have been termed M2d (Wang et al. 2010). Different phenotypes have also been described after simulation with vascular endothelial growth factor (VEGF) (Mayer et al. 2012; Medina et al. 2011) and PF4 (Gleissner 2012), and osteoclasts are prepared in vitro by adding RANKL (Kobayashi et al. 2000).

Although a number of studies have compared M2 sub-phenotypes at genomic and protein secretion levels (Ambarus et al. 2012; Becker et al. 2012; Lu et al. 2013b), similarities and differences among macrophage subtypes are still under investigation and their role in various physiologic and pathophysiologic conditions is only beginning to emerge. For instance, M2a and M2c macrophages have been shown to differ substantially with respect to their ability in phagocytosis of apoptotic cells (Zizzo et al. 2012; Leidi et al. 2009). Importantly, macrophages can also switch back and forth between phenotypes in a relatively quick and easy way (Stout et al. 2005; Porcheray et al. 2005; Arnold et al. 2007). Such plasticity not only enables macrophages to quickly adapt to their surrounding environment and perform their protective role as key players of the innate immune system, but also makes them a potential therapeutic target in cases of dysfunctional macrophage activation. For example, Rao et al. (2012) recently reported that patients receiving revision TJR because of aseptic loosening displayed elevated levels of M1 macrophages relative to M2 macrophages in the periprosthetic tissue, consistent with numerous studies that have demonstrated in vitro that macrophages polarize to the M1 phenotype in response to clinically relevant wear debris (Pajarinen et al. 2013). When IL4 was added in vitro to macrophages stimulated to the M1 state with PMMA particles, they efficiently switched to the M2 phenotype (Rao et al. 2012; Antonios et al. 2013), suggesting that therapies that induce M2 polarization of periprosthetic macrophages might inhibit osteolysis and reduce implant loosening in TJR.

5 Strategies to Actively Manipulate Macrophage Behavior

While M1 polarization of macrophages at early times after bone fracture is beneficial for repair, they must transition to the M2 phenotype in order to resolve the healing process. Successful conversion of M1 macrophages to an M2 phenotype with positive effects on healing has already been described in animal models to facilitate repair of multiple tissues, including injured spinal cord (Mokarram et al. 2012), kidney (Jung et al. 2012), and bone (Das et al. 2013a). The main strategies that have been explored to manipulate macrophage behavior include the delivery of cells that can exert effects on macrophages, the delivery of drugs and proteins from drug delivery systems or

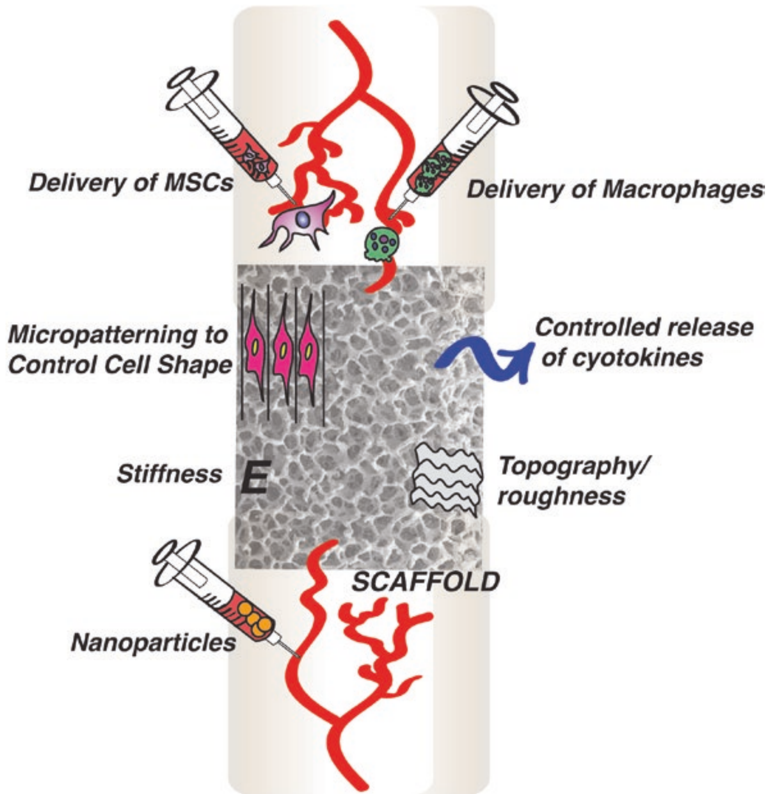

Fig. 4 Strategies to actively control macrophage phenotype

biomaterial scaffolds, gene delivery strategies, and physical modification of scaffold properties (Fig. 4). In the following section, these strategies are described with particular emphasis on those that have been employed in bone regeneration.

5.1 Cell Delivery

Cell-based therapy is an active area of research in tissue engineering in various applications (Telukuntla et al. 2013; Menzel-Severing et al. 2013; Kaigler et al. 2013). Given their prominent role in wound healing and tissue regeneration, macrophages are an ideal cell source for cell-based therapy. Delivery of unactivated macrophages has been shown to increase angiogenesis in animal models of ischemic tissue (Hisatome et al. 2005; Hirose et al. 2008). Recently, macrophages activated ex vivo to the M2 phenotype were administered intravenously to rats with an experimental autoimmune encephalitis model of multiple sclerosis at 3

and 5 days after clinical onset (Mikita et al. 2011). M2 macrophages ameliorated clinical symptoms compared to unactivated control macrophages. A similar strategy protected against renal injury in rats (Mikita et al. 2011). However, challenges face the delivery of macrophages for therapeutic use, including their limited survival time in vitro as well as obstacles to manufacturing and quality assessment. Nonetheless, advances in mimicry of the in vivo cell niche combined with sophisticated bioreactor design make the promise of macrophages as a therapeutic cell source a realizable goal.

While macrophages are yet to be fully appreciated as a source for cell therapy, the delivery of MSCs is becoming increasingly popular for treatment of numerous diseases (Kaplan et al. 2011). It is now known that MSCs are immunomodulatory, causing M2 activation of macrophages (Kim and Hematti 2009), a phenomenon that has been confirmed in vivo (Zhang et al. 2010). For example, infusion of MSCs after induction of myocardial infarction in a mouse model caused M2 polarization, coincident with reduced numbers of apoptotic cardiomyocytes and increased cardiac function (Dayan et al. 2011). When MSC-seeded scaffolds were implanted into mice as venous interposition grafts, the MSC-mediated vascularization of polymeric scaffolds was entirely dependent on the action of recruited macrophages, shown by simulating the effects of MSCs with alginate microparticles that released monocyte chemoattractant protein (Roh et al. 2010). Interestingly, after osteogenic differentiation of MSCs on collagen scaffolds over 4 weeks in vitro, they elicited a predominantly M1 response compared to non-cell-seeded scaffolds in calvarial defects in rats, suggesting that they may lose their immunomodulatory potential as they differentiate in culture (Lyons et al. 2010).

5.2 Drug and Protein Delivery

Controlled delivery of proteins, especially bone morphogenetic proteins, from drug delivery systems and biomaterial scaffolds is widely used clinically to promote bone regeneration (Spiller and Vunjak-Novakovic 2013). The delivery of macrophage-manipulating cytokines is only recently being explored. For example, when IL33 was administered twice weekly to transgenic mice that overexpress human *TNFa*, which develop spontaneous joint inflammation, bone macrophages shifted toward the M2 phenotype and bone loss was reduced (Zaiss et al. 2011).

For large bone defects, an osteoconductive and biodegradable scaffold is required to support the growth of new bone tissue, so strategies that incorporate immunomodulation directly into scaffold design would be highly desirable. In one approach, recruitment of macrophages was achieved by delivering FTY720, an S1P receptor-targeted drug, from biomaterial scaffolds (Das et al. 2013a, b). S1P is a signaling lipid mediator shown to be involved in trafficking and migration of immune cells and osteoblasts (Sefcik et al. 2011; Pederson et al. 2008). Sustained release of FTY720 from poly(lactic-co-glycolic acid) (PLGA)-based scaffolds caused increased recruitment of M2 macrophages to scaffolds implanted

in mandibular and tibial defects in rodents, leading to increased scaffold integration, vascularization, and formation of new bone (Das et al. 2013a; Petrie Aronin et al. 2010; Awojoodu et al. 2013).

Kim et al. (2014) used another S1P agonist, SEW2871, to enhance bone regeneration. SEW2871-loaded gelatin hydrogels recruited more macrophages than control hydrogels, with resulting increases in new bone regeneration in critical sized ulna defects in rats. Importantly, the authors noted increased gene expression of the M1 gene *TNFa* at 3 days, which was replaced by increased expression of M2-associated genes *OPG*, *IL10*, and *TGFb*. This sequential M1–M2 activation is the sequence observed in normal wound healing (see Sect. 4). To actively promote this sequential activation profile, we have recently prepared scaffolds based on decellularized bone that released M1-promoting interferon-γ within 24 h followed by sustained release of IL4, which was attached to the scaffolds using biotin–avidin binding (Spiller et al. 2013). Primary human macrophages seeded on these scaffolds exhibited sequential M1 and M2 activation, with sequential release of VEGF and platelet-derived growth factor (PDGF), which is consistent with the natural order observed in angiogenesis. This system is currently under investigation in a murine subcutaneous implantation model.

5.3 Physical Modification of Scaffold Properties

Manipulation of scaffold composition and processing has a significant effect on macrophage polarization, with resulting implications for biomaterial acceptance or rejection (Brown et al. 2012, 2009; Badylak et al. 2008). We recently showed that glutaraldehyde crosslinking of collagen scaffolds increased scaffold vascularization in a murine subcutaneous implantation model, coincident with a mixed M1/M2 population of macrophages (Spiller et al. 2014). Chen et al. (2014) recently reported that extracts from β-tricalcium phosphate (β-TCP) scaffolds induced a phenotypic switch of murine RAW 264.7 macrophages to an M2 phenotype, with upregulation of bone morphogenetic protein 2 (BMP2), which was attributed to the activation of the calcium-sensing receptor pathway. Similarly, nanoscale crystalline calcium phosphate-modified Ti surfaces have been shown to downregulate pro-inflammatory gene expression by macrophage-like RAW 264.7 cells in vitro when compared to nanoscale-modified pure Ti (Hamlet and Ivanovski 2011).

Manipulation of physical properties of scaffolds, especially topography and stiffness, can also exert a significant influence on the function of macrophages (Paul et al. 2008). The effects of changing surface topography at both micro- and nanoscales have been examined on macrophage adhesion, spreading, proliferation, cytokine secretion, and fusion into foreign body giant cells, with huge potential for the design of implants (Mohiuddin et al. 2012; Ghrebi et al. 2013; Lamers et al. 2012; Bartneck et al. 2012; Chen et al. 2010; Bota et al. 2010). Macrophage accumulation in vivo has been shown to precede bone formation on rough but not smooth surfaces (Chehroudi et al. 2010). Recently, Ti particles with

rough surfaces elicited upregulation of M2-associated genes and downregulation of M1 genes in murine RAW 264.7 macrophages (Barth et al. 2013). It has also been reported that surface topographies differentially activate components of the pro-inflammatory ERK1/2 pathway in RAW 264.7 macrophages that affect cell function (Ghrebi et al. 2013), suggesting that topography can be designed to optimize immune responses. In addition, surface-modified Ti discs displaying increased hydrophilicity induced an overall downregulation of macrophage gene expression for pro-inflammatory mediators that are significantly upregulated by surface roughness alone (Alfarsi et al. 2013; Hamlet et al. 2012), indicating that surface hydrophilicity attenuates the immunostimulatory effect of Ti surface microroughness.

Intriguingly, it was recently shown that cell shape could be used for modulation of macrophage phenotype (McWhorter et al. 2013). M2a macrophages naturally have an elongated shape compared to other phenotypes, and when they were coaxed into this shape using micropatterning techniques, M2a genes were upregulated even in the presence of inflammatory stimuli.

In addition to cell shape and surface topography, substrate stiffness also has the potential to modulate macrophage phenotype. When cultured in vitro on poly(ethylene glycol) (PEG)-based hydrogels with lower elastic moduli (130 kPa vs. 840 kPa), RAW264.7 macrophages expressed lower levels of M1 genes, which translated to thinner fibrous capsules upon subcutaneous implantation in mice in vivo (Blakney et al. 2012). Taken together, these studies suggest that physical cues including cell shape, surface topography, and elasticity of the substrate can be tailored to induce certain responses in macrophages either individually or in conjunction with chemical and soluble cues.

5.4 Selectively Delivery to Macrophages Using Nanoparticles

Circulating mononuclear cells and macrophages selectively phagocytose nanoparticles in the bloodstream, making intravenous administration of nanoparticles an efficient and effective means to target macrophages. Nanoparticles containing bisphosphonates such as clodronate and alendronate in liposomes are a popular treatment of osteoporosis in postmenopausal women (Eslami et al. 2011). These nanoparticles selectively induce apoptosis in macrophages, especially those in bone because of the calcium-chelating properties of bisphosphonates, thus inhibiting not only bone resorption but also the growth of tumors that exploit macrophage behavior (Roelofs et al. 2010; Zeisberger et al. 2006). Unfortunately, this treatment often leads to osteonecrosis in the jaw, where bisphosphonates tend to accumulate due to the high local density of macrophages in this region (Pazianas 2011).

The potential to modify the size, shape, surface charge, and other characteristics of nanoparticles, with resultant effects on phagocytosis by macrophages

(Bartneck et al. 2010) makes them an attractive strategy to manipulate macrophage behavior. In one study, liposomal preparations of M2-polarizing glucocorticoids were delivered to synovial macrophages in experimental arthritis in mice, which resulted in decreased inflammation and M1 gene expression, but did not affect M2 gene expression (Hofkens et al. 2013). These results were in stark contrast to in vitro results, which showed increased M2 gene expression in primary mouse macrophages, suggesting that it may prove challenging to translate results from in vitro to in vivo experiments. As our knowledge increases concerning ways to control macrophage phenotypes, their phagocytic activity, and surface receptors available for targeting, selective delivery of therapeutics to a particular macrophage subtype may soon be plausible.

6 Conclusions

The natural inflammatory response to injury or to an implanted biomaterial, or both, is a powerful force that determines the course of repair of all tissues. In particular, bone possesses the unique ability to repair itself perfectly, without scarring, in most defects. The macrophage has emerged as the central regulator of bone healing, orchestrating intricate interactions between osteoblasts, osteoclasts, and blood vessels.

One potential obstacle is that the temporal sequence of macrophage activation in bone has not been described. Even in studies that have thoroughly described macrophage dynamics in the healing of other tissues, the difference between M2 subtypes was not addressed. At a minimum, it is highly likely that simple administration of anti-inflammatory drugs or cytokines will not be successful for enhancing bone regeneration, because an early inflammatory response is beneficial for bone repair. Another challenge is that biomaterial strategies are first evaluated in vitro and in animal models, even though correlation between these conditions and clinical application has not been demonstrated. On the contrary, studies have shown limited correlation between in vitro and animal models of macrophage activation with results in humans (Hofkens et al. 2013; Seok et al. 2013). In addition, humans exhibit considerable patient-to-patient variation, especially in terms of their baseline inflammatory response, which has made it difficult to achieve statistical significance in clinical trials of tissue engineering strategies (Spiller and Vunjak-Novakovic 2013).

Despite these challenges, immunomodulatory strategies to promote tissue regeneration have significant advantages over others. For one, they utilize the body's own healing potential, which is particularly promising in a tissue such as bone with such a great capacity for self-repair. In addition, immunomodulatory strategies mobilize the body's own cells, so that costly and time-consuming cell-seeding of scaffolds may not be necessary. Future studies of macrophage–biomaterial interactions will be key for harnessing the inflammatory response for therapeutic strategies.

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Cartilage Regeneration Using Induced Pluripotent Stem Cell Technologies

Noriyuki Tsumaki

Abstract The loss of articular cartilage due to trauma or the degeneration caused by aging can result in debilitating conditions and osteoarthritis, because hyaline cartilage has a poor intrinsic capacity for healing. Articular cartilage defects are currently treated by several procedures, including microfracture and autologous chondrocyte transplantation, although fibrocartilaginous tissue is frequently formed instead of true hyaline cartilage. The development of induced pluripotent stem cells (iPSCs) offers a new cell source that is free of the ethical issues associated with the use of embryonic stem cells. In addition, the methods used to generate iPSCs and their differentiation into chondrocytes have been improved. As another cell source, a method for the direct conversion of fibroblasts to chondrocytes, which can generate hyaline cartilage, is also being developed.

1 The Structure and Limited Repair Capacity of Cartilage

Articular cartilage covers the ends of each skeletal element and provides shock absorption to diarthrodial joints. It is an avascular tissue that consists of chondrocytes embedded in a large amount of extracellular matrix (Fig. 1a). The mechanical function of articular cartilage is defined by the properties of the extracellular matrix which is produced and maintained by chondrocytes. Cartilage collagen fibrils form a three-dimensional network which provides scaffolding for proteoglycan (Fig. 1b). This structure defines the tensile and compressive properties of the cartilage. Cartilage collagen fibrils are heterotypic fibrils composed of type II and XI collagen molecules. The amount of type XI collagen is around one-tenth of that of type II collagen, and the type IX collagen molecules associate on the surface

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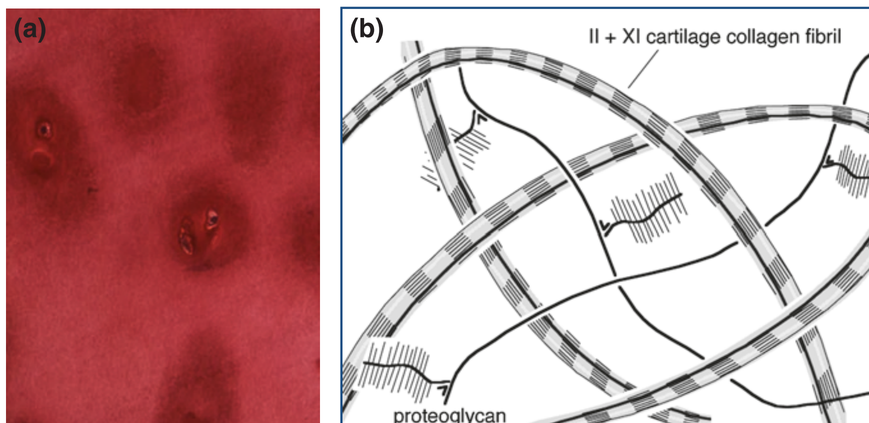

Fig. 1 The structure of articular cartilage. **a** A histological section of human knee articular cartilage stained with Safranin O. Chondrocytes are embedded in an abundant extracellular matrix, which the chondrocytes produce. The Cartilage extracellular matrix is strongly stained with Safranin O due to presence of proteoglycan; **b** a schematic representation of the structure of the cartilage extracellular matrix

of the fibrils. Healthy articular cartilage is called hyaline cartilage, and does not contain type I collagen, which is found in most of other connective tissues, such as the dermis, bone, ligament, and tendon.

Articular cartilage has only a limited capacity for repair (Buckwalter and Mankin 1998), probably because it is avascular, and thus is provided with minimal cells and nutrition which are generally required for tissue repair (Huey et al. 2012). Therefore, defects within articular cartilage seldom heal when the cartilage is injured. The use of joints with injured cartilage further damages the adjacent areas of cartilage, and can eventually lead to diffuse degeneration of the cartilage and debilitating conditions such as osteoarthritis.

When a cartilage injury penetrates the subchondral bone, defects reach the bone marrow, and mesenchymal cells within the bone marrow fill the defects, creating repaired tissue. But such repair tissue contains type I collagen, exhibits a fibrous structure histologically, and thus is called fibrocartilage. The existence of type I collagen may interfere with the normal structure of the cartilage extracellular matrix. Fibrocartilage is inferior to hyaline cartilage in terms of its mechanical properties, and will be eroded and degenerated gradually.

Currently, there are no drugs available for articular cartilage repair. The joint pain caused by the dysfunction of articular cartilage is controlled by limiting daily activities and the administration of anti-inflammatory drugs. When the cartilage degeneration reaches an end stage, patients undergo joint replacement surgery. Joint replacement is an effective treatment, especially for controlling pain, but is associated with some limitations in the range of motion and a risk of loosening of the components, which can lead to additional replacement. Regenerative medicine for cartilage defects is expected to be able to treat patients in earlier stages.

2 Cell Transplantation into Articular Cartilage Defects

Microfracture is relatively widely used procedure for the treatment of articular cartilage defects. In the microfracture technique, multiple holes across subchondral bone are created by drilling, recruiting cell populations that include mesenchymal stem cells in the bone marrow into the articular cartilage defects. These mesenchymal cells create repair tissue, which includes fibrous tissue. The technique is also associated with concerns about the overgrowth of subchondral bone, which could damage the surface of opposing cartilage (Fortier et al. 2012; Mithoefer et al. 2009).

The goal of treating defects or degeneration of the articular cartilage is to induce the regeneration of hyaline cartilage. There are two approaches used for cartilage regeneration (Luyten and Vanlauwe 2012). One is the promotion of endogenous repair and the other is exogenous repair, which employs cell transplantation into the defects. Endogenous repair can be promoted by the application of growth factors to recruit and stimulate endogenous progenitor cells which will proliferate and differentiate, forming tissues and enhancing tissue remodeling. Clinical trials of the application of OP1/BMP7 or FGF18 to treat osteoarthritis or cartilage injury are currently underway (http://www.clinicaltrials.gov/ct2/home).

The most commonly used exogenous repair approach for articular cartilage defects includes autologous chondrocyte transplantation (Brittberg et al. 1994) (Fig. 2). During this procedure, small pieces of cartilage are harvested from a less weight-bearing area of articular cartilage, and are subjected to enzymatic digestion to prepare primary cultures of chondrocytes. The chondrocytes are expanded in monolayer culture to be transplanted into the defects of articular cartilage, which are much larger in size than the size of the cartilage harvested. Autologous chondrocyte transplantation is a well-accepted treatment method, and is performed worldwide.

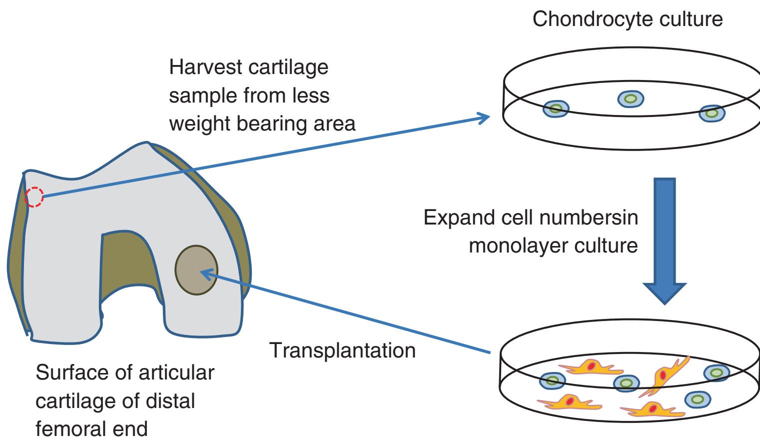

Fig. 2 Autologous chondrocyte transplantation. The required expansion of chondrocytes causes the dedifferentiation of chondrocytes, resulting in the repaired region containing some fibrocartilaginous tissue

The major limitation of autologous transplantation is that cells lose their chondrocyte-specific characteristics, such as expression of chondrocyte markers types II, IX, and XI collagen and aggrecan, and start to express fibroblasts markers like type I collagen when expanded in monolayer culture (Layman et al. 1972; von der Mark et al. 1977; Benya et al. 1978; Marlovits et al. 2004). This process is called the "dedifferentiation of chondrocytes." The word "dedifferentiation" indicates that the cells return to an undifferentiated state. However, in the case of chondrocyte dedifferentiation, the chondrocytes lose their chondrocytic characteristics and obtain fibroblastic characteristic, but do not become undifferentiated. Dedifferentiated chondrocytes undergo increased cell senescence, because the cells undergo multiple divisions. The repair tissue generated by autologous transplantation contains some fibrocartilaginous tissue (Roberts et al. 2009), because dedifferentiated chondrocytes produced during the required expansion are transplanted into the defects.

To observe the process of chondrocyte dedifferentiation, we created transgenic mice expressing EGFP in chondrocytes under the control of the promoter and enhancer sequences of the type XI collagen α2 chain gene (*Col11a2*). Time-lapse observations confirmed that chondrocytes gradually lose EGFP fluorescence, indicating that they turn to nonchondrocytic cells as they undergo cell division (Minegishi et al. 2013). Thus, the possibility that fibroblasts existing at the start of chondrocyte expansion culture proliferate faster and prevail in the culture was ruled out. In addition, we found that chondrocyte dedifferentiation may not be associated with cell division, because mitomycin C-treated chondrocytes still lost their chondrocytic characteristics, although they did not undergo division during culture (REF). These results may provide basis for rationality for exploring methods for the expansion of chondrocytes without losing chondrocytic characteristics.

There is a continuous need for new cell sources for chondrocytes to use in regenerative medicine. Mesenchymal cells from bone marrow and other tissues have been shown to have multipotency, and can differentiate into chondrocytes. These cells are good candidates for transplantation into defects of articular cartilage to generate repair tissue. Use of mesenchymal cells include the microfracture and transplantation of in vitro cultured mesenchymal cells prepared from bone marrow or synovium. Mesenchymal cells are heterogenous, and thus, the repair tissue generated from mesenchymal cells contains cartilage, as well as fibrocartilaginous and hypertrophic tissues (Steck et al. 2009; Mithoefer et al. 2009). It has been reported that chondrocytes derived from bone marrow-derived mesenchymal stem cells tend to exhibit hypertrophy (Pelttari et al. 2006).

3 The Use of iPSC-Derived Chondrocytes

3.1 Generation of iPSCs

The limitations of expanded chondrocyte and mesenchymal cells derived from bone marrow or the synovium appear to be associated with their limited capacity for differentiation to produce hyaline chondrocytes and with their cellular

senescence. Mesenchymal cells isolated from bone or synovium are sometimes termed "mesenchymal stem cells," although their self-renewal is not clearly determined. They appear to have limited proliferation capacity and show decreased multipotency and increased cell senescence as they undergo cell division. On the other hand, embryonic stem cells (ESCs) are pluripotent, and are capable of differentiating into any type of cell and have virtually infinite proliferative activity, and thus have the potential to overcome these limitations and provide a source of cells for chondrocytes that can generate hyaline cartilage. The induced pluripotent stem cells (iPSCs) (Fig. 3) are also pluripotent and have the virtually infinite proliferative activity of ESCs. iPSCs were originally generated from mouse dermal fibroblasts by Takahashi and Yamanaka in 2006 (Takahashi and Yamanaka 2006). They selected 24 factors that were highly expressed and functional in ESCs, transduced mouse fibroblasts with the 24 factors, and found that cells showing characteristics similar to those of ESCs were induced. They selected such ESC-like cells using the activity of a reporter gene put into the locus of *Fbx15,* which is expressed in ESCs. After testing various combinations of factors from among the 24 factors, the combination of c-Myc, Klf4, Oct3/4, and Sox2 was found to be necessary and sufficient to induce ESC-like cells. The selected cells formed teratomas when implanted into immunodeficient mice, and contributed to embryonic development when microinjected into blastocysts, although live chimeric mice were not obtained after birth.

Subsequently, germline-competent mouse iPSCs were generated using a reporter which was put into the *Nanog* locus (Okita et al. 2007). Adult chimeric mice were obtained from the iPSCs, and the iPSCs were transmitted through the germline to the next generation. Nanog is expressed in ESCs, but not differentiated cells, making it more specific than *Fbx15*. In 2007, iPSCs were generated from human somatic cells by introducing the same combination of factors (c-MYC, Klf4, Oct3/4, and Sox2) (Takahashi et al. 2007) or a different combination (OCT3/4, SOX2, NANOG, and LIN28) (Yu et al. 2007). Later on, the depletion of endogenous factors (e.g., p53 or Mbd3) or the expression of an additional

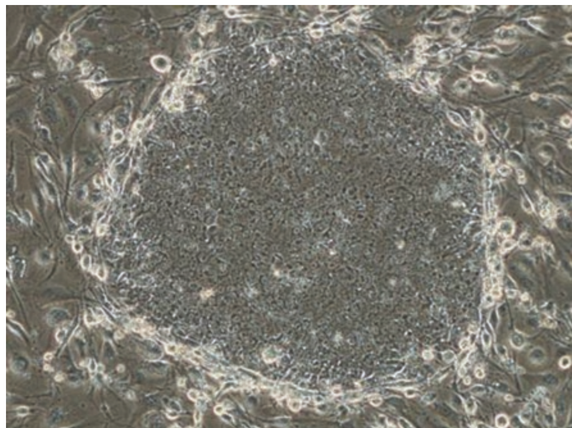

Fig. 3 A phase contrast image of human iPSCs. These iPSCs were generated by transducing human dermal fibroblasts with c-Myc, Klf4, Oct3/4, and Sox2 using episomal vectors

exogenous factor (Glis1) was found to the increase the efficiency of generation of iPSCs (Hong et al. 2009; Kawamura et al. 2009; Li et al. 2009; Marion et al. 2009; Utikal et al. 2009; Maekawa et al. 2011; Rais et al. 2013).

3.2 Improvement of the Safety of iPSCs

iPSCs were initially generated by introducing factors into cells using retroviral or lentiviral vectors. These vectors cause the integration of transgenes into the genome, which increases the risk of tumor formation. To overcome this problem, integration-free human iPSCs have been generated by several methods, including adenovirus infection (Zhou and Freed 2009), Sendai virus infection (Fusaki et al. 2009), the piggyBac system (Woltjen et al. 2009), the minicircle vector (Jia et al. 2010), episomal vectors (Yu et al. 2009), direct protein delivery (Kim et al. 2009), and synthetic mRNA (Warren et al. 2010). Efficient method for generation of human iPSCs by using episomal vectors has been reported (Okita et al. 2011).

3.3 Transplantation of iPSC-Derived Chondrocytes into Articular Cartilage Defects of Patients

iPSCs are expected to be used to treat patients with articular cartilage defects using the following procedure: First, a skin fragment is harvested from the patient under local anesthesia. Dermal fibroblasts are prepared from the skin fragment and expanded in culture. Reprogramming factors are introduced into the dermal fibroblasts to generate iPSCs by an integration-free method, such as using episomal vectors. As an alternative to dermal fibroblasts, blood cells can be converted to iPCSs (Okita et al. 2013). The collection of blood cells is less invasive for patients than harvesting skin fragment. The iPSCs are then differentiated into chondrocytes, which will be transplanted into articular cartilage defects (Fig. 4). The use of iPS cells can avoid the ethical issues associated with sacrificing early embryos, which is necessary for the preparation of ES cells. In addition, the use of iPS cells makes it possible to prepare patient-specific iPS cells.

Several methods have been reported for the differentiation of human ESCs and iPSCs into chondrocytes (Medvedev et al. 2011; Koyama et al. 2013; Toh et al. 2010; Nakagawa et al. 2009; Bigdeli et al. 2009; Hwang et al. 2008a; Koay et al. 2007; Barberi et al. 2005; Oldershaw et al. 2010; Vats et al. 2006; Umeda et al. 2012; Bai et al. 2010). These chondrogenic differentiation methods can be classified into three (Park and Im 2013) or four categories (Oldershaw 2012). These are: 1) co-culture of ESCs/iPSCs with primary chondrocytes. Factors secreted from primary chondrocytes are considered to stimulate and lead to the differentiation of ESCs/iPSCs into chondrocytes; 2) generation of embryoid bodies (EB) from ESCs/iPSCs, followed by differentiation of the mesodermal cells in EB into chondrocytes by treatment with

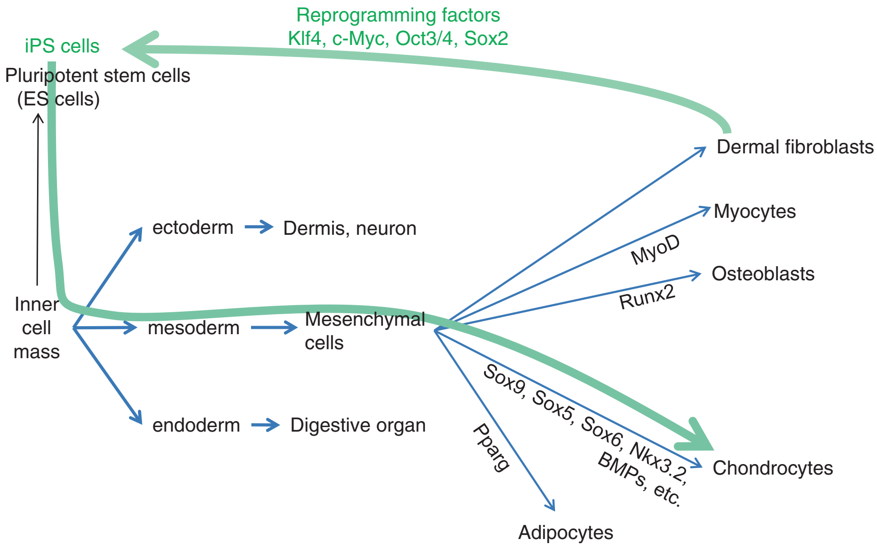

Fig. 4 A method used to generate chondrocytes from dermal fibroblasts through iPSCs. The developmental pathways are drawn from left to right. Chondrocytes, as well as myocytes, osteoblasts, adipocytes, and dermal fibroblasts, originate from mesodermal mesenchymal cells during development. Dermal fibroblasts are converted to iPSCs by expressing four reprogramming factors (c-Myc, Klf4, Oct3/4, and Sox2), followed by the differentiation of the cells into chondrocytes

growth factors; 3) induction of mesenchymal stem cell-like cells from ESCs/iPSCs, followed by their differentiation into chondrocytes by chondrogenic medium, which is known to differentiate mesenchymal stem cells into chondrocytes; 4) differentiate ESCs/iPSCs toward chondrocytes through intermediate populations which consist of mesendoderm, mesoderm, and chondrocyte stages by treating them with a series of appropriate media mimicking normal developmental pathways. This targeted differentiation method produces a cell population in which 74–97 % of cells are Sox9-positive (Oldershaw et al. 2010).

The most important point is that undifferentiated cells should not be left behind after the differentiation of iPSCs into chondrocytes, in order to eliminate the risk of teratoma formation when the cells are implanted in vivo. So far, a limited number of studies have performed implantation of human ESC-derived chondrocytes into immunodeficient mice and nude rats (Hwang et al. 2008b), or to rats administrated with immunosuppressive drugs (Toh et al. 2010). Cartilage was formed in the defects created in rat articular cartilage, without any teratoma formation, suggesting that ESC-derived chondrocytes are a promising source of cells for transplantation. Similar experiments need to be performed using iPSC-derived chondrocytes, because iPSCs have another risk of tumor formation due to the reprograming process. Ideally, the efficacy and safety need to be investigated in large animal models to more accurately assess the repair capacity.

3.4 Development of an iPSC Library

One of the advantages of using iPSCs is the possibility of generating iPSCs from each patient so that he or she can undergo autologous transplantation. However, such individual preparation of iPSCs is costly, and must be produced to conform to good manufacturing practice (GMP) guidelines. To overcome these issues, the generation of a bank of allogenic clinical GMP cell lines is being considered (Turner et al. 2013; Okita et al. 2011). It is estimated that a bank of 100 cell lines homozygous for common HLA types from each population would match around 78 % of Northern Europeans, 63 % of Asians, 52 % of Hispanics, and 45 % of African Americans (Gourraud et al. 2012). An international assessment of how immune incompatibility can best be managed and how a network of GMP HLA homozygous haplobanks could be operated is being performed (Turner et al. 2013).

4 Use of Chondrogenic Cells Generated by Direct Conversion

4.1 Cell-Type Conversion Without the Need for iPS Cells

Converting one cell type to another is one of the aims of regenerative medicine. One approach is to generate iPSCs from accessible cells, such as dermal fibroblasts, followed by differentiation of the cells into those that can be used to repair diseased organs. Another approach is the direct conversion in one step, without going through the generation of iPSCs. The resultant cells can be transplanted into the lesions of diseased organs (Fig. 5).

Cell type conversion had been demonstrated in some cell types before generation of iPS cells. For example, the transduction of fibroblasts with MyoD causes their conversion into myoblasts (Davis et al. 1987). The transduction of fibroblasts with microphthalmia-associated transcription factor (MITF) causes their conversion into melanocytes (Tachibana et al. 1996). On the other hand, cell-type conversion had been difficult in other cell types. Generation of iPS cells showed that cell-type conversion into iPS cells can be achieved by transduction of somatic cells with plural factors, which are abundantly expressed in ES cells and belong to the category of transcriptional factor. After the generation of iPS cells, cell-type conversions have been achieved by expressing plural transcriptional factors which play important roles in the target cells. For example, fibroblasts were converted into neurons by misexpression of Ascl1, Brn2 (also called Pou3f2), and Myt1 l (Vierbuchen et al. 2010). Fibroblasts were converted into cardiomyocytes by misexpression of Gata4, Mef2c, and Tbx5 (Ieda et al. 2010).

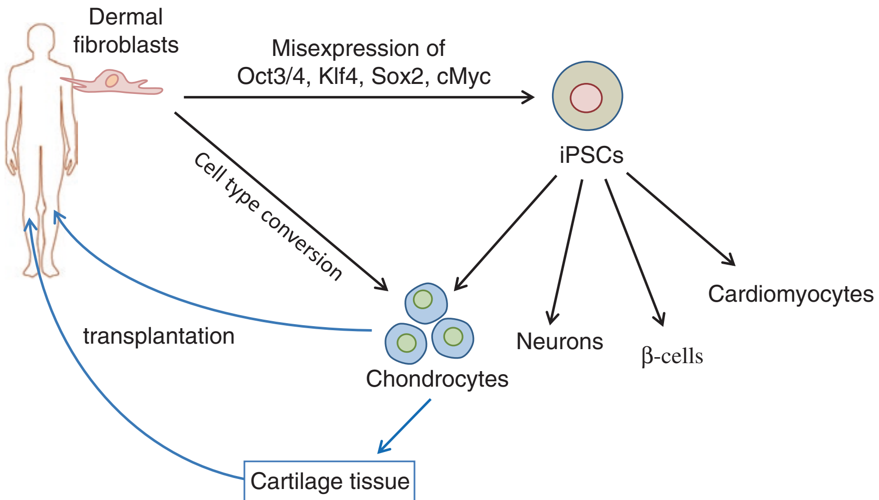

Fig. 5 Two approaches for converting cell types. One approach involves the generation of iPSCs from accessible cells, such as dermal fibroblasts, followed by the differentiation of these cells into cells specific for the affected tissue. The other approach is direct conversion in one step, without going through iPSCs

4.2 Direct Conversion of Dermal Fibroblasts into Chondrogenic Cells

Mouse and human genetic studies have revealed that there are several important factors, such as Sox5, Sox6, Sox9, Nkx3.2, BMPs, and TGFβs, that are required for the differentiation of undifferentiated mesenchymal cells into chondrocytes during development. Misexpression of Sox5, Sox6, and Sox9 in fibroblasts (Ikeda et al. 2004) and treatment of fibroblasts with TGFβ (Sudo et al. 2007) leads to the generation of chondrocytic cells. However, the resultant cells continued to express fibroblast marker genes, such as type I collagen genes, because the characteristics of fibroblasts are stable. This expression of type I collagen is an obstacle for producing hyaline cartilage.

The studies of iPS cells have shown that the expression of four reprogramming factors (c-Myc, Klf4, Sox2, and Oct3/4) can convert fibroblasts into iPS cells, erasing the fibroblastic characteristics completely. We hypothesized that hyaline chondrogenic cells could be directly induced from dermal fibroblast cultures by the combined expression of some reprogramming factors along with chondrogenic factors. Under this hypothesis, we performed experiments, and found that the transduction of mouse dermal fibroblasts with two reprogramming factors (c-Myc and Klf4) and one chondrogenic factor (Sox9) resulted in the induction of chondrogenic cells (Hiramatsu et al. 2011) (Fig. 6). Misexpression of the same factor

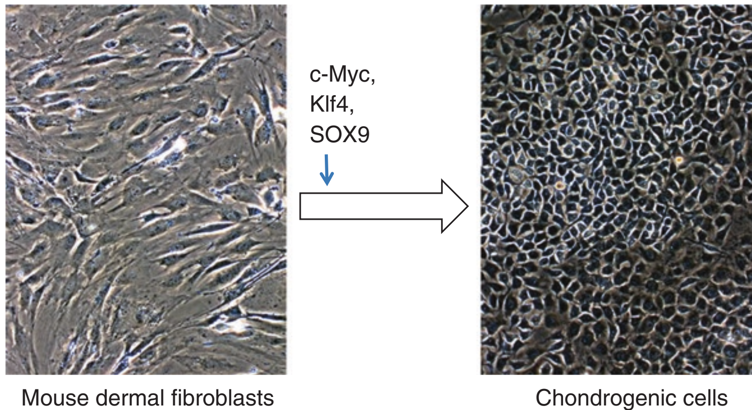

Fig. 6 The transduction of mouse dermal fibroblasts with c-Myc, Klf4, and Sox9 caused conversion toward chondrogenic cells. Phase contrast images are shown

combination in human dermal fibroblasts also induced chondrogenic cells (Outani et al. 2013). When the resultant chondrogenic cells were implanted into the subcutaneous spaces of immunodeficient mice, they produced hyaline cartilage which expressed type II collagen, but not type I collagen.

The cells do not go through a pluripotent state during the conversion from mouse dermal fibroblasts into chondrogenic cells, as shown by the negative Nanog-GFP reporter expression confirmed by time-lapse observations (Outani et al. 2011). Since Nanog is a marker for pluripotency, this result indicates that teratomas should theoretically not be formed when the directly induced chondrogenic cells are transplanted in vivo.

5 Conclusions

Following the generation of iPS cells, it has become possible to generate chondrocytes from other cell types. In addition, direct conversion techniques provide additional options for obtaining chondrocytes. Cell reprogramming technology may allow large numbers of chondrocytes to be obtained and used to produce hyaline cartilage, which is difficult to achieve by current methods. On the other hand, cell type conversion, either with or without going through iPS cells, requires multiple steps, including gene transduction and a long period of culture, which increase the risk of tumor formation when the cells are implanted in vivo due to possible alterations of the genomic sequences. Systematic examinations for safety could minimize such risks. Efforts are being made to improve these technologies to ensure that the benefits exceed the risks, and cartilage replacement via regenerative medicine using cell reprogramming technologies may be possible in future.

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Alveolar Augmentation: Focus on Growth Factors (BMPs)

Ulf M.E. Wikesjö, Cristiano Susin and Jaebum Lee

Abstract Surgical placement of dental implants is governed by the prosthetic design and the morphology and quality of the alveolar bone. Often, implant placement may be difficult, if at all possible, due to alveolar ridge aberrations. In consequence, prosthetically dictated implant positioning commonly entails bone augmentation procedures. We herein discuss the unique biologic potential, the clinical relevance, and perspectives of bone morphogenetic protein (BMP) technologies (focus on rhBMP-2) for alveolar bone augmentation. We also address merits and short-comings of current treatment protocol including bone biomaterials and guided bone regeneration (GBR). In perspective, our studies suggest that BMPs have an unparalleled, dose-dependent potential to augment alveolar bone and in turn support dental implant fixation and functional loading. Inclusion of BMPs for alveolar augmentation to facilitate dental implant fixation may thus not only enhance predictability of existing clinical protocol but radically change current treatment paradigms making conventional "grafting" and GBR procedures altogether obsolete.

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1 Introduction

Prosthetic rehabilitation of the edentulous or partially edentulous patient presents considerable clinical as well as patient-centered challenges. Alveolar ridge aberrations, a sequel to bone remodeling following tooth extractions, periodontal disease, resective surgery, or traumatically induced or of congenital origin must be mastered in addition to prosthetic technical challenges. Patient expectations regarding esthetic and functional outcomes as well as expectations of a minimally invasive, painless, and rapid completion of the prosthetic rehabilitation must equally effectively be mastered. As bone-anchored (osseointegrated) dental implant-based prosthetic rehabilitation supported by favorable long-term survival rates (Albrektsson et al. 1988; Adell et al. 1990; Henry et al. 1996; Lekholm et al. 1999) has become a preferred approach, surgical augmentation of the deficit alveolar ridge and adjoining mucosal tissues has increasingly become a required addition as much as dental implant installation in itself represents a surgical event. In perspective, it is estimated that approximately 12 million dental implants are sold/placed annually worldwide of which 2 million units in the U.S. alone (iData Research Inc.) indicating a substantial need for patient-centered, clinically-relevant, and evidence-based routines for dental implant surgery and alveolar augmentation.

Current surgical protocol includes inlay/onlay access flap procedures for alveolar preservation and horizontal or vertical alveolar ridge augmentation (Simion et al. 2007; de Freitas et al. 2014a). Modified Caldwell-Luc and transalveolar osteotomy protocols have been introduced to gain access and augment the subantral space to increase the vertical dimension of the alveolar base for implant anchorage in the posterior maxilla (Boyne and James 1980; Summers 1994). As these procedures have gained general acceptance, a number of autogenous bone preparations, cadaver-sourced or synthetic bone biomaterials, as well as membranes for guided tissue/bone regeneration as standalone protocols or in combinations have been introduced for alveolar augmentation (Fig. 1). Controlled clinical studies examining their capacity to support alveolar augmentation, dental implant osseointegration, and survival have been conducted and subjected to systematic reviews (Esposito et al. 2009, 2010; Horvath et al. 2013).

Considered the gold standard or benchmark, autogenous bone preparations require a donor site adding undesirable morbidity to the surgical event as well as present limitations relative to graft volumes attenuating their clinical attraction for alveolar augmentation (Clavero and Lundgren 2003; Andersson 2008). While readily commercially accessible, cadaver-sourced allogeneic or xenogeneic bone biomaterials, and synthetic biomaterials, should not be expected to support osteogenic bone formation as discerned from an expanding portfolio of histological evaluations (Pinholt et al. 1992; Caplanis et al. 1997; von Arx et al. 2001; Pöhling et al. 2006; Hong et al. 2014). As an example, such studies unequivocally demonstrate that a bovine bone mineral, a biphasic calcium phosphate, and a ß-tricalcium phosphate biomaterial delay, if not obstruct, osteogenic bone formation rendering them unattractive surrogates for autogenous bone grafts (Pöhling et al. 2006; Hong et al. 2014). Similarly, allogeneic demineralized bone matrix (DBM) preparations apparently should not be expected to enhance osteogenic bone formation, even under

Grafts

- Autogenous bone (fresh/frozen)
- Allogeneic bone (fresh/frozen; not used in dentistry)
- Xenogeneic bone (fresh/frozen; not used in dentistry)
- Osteoblast cell constructs (experimental)

Biomaterials

- Bone derivatives (processed cadaver bone)
  - **Allogeneic bone implants**
    - (un)demineralized freeze-dried bone matrix
  - **Xenogeneic bone implants**
    - deproteinized bovine/equine/porcine bone mineral (BioCera™,BBP®, Bio-Oss®)
    - $CaCo_3$ coral exoskeleton (Biocoral®)
- Bone substitutes (“synthetic bone”)
  - **Ceramics**
    - β-tricalcium phosphate (β-TCP)
    - hydroxyapatite (HA)
    - Ca_2So_4 (plaster of Paris)
    - calcium phosphate cements (α -BSM®, Ceredex™)
    - bioactive glass (BioGran®, PerioGlas®)
  - **Polymers**
    - methylmethacrylate (HTR Synthetic Bone)
    - poly-α-hydroxy acids (PLA, PLGA)

Biologics

- Matrix factors (fibronectin, vitronectin, thrombospondin-1, amelogenins)
- Growth Factors (PDGF, TGF-β, IGF 1, VEGF)
- Differentiation factors (BMP-2, BMP-7, GDF-5)
- Peptides (thrombin peptide TP508)
- Small molecules (PGE receptor antagonists)
- Platelet-Rich-Plasma

GTR/GBR Devices

- Nonresorbable (ePTFE, Ti-mesh)
- Bioresorbable (PLA, PLGA, collagen, hyaluronan)

Combinations

Fig. 1 Bone grafts, biomaterials, biologics, and devices used for alveolar augmentation

optimal circumstances for wound healing (Caplanis et al. 1997). In perspective, it appears that bone biomaterials—whether cadaver-sourced or synthetic—become encapsulated in dense connective tissue without projecting any meaningful bone metabolic activity to eventually or not undergo biodegradation (Fig. 2). Thus, projected clinical success appears limitedly, if at all, influenced by osteoconductive or other properties generated by implanted bone biomaterials.

The intuitive observation (Levander 1938; Lacroix 1945), the critical discovery (Urist 1965), and the eventual purification, cloning, and characterization of bone morphogenetic proteins (BMPs) (Wozney et al. 1988; Wang et al. 1990; Celeste et al. 1990; Özkaynak et al. 1990; Sampath et al. 1992; Hötten et al. 1994, 1996) prompted research evaluating treatment concepts using purified or recombinant forms of BMPs in support of local bone formation for orthopedic, spine, and in turn craniofacial indications (Bishop and Einhorn 2007; Hsu and Wang 2008;

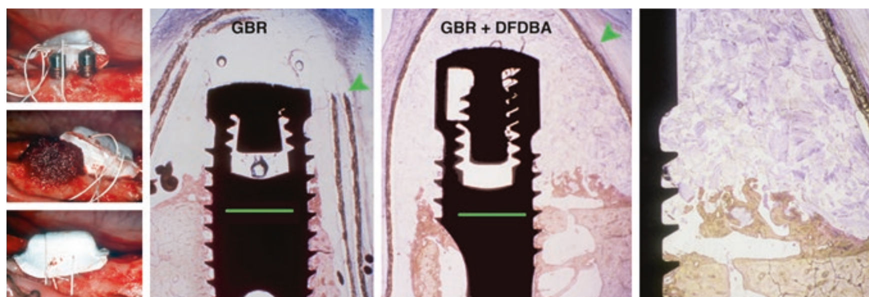

Fig. 2 Critical-size, supraalveolar, peri-implant defect treated with guided tissue/bone regeneration (GBR) using an occlusive space-providing ePTFE membrane (*green arrowheads*), with or without an allogeneic demineralized bone matrix (DBM/DFDBA). Clinical panels show the supraalveolar defect with the 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 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. (1997), figure copyrighted by and modified with permission from Quintessence Publishing

Wikesjö et al. 2009). Recombinant human BMP-2 (rhBMP-2) combined with an absorbable collagen sponge (ACS) carrier was approved in 2002 for spine fusion and long bone fracture repair by the US Food and Drug Administration, as it also was approved for craniofacial indications in 2007 (McKay et al. 2007).

2 Alveolar Augmentation

Compiled over the last decades, a number of studies using clinically relevant translational models and canine, porcine, and nonhuman primate platforms including discriminating critical-size supraalveolar peri-implant defects (Wikesjö et al. 2006) and clinical modeling (Hanisch et al. 1997a, b, c; Jovanovic et al. 2007; Lee et al. 2013a) illuminate the potential of BMPs to augment alveolar bone in craniofacial settings. This text selectively focuses on advances that display the remarkable biologic and clinical potential BMPs, in particular rhBMP-2, may bring to alveolar augmentation and in turn implant dentistry.

Using a discriminating onlay defect model for vertical alveolar ridge augmentation, Sigurdsson and colleagues first showed that a BMP construct—rhBMP-2 soak-loaded onto an ACS carrier—has the potential to induce clinically relevant bone formation (Sigurdsson et al. 1997). Ten-mm dental implants placed 5 mm into the surgically reduced edentulous mandibular alveolar ridge leaving 5 mm of the implant residing above the alveolar crest were draped with rhBMP-2/ACS (*rhBMP-2* at 0.4 mg/mL) or in contralateral jaw quadrants ACS soak-loaded with buffer (control) and then submerged underneath the mucoperiosteal flaps for primary intention healing (Fig. 3). The experimental sites were subject to histometric evaluation following a 16-week healing interval. Sites receiving rhBMP-2/ACS displayed

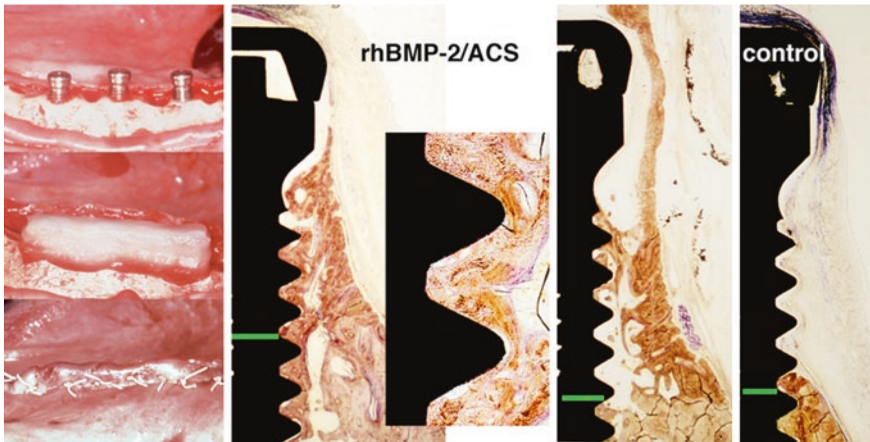

Fig. 3 Critical-size, supraalveolar, peri-implant defect implanted with rhBMP-2/ACS (0.4 mg/mL) or ACS without rhBMP-2 (control). Clinical panels show the supraalveolar defect with rhBMP-2/ACS before and after wound closure for primary intention healing. The photomicrographs show defect sites implanted 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. (1997), figure copyrighted by and modified with permission from Wiley-Blackwell

significant bone formation intimately anchored to the implant surface reaching the top of the exposed dental implants. The control showed as expected limited, if any, bone formation. These observations should be compared with the limited native regenerative potential of the alveolar bone in this defect model shown following the use of space-providing membranes for guided tissue/bone regeneration also including implantation of an allogeneic demineralized bone matrix preparation (Caplanis et al. 1997; Wikesjö et al. 2004). Nevertheless, rhBMP-2/ACS-induced bone formation exhibited considerably variable geometry; at times only a thin layer of bone wallpapered the root of the threads of the bone-anchoring implant surface. Apparently, the ACS carrier was ineffective to consistently support relevant rhBMP-2 induced bone formation also shown in parallel studies using a panel of rhBMP-2 concentrations (Tatakis et al. 2002; Wikesjö et al. 2003; Lu et al. 2013). This apparent variability in bone formation could have several geneses including rhBMP-2 dose and release kinetics or bioavailability, but also reflect ACS structural integrity, biodegradation, degree of soak-load, or any combination(s) thereof.

Several approaches have been explored in an effort to enhance the performance of rhBMP-2/ACS for onlay indications such as alveolar augmentation. They have included dose-variation (Tatakis et al. 2002), the use of space-providing macroporous membranes or titanium mesh devices to shield the rhBMP-2/ACS from compressive forces reducing the potential volume for tissue to form into (Wikesjö et al. 2003, 2004; Lee et al. 2013b), as well as supplementing the ACS with bulking agents to withstand compressive forces compromising bone augmentation (Barboza et al. 2000, 2004; Miranda et al. 2005; Lu et al. 2013). Whereas

dose-variation (*rhBMP-2* at 0.05, 0.1, and 0.2 mg/mL) significantly failed to influence rhBMP-2/ACS-induced bone formation (Tatakis et al. 2002), the use of macroporous space-providing devices allowed significantly enhanced rhBMP-2/ACS (*rhBMP-2* at 0.2 mg/mL)-induced bone formation/alveolar augmentation compared with unshielded sites supporting the tissue engineering principle that the geometry/volume of new bone formation can be ascertained in the design of a space-providing device/matrix (Fig. 4) (Wikesjö et al. 2003, 2004).

The use of bulking agents including granular hydroxyapatite and ß-tricalcium combinations has likewise been shown to significantly support enhanced rhBMP-2/ACS-induced alveolar augmentation. However, bulking agents also introduce compromises related to biodegradation; slowly or nonresorbable biomaterials may compromise the structural integrity of the newly formed bone including osseointegration of dental implants (Barboza et al. 2000, 2004; Miranda et al. 2005; Lu et al. 2013) while for bioresorbable conduits the resorption process per se may solicit inflammatory reactions compromising bone formation and/or maintenance (Sigurdsson et al. 1996).

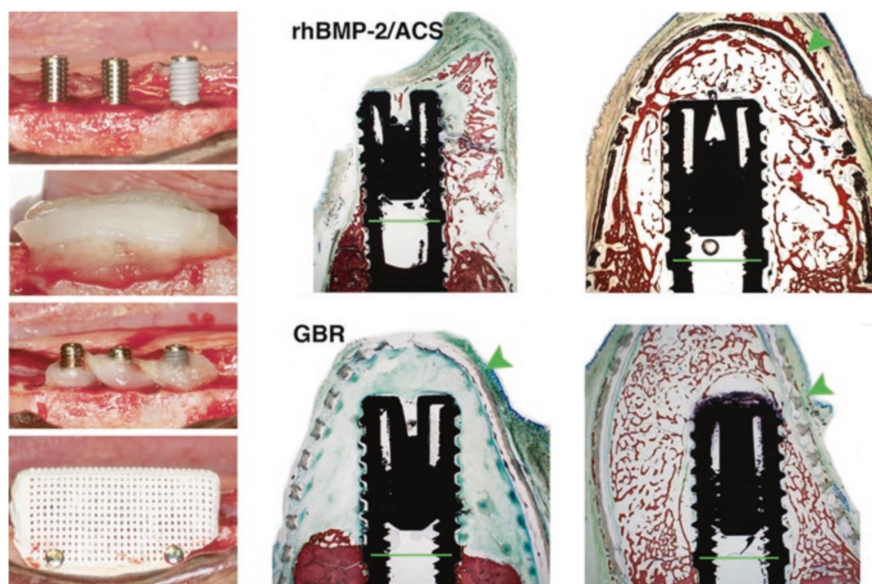

Fig. 4 Critical-size, supraalveolar, peri-implant defects treated with rhBMP-2/ACS (0.2 mg/mL), a porous, space-providing ePTFE membrane for guided tissue regeneration, or rhBMP-2/ACS combined with the porous, ePTFE membrane. The clinical panels show the supraalveolar defect with rhBMP-2/ACS and with 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. (2003, 2004), figure copyrighted by and modified with permission from Wiley-Blackwell

3 Clinical Modeling

Several studies have used clinical modeling as a proxy to illuminate potential utility of rhBMP-2/ACS in clinical settings, that is evaluating rhBMP-2/ACS using typified clinical defects applied to large animal, usually canine, porcine or nonhuman primate platforms. Such studies have used alveolar saddle-type defects (Hunt et al. 2001; Jovanovic et al. 2003, 2007), chronic post-extraction defects (Barboza et al. 2000, 2004), dental implant dehiscence defects (Hanisch et al. 2003), chronic peri-implantitis defects (Hanisch et al. 1997a, b), and sinus augmentation for extended dental implant bone anchorage in the posterior maxilla (Hanisch et al. 1997c; Lee et al. 2013a).

Comparing rhBMP-2/ACS (*rhBMP-2* at 0.20 mg/mL)-induced bone formation with that of the innate regenerative potential in saddle-type defects using a guided tissue regeneration membrane shielding the defect site from competing mucosal ingress, Jovanovic and coworkers showed that rhBMP-2/ACS outperformed the membrane predicate benchmark (Jovanovic et al. 2007). Defect sites receiving rhBMP-2/ACS showed complete to almost complete defect resolution whereas sites receiving the membrane commonly experienced exposures and compromised wound healing/regeneration. In turn, sites receiving an rhBMP-2/ACS-membrane combination also became subject to exposures and compromised healing. In parallel studies, Jovanovic et al. (2003) showed that the geometry/volume of induced bone formation allowed placement and dental implant osseointegration allowing long-term (12 months) functional loading comparable to that in the adjoining resident bone.

Chronic post-extraction alveolar defect sites were used in other studies to evaluate rhBMP-2/ACS (*rhBMP-2* at 0.40 mg/mL) combined with various bulking agents including hydroxyapatite, bioactive glass and demineralized/mineralized bone matrix (Barboza et al. 2000, 2004). Wrapped into the ACS matrix slowly or nonresorbable adjuvants supported “clinically relevant augmentation however, the quality of bone is compromised” questioning the relevance of at least these common biomaterials for sites intended for osseointegration of dental implants.

In still other studies, Hanisch et al. (1997a, b) using a nonhuman primate platform evaluated rhBMP-2/ACS as a stand-alone therapy for resolution of chronic peri-implantitis defects (*rhBMP-2* at 0.43 mg/mL), peri-implantitis representing a biofilm-induced inflammatory lesion progressively depriving dental implants from their alveolar support. rhBMP-2/ACS supported significant resolution of the advanced chronic peri-implantitis defects, mean defect fill approximating 77 % of the 3.4 mm peri-implant defect versus 24 % for the sham-surgery control. Importantly, the newly formed bone osseointegrated to a titanium implant surface that had been exposed to a biofilm-induced inflammatory lesion over 12 months, osseointegration reaching 40 % following a 16-week healing interval. These singularly unique observations become even more critically important considering the increasing awareness of peri-implantitis and the hereto absence of effective clinical solutions.

One main clinical indication for bone augmentation in implant dentistry includes augmentation of the maxillary sinus to extend the alveolar base coronally

to allow placement and anchorage of dental implants for prosthetic reconstruction in the posterior maxilla. Unlike alveolar onlay grafts, maxillary sinus augmentation is considered an inlay indication, implanted graft materials secured within the sinus antral walls without interference from local environmental factors such as for alveolar onlay indications. Hanisch et al. (1997c) evaluated rhBMP-2/ACS (*rhBMP-2* at 0.43 mg/mL) for maxillary sinus augmentation using a nonhuman primate platform. Following a staged protocol, dental implants were placed 3 months following implantation of the rhBMP-2/ACS construct into the maxillary sinus and allowed to osseointegrate for 3 months. Sites receiving rhBMP-2/ACS showed a two-fold mean increase in vertical bone augmentation compared with the ACS carrier control (6 vs. 2.6 mm), newly formed bone exhibiting the same density and osseointegration as the adjoining resident bone. This study first provided the evidence of clinically relevant bone augmentation by rhBMP-2/ACS in maxillary sinus serving as a baseline for clinical evaluations and eventual regulatory approval of this indication.

Autogenous cancellous bone has long been regarded the gold standard for bone grafting due to its content of bone forming cells and serving as a matrix for bone growth. Lee et al. (2013a) compared bone formation/osseointegration following sinus augmentation using rhBMP-2/ACS (*rhBMP-2* at 0.43 mg/mL) versus a particulated fresh autogenous cancellous bone graft harvested from the iliac crest using a Yucatan mini-pig platform. This study used a protocol placing dental implants in conjunction with the augmentation procedure rather than using a staged protocol. Histologic evaluation following an 8-week healing interval revealed significant augmentation of the maxillary sinus following implantation of rhBMP-2/ACS covering most of the dental implant bone-anchoring surfaces compared with irregular bone formation including active resorption in sites receiving the autogenous bone graft. Notably, the rhBMP-2/ACS-induced bone exhibited significantly greater density compared with that of the autogenous bone graft (52 % vs. 33 %). The observations in this study imply significant clinical time-savings using the rhBMP-2/ACS technology due to the augmentation protocol can be used in parallel with implant placement with superior outcomes without need to access a donor site and associated morbidity; greater bone density of predicable geometry without evidence of osteoclastic resorption overall suggesting that rhBMP-2/ACS appears a realistic and effective alternative to autogenous bone grafts in patients requiring maxillary sinus augmentation and should thus be considered the new standard for this indication.

4 Alternative Carrier Technologies

Ideal delivery systems for growth factors/BMPs for alveolar augmentation should meet several criteria conceptually critical to successful regeneration. They should be injectable for ease-of-use implantable and minimally invasive approaches; they should be space-providing allowing structurally integrity/wound stability for

a regenerate to form also in noncontained sites (onlay indications); they should be macroporous for rapid ingrowth of cells and vascular support from adjoining tissue resources; they should allow appropriate release/bioavailability of the biologic; and they should feature a biodegradation profile/rapid clearance allowing the regenerate uneventful maturation (Herberg et al. 2008).

Only few rhBMP-2 delivery systems exhibiting structural integrity have been evaluated in discriminating craniofacial models. An early proof-of-principal report describes the application of rhBMP-2 (*rhBMP-2* at 0.2 mg/mL) in an allogeneic DBM/fibrin clot construct to unsupported augment difficult to treat horizontal alveolar defects using a canine platform (Fig. 5) (Sigurdsson et al. 2001). Ten-mm, dental implants were placed into the rhBMP-2-induced alveolar ridge at 8 and 16 weeks. Block biopsies for histometric analysis were collected at 24 weeks. Roughly 90 % of the bone-anchoring implant surfaces were invested in rhBMP-2-induced bone leaving not more than the apex of the implants interfacing resident bone; all dental implants showing a high degree (~55 %) of osseointegration. There were no significant differences in bone density between rhBMP-2-induced and resident bone. However, the use of cadaver-derived biomaterials such as the allogeneic demineralized bone matrix may with difficulty receive public acceptance for elective procedures in preference for synthetic carrier technologies.

A subsequent study thus evaluated a synthetic calcium phosphate cement (α-BSM®, ETEX Corporation, Cambridge, MA) as a candidate carrier for rhBMP-2 using the critical-size supraalveolar defect model (Fig. 6) (Wikesjö et al. 2002). Block biopsies for histometric analysis collected following a 16-week healing interval showed that rhBMP-2/α-BSM® (*rhBMP-2* at 0.40 and 0.75 mg/mL) induced

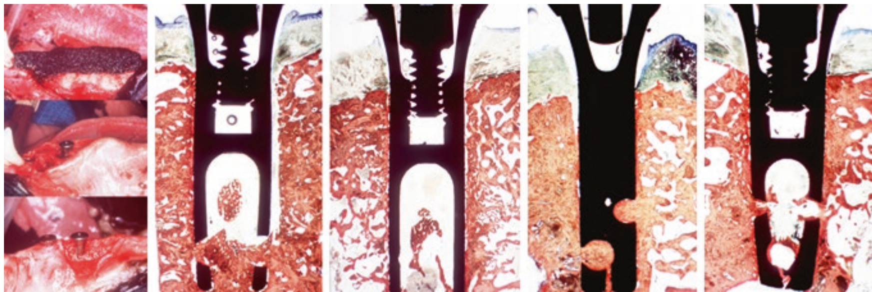

Fig. 5 Surgically created horizontal alveolar ridge defect implanted with rhBMP-2 combined with allogeneic DBM rehydrated in autologous blood. Clinical panels show the rhBMP-2 construct placed onto the surgically reduced alveolar ridge prior to wound closure for primary intention healing. Endosseous dental implants were placed into the rhBMP-2 induced alveolar ridge at 8 and 16 weeks. The animals were euthanized at 24 weeks. *Left* and *right photomicrographs* show implants placed at 8 and 16 weeks, respectively. Approximately 90 % of the bone-anchoring surface of the implants was housed in rhBMP-2 induced bone. There was no significant difference in bone density between rhBMP-2 induced and the contiguous resident bone. Also osseointegration (approximately 55 %) was similar in induced and resident bone irrespective of whether the implants were placed at week 8 or 16. From Sigurdsson et al. (2001), figure copyrighted by and modified with permission from Quintessence Publishing

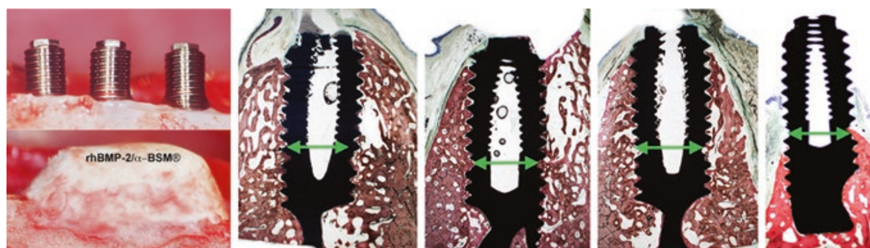

Fig. 6 Critical-size, supraalveolar peri-implant defect treated with rhBMP-2 in a calcium phosphate cement (α-BSM®) or α-BSM® without rhBMP-2 (control). Clinical panels show the supraalveolar peri-implant defect before and after application of α-BSM®. Photomicrographs show representative observations for jaw quadrants receiving rhBMP-2/α-BSM® at 0.4 mg/mL. Note substantial new bone formation at sites treated with rhBMP-2/α-BSM® compared to the control (*far right*) exhibiting limited, if any, evidence of new bone formation. The rhBMP-2-induced bone exhibits similar trabeculation, osseointegration, and cortex formation as the contiguous resident bone. *Green arrows* delineate the apical extension of the supraalveolar peri-implant defects. Healing interval 16 weeks. From Wikesjö et al. (2002), figure copyrighted by and modified with permission from Wiley-Blackwell

substantial and clinically relevant augmentation of the alveolar ridge while control sites receiving α-BSM® without rhBMP-2 exhibited limited, if any, new bone formation. Vertical alveolar augmentation comprised almost the entire 5-mm exposed implants; the newly formed bone density approximating 60 %, cortication, bone–implant contact approximating 27 %, and limited α-BSM® residuals. Clearly, this calcium phosphate cement technology presents considerable promise for a number of indications in the craniofacial skeleton since the α-BSM® may easily be shaped to desired contour and sets to resist compression to provide space for rhBMP-2-induced bone formation. Moreover, the α-BSM® is injectable for ease-of-use and may well prove useful for augmentation of the maxillary sinus in conjunction with placement of dental implants pin-pointing bone formation at the implant body using either a modified Caldwell–Luc or transalveolar osteotomy approach.

5 rhBMP-2 Coated Dental Implants

Conceptually, dental implants coated with a bone inductive factor may stimulate local bone formation and osseointegration (Hall et al. 2007). This hypothesis has engaged our laboratories in a series of studies with the intent to develop a dental implant coated with rhBMP-2. Initial in vitro retention assays evaluating a panel of dental implant surface technologies demonstrated that an anodized titanium surface with open pores appeared the most effective vehicle for rhBMP-2 (Hall et al. 2007). Subsequent in vivo evaluations of rhBMP-2-coated titanium disk implants inserted into the ventral thoracic region in rats showed significant bone formation within a 14-day healing interval engaging the anodized titanium disk implants

(Hall et al. 2007). Studies evaluating rhBMP-2-coated anodized screw-type dental implants placed into the edentulated posterior mandible (Type II bone) in dogs (Wikesjö et al. 2008a) or into the edentulated posterior maxilla (Type IV bone) in nonhuman primates (Wikesjö et al. 2008b) showed robust bone formation in a dose-dependent order. Collectively, these initial proof-of-concept studies using qualified ectopic and orthotopic small and large animal models demonstrate that rhBMP-2 can be successfully delivered to induce local bone formation and osseointegration using a dental implant as a carrier.

Subsequent studies focused on indications for the rhBMP-2-coated implant including alveolar augmentation using the critical-size supraalveolar peri-implant defect model (Wikesjö et al. 2008c; Leknes et al. 2008). Anodized screw-type dental implants soak-loaded with rhBMP-2 at 0.75, 1.5, and 3.0 mg/mL were evaluated following an 8-week healing interval. Jaw quadrants receiving control implants showed limited new bone formation whereas implants coated with rhBMP-2 at 0.75 and 1.5 mg/mL showed clinically relevant bone formation/alveolar augmentation reaching the implant platform (Fig. 7). The quality of the newly

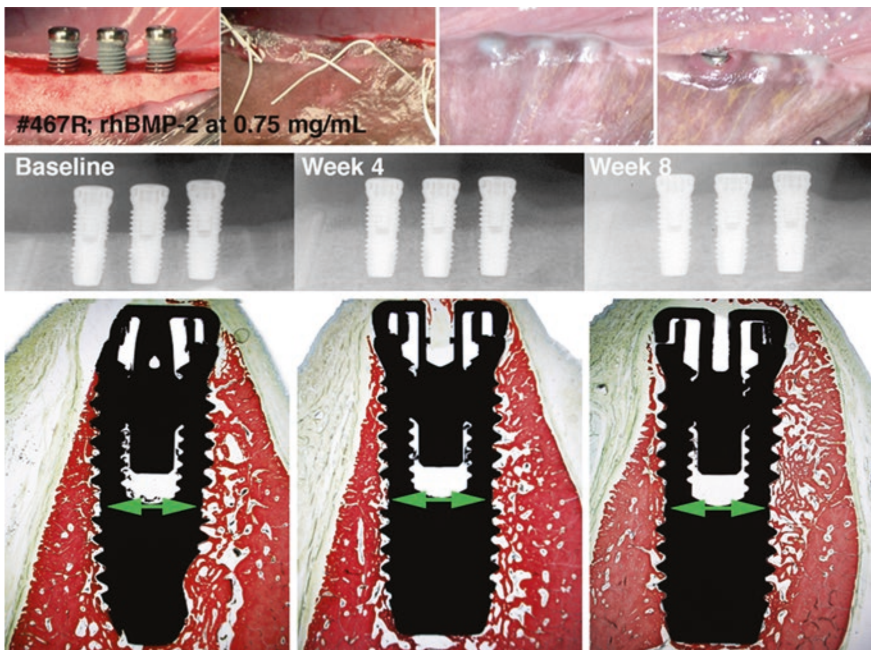

Fig. 7 Critical-size, supraalveolar peri-implant defect including dental implants coated with rhBMP-2 at 0.75 mg/mL following placement and wound closure, and healing at 4 and 8 weeks. Radiographs show bone formation reaching the implant platform at 4 and 8 weeks. Photomicrographs show bone formation with an established cortex reaching or exceeding the implant platform. *Green arrows* delineate a 5 mm notch placed level with the resident alveolar bone. From Wikesjö et al. (2008c) and Leknes et al. (2008), figure copyrighted by Wiley-Blackwell

formed bone approximated that of the adjoining mature resident bone including cortex formation within 8 weeks. In contrast, implants coated with rhBMP-2 at 3.0 mg/mL showed sparsely trabecular immature bone formation exceeding the implant platform without cortication. These studies evaluating rhBMP-2 coated dental implants using the critical-size supraalveolar peri-implant defect model thus reveal an inverse relationship between rhBMP-2 concentration/dose and induced bone formation/maturation. Whereas lower rhBMP-2 concentrations support clinically relevant vertical/horizontal alveolar augmentation, the higher concentration apparently extends/delays bone formation/maturation. These studies also imply that rhBMP-2-induced bone formation benefits from space provision, the lingual aspects of the implants exhibiting a wider alveolar base generally display more robust bone formation than corresponding buccal surfaces, important to the clinical surgical management.

6 Alveolar Augmentation in Clinical Settings

rhBMP-2 soak-loaded onto the ACS carrier has met increasing yet guarded acceptance for the management of craniofacial indications including alveolar augmentation for implant dentistry. We recently conducted a systematic review of the field; some of our major findings summarized herein (de Freitas et al. 2014b). To date, relatively few clinical trials have been conducted to evaluate the on-label clinical efficacy and safety of rhBMP-2/ACS or its off-label use combined with other biomaterials for alveolar ridge augmentation. Interpretation of the results is convoluted by the use of varying rhBMP-2 concentrations, and a wide absolute dose range for the same indication. Whereas rhBMP-2 concentrations at 0.43, 0.75, and 1.5 mg/mL have been tested in experimental clinical settings, the only US. Food and Drug Administration-approved and commercially available concentration to date is rhBMP-2 at 1.5 mg/mL. rhBMP-2/ACS kits are commercially available in sizes ranging from 0.7 to 8.0 cc including rhBMP-2 doses ranging from 1.05 to 12.0 mg, respectively, allowing the clinician to tailor the rhBMP-2 dose applied to the surgical site by increasing the number of rhBMP-2 soak-loaded ACSs. For simplicity, we chose to only present results related to the commercially available product in Table 1, i.e., rhBMP-2/ACS at the 1.5 mg/mL concentration.

Maxillary sinus augmentation using rhBMP-2/ACS as a stand-alone therapy has been evaluated in three studies using rhBMP-2 concentrations at 0.43, 0.75, and 1.5 mg/mL (Boyne et al. 1997, 2005; Triplett et al. 2009). All surgeries were performed using a lateral window modified Caldwell–Luc approach. The mean rhBMP-2 dose ranged between 2.9 and 20.8 mg per site complicating interpretation of the results. Nevertheless, implantation of rhBMP-2/ACS yielded clinically meaningful bone augmentation ranging between 7.8 and 10.2 mm. No consistent differences in bone formation could be observed among rhBMP-2 concentrations and no specific analysis was performed regarding dose variations. Compared with autogenous bone graft, rhBMP-2/ACS yielded 1.6 mm (95 %CI: 0.5–2.7)

Table 1 Summary of randomized clinical trials assessing the radiographic effect of rhBMP-2/ACS at 1.5 mg/mL for sinus and alveolar ridge augmentation

Study and indication	Main outcome	Main results		
Boyne et al. (2005)	CT scans		Autogenous bone graft ($n = 13$)	rhBMP-2/ACS ($n = 17$)
Sinus augmentation	New bone height and width at 6 months post-surgery	New bone height (mm)	11.3 ± 4.2	10.2 ± 4.7
		New bone width at subcrestal level (mm)*	4.7 ± 2.8	2.0 ± 2.4
		New bone width at mid-crestal level (mm)	10.2 ± 3.0	7.8 ± 3.9
		New bone width at apical crestal level (mm)	10.6 ± 3.2	10.8 ± 4.6
Triplett et al. (2009)	CT scans		Autogenous bone graft ($n = 78$)	rhBMP-2/ACS ($n = 82$)
Sinus augmentation	Bone height gain at 6 months post-surgery	Sites with ≤4 mm at baseline (mm)*	12.7 ± 6.0	10.4 ± 5.0
		Sites with ≤6 mm at baseline (mm)*	12.1 ± 5.8	9.7 ± 4.7
		Overall new bone height (mm)*	9.5 ± 4.1	7.8 ± 3.5
Fiorellini et al. (2005)	CT scans		No treatment ($n = 20$)	rhBMP-2/ACS ($n = 20$)
Alveolar ridge preservation post-extraction	New bone height and width at 4 months post-surgery	New bone height (mm)	−1.2 ± 1.2	−0.02 ± 1.2
		New bone width at subcrestal level (mm)	0.6 ± 2.6	3.3 ± 2.5
		New bone width at mid-crestal level (mm)	1.6 ± 2.5	3.97 ± 2.5
		New bone width at apical crestal level (mm)	1.7 ± 2.1	2.7 ± 1.4
de Freitas et al. (2013)	CBCT scans		Autogenous bone graft ($n = 12$)	rhBMP-2/ACS ($n = 12$)

(continued)

Table 1 (continued)

Study and indication	Main outcome	Main results		
Horizontal ridge augmentation	New bone width at 6 months post-surgery	New bone width at subcrestal level (mm)	0.5 ± 0.9	1.5 ± 0.7**
		New bone width at mid-crestal level (mm)	2.9 ± 0.9	2.9 ± 0.8^{NS}
		New bone width at apical crestal level (mm)	1.8 ± 1.1	1.7 ± 0.9^{NS}

*$p < 0.05$; **$p < 0.01$
NS not significant, *CT* computed tomography, *CBCT* cone-beam computed tomography

less bone formation (de Freitas et al. 2014a); yet both treatments allowed implant placement. Radiographic bone density at 4–6 months post-surgery was significantly greater for the autogenous bone graft, whereas a reversal was observed after implant loading; rhBMP-2/ACS yielding the greater bone density.

rhBMP-2/ACS has also been evaluated for preservation of the alveolar ridge following tooth extractions, an inlay application. In a randomized clinical trial, Fiorellini et al. (2005) demonstrated that surgical implantation of rhBMP-2/ACS at the commercially available 1.5 mg/mL concentration maintained the height of the alveolar ridge (mean ± SD: 0.0 ± 1.2 vs. −1.2 ± 1.2 mm), while also yielding a wider alveolar ridge at the sub- (3.3 ± 2.5 vs. 0.6 ± 2.2 mm) and mid-crestal (4.0 ± 2.5 vs. 1.6 ± 2.5 mm) levels compared with untreated tooth extraction sockets. The mean rhBMP-2 dose per site was 1.9 mg; a dose–effect relationship was reported.

Recently, de Freitas et al. (2014a) reported a study evaluating application of rhBMP-2/ACS for alveolar ridge augmentation—onlay application—in the atrophic anterior maxilla. This randomized clinical trial compared rhBMP-2/ACS (*rhBMP-2* at 1.5 mg/mL) and the "gold standard" autogenous bone graft for horizontal augmentation. The surgical protocol also included the placement of a customized titanium mesh device to provide for space provision, wound stability, and conditions for primary intention healing. At the subcrestal level, rhBMP-2/ACS yielded significantly greater radiographic horizontal bone augmentation compared with autogenous bone graft (1.5 ± 0.7 vs. 0.5 ± 0.9 mm); no differences between treatments were observed at the mid- and apical crestal levels. Whereas the sample size in this study was limited ($n = 24$), the authors were still able to conclude: "rhBMP-2/ACS appears a realistic alternative for augmentation of the edentulous atrophic anterior maxilla."

Some of the studies have included histological evaluations of core biopsies obtained in conjunction with dental implant placement (Boyne et al. 2005; Triplett et al. 2009; Kao et al. 2012; de Freitas et al. 2014b). General histological findings demonstrate limited or no residual ACS, woven and lamellar bone including

a cell-rich fibrovascular marrow, limited number of osteoblasts and osteoclasts, and no or limited inflammatory infiltrates. These findings validate the preclinical results described earlier in this chapter.

Safety is always a major concern regarding the clinical use of biologics and this has become particularly true for rhBMP-2/ACS. Adverse effects related to on- and off-label rhBMP-2/ACS use for spine surgery have gained considerable attention and publication of independent reviews of earlier publications and data disclosure under the Yale Open Data Access (YODA) project (Carragee et al. 2012; Laine et al. 2013; Resnick and Bozic 2013). Safety data for application of rhBMP-2/ACS in craniofacial settings are mostly limited to that reported in industry-sponsored studies. Frequent post-surgery events include transient oral and facial erythema, edema, sensory loss and pain; some subjects experiencing significant facial swelling, findings in agreement with anecdotal reports from clinicians using rhBMP-2/ACS. rhBMP-2 antibody formation appears a rare event (<3 %) with most individuals exhibiting antibodies to bovine type I collagen used in the carrier (23 %) (Boyne et al. 1997, 2005; Fiorellini et al. 2005; Triplett et al. 2009). As evidenced by an increasing off-label use of rhBMP-2/ACS for craniofacial applications, safety concerns are likely to heighten.

The debate generated by the YODA project is critical for the future use of biologics in general and BMPs in particular. However, extrapolating findings and conclusions from orthopedic settings to craniofacial applications is unwarranted. Whereas the use of autogenous bone grafts for posterolateral spine procedures may not need a second surgical/donor site, this is rarely the case for craniofacial applications. Intraoral sites yield limited amounts of autogenous bone, generally constrained to cortical bone, and access to extraoral donor sites incurs increased costs and morbidity that is not usually expected for outpatient procedures like implant dentistry. Thus, any biologics intended for bone augmentation in craniofacial settings does not need to surpass the clinical efficacy offered by autogenous bone grafts as long as treatment complexity and morbidity are reduced.

In conclusion, rhBMP-2/ACS appears a promising alternative to autogenous bone grafts and other biomaterials for alveolar ridge augmentation also including the maxillary sinus. Safety reports do not appear to represent major concerns for the proposed indications. Further research and development is needed for dose and carrier optimization. Caution should be exercised since most clinical data available are derived from few randomized clinical trials of limited follow-up.

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Bone-Biomimetic Biomaterial and Cell Fate Determination

ZuFu Lu, Jiao Jiao Li and Hala Zreiqat

Abstract Despite its remarkable capacity to undergo self-repair, bone tissue cannot regenerate across critical-sized defects, and their successful reconstruction remains a major clinical challenge. Current treatment options are limited and often associated with a high incidence of complications, which may result in non-union or re-fracture. There is a great and growing need for alternative techniques to replace, restore or regenerate damaged or diseased bone. Biomaterials-based bone tissue engineering via the use of synthetic bone substitutes represents a particularly promising alternative, which circumvents the drawbacks of conventional treatments. To achieve successful reconstructive outcomes, synthetic bone substitutes need to be biocompatible and provide necessary signals to osteoprogenitor cells to control downstream cell responses including adhesion, migration, proliferation and differentiation into osteoblasts. One feasible approach to develop synthetic bone substitutes with such biological properties is to mimic the innate physical and/or chemical properties of bone. In this chapter, we discuss the design aspects of bone-biomimetic biomaterials that provide the signals necessary for bone regeneration, and the underlying mechanisms by which bone-biomimetic biomaterials determine the fate of mesenchymal stem cells/osteoprogenitor cells. Protein adsorption to biomaterial surfaces and their subsequent influence on cell adhesion and intracellular signal transduction will be discussed in detail, with particular emphasis on the key molecules and signalling pathways involved in directing the osteogenic development of cells.

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1 Introduction

Bone tissue has innate regenerative ability and undergoes constant remodelling throughout life. However, the repair and regeneration of critical-sized bone defects requires clinical intervention and remains an unresolved challenge. Accordingly, bone is the second most common transplanted tissue, and more than 500,000 bone-grafting procedures are performed annually in the United States alone (Baroli 2009; Amini et al. 2012). The current gold standard for the treatment of critical-sized bone defects is autologous bone grafting, but this procedure faces major drawbacks including limited availability and second site surgery, which leads to donor site morbidity in 20–30 % of cases (Schwartz et al. 2009). Allogeneic bone grafts may be used as an alternative if insufficient autologous bone graft material can be harvested, which is often the case for extensive bone defects, but these are complicated by variable bioactivity and the risk of immune rejection or disease transmission (Laurie et al. 1984; Quarto et al. 2001; Lord et al. 1988; Mankin et al. 1996). Therefore, there is a growing unmet clinical need for new and effective alternatives to circumvent the drawbacks of autologous and allogeneic bone grafting in bone repair and regeneration (Crowley et al. 2013; Kosuge et al. 2013). To address this need, a promising approach is to develop synthetic bone substitutes composed of one or more biomaterials.

Successful regeneration of critical-sized bone defects in load-bearing applications requires the use of a scaffolding material that has the mechanical strength to support bridging of the defect under load, has highly porous and interconnected architecture to promote new bone growth across the entire defect and is biodegradable at a controlled rate that is coupled to the rate of new bone formation with no release of toxic or inhibitory products. More importantly, the scaffolding material must also be biocompatible and capable of providing signals to direct the recruitment and differentiation of mesenchymal stem cells (MSCs) and osteoprogenitor cells, including adhesion, migration, proliferation and differentiation into osteoblasts. Recently, there has been increasing research focused on the development of bone-biomimetic biomaterials which mimic the physical and chemical characteristics of bone (Drevelle and Faucheux 2013; McMahon et al. 2013; Holzwarth and Ma 2011; Liu et al. 2009; Roohani-Esfahani et al. 2010, 2011, 2013). By mimicking the native bone microenvironment, these novel bone-biomimetic biomaterials are designed to provide signals for the recruitment and differentiation of local and systemic osteoprogenitor populations in order to achieve successful defect reconstruction.

In this chapter, we will discuss two main aspects of cell fate determination using bone-biomimetic biomaterials: (i) design aspects of bone-biomimetic biomaterials that provide the signals necessary for bone regeneration and (ii) the underlying

mechanisms by which bone-biomimetic biomaterials determine the lineage commitment and differentiation of MSCs/osteoprogenitor cells into osteoblasts, with focus on the key molecules and necessary signalling pathways involved.

2 Biomaterial Design for Bone Regeneration

The minimal essential requirements for a biomaterial scaffold for bone regeneration across a critical-sized defect, is its ability to act as a filler to bridge the defect and as a carrier or guide through which cells can migrate to heal the defect. An ideal bone scaffold possesses both osteoconductive and osteoinductive properties. An osteoconductive scaffold allows the attachment, growth and extracellular matrix formation of bone-related cells on its surface and pores, while an osteoinductive scaffold can actively induce new bone formation via biomolecular signalling and recruitment of osteoprogenitor cells (Albrektsson and Johansson 2001). Optimal bone regeneration relies on the ability of the biomaterial scaffold to communicate with osteoprogenitor cells and direct their migration, differentiation and osteogenic activity. To achieve this aim, biomaterials have been developed using design strategies to mimic both the physical and chemical characteristics of bone.

2.1 Designs to Mimic Physical Characteristics of Bone

Bone has unique physical characteristics in terms of architecture, topography and mechanical properties, which fulfil its function and serve as important design targets for scaffold-based bone regeneration.

2.1.1 Designs to Mimic Architecture of Bone

A number of architectural characteristics including porosity, pore size and pore interconnectivity of the scaffold make a significant contribution to bone regeneration outcomes. Critical-sized bone defects often require regeneration of large amounts of cancellous bone, which is an interconnected network of small bone trabeculae containing vasculature and bone marrow with 50–90 % porosity (Sikavitsas et al. 2001). Scaffolds designed for bone regeneration generally attempt to match the porosity of cancellous bone (Karageorgiou and Kaplan 2005), and pore sizes within the range of 100–500 μm are considered as optimal for encouraging cell attachment, migration and ingrowth throughout the scaffold (Ikada 2006). In vitro and in vivo studies investigating osteogenic outcomes in polymer scaffolds with a range of different pore sizes have established optimal pore sizes of around 300 μm for bone regeneration (Murphy et al. 2010; Oh et al. 2007). Numerical and experimental studies have also underlined the importance

of scaffold porosity and interconnectivity in bone regeneration, which determines the spatial distribution of new bone formation (Mastrogiacomo et al. 2006; Sanz-Herrera et al. 2010). In vivo, higher porosity and pore sizes generally result in greater bone ingrowth as the processes of bone formation and remodelling are intimately linked to vascularisation. Scaffold architecture can therefore influence the progression of osteogenesis, as small pores introduce hypoxic conditions which tend to induce the formation of osteochondral tissue before osteogenesis occurs. In contrast, larger pores promote rapid vascularisation leading to direct osteogenesis, as higher oxygen tension favours the differentiation of MSCs into the osteoblast lineage (Santos and Reis 2010).

Recently, several studies have indicated that scaffolds with multi-scale porosity, consisting of both macropores (>100 μm) and micropores (0.1–10 μm), can significantly improve bone regeneration in vivo due to their microstructural imitation of cancellous bone (Woodard et al. 2007; Pek et al. 2008; Lan Levengood et al. 2010). The mechanism of enhanced bone regeneration in the presence of scaffold micropores has been elucidated as increased surface area for cellular interaction in hydroxyapatite scaffolds with 2–8 μm micropores (Woodard et al. 2007), multi-scale osteointegration with micropores filled by osteogenic cells which proceed to form osteoid and mineralised matrix in biphasic calcium phosphate scaffolds with 1–10 μm micropores (Levengood et al. 2010) and improved protein adhesion and interfacial dynamics inducing osteoblastic differentiation of MSCs in collagen-apatite nanocomposite scaffolds with 50–100 nm ultrafine pores (Pek et al. 2008). Control of both macroporosity and microporosity is becoming a new paradigm in the architectural design of bone scaffolds.

2.1.2 Designs to Mimic Topography of Bone

Bone has a nanocomposite structure consisting of an organic matrix (30 wt%) mainly composed of collagen fibrils which are around 15 μm in length and 40–70 nm in diameter, and inorganic hydroxyapatite nanocrystals (70 wt%) which are typically 20–80 nm long and 2–5 nm thick (Rogel et al. 2008; Zhang and Webster 2009). Scaffold design for bone regeneration has aimed at mimicking the nanoscale topography of bone, as the nanostructured extracellular matrix (ECM) closely surrounds bone-related cells and is believed to play an important role in regulating cell attachment, proliferation and differentiation. A range of nanofibrous polymer scaffolds have been investigated for their efficacy in promoting bone regeneration (Chen et al. 2006; Tuzlakoglu et al. 2005; Woo et al. 2007a), with the expectation that they would mimic the morphological function of collagen fibrils to create a more favourable microenvironment for osteogenesis. Compared to control scaffolds without nanofibrous structure, nanofibrous scaffolds were shown to significantly enhance the manifestation of osteogenic markers in osteoprogenitor cells, including both early markers such as alkaline phosphatase activity and runx2 mRNA expression (Tuzlakoglu et al. 2005; Woo et al. 2007a), and late markers such as bone sialoprotein and osteocalcin mRNA expression (Chen et al. 2006;

Woo et al. 2007a). Nanofibrous scaffolds also promoted a greater extent of mineralisation and more uniform distribution throughout the scaffold (Chen et al. 2006; Woo et al. 2007a). Furthermore, one study has noted that osteoprogenitor cells cultured on nanofibrous scaffolds exhibited increased expression of integrins associated with collagen, fibronectin and vitronectin (Woo et al. 2007a). Coupled with the observation that nanofibrous scaffolds can selectively enhance protein adsorption including fibronectin and vitronectin (Woo et al. 2003), the nanofibrous structure may encourage the adhesion of osteoprogenitor cells and provide these cells with an ECM which more closely resembles in vivo conditions, thereby inducing increased bone formation. Other than mimicking the organic component of bone, some scaffold designs have aimed at mimicking the mineral component by the incorporation of hydroxyapatite nanocrystals, either dispersed in a polymer matrix (Thein-Han and Misra 2009) or deposited on a ceramic scaffold as part of a coating (Roohani-Esfahani et al. 2010). Significant improvements in the attachment and proliferation of osteoprogenitor cells and their differentiation into osteoblasts were observed, which were attributed to the hydroxyapatite nanocrystals providing a larger specific surface area for cell interactions, as well as causing changes in cell morphology which induced osteoconductive signals.

Recently, some studies have explored the effect of precisely controlled nanotopographies produced by lithography on the activities of osteoprogenitor cells (Biggs et al. 2009; Dalby et al. 2006, 2007). Nano-sized surface pits and grooves several hundred nanometres in depth have been found to profoundly affect cell-surface interactions and modulate osteoprogenitor cell activities and functions. Various nanotopographies were shown to direct cytoskeletal changes and allow control of cell adhesion, growth and production of osteoblast markers including osteocalcin and osteopontin (Biggs et al. 2009; Dalby et al. 2006). By producing nanotopographies with differentiation of osteoprogenitor cellstures, it was also possible to induce in vitro osteogenic differentiation of MSCs with mineral production in absence of osteogenic supplements (Dalby et al. 2007). These studies represent an important step in the topographical design of bone scaffolds to direct in vivo bone regeneration outcomes.

2.1.3 Designs to Mimic Mechanical Properties of Bone

The mechanical properties of cancellous bone vary widely with density, with mid-range values of 5–10 MPa for strength and 50–500 MPa for modulus which can serve as design goals for biomaterials for bone regeneration (Yaszemski et al. 1996; Rezwan et al. 2006). The major challenge in designing biomaterial scaffolds for bone regeneration lies in matching the mechanical properties of bone to satisfy the initial mechanical requirements of the bone defect (often load-bearing) but without excessive mechanical properties sufficient to cause stress shielding, while incorporating other necessary properties such as bioactivity, sufficient porosity and adequate rate of degradation. Matching the mechanical properties of bone with monolithic polymer or ceramic scaffolds has proven to be difficult.

Polymer scaffolds generally lack mechanical competence for load-bearing applications, while ceramic scaffolds suffer from high brittleness and low flexural strength, with the drawbacks of each exacerbated by the highly porous architecture required for bone regeneration (Mistry and Mikos 2005). To improve the mechanical properties and also low bioactivity of polymer scaffolds, attempts have been made to reinforce the polymer matrix with various fillers including hydroxyapatite particles (Shor et al. 2007; Bhumiratana et al. 2011) and nanoparticles (Kim et al. 2006), bioactive glass particles (Boccaccini and Maquet 2003) and nanoparticles (Hong et al. 2008), carbon nanotubes (Shi et al. 2007) and polymer particles (Rockwood et al. 2011). Mechanical properties of these reinforced polymer scaffolds were significantly improved compared to the unreinforced controls, but were generally still less than that of cancellous bone. For ceramic scaffolds, attempts have been made to reduce brittleness and enhance mechanical performance mainly by reinforcement with coating layers of polymers and/or ceramics. Several biocompatible and biodegradable polymers have been used to coat ceramic scaffolds, including poly(lactic-co-glycolic acid) (PLGA) (Miao et al. 2007, 2008), poly(D,L-lactic acid) (PDLLA) (Chen and Boccaccini 2006; Tian et al. 2008; Lu et al. 2008b; Zhao et al. 2009), polycaprolactone (PCL) (Kim et al. 2004; Zhao et al. 2008; Roohani-Esfahani et al. 2011), poly(3-hydroxybutyrate) (PHB) (Bretcanu et al. 2009) and silk fibroin (Wu et al. 2010; Roohani-Esfahani et al. 2012; Li et al. 2013b). Some of these polymer coatings have an additional ceramic component in the form of powder or nanoparticles for bioactivity and further strength enhancement (Miao et al. 2007; Roohani-Esfahani et al. 2011). Polymer-coated ceramic scaffolds generally showed significant improvements in mechanical properties, particularly in terms of strength and toughness. These improvements can be attributed to a micron-scale crack bridging mechanism (Pezzotti and Asmus 2001), where the polymer fills existing cracks in the ceramic microstructure and lowers the chance of crack propagation under load. The use of silk fibroin as a coating material for ceramic scaffolds in bone regeneration is a recent advancement, and as a natural polymer, offers the additional benefit of imparting some bioactivity to the coating.

Recently, some studies have explored the microstructural design of ceramic scaffolds to produce more solid scaffold struts with few cracks and defects, which results in high-strength ceramic scaffolds suitable for bone regeneration at load-bearing sites without need for further modification. In one study, high strut densification and reduction in microporosity greatly increased the compressive strength of glass–ceramic scaffolds (Vitale-Brovarone et al. 2009). Another study produced a unique ceramic microstructure and composition consisting of bioactive grains reinforced by a glass phase wetting the grain boundaries, with dispersed submicron crystals which function in crack deflection (Fig. 1). The result is a high-strength ceramic scaffold with improved toughness compared to conventional ceramic scaffolds, and mechanical properties comparable to cancellous bone even at 80–90 % porosity (Roohani-Esfahani et al. 2013). Such microstructural design strategies hold promise for the development of biomaterial scaffolds which satisfy the mechanical requirements for load-bearing bone regeneration without compromising bioactivity and porous architecture.

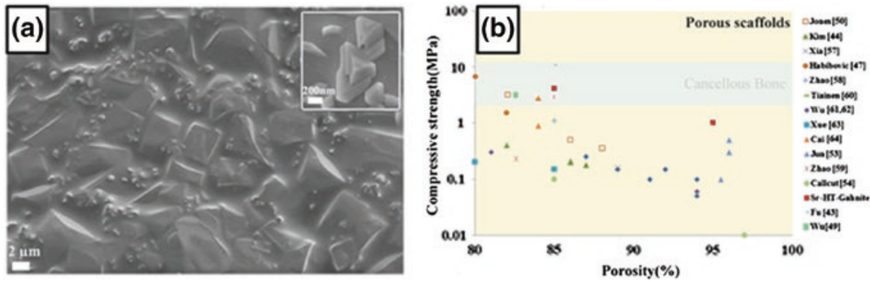

Fig. 1 **a** Unique microstructure of a high-strength Sr-HT-Gahnite ceramic scaffold, **b** compressive strength of Sr-HT-Gahnite ceramic scaffolds match cancellous bone at 85 % porosity (Roohani-Esfahani et al. 2013)

2.2 Designs to Mimic Chemical Composition of Bone

The extracellular matrix of bone consists of an organic phase comprising collagen fibres and non-collagenous proteins, and a mineral phase comprising hydroxyapatite crystals. Many scaffold design strategies for the regeneration of bone have focussed on mimicking the chemical composition of its two phases.

2.2.1 Designs to Mimic Organic Phase of Bone

The organic phase of the bone extracellular matrix is composed primarily of type I collagen fibrils. Collagen molecules are secreted by osteoblasts and self-assemble into fibrils with a specific tertiary structure. The organic matrix also contains small amounts of non-collagenous proteins which may function to regulate mineralisation, including osteopontin, bone sialoprotein, osteonectin and osteocalcin (Rho et al. 1998). Biomimetic scaffold design strategies have explored the utilisation and/or incorporation of the organic matrix components of bone to improve regenerative outcomes.

Collagen has been extensively studied as a scaffold material for bone regeneration as it is the main component of the extracellular matrix. Collagen substrates can provide a native surface for cell attachment, and may influence the morphology, migration and even differentiation of cells (Kleinman et al. 1981). Collagen matrices used for bone tissue engineering in the form of gels or sponges were able to induce favourable osteoblastic differentiation in vitro and bone formation in vivo, particularly when coupled with mechanical stimulation or growth factor release (Ueda et al. 2002; Ignatius et al. 2005). However, a major drawback of using pure collagen as a biomaterial for tissue repair is its high degradation rate, which leads to rapid loss of mechanical properties (Puppi et al. 2010). To overcome this problem, various materials have been combined with collagen substrates both for stabilisation and to improve scaffold properties for bone regeneration, including natural

polymers, synthetic polymers, mineral crystals, or combinations of these. The collagen–glycosaminoglycan scaffold consists of natural polymers and represents a biomimetic structure which supported the growth of osteoprogenitor cells and could direct the osteogenic differentiation of MSCs with subsequent mineralisation (Farrell et al. 2006; Murphy et al. 2010). A collagen–PLGA scaffold represents a natural–synthetic polymer hybrid and provided surface properties which promoted the adhesion and proliferation of embryonic stem cells and osteoblasts (Lee et al. 2006). Collagen–apatite scaffolds consist of a collagen matrix mineralised with calcium phosphate crystals, giving a biomimetic system that resembles the composition of native bone matrix. These scaffolds were osteoinductive and showed ability to heal critical-sized defects in mouse calvaria and pig tibia over 4 weeks and 6 months, respectively (Pek et al. 2008a; Xia et al. 2013). More complex systems consisting of collagen combined with hydroxyapatite and a synthetic biocompatible polymer were able to encourage the attachment, proliferation and osteogenic activity of osteoprogenitor cells (Akkouch et al. 2011; Liao et al. 2004), as well as bridge a radial segmental defect in the rabbit over 12 weeks (Liao et al. 2004). The ability of collagen to directly affect cell behaviour in bone regeneration is demonstrated by the binding of its specific motif, GFOGER, to $\alpha_2\beta_1$ integrin which is involved in osteogenesis (Knight et al. 2000). Following on from this, the collagen-mimetic peptide GFOGER has been used to coat synthetic scaffolds to promote bone formation in a critical-sized segmental defect in rats (Wojtowicz et al. 2010).

Other strategies aimed at mimicking the organic matrix of bone have explored surface modification of scaffolds using biomimetic peptides to enhance cell adhesion and osteogenesis. Arg-Gly-Asp (RGD) is the most effective and frequently employed peptide sequence for stimulating cell adhesion on synthetic scaffold surfaces. It is present in many ECM proteins and promotes integrin-mediated cell adhesion in multiple cell types, which in turn activates cell-ECM signal transduction to influence cell behaviour including migration, proliferation, differentiation, apoptosis and survival (Ruoslahti 1996; Takada et al. 2007). RGD sequences immobilised on a variety of polymer scaffolds including poly(lactic acid) (PLA) (Hu et al. 2003), silk (Chen et al. 2003) and PCL (Zhang et al. 2009) enhanced the attachment of MSCs and osteoprogenitor cells, leading to increased cell survival and growth. Improved bone formation was also observed in some cases (Chen et al. 2003; Hu et al. 2003). Considering that RGD peptides interact with multiple cell types, peptide sequences that elicit more specific responses from selected cell types for bone regeneration have been identified. For example, hydrogels modified with an osteopontin-derived peptide were able to modulate osteoblast proliferation and migration, and the extent of modulation was dependent on peptide concentration (Shin et al. 2004). Other than biomimetic peptides, a recent study extracted non-collagenous proteins directly from the long bones of rats and integrated them with nanofibrous gelatin scaffolds (Sun et al. 2013). The mixture of non-collagenous proteins included bone sialoprotein, osteopontin and osteonectin, and their incorporation into the scaffold led to significantly enhanced osteoblast gene expression and mineralisation by osteoblasts, as well as improved reconstruction of a rat calvarial defect.

2.2.2 Designs to Mimic Mineral Phase of Bone

The mineral phase of bone consists of plate-like hydroxyapatite crystals which occupy discrete spaces within the matrix of collagen fibrils (Rho et al. 1998). Due to their chemical similarity to the composition of bone mineral, calcium phosphate ceramics have had a long history of application in bone regeneration. Calcium phosphate-based scaffolds are inherently bioactive, and can encourage bone formation in vivo via the formation of a hydroxyl carbonated apatite (HCA) layer at the bone-scaffold interface (LeGeros 2002). This is thought to be caused by a cell-mediated dissolution and precipitation process, where calcium and phosphate ions are released from the ceramic into the microenvironment and encourages the precipitation of HCA microcrystals. The extracellular matrix surrounding the scaffold therefore becomes richly mineralised and creates a favourable environment for bone formation. Furthermore, the high concentration of calcium ions adjacent to the scaffold may exert a chemotactic effect on osteoblasts, while phosphate is believed to play a critical role in bone matrix mineralisation (Chai et al. 2012). The most commonly used calcium phosphate-based ceramics for bone regeneration are synthetic hydroxyapatite, β-tricalcium phosphate (β-TCP) and biphasic calcium phosphate (BCP).

Synthetic hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) has a calcium to phosphate ratio of 1.67 and is the closest in composition to bone mineral (Samavedi et al. 2013). Early uses of hydroxyapatite as a bone graft substitute showed good functional recovery over long-term follow-up (Heise et al. 1990; Kitsugi et al. 1993). However, synthetic hydroxyapatite has very low solubility, exemplified by little degradation after more than 5 years of implantation in the long bone segmental defects of three patients (Quarto et al. 2001; Mastrogiacomo et al. 2005). Persisting hydroxyapatite at the implantation site interferes with bone formation and is prone to mechanical failure. Furthermore, synthetic hydroxyapatite is osteoconductive but not osteoinductive (Habibovic et al. 2008). In comparison, β-TCP possesses both osteoconductive and osteoinductive properties. β-TCP is the low temperature phase of tricalcium phosphate ($Ca_3(PO_4)_2$) and has high degradability, which allows rapid precipitation of a surface HCA layer in physiological solution (Samavedi et al. 2013). A range of studies have demonstrated the ability of β-TCP scaffolds to promote bone formation in vivo, both in animal models (Dong et al. 2002; Kondo et al. 2005) and in patients (Gaasbeek et al. 2005; Galois et al. 2002) with good short- and long-term regenerative outcomes. However, the rapid degradation of β-TCP scaffolds in vivo accompanied by loss of scaffold integrity may hinder bone formation (Hing et al. 2007). One study reported less than 5 % of β-TCP scaffolds remaining after being implanted for 24 weeks in the cancellous bone of sheep (von Doernberg et al. 2006). Excessive solubility may lead to decoupling of scaffold degradation and bone formation, resulting in net bone loss at the defect site due to imbalances in bone remodelling (Okuda et al. 2007). BCP is a two-phase ceramic containing hydroxyapatite and β-TCP phases (obtained by sintering calcium-deficient apatite at high temperatures), which combines the low solubility and osteoconductivity of hydroxyapatite with the high solubility and

osteoinductivity of β-TCP (Samavedi et al. 2013). The result is an osteoconductive and osteoinductive ceramic with HA/β-TCP ratios typically adjusted within 20/80 to 40/60 for optimal degradation to match the rate of bone formation (LeGeros et al. 2003). BCP scaffolds have induced superior in vivo bone formation in a range of animal models compared to hydroxyapatite or β-TCP scaffolds (Arinzeh et al. 2005; Bodde et al. 2007; Habibovic et al. 2008; Yuan et al. 2010). The osteoinductive properties of BCP ceramics have been demonstrated by in vitro and in vivo studies investigating the interactions between BCP and MSCs. An in vitro study showed that BCP surfaces were able to stimulate development of osteoblast features in MSCs in expansion medium without osteogenic supplements (Müller et al. 2008). An in vivo study in a canine model also provided evidence for the ability of BCP to induce the homing of MSCs from circulation to participate in ectopic bone formation at the implant site without growth factor delivery (Song et al. 2013).

An interesting set of design strategies to more closely mirror the chemical composition of bone mineral is by making atomic substitutions in the structure of hydroxyapatite, which also leads to improvements in bioactivity and degradability. Cationic substitutions for calcium include zinc (Zn-HA), strontium (Sr-HA) and magnesium (Mg-HA), while anionic substitutions for phosphate include silicate (Si-HA). These ions represent essential trace elements in the human body with ability to stimulate bone formation and/or reduce bone resorption (Shepherd et al. 2012). Zn-HA showed enhanced osteoblast proliferation and differentiation as a coating on porous titanium surfaces (Yang et al. 2012), as well as antimicrobial activity (Stanić et al. 2010). Sr-HA promoted osteogenic activity and mineralisation in osteoblasts and inhibited the proliferation of osteoclasts, and these effects were more prominent at higher strontium contents (Capuccini et al. 2009; Ni et al. 2011). Mg-HA demonstrated improved osteoconductivity and resorption compared to stoichiometric hydroxyapatite as bone fillers in a rabbit model (Landi et al. 2008). Si-HA was found to influence the differentiation of osteoblasts in vitro depending on the level of silicon substitution (Botelho et al. 2006), and promote bone remodelling at the bone-implant interface in an ovine model with apposition of organised collagen fibrils and apatite crystals (Porter et al. 2004). These modified hydroxyapatite materials hold potential for use in bone reconstruction as bioactive scaffolds imitating the mineral phase of natural bone.

3 Mechanisms of Cell Fate Determination by Bone-Biomimetic Biomaterial

One essential goal in the design of bone-biomimetic biomaterials is to provide osteoconductive and/or osteoinductive signals to osteoprogenitor cells and control their activity and fate to favour bone formation. Optimal bone regeneration outcomes therefore rely on the ability of the biomaterial to communicate with

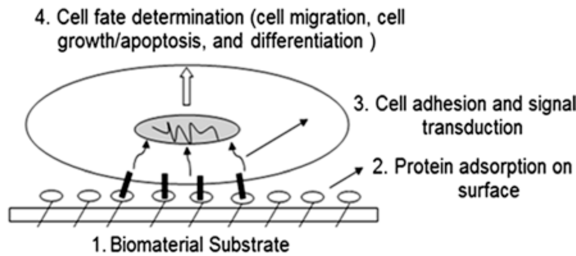

Fig. 2 Cell fate determination by biomaterial substrate. There are four key steps by which the biomaterial substrate determines cell fate: (*1*) biomaterial design, (*2*) protein adsorption on surface, (*3*) cell adhesion and signal transduction and (*4*) cell fate determination

osteoprogenitor cells. Figure 2 illustrates the process by which a biomaterial substrate can communicate with cells and subsequently determine cell fate. First, there is rapid protein adsorption on the biomaterial surface once the biomaterial comes into contact with serum-containing culture medium or body fluids such as blood after being implanted into the body. Second, the adsorbed proteins selectively bind to cellular receptors on the cell membrane. Third, the binding of extracellular proteins to cell receptors specifically activates a cascade of signalling events. Finally, the activated signals determine cell fate.

3.1 Protein Adsorption

Blood proteins have long been regarded as key factors in determining the in vivo acceptance of implants (Rosengren et al. 2002; Horbett 1982). Upon in vivo implantation, the biomaterial surface is almost immediately coated with various proteins such as fibronectin from blood before cells sense the surface and attach to it (Shin et al. 2012). This rapid protein adsorption implies that the cell–biomaterial interaction might actually occur between cells and the adsorbed protein layer rather than directly with the material itself (Horbett 1982; Wilson et al. 2005). Thus it is critical to understand the relation between nature of the biomaterial and protein adsorption on its surface, which subsequently modulates cell behaviour including cell attachment, growth, migration and differentiation. Much research effort has been directed towards chemically and/or physically modifying biomaterial surface properties such as surface roughness (Deligianni et al. 2005; Wang et al. 2013), wettability (Wei et al. 2009), and surface energy (Zhao et al. 2005; Michiardi et al. 2007), to facilitate the adsorption of specific proteins that will determine cell adhesion and signal transduction ultimately leading to control of cell fate (Samavedi et al. 2013; Baxter et al. 2010; Wilson et al. 2005).

There are dozens of different proteins in blood, including albumin, globulins, fibrinogen, vitronectin and fibronectin. Among these, fibronectin and vitronectin are of particular interest, as they are also found in bone extracellular matrix,

and can induce the reorganisation of actin microfilaments and promote cell adhesion and spreading, which in turn modulates cell behaviour such as cell shape and migration (Scotchford et al. 2003; Howlett et al. 1994). As a result, many studies have attempted to increase the deposition of fibronectin and vitronectin to improve the attachment, growth and osteoblastic differentiation of osteoprogenitor cells (Tran et al. 2012; Brun et al. 2013; Alves et al. 2008; Woo et al. 2007b). For example, nanoporous titanium surfaces have been designed to specifically increase the adsorption of fibronectin and vitronectin, which promoted osteoblast attachment and proliferation (Rivera-Chacon et al. 2013). Hydroxyapatite coated with iron oxide nanoparticles also resulted in enhanced osteoblast proliferation and differentiation by increasing fibronectin adsorption on the surface (Tran et al. 2012).

Apart from efforts on modulating the composition of adsorbed proteins, protein conformation is another important aspect that researchers have been attempting to address. Protein conformation includes the secondary, tertiary and quaternary structures, which dramatically affect protein interaction with receptors on the cell membrane leading to changes of material bioactivity. A number of studies have attempted to alter the bioactivity of biomaterials by changing the conformation of adsorbed surface proteins (Binazadeh et al. 2013, Depan and Misra 2013; Assal et al. 2013; Vasita and Katti 2012). One study investigated the influence of protein conformation adsorbed onto the surface of amorphous and crystallised bioactive glass on stem cell adhesion and spreading (Buchanan and EI-Ghannam 2010). It was found that the surface of amorphous bioactive glass led to significant expression of unordered secondary structure in the conformation of fibronectin, which increased cell adhesion and spreading. In contrast, the surface of crystallised bioactive glass resulted in exposure of the stable beta-sheet structure and alpha-helix conformation of fibronectin, which limited cell adhesion and spreading.

3.2 Integrin Signalling

Cell adhesion to biomaterials normally occurs via binding of cellular receptors to the ligands of the proteins adsorbed to the biomaterial surface. The adhesive processes can then trigger a cascade of intracellular signalling events leading to changes in cellular behaviour, such as migration, growth and differentiation. Integrins, the most important and extensively studied cell adhesion molecules, are a family of receptors characterised by transmembrane molecules composed of α and β chains that assemble noncovalently as heterodimers. Currently, 8 β and 18 α subunits have been identified, which form 24 distinct αβ integrin combinations each with unique binding property. These combinations possess dual functionalities of “outside-in” and “inside-out”, which are transducing signals in both directions through the cell membrane. “Inside-out” signalling occurs when integrins are activated by intracellular signals, which leads to conformational changes and promotes their binding affinity for extracellular ligands (Humphries et al. 2004). On the other hand, “outside-in” signalling takes place when extracellular ligands bind

to integrins and initiate integrin clustering, cell adhesion and downstream intracellular signalling pathways (Cabodi et al. 2010; Schneider and Engelman 2004).

Over the past decades, a large number of integrin members have been identified in osteoprogenitor cells and osteoblasts, and their roles in mediating bone formation are highly appreciated (Marie 2013). Among these, β1 integrins are the most abundantly expressed by osteoprogenitor cells and serve as the predominant mediators for cell adhesion to bone ECM molecules including type I collagen and fibronectin (Marie 2013). The critical role of β1 integrins in bone formation is evidenced by a genetic functional study, which demonstrated that transgenic mice with a dominant-negative β1 integrin subunit have reduced bone mass, increased cortical porosity in long bones and thinner flat bones in the skull (Zimmerman et al. 2000). Within the β1 subfamily, α5β1 and α2β1 integrins have received considerable research attention for their roles in promoting osteoblast differentiation, which is attributed to their binding affinity for fibronectin and type I collagen (the predominant molecule in bone ECM). Functional studies demonstrated that the osteoblast-fibronectin interaction is a critical event in the differentiation of rat osteoblasts and involves the interaction between α5β1 and fibronectin (Moursi et al. 1997; Damsky 1999). These results are in agreement with studies demonstrating the roles of α5β1 in the osteogenic differentiation of human osteoblastic cells (Dedhar et al. 1987). The critical role of type I collagen-α2β1 integrin signalling in osteoblastic differentiation has also been demonstrated in several studies (Takeuchi et al. 1997; Schneider et al. 2001; Gronthos et al. 2001; Petrie et al. 2008). For instance, α2β1 integrin–collagen interaction is required for the induction of osteoblast-specific gene expression through a post-translational pathway (Xiao et al. 1998). In addition, other integrin members, including α1β1, α4β1 α11β1 and αvβ3 also participate in osteoblastogenesis (Marie 2013; Martino et al. 2009).

Consistent with the key role of integrins in bone formation, studies have found that the induction of relevant integrin signalling pathways is the underlying mechanism by which bone-biomimetic biomaterials promote osteogenic differentiation of osteoprogenitor cells (Lu et al. 2012; Lu and Zreiqat 2010a, b; Liu et al. 2013a; Woo et al. 2007a). We recently demonstrated that scaffolds coated with hydroxyapatite nanoparticles can promote the differentiation of MSCs into osteoblasts by inducing α2β1 integrin signalling (Lu et al. 2012). On the other hand, the critical roles of integrins for bone formation have inspired researchers to pre-design scaffolding materials with tailored integrin-mediated signals to promote bone repair and regeneration. When α2β1 integrin-specific collagen-mimetic peptide glycine-phenylalanine-hydroxyproline-glycine-glutamate-arginine was coated onto titanium surfaces, this specific integrin-targeted coating not only promoted in vitro osteoblast differentiation and mineral deposition in stem cells, but also significantly improved peri-implant bone regeneration and osseointegration in vivo (Reyes et al. 2007; Wojtowicz et al. 2010). α5β1 integrin signalling is another key target which can be employed for bone regeneration (Hamidouche et al. 2009; Martino et al. 2009; Keselowsky et al. 2005). The activation of endogenous α5β1 integrin using agonists such as a specific antibody has been shown to promote

osteoblast differentiation and osteogenic capacity of MSCs (Hamidouche et al. 2009). The above findings demonstrate that control of integrin binding specificity to elicit desired cellular activities is of great value in the design of informative biomaterials for bone tissue repair and regeneration.

3.2.1 Integrin Downstream Signalling Pathways

Signal transduction occurs when an extracellular signalling molecule binds to cell surface receptors and initiates a cascade of intracellular responses, which can then be dramatically amplified. This signal amplifying process is well exemplified by integrin-mediated intracellular signalling pathways. In general, integrin binding to ECM proteins initiates integrin clustering, cell adhesion and activation of multiple downstream intracellular signalling pathways, which ultimately determines cell fate including migration, proliferation and differentiation. First, integrin clustering results in the binding of integrin cytoplasmic tails to a large complex of proteins such as focal adhesion kinase (FAK), talin, paxilin, vinculin and α-actinin, to form so-called focal adhesion complexes. Second, proteins in the focal adhesion complexes are activated by phosphorylation, which creates docking sites for the activation of other cytosolic protein kinases/phosphatases. Finally, focal adhesion complexes serve as a bridge to connect ECM molecules to their downstream intracellular signalling pathways.

FAK is one of the most important components of focal adhesion complexes which are recruited by integrin clustering. The dependence of FAK phosphorylation and activation on integrin binding to their extracellular ligands has been demonstrated in a variety of cell types (Schwartz et al. 1995). The binding of FAK to cytoplasmic domains of chimeric integrin receptors can automatically activate FAK via phosphorylation (Akiyama et al. 1994). In other words, information coded within the cytoplasmic domain of integrins is possibly sufficient for FAK activation. The activated FAK then phosphorylates and activates a variety of molecules, including Rho, Rac Src, phosphoinositide 3-kinase (PI3K), threonine–protein kinase (Akt) and mitogen-activated protein kinases (MAPK) (Marie 2013; Thompson et al. 2012; Li et al. 2013a), which exert their biological functions of regulating cytoskeletal organisation, cell migration, cell proliferation and differentiation (Wozniak et al. 2004). Here we will specifically discuss three signalling pathways: extracellular signal-regulated kinase (ERK/MAPK), Rho/Rock and PI3K-Akt (illustrated in Fig. 3), which play crucial roles in regulating cell behaviour involved in osteogenesis.

ERK/MAPK Signalling Pathway

MAPK signal pathways, including ERK1/2, c-Jun N-terminal kinase (JNK) and p38 MAPK (p38), are regulated by a diverse group of extracellular stimuli and mediate a variety of cellular responses. In particular, ERK1/2 signalling has been

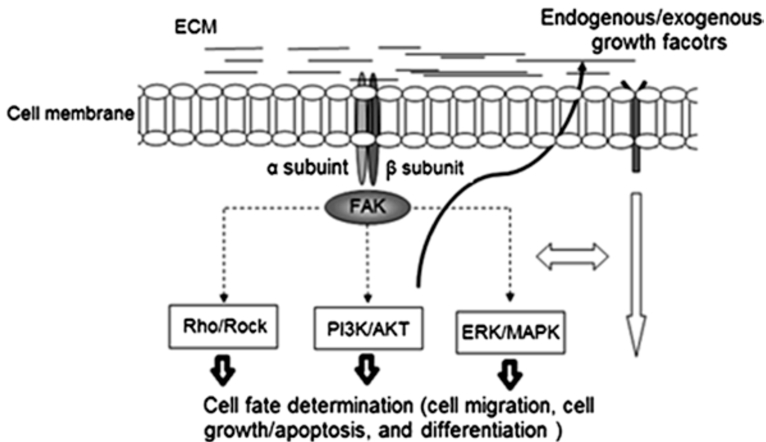

Fig. 3 Integrin signalling modulation of cell fate. The binding of ECM ligands to integrins triggers a cascade of downstream signalling pathways, mainly involving FAK-Rho/Rock, FAK-PI3K/AKT and FAK-ERK/MAPK. Meanwhile, integrin signalling pathways promote the production of endogenous growth factors and coordinate with endogenous/exogenous growth factor-mediated signalling pathways to accomplish cell fate determination

shown to favour osteoblastic cell proliferation and differentiation (Binetruy et al. 2007; Geest and Coffer 2009). In bone, the ERK/MAPK pathway is a major conduit for conveying signals from the extracellular environment to the nucleus, and is also implicated in the response of bone to a variety of signals, including hormone/growth factor stimulation, extracellular matrix–integrin binding and mechanical loading (Zeng et al. 2013; Shi et al. 2012; Lu et al. 2008a; Lu and Zreiqat 2010a, b). A study in transgenic mice found that skeletal size and calvarial mineralisation are decreased in ERK1/2/MAPK knock-down mice but increased in ERK1/2/MAPK induced mice, and the process of endochondral ossification in diaphyseal regions of long bones is also drastically delayed with only early bone collar formation being visible in ERK1/2/MAPK knock-down mice (Ge et al. 2007). In agreement with the key roles of ERK1/2/MAPK signalling in bone development, different osteoconductive/osteoinductive components of bone ECM can induce differentiation of osteoblasts from MSCs by activating ERK1/2/MAPK associated signalling pathways, including type I collagen (Tsai et al. 2010), fibronectin (Ding et al. 2006) and bone sialoprotein (Gordon et al. 2009). We previously demonstrated that β-TCP promotes the differentiation of human osteoblasts by activating ERK1/2/MAPK signalling (Lu and Zreiqat 2010a).

Rho/ROCK Signalling Pathway

RhoA, a member of the large Rho-family of GTPases, has been widely implicated in integrin-mediated signalling (Schoenwaelder and Burridge 1999; Clark et al. 1998).

RhoA can exert its biological function through one of its downstream effectors, the Rho-associated protein kinase or ROCK, to control cell migration and differentiation in response to different stimuli (Clark et al. 1998; Kalaji et al. 2012; Seo et al. 2011; Xu et al. 2012; Lu et al. 2008a, 2010). Cell migration is a key step in tissue repair and regeneration and involves recruitment of progenitor cells to injury sites. The involvement of Rho/Rock signalling pathway in mediating cell migration is evidenced during the development of various tissues including bone (Li et al. 2006; Breyer et al. 2012; Ichida et al. 2011; Montanez et al. 2009; Benoit et al. 2009), and Rho/ROCK signalling inhibition can increase cell movement into bone formation sites in a mouse model of ectopic bone formation (Ichida et al. 2011). In addition, a line of evidence suggests that Rho/Rock signalling plays a key role in directing osteoprogenitor cells into the osteoblast lineage in different models of osteoinduction (Shih et al. 2011; Santos et al. 2010; Khatiwala et al. 2009). Using micropatterned substrates to progressively restrict cell spreading and flattening, Rho/ROCK signalling has been shown to regulate BMP-induced signalling and osteoblast differentiation of MSCs (Wang et al. 2012), and matrix stiffness has also been shown to control the osteogenic phenotype of MSCs by affecting Rho/ROCK intracellular signalling (Shih et al. 2011). The mechanism by which the Rho/ROCK signalling pathway influences the differentiation of osteoblasts has been largely attributed to its ability of assembling actin fibres and regulating cell shape (Guilak et al. 2009; Mathieu and Loboa 2012). When MSCs are allowed to adhere, flatten and spread, they undergo differentiation into osteoblasts; in contrast, the unspread and round cells become adipocytes (McBeath et al. 2004).

PI3K-Akt Signalling Pathway

The PI3K-Akt signalling pathway can be activated by extracellular signals as well as growth factors, and regulates many fundamental cellular processes including cell growth, proliferation and survival (Cantrell 2001; Guntur and Rosen 2011). Following PI3K activation, the lipid product of phosphatidylinositol 3,4,5 triphosphate (PI3) recruits both Akt and PI-dependent kinase 1 (PDK1) to the plasma membrane. Akt is then phosphorylated on T308 by PDK1 and on S473 by mTORC2, leading to full activation (Guntur and Rosen 2011). Activated Akt, in turn, regulates several downstream pathways including Runx2, the master transcription factor for osteogenesis (Kita et al. 2008; Liu et al. 2013b). Recent studies revealed that Akt and its downstream targets are critical regulators of bone formation and remodelling (Peng et al. 2003; Ulici et al. 2009). In vivo, Akt1 knockout mice have shorter bones and delayed formation of secondary ossification centres (Ulici et al. 2009). In vitro, osteoblasts lacking the negative regulator of PI3K/AKT signalling have a strikingly decreased susceptibility to apoptosis and accelerated differentiation capacity in association with markedly increased levels of phosphorylated Akt (Vinals et al. 2002). The important role of the PI3K-Akt signalling pathway in bone formation is also reflected by biomaterial surface modification studies. For instance, collagen I surface treatment promotes the

proliferation and osteogenesis of MSCs via activation of ERK and Akt pathways (Tsai et al. 2010). Altered surface microroughness and hydrophilicity also affects osteoblast proliferation and the early stage of osteoblast differentiation by activating the PI3K/Akt signalling pathway (Gu et al. 2013).

3.2.2 Crosstalk Between Integrin and Growth Factor Signalling

Integrins can determine cell fate by activating several signalling pathways independently as discussed above, but they are also frequently coupled with growth factor receptor-mediated signalling, including vascular endothelial growth factor (VEGF) receptor, transforming growth factor beta (TGF-β) receptor, insulin receptor, type 1 insulin-like growth factor (IGF) receptor, BMP-2 receptor and others (Fig. 3) (Schneller et al. 1997; Kisiel et al. 2013; Hudalla et al. 2011; Massuto et al. 2010; Rapraeger et al. 2013). One recent study reported that the combination of BMP-2 with a hydroxyapatite/fibronection hydrogel mediated integrin signalling resulted in the formation of twice as much bone with better organisation of collagen fibres, compared to delivering the growth factor in a non-functionalised HA hydrogel (Kisiel et al. 2013). Research in the field all points to the fact that integrin signalling pathways are able to modulate cell behaviour by inducing the production of endogenous growth factors, which then exert autocrine and/or paracrine effects (Lu and Zreiqat 2010b; Lu et al. 2011; Hudalla et al. 2011; Moyano et al. 2010; Liu et al. 2013a). We recently found that β-TCP scaffolds promote osteoblastic differentiation by increasing endogenous BMP-2 production through a process involving α2β1 integrin and MAPK/ERK signalling pathways (Lu and Zreiqat 2010b). Similar results were shown in another study which demonstrated that hydroxyapatite/chitosan scaffolds promote MSC adhesion, proliferation and osteoblast differentiations by activating integrin-mediated BMP signalling pathways (Liu et al. 2013a). Thus, the substrate-integrin-endogenous growth factor loop indicates the potential feasibility of designing a smart biomaterial for bone tissue regeneration while avoiding the use of exogenous growth factors.

4 Summary, Conclusion and Perspectives

To circumvent problems associated with current clinical methods of bone reconstruction, including autografting and allografting, the design and development of synthetic biomaterial scaffolds has been an area of great interest. By mimicking the physical and chemical characteristics of natural bone tissue, significant achievements have been made in designing biomaterials that meet the requirements for bone repair and regeneration. One of the key requirements for ideal bone scaffold materials is that they should have the properties to recruit MSCs and osteoprogenitor cells and direct their differentiation into osteoblasts, which require appropriate cell–biomaterial communication. Understanding and identifying the

key molecules and signalling pathways involved in the cross-talk between biomaterials and osteoprogenitor cells will bring substantial benefit to the development of ideal bone scaffold materials with excellent bioactivity.

In this book chapter, we have summarised various design strategies which aim to optimise the osteoconductive and osteoinductive properties of bone scaffolds by mimicking the physical and chemical characteristics of bone, namely architecture, topography, mechanical properties and composition of its organic and mineral phases. This was followed by detailed discussion of the underlying mechanisms by which biomaterials determine cell fate. These include modulating protein adsorption on biomaterial surfaces, eliciting cell adhesion to biomaterials by binding to specific cellular receptors, and triggering a cascade of downstream intracellular signalling events. A range of key molecules (e.g. α2β1 integrin and α5β1 integrin) and signalling pathways (e.g. ERK/MAPK, Rho/Rock and PI3K-Akt) have been identified as being critical in the determination of cell fate when cells come into contact with a biomaterial intended for bone regeneration. The control of biomaterial binding specificity, such as binding to specific integrins to elicit desired cellular activities, has become a powerful tool in the design of bone informative scaffolding materials.

In the future, further exploration of developmental bone biology and the underlying mechanisms by which biomaterials communicate with relevant cells will continually contribute to biomaterial- and cell-based strategies for bone repair and regeneration. As increasing numbers of cell types, including osteoblasts, osteoclasts, MSCs, endothelial progenitor cells and macrophages, and their interactions have been shown to be critical for bone regeneration (Pirraco et al. 2010), a systematic methodology might be needed in order to assess the effect of biomaterial modulation on the behaviour of all of these cell types and their interactions. In addition, it is also imperative to identify the signals in each cell type that are spatially and temporally necessary for bone repair and regeneration, such that they can be incorporated into the design of biomaterial scaffolds to achieve optimal bone regeneration and thus functional repair of bone defects.

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Biomimetic Scaffolds for Craniofacial Bone Tissue Engineering: Understanding the Role of the Periosteum in Regeneration

Michael E. Frohbergh and Peter I. Lelkes

Abstract The role of the periosteum in bone tissue engineering is a new and exciting development. Although its regenerative capacity is known and its role in initiating wound healing is well-documented, a complete understanding of the underlying mechanisms and specific cues that cause healing induction is still unknown. Recently, a number of different studies have begun to explore how stimulating periosteal recruitment is involved in regeneration. In this chapter we review the importance of the periosteum as well as a number of different materials used to activate and initiate the healing process indicative of the periosteum. Our own work has focused on using electrospun chitosan/hydroxyapatite composite scaffolds in order to integrate the native periosteal tissue with our material and instigate the healing process in critical size calvarial bone defects. Critical size defects remain elusive and problematic in the clinic to date and tissue engineering is a promising candidate to alleviate such problems. In this chapter we will briefly review our material and its ability to induce osseointegration, osteoinduction and support the formation of new, mineralized tissue in a murine model. This material, along with others, reflect promising and auspicious developments in musculoskeletal tissue engineering and are helping to pave the way in understanding how the periosteum is involved in wound healing.

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1 Introduction

Regenerative bone tissue engineering encompasses a wide range of different strategies, materials and therapies aimed at repairing, restoring and regenerating tissue rather than replacing it. Since there are many different types of bones with different structures and diverse requirements for specific mechanical strengths, depending on the location and micro-scale composition/ arrangement of specific bones, there is no one "universal approach" to regenerative bone tissue engineering: Successful, tissue-engineered constructs for repairing bone after injury and/ or in the wake of the many bone disorders, will have to be tailored to the specifics of all of these different factors. For example, the Young's modulus in the longitudinal direction of a human femur can range from 15–20 GPa as determined from 3 point bending tests (Cuppone et al. 2004), whereas the Young's modulus for cranial bones is closer to 10 GPa (Motherway et al. 2009). Amongst the important features when engineering regenerative bone scaffolds are the mechanical properties at the onset of bone healing following a fracture. Regenerating bone is characterized by the presence of woven, or immature bone, with Young's moduli that range from ~30–1,000 MPa depending on the distance from the fracture point, with a median of ~130 MPa (Leong and Morgan 2008). This unique microenvironment harbors the osteoblasts that begin the healing process of bone repair. Understanding the mechanisms of bone development, maintenance and repair of specific bone types are crucial to developing successful, integrative materials and therapies.

An essential, yet often neglected component for successful regeneration of any injured bone is its outer living tissue envelope, called the periosteum. The outer fibrous layer of the periosteum contains mainly fibrous ECM proteins, mostly collagens and elastin, as well as fibroblasts and is highly vascularized, while the inner cambium layer is composed of osteoblasts and periosteal (stem-like) cells (Lin et al. 2014). The latter cells are multipotent cells that can differentiate into osteoblasts and chondrocytes (Hutmacher and Sittinger 2003; Lin et al. 2014). Sharpey's fibers are large bundles of collagen fibers that affix the periosteum to the outer layer of the cortical bone. During development, Sharpey's fibers are low in number, allowing the periosteum to move more freely, causing a much more highly activated layer of osteoprogenitor cells to induce tissue formation. Periosteum plays a large role in the initiation of bone regeneration during injury (Clark 2005; Clarke 2008; Zhang et al. 2008a; Rios et al. 2009). The inner layer of cortical bone, the endosteum, is a thin layer of osteoprogenitor cells, osteoblasts and connective tissue that attaches the cortical bone to the trabecular bone, as seen in Fig. 1 (Clark 2005).

The periosteum forms during the early stages of development during intramembranous ossification in flat bones, such as the skull. Mesenchymal stem cells (MSCs) from the neural crest proliferate and begin to differentiate into capillary forming

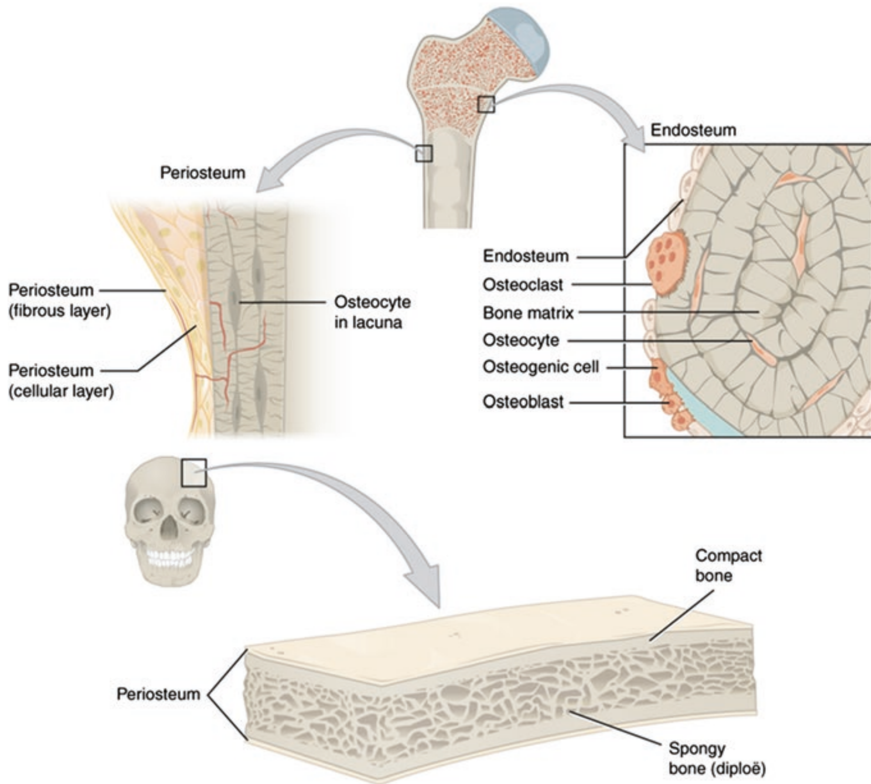

Fig. 1 Micro-scale bone anatomy. The top of the image depicts the hierarchical organization of bone tissue, with the periosteum surrounding the outer layer cortical bone, the presence of numerous cell types embedded in a calcified matrix and the inner endosteum separating the inner layer of the cortical bone from the trabecular bone. The bottom depicts the gross anatomy of cranial bone, showing the two outer layers of cortical bone and the inner trabecular bone, or diploe. Download for free at http://cnx.org/contents/9306de62-3f52-46f8-ab1a-94263c480eda@3

cells and osteoblasts. These osteoblasts begin depositing a collagen- and proteoglycan-rich microenvironment that later becomes mineralized. The early bone matrix (osteoid) becomes calcified through this mineralization process and matures into functional bone tissue. Osteoblasts and MSCs stay to the periphery of the calcified tissue and create new layers of bone, while osteoblasts that become entrapped in the matrix mature and differentiate into osteocytes. As the bone develops, dense groups of MSCs gather around the outer edges of the bone and form into the periosteum (Gilbert 2010). Upon complete maturity, the cranial bones contain two layers of cortical bone (outer and inner layers of the skull) which surround a thick layer of trabecular bone, called the diploe, as seen in Fig. 1 (Lynnerup et al. 2005).

2 The Role of the Periosteum in Bone Development and Regeneration

2.1 Periosteal Involvement in Wound Healing Initiation

The current gold standard for craniofacial reconstruction involves autografts due to the presence of an intact and functional periosteal layer (Allen et al. 2004; Zhang et al. 2008a). However, this introduces a secondary operative site which is often accompanied by surgical complications, donor morbidity/pain and a decreased quality of life. Methods for manufacturing bone grafts from either synthetic/natural materials or the use of cadaveric donor grafts are suboptimal due to the lack of a functional periosteum (Zhang et al. 2008a). Engineered materials typically lack the ability to successfully integrate with the host tissue and fail to induce osseointegration. Integration between the host and the graft is critical, since this integration will facilitate the migration of osteoprogenitor cells from the host into the graft and induce quicker, more regenerative responses and bone formation.

Focusing on craniofacial regenerative engineering, the inner layer of the periosteum in the skull harbors multipotent cells that have a fibroblast-like morphology and can differentiate towards either a chondrogenic or an osteogenic lineage (Zhang et al. 2005). The outer fibrous layer of the periosteum consists of fibroblasts and Sharpey's fibers, which are responsible for binding the cranial bones firmly, but at the same time allowing them to move and absorb shock or trauma. These fibers are most abundant where shock and force are common (Hutmacher and Sittinger 2003).

Cell labeling and tracking experiments have shown the pivotal contribution of the periosteum and endosteum to the initiation of bone healing, where other stromal cells from the marrow in trabecular bone are more involved in the later stages of wound healing (Hutmacher and Sittinger 2003). For example, the importance of the periosteum in bone callus formation was demonstrated by removing the periosteum from an autograft prior to implantation, which resulted in a substantial decrease in new bone formation as well as a 10-fold decrease in neovascularization (Tiyapatanaputi et al. 2004).

Using β-Galactosidase as a tag, Zhang et al. (2005) reported that the periosteal cells migrated from the host onto and localized on and around the graft, differentiating into osteoblasts, chondrocytes, osteocytes and perivascular vessel cells. This study demonstrated the multipotency of these cells and that they tend to remain on the surface of the graft rather than migrating into it (Zhang et al. 2005).

2.2 BMP Signaling

Although the molecular signaling involved in the initiation and morphogenesis of periosteal bone healing is not well defined, a number of molecules, such as proteins of the BMP (Sun et al. 2013), Hedgehog (Huang et al. 2014), and Wnt (Almeida et al. 2013) families, actively participate in this process.

Members of the FGF and IGF families are also upregulated in bone healing (Zhang et al. 2008a). There is a general consensus that wound healing shares some similarities with the natural fetal limb budding and normal bone development (Mariani 2010). During development, BMP-2, 4 and 7 are involved in the activation of core-binding factor α1 (CBFA1), a crucial transcription factor that induces osteogenesis in MSCs (Nishimura et al. 2002). Some studies suggest that BMP-2 is upregulated during the formation of the periosteal callus, which is the initiator to bone healing following cortical bone fracture (Bostrom et al. 1995). Knockout of BMP-2 during organogenesis disrupts the progression of healing following injury in BMP-$2^{-/-}$ mice, in spite of the presence of other osteogenic factors, indicating the pivotal role of this particular factor in fracture repair (Tsuji et al. 2006). BMP2 also plays an important role in angiogenesis and vascularization of the periosteum, as inferred from a decrease in VEGF levels and in specific MSC markers α-smooth actin, CD146 and angiopoietin-A, in a mouse model in which BMP-2 was selectively knocked in osteoblasts (Yang et al. 2013). Addition of BMP-2-transfected periosteal cells to an allogeneic implant yielded increased levels of ALP and accelerated wound defect healing in a rabbit mandibular injury model (Sun et al. 2013). As a caveat, BMPs induce bone formation and osteogenic differentiation in animal models, but in human studies BMPs fail to induce bone formation except at very high doses and following sustained release. BMPs have also had very little effect on non-union fractures (Aspenberg 2013).

2.3 Hedgehog Signaling

The hedgehog signaling pathway is a crucial signaling mechanism involved in development and injury repair. Recently, it has been shown to play a crucial role in stimulating periosteal healing initiation. Sonic hedgehog transfected periosteal cells showed significant increases in both osteogenic and chondrogenic differentiation of MSCs derived from autograft periosteum. Both Indian and sonic hedgehog were significantly upregulated in these cells, leading to a more developed, robust bone formation in vivo. Deletion of Smoothened, a receptor of the hedgehogs, resulted in a significant decrease in osteogenic differentiation and periosteal callus formation (Wang et al. 2010). Furthermore, osteophyte formation in osteoarthritis mouse models was significantly reduced by blocking Smoothened and inhibiting the hedgehog pathway (Ruiz-Heiland et al. 2012). Osteophytes are calcified bone formations in the subchondral regions of bone defects; hence, inhibiting their formation by blocking hedgehog is an indication for its role in bone tissue formation. Overexpressing sonic hedgehog in periosteal progenitor cells resulted in enhanced wound healing in a critical size mouse defect model. Seeding transfected periosteal-derived mesenchymal progenitor cells on scaffolds resulted in a marked increase in endothelial progenitors and microvessel formation (revascularization) and significantly enhanced donor site periosteal cell survival and migration into the construct (Huang et al. 2014).

2.4 Wnt Signaling

The Wnt signaling pathway is a ubiquitous and critical signaling pathway in a multitude of developmental process. In bone development and healing, the non-canonical Wnt/calcium pathway is pivotal for the induction of osteogenesis in the presence of calcium phosphate. Seeding of decalcified graft materials leads to a significant decrease in bone formation. Similarly, blocking of BMP and Wnt pathways using Noggin and Frizzled receptor antagonists also showed a comparable decrease in bone formation (Eyckmans et al. 2010). In the periosteum, down-regulation of the Wnt/β-catenin pathway by recombinant BMPs increased th levels of Sox9, a pro-chondrogenic marker, which ultimately led to chondrogenic, rather than to osteogenic differentiation of the periosteal progenitor cells (Minear et al. 2010). These studies not only demonstrate the importance of these factors for bone development and healing, but also show how they can be employed for as part of the strategy for the use of tissue engineered constructs.

2.5 Periosteal Cell Recruitment and Function

The main constituents of the periosteum responsible for healing are the periosteal cells. These adult stem-like progenitor cells are mainly responsible for instigating the healing process and are also indicative as to why in contrast to using functional autografts, cadaveric allografts lacking such a layer are inadequate for inducing appropriate healing (Allen et al. 2004). An engineered periosteal sleeve can be used to enhance the regenerative abilities of allografts. The three main prerequisite for engineering a periosteal sleeve around a graft material are (a) live osteogenic cells, (b) osteoinductive genes or factors and (c) an osteoconductive scaffolding material. In terms of cell sourcing, the most common choices are MSC derived from the bone marrow or adipose derived stem cells, as well as periosteal cells (Zhang et al. 2008a). These cell types offer a unique opportunity to avoid ethical issues involved with the use of embryonic stem cells as well as provide a renewable and autologous cell source. For example, Long and colleagues used MSCs cultured to form periosteal sheets to revitalize an allograft implant which then functioned like an autograft with an active periosteal layer (Long et al. 2014). These MSC-sheet wrapped allografts demonstrated superior periosteal callus formation, endochondral tissue formation around the periphery of the scaffolds and enhanced osseointegration.

2.6 Vascularization and Extracellular Environment

Bone wound healing and repair requires proper and appropriate vascularization, which has been shown to have a reciprocal effect on osteogenesis. Angiogenic factors, such as VEGF and PDGF not only aid in vascularization, but also aid in bone

formation as well (van Gastel et al. 2012; Ferretti et al. 2012). Like wound healing in other tissues, initiation of bone healing also requires appropriate blood clotting, vessel and callus formation to stimulate the healing process. Periosteal cells are not only influential in the early steps leading to osteogenesis, but also in inducing angiogenesis (van Gastel et al. 2012; Ferretti et al. 2012). Further, incorporation of endothelial cells with MSCs seeded onto implants greatly enhances the initiation of wound healing and leads to healthy functional bone tissue long term (Zigdon-Giladi et al. 2013).

The microenvironment in which stem/progenitor cells reside is called a niche. The niche for bone/periosteal stem/progenitor cells is composed of nanofibrous extracellular matrix proteins, including collagens and elastin, and contains also other cell types, including fibroblasts and osteoblasts and sympathetic nerves/microvasculature (Lin et al. 2014). One of the goals of engineered regenerative tissue scaffolds is to confer biomimetic properties to these scaffolds. One of those properties is their nanofibrous structure, which can be obtained by diverse manufacturing processes, such as electrospinning (Frohbergh et al. 2012; Son et al. 2013), self-assembly (Kocabey et al. 2013; Cakmak et al. 2013) and phase separation (Hsu et al. 2013; Zhao et al. 2012). The goal is to create a tissue-specific environment that can emulate this niche and its unique components. Structure and mechanics are shown to be two of the main causes to induce context-dependent cellular instructions, like maintenance of stemness (Hashemi et al. 2011), proliferation (Li et al. 2013), or tissue-specific differentiation (Liu et al. 2014; Novotna et al. 2013).

3 Tissue Engineered Electrospun Hydroxyapatite Containing Chitosan Scaffolds

3.1 Key Features of Tissue Engineered Bone Scaffolds

Physical properties, such as elasticity, tensile strength, toughness, etc. also induce changes in bone patterning and morphogenesis during development, and these cues also aid in repair and remodeling (Hutmacher and Sittinger 2003). For example, incorporation of hydroxyapatite increases the mechanical properties (stiffness/Young's modulus) of poly-caprolactone (PCL) fibers and enhances osteogenic expression in vitro and new bone formation in vivo (Ba Linh et al. 2013). Bi-layer hydroxyapatite scaffolds have mechanical properties similar to mandibular trabecular bone as well as a porous architecture suitable for osseointegration (Guda et al. 2012).

In our own work we focused on periosteal regenerative engineering and aimed at developing a biomimetic/bioactive material that could be used to induce bone regeneration in critical size defects by stimulating/recruiting the cells from the periosteum of the surrounding tissue to initiate wound healing. Our biomaterial of choice was a composite scaffold generated by co-electrospinning pure chitosan and hydroxyapatite nanoparticles to mimic the biphasic nature of bone (Frohbergh et al. 2012). The nanofibrous ultrastructure of electrospun scaffolds closely mimics that

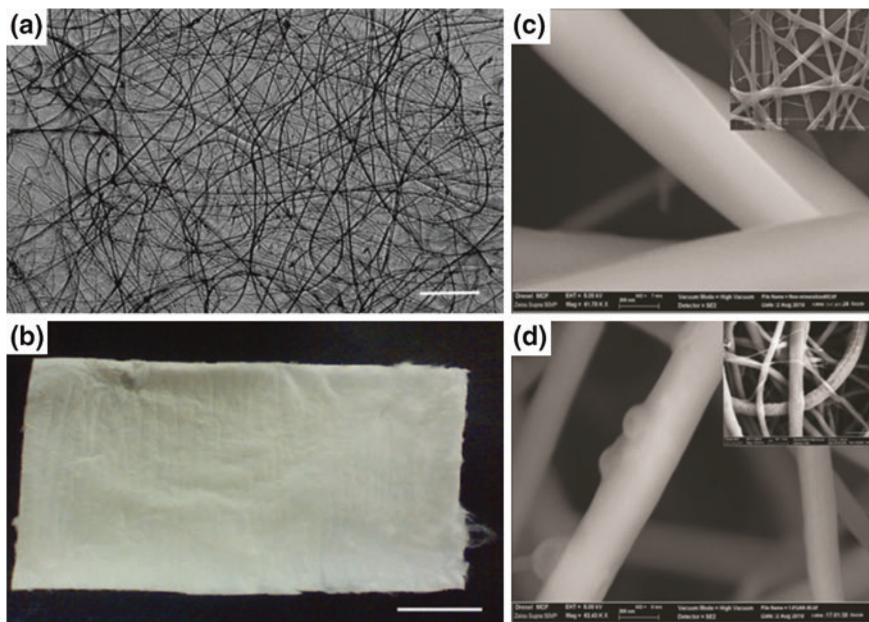

Fig. 2 Electrospun nanofiber morphology. **Panel a** microscopic view of the electrospun chitosan/hydroxyapatite/genipin fibers. **Panel b** gross macroscopic view of an electrospun scaffold. **Panels c** and **d** show the differences between the smooth surface of electrospun fibers without hydroxyapatite and the rougher surface of fibers studded with hydroxyapatite nanoparticles respectively (Frohbergh et al. 2012)

of natural ECM in most tissues, including bone (Fig. 2). Inclusion of hydroxyapatite nanoparticles (in the absence of any fiber forming agents, such as ultrahigh molecular weight polyethylene oxide (Zhang et al. 2008b) in the electrospinning process not only simplifies the manufacturing process, but also instantly enhances both the mechanical properties as well as the bioactivity of our scaffolds. Crosslinking with a natural, non-toxic cross-linker genipin (Torricelli et al. 2014; Bavariya et al. 2013) resulted in a further increase in the Young's modulus and tensile strength of the scaffolds, reaching 147 ± 22 MPa, which is very similar value to the mechanical properties of the periosteum at the periphery of a wound callus., rendering our scaffolds suitable for craniofacial bone tissue engineering. Finally, the scaffolds supported adhesion and proliferation of 7F2 mouse osteoblast-like cells and enhanced their histiotypic differentiation (Fig. 3).

3.2 Electrospinning and Scaffold Fabrication

Electrospinning of natural biopolymers, such as collagen or chitosan may not necessarily be ideal manufacturing process for fracture healing in load-bearing bones, which require stiff and rigid scaffolds in order to provide for the mechanical

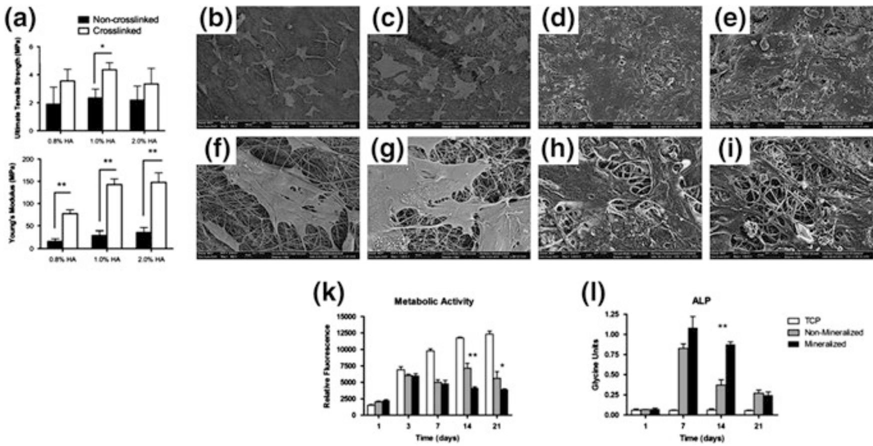

Fig. 3 In vitro characterization of 7F2 osteoblast-like cells on chitosan based scaffolds. Hydroxyapatite-containing scaffolds show mechanical properties similar to those of the periosteum at the formation of a wound callus in natural bone healing processes **a**. 7F2 cells attached and spread after 7 days of culture on both scaffolds without hydroxyapatite **b** and **f** and scaffolds with hydroxyapatite **c** and **g**. The cells remained viable for up to 21 days on both scaffolds without **d** and **h** and with **e** and **j** hydroxyapatite and proliferated on both scaffold types over a 21-day period **k**. ALP expression peaked at day 7 **l**

support required for movement and stability. However, in non-load bearing bones with critical size defects that will not heal spontaneously, scaffolds made of electrospun biomaterials may serve as bioactive “bridges” to cover the defects and induce healing. Mimicking the natural ECM fibrillar structure, electrospun nanofibers promote enhanced cell attachment and spreading and are easily tunable both mechanically (crosslinking) and structurally (coatings, fiber modifications, blended materials, etc.) (Bhardwaj and Kundu 2010; Chew et al. 2006; Huang et al. 2011; Ito et al. 2005; Li et al.; 2002, 2005). These integrative properties are exactly what most inert materials and cadaveric implants are lacking.

Successfully engineered tissue constructs will mimic certain features of native tissues including their unique mechanical properties. While electrospun scaffolds made of “natural” biopolymers such as collagen or chitosan morphologically resemble the fibrous structure of the ECM, their mechanical properties make them less suitable for use as bone analogs. Although non-load bearing bones do not undergo much physical exertion, they still have the biphasic composite strength of bone, i.e., the mineralized collagen/hydroxyapatite ECM represents an organic/inorganic interface designed to withstand trauma. Achieving similar features in electrospun fiber scaffolds is crucial for the development of a suitable bone implant. Crosslinking can be used to enhance the mechanical properties of the constructs and fine-tune them to approximate the properties of bone ECM. Crosslinking can be physical, enzymatic or chemical. For our studies we used genipin as a natural, non-toxic chemical crosslinker (Bispo et al. 2010; Solorio et al. 2010; Zhang et al. 2010). Crosslinking with genipin increases the mechanical

properties (tensile strength) of electrospun chitosan fibers, as assessed for example by a suture pullout strength test (Norowski et al. 2012). While the complete mechanism of how genipin crosslinks chitosan is still not fully understood it is believed to involve a spontaneous reaction between genipin and the NH_2 subunits on the chitosan chain, creating partial covalent bonds and increased stability of the polymer chains (Austero et al. 2012), which in turn causes an increase in the scaffold stiffness. The Young's modulus of our scaffolds increased 4–5 fold upon cross-linking, while the ultimate tensile strength increased by 50 % (Frohbergh et al. 2012).

3.3 HA Containing Chitosan Scaffolds are Osteoinductive

In terms of functional tissue engineering, our aim was to fabricate a scaffold with structural and mechanical properties similar to those of non-load bearing bone and which emulates the regenerative capacity of periosteum. Specifically, our goal was to generate a bioactive scaffold capable of inducing/accelerating osteogenic differentiation in vivo similar to what occurs when osteoprogenitor cells from the periosteum migrate to damaged bone tissue. The osteogenic capacity of our fibrous scaffolds was assessed in vitro using 7F2 mouse osteoblast like cells. The cells attached to all of our scaffolds, mineralized or not, and proliferated over a 14-day period and covered the scaffold in a multilayered fashion. At the same time, the metabolic activity decreased over time, especially in cells cultured on hydroxyapatite-containing scaffolds, which is indicative of cells undergoing differentiation while ceasing proliferation (Moore and Lemischka 2006). Recently, (Venugopal et al. 2011) showed that mineralization of the electrospun scaffolds by inclusion of hydroxyapatite nanoparticles during the spinning process caused a significant increase in osteoblast mineralization and concluded that hydroxyapatite nanoparticles act as nucleation sites for osteogenic induction and maturation in vitro. Our recent in vitro studies yielded comparable results (Frohbergh et al. 2012).

These and similar studies suggest that electrospun composite materials can be considered osteoinductive in vitro by promoting the histiotypic differentiation of cultured osteoblasts or other progenitor cells towards functional osteocytes (Rajzer et al. 2014; Dong et al. 2014; Patlolla and Arinzeh 2013). In lieu of using allogeneic or autologous progenitor cells, periosteal osteoprogenitors would be an ideal cell source, however obtaining these cells is quite difficult and not practical in terms of the number of cells one would have to harvest for a suitable implant in a critical size defect. As an alternative, MSC can be isolated fairly easily from the bone marrow or adipose tissue and differentiated into osteoblasts by simple chemical differentiation protocols (Delorme and Charbord 2007; Frohlich et al. 2008; Giordano et al. 2007; Jaiswal et al. 1997). MSCs are lineage-restricted multipotent cells that are derived from the bone marrow, umbilical cord blood or adipose tissue and have the potential to differentiate into bone, cartilage and adipose (Delorme and Charbord 2007). Due to technical and ethical issues associated with ESCs and of induced pluripotent stem cells (iPSCs), especially their potential for

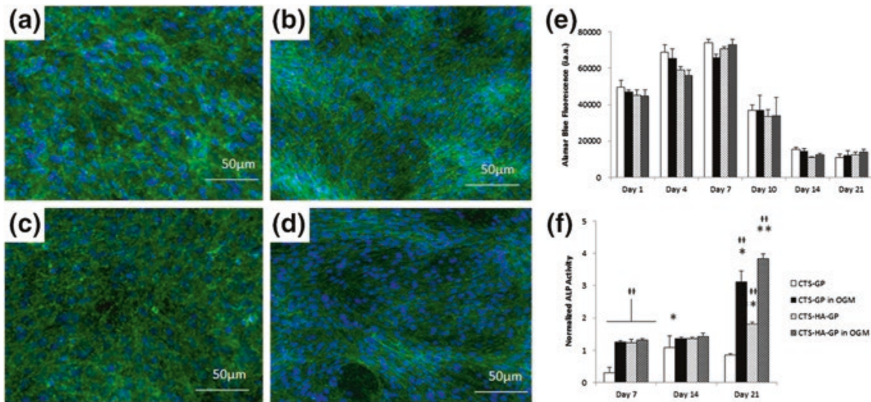

Fig. 4 In vitro assessment of osteogenic differentiation of mouse mesenchymal stem cells. Cell morphology was observed using DAPI/phalloidin (*blue*/*green*) staining and indicated formation of cellular multilayers on both scaffolds without hydroxyapatite **a** and **c** and with hydroxyapatite **b** and **d** at days 7 and 21 respectively. Reduction of Alamar blue activity between 14 and 21 days **e** coupled with an elevation in ALP activity at day 21 **f** is indicative of the mMSCs leaving the proliferative phase and entering the differentiation phase (Frohbergh et al. 2014)

immunogenicity and teratoma formation (Alvarez et al. 2012), lineage restricted, MSCs are preferentially used for bone tissue engineering (Ngiam et al. 2011).

In extending our *in vitro* studies, we tested the ability of our electrospun genipin-crosslinked scaffolds to promote osteogenic differentiation of murine MSCs (Frohbergh et al. 2014). As seen in Fig. 4, the scaffolds promote the assembly of multi-layer cell sheets on the surface, indicating appropriate adhesion of MSCs on the scaffold and the ability to form tissue-like structures on the scaffold surface (Fig. 4). They also induce initial osteogenic differentiation of MSCs which is further significantly enhanced in the presence of an osteogenic medium, indicating that the physicochemical cues from the material play a significant role in instigating MSC differentiation (Fig. 4).

3.4 HA Containing Chitosan Scaffolds are Osseointegrative/Osteoconductive

Osteoconduction is an important and substantial finding, indicating that these scaffolds can support osteogenesis. However, it is equally, if not more important to ensure that engineered materials are also integrative with the host/patient and can promote substantial tissue/scaffold interactions to induce self-healing and regeneration. To show the osseointegrative capacity of our electrospun scaffolds, we used a cranial defect murine model induced by micro-drilling and removal of a section of the skull (Fig. 5). Scaffolds were implanted with and without naïve MSCs in order to compare the healing competence of the scaffolds alone and in the presence of cytokine signaling from implanted cells (Frohbergh et al. 2014).

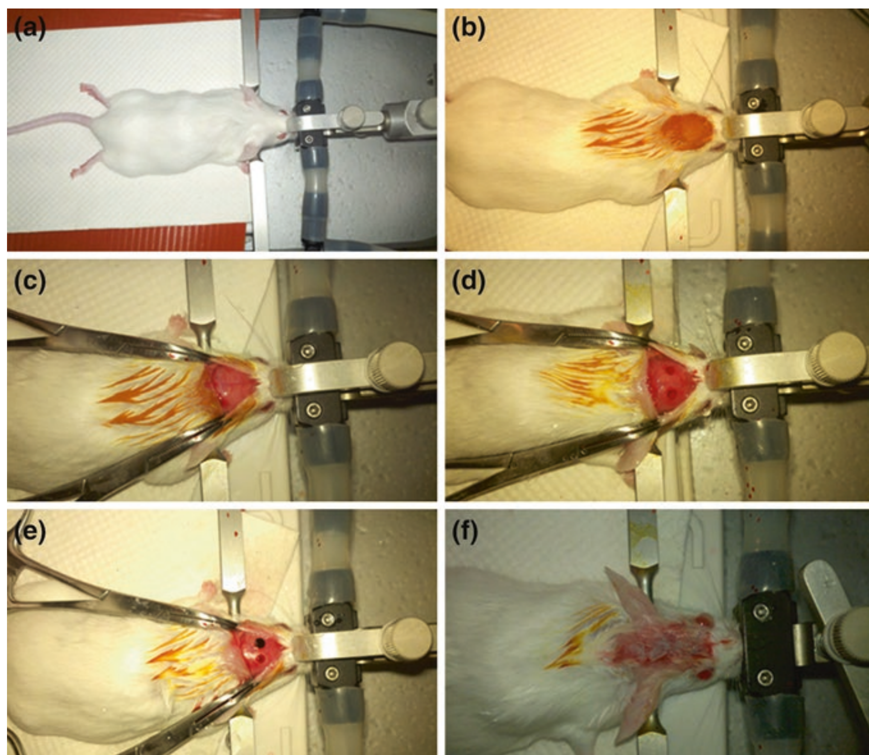

Fig. 5 Surgical Procedure to Generate Calvarial Defects. The animal was appropriately anesthetized and positioned in a stereotaxic fixture (**a**). The wound was shaved and sterilized (**b**). A distal incision was made exposing the parietal bones of the skull (**c**). Two critical size defects were drilled on either side of the sagittal suture, one for control (**d**) and the other fitted with a scaffold (**e**). Wounds were sutured and bio-glue was applied for extra stability (**f**)

Three month post-surgery, optimal osseointegration with the host tissue was provided by mineralized scaffolds that had been pre-seeded with MSCs, as inferred from both the presence of mineralized tissue in the defect area (microCT, Fig. 6 panel a) and of new, healthy tissue growing from the periphery of the wound onto the scaffold (histology, Fig. 6 panel d). In the absence of MSCs, the non-mineralized scaffold was essentially ineffective in inducing bone healing (Fig. 6 panel b), where as addition of MSCs to the non-mineralized scaffolds resulted in modest healing and bone regeneration (Fig. 6 panel c).

An ideal bioactive bone tissue scaffold will demonstrate two distinct properties: (1) the ability to induce host tissue migration and (2) minimize inflammation and immune rejection in the host. Crucial for the induction of bone tissue regeneration and healing is the migration osteoprogenitor cell from the periosteum (Allen et al. 2004; Hutmacher and Sittinger 2003; Zhang et al. 2008a; Zhang et al. 2005). Critical size defects in bone injuries do not effectively heal because there is no permissive tissue in the defect area for the osteoprogenitor cells to migrate onto in order

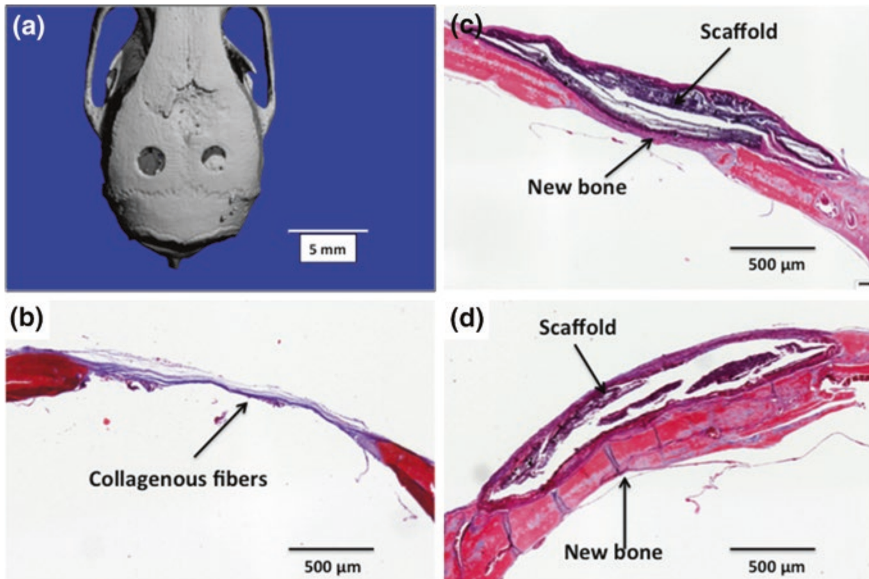

Fig. 6 Healing of critical size calvarial defects in a mouse model. Three months after surgery, microCT analysis shows significant formation of mineralized bone in critical size defects treated with mineralized genipin-crosslinked chitosan scaffolds (*right*) pre-seeded with murine mesenchymal stem cells (MSCs), but not in the untreated contralateral lesions (**a**). Panels B–D: Mason Trichrome staining of critical defects treated with non-mineralized scaffolds (**b**), non-mineralized scaffolds, pre-seeded with MSCs (**c**) and mineralized scaffolds pre-seeded with MSCs (**d**). Non-mineralized scaffolds failed to induce the healing process; the defects were covered with a collagenous matrix only (*blue*), as also seen with untreated samples (not shown). The MCSs had a minor beneficial effect in non-mineralized scaffolds. Note the significant enhancement of bone formation (*red*) in induced by MSCs when used in conjunction with mineralized scaffolds (Frohbergh et al. 2014)

to begin depositing matrix and initiate healing (Zhang et al. 2008a). Suitable biomaterials, such as genipin-cross-linked, mineralized chitosan, fulfill both the above requirements and can be used to bridge this gap and provide a template that will initiate and support the healing process to begin (Frohbergh 2013; Frohbergh et al. 2014).

In our studies untreated defects were covered by a thin acellular fibrous layer. In the absence of an appropriate scaffolding material, the critical size bone defect is will not heal on its own. The tissue growing on the scaffolds exhibits matrix formation and contain collagen type I, the main ECM component of newly forming bone tissue (Gentili and Cancedda 2009), as inferred from the Masson's Trichrome stain. Other studies have observed similar regenerative responses when using chitosan-based implants in vivo. For example, blended poly(vinyl alcohol)/N-methylene phosphonic chitosan scaffolds significantly increased ALP and collagen I levels in cultured MG-63 cells, a human osteosarcoma cell line and enhanced wound healing by 300 % when compared to untreated wounds in a rabbit tibia model (Datta et al. 2013). Liu and colleagues (2013) showed the ability of chitosan/hydroxyapatite/ultra-high molecular weight poly (ethylene oxide) scaffolds

to support MSC proliferation and osteogenic differentiation in vitro via the BMP/SMAD pathway. These authors also showed that their scaffolds promoted bone healing in a rat calvarial defect model more effectively than chitosan alone and chitosan/hydroxyapatite membranes (Liu et al. 2013).

Numerous preclinical studies demonstrated that implanting osteoinductive scaffolds seeded with naïve or pre-differentiated allogeneic or autologous progenitor cells results in enhanced regenerative capabilities of the cell-seeded versus the cell-free constructs (Mestak et al. 2013; Tasso et al. 2009; Jin et al. 2009). While the outcomes of these studies generally support the notion that the presence of progenitor (or even differentiated cells) will benefit wound repair and tissue regeneration, the clinical implementation of this concept may still be limited by numerous problems surrounding the use of cells, such as cell sourcing (what kind of cells to use, at what stage of differentiation, how to obtain enough of them, etc.) and potential immunogenicity and teratogenicity in the case of stem cells (whether embryonic or iPS). Moreover, from a translational standpoint, handling, storing, transporting cell-based tissue engineered constructs, is complex, to say the least, and may thwart the commercial success of technically/scientifically/clinically promising regenerative biomaterials, e.g. recently happened with some "living" skin substitutes.

3.5 Conclusions

The induction of de novo tissue formation around the scaffold suggests that our scaffolds *per se* are permissive and promote proper host integration. Given their mechanical properties, these scaffolds hold potential promise for treating non-load bearing bone injuries. While tissue integration and immunosuppression are of upmost concern, the end goal is to engineer a scaffold that is osteoconductive and will lead to fully function bone tissue. Our results suggest that the presence of hydroxyapatite greatly enhances the osteogenic capacity of these scaffolds and leads to mineralized tissue formation by month 3. Osteoconduction can be improved with the presence of MSCs. Quantitatively there was up to a 5 fold increase in defect closure versus scaffolds without hydroxyapatite and MSCs. Further, MSC seeded hydroxyapatite-containing scaffolds only showed ~10 % more wound healing than hydroxyapatite-containing scaffolds without cells, indicating that the mineralized scaffolds by themselves were fully capable of inducing enhanced wound healing without the need for a cellular component. This makes these scaffolds clinically relevant with the added benefit of off-the-shelf availability and no time (and additional expenses) required for cell culture and scaffold preparation prior to implant. Combined with the findings of endochondral tissue formation on the composite scaffolds after 3 months of implantation, we can conclude that this de novo generated tissue is in the early stages of endochondral ossification and that mineralized ECM is beginning to replace cartilage tissue. Interestingly, the normal development process of cranial bone is intramembranous ossification.

Further studies into the mechanisms involved in tissue formation on these genipin-cross-linked mineralized chitosan scaffolds are warranted and may yield a new and improved manner to initiate endochondral bone healing in cranial bones.

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Biomaterials Used for Maxillofacial Regeneration

Xinquan Jiang and Zhiyuan Zhang

Abstract Critical-sized bone defects in the maxillofacial region attributed to congenital maldevelopment, trauma, periodontal disease, or surgical ablation, as in the case of tumor surgery, and progressive resorption of the alveolar bone after tooth loss can cause damage to their structures, leading to noticeable deformity and dysfunction. Therefore, maxillofacial bone regeneration has been attracting great interest of many surgical specialties, specialties of dentistry, and experts in the region of stem cell and biomaterial. Clinical imperatives for maxillofacial bone regeneration require new therapies or procedures instead of autologous/allogeneic bone grafts. A variety of biomaterials have been developed as alternatives over a short period of time. This chapter reviews current clinical treatments and the biomaterials clinically used for maxillofacial bone regeneration. Moreover, recent advances and future directions in biomaterials used for maxillofacial bone regeneration have been discussed in the present chapter.

1 Introduction

The bones in the maxillofacial region include the mandible (1), the vomer (1), the maxilla (2), the zygomata (2), the nasal (2), the lacrimals (2), the palatines (2), and the inferior nasal conchae (2). Unlike the long bones in other sites, maxillofacial bones are small, delicate, and located near areas that are highly contaminated with bacteria. Their proper function depends not only on load bearing but also on permanently maintaining a specific three-dimensional (3D) shape. They support soft tissue structures and specialized organs essential for many

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basic life functions—breathing, speaking, chewing, and swallowing. They form the basis for the unique physical appearances of every human being (Sutradhar et al. 2010).

Critical-sized bone defects in the maxillofacial region attributed to congenital maldevelopment, trauma, periodontal disease, or surgical ablation, as in the case of tumor surgery, and progressive resorption of the alveolar bone after tooth loss can cause damage to their structure, leading to noticeable deformity and dysfunction (Kinoshita and Maeda 2013). Unlike bone defects in other sites, bone defects in the maxillofacial region require full restoration of complicated three-dimensional structures, which may necessitate follow-up dentures or dental implants for restoration of function (Kinoshita and Maeda 2013). Therefore, the ultimate goal of maxillofacial bone repair is the regeneration of physiological bone to simultaneously achieve restoration of both morphology and function.

2 Current Clinical Treatments for Maxillofacial Bone Regeneration

2.1 Bone Grafting

Bone grafting is a surgical procedure that places new bone into spaces between or around broken bone (fractures) or in holes in the bone (defects) to aid in healing. In general, bone grafts that are clinically used for repairing maxillofacial bone defects can be categorized as autografts, allografts, and xenografts depending on their source (Sàndor et al. 2003a).

2.1.1 Autografts

An autograft, which is autogenous bone harvested from the patient's own body, is considered excellent because of its osteoconductive and osteoinductive properties stemming from the osteoprogenitor cells contained inside. More importantly, it is the gold standard by which all techniques for the osseous reconstruction of maxillofacial bone must be judged (Rosenberg and Rose 1998)

Autografts can be vascularized or nonvascularized. Vascularized autografts are much more complicated to harvest and have a great deal of donor site morbidity associated with their application. Nonvascularized autografts are considerably easier to harvest and apply if they are placed into a well-vascularized recipient bed (Marx 1993). Generally, nonvascularized autografts are composed of either cortical or cancellous bone. Cortical grafts, which are harvested from the cranial vault, the ribs, the medial or lateral table of the anterior aspect of the iliac crest, or the posterior iliac crest or the mandibular symphysis, are able to withstand mechanical forces earlier; however, these grafts may need more time to revascularize (Sàndor et al. 2003a; Boyne and Peetz 1997). Cancellous grafts, usually harvested from the

anterior or posterior iliac crest, are considered easier to manipulate and revascularize more rapidly (Marx 1993). Cancellous grafts impart no mechanical strength, meaning that when they are applied to reconstruct large continuity defects, additional stability and rigid fixation is required. In maxillofacial bone repair, these grafts can be packed into bone defects, such as alveolar clefts and maxillary sinus floor elevations (Boyne and James 1980). However, the major disadvantage of autografts is the need for a second surgical site and the morbidity resulting from harvesting. Moreover, autografts are not a limitless resource. A point may be reached in reconstruction at which the donor site morbidity may exceed the discomfort of the presenting complaint. Moreover, such potential discomfort is a serious reason why patients avoid presenting themselves for reconstructive procedures (Sàndor et al. 2003a).

2.1.2 Allografts

An allograft is allogeneic nonvital osseous tissue taken from one individual and transferred to another individual of the same species. It includes freeze-dried bone allografts (FDBA) and demineralized freeze-dried bone allografts (DFDBA).

FDBA, which are processed to remove moisture from the bone, possess mechanical strength, allowing them to be implanted in onlay areas or as a crib to retain autogenous bone (Marx 1993). This bone graft can act as an osteoconductive scaffold but lacks osteogenic or osteoinductive capabilities and consequently requires a source of osteocompetent cells. Therefore, FDBA are usually implanted in conjunction with autografts for repairing maxillofacial bone defects (Sàndor et al. 2003a). DFDBA lacks mechanical strength, but can retain some osteoinductive properties through the exposure of osteogenic proteins, such as bone morphogenetic protein (BMP), upon removal of the mineral component (Urist 1965; Zhang et al. 1997a and 1997b). Recent studies have shown that DFDBA can be incorporated into various carriers, such as collagen or selected polymers (Helm et al. 1997; Babbush 1998). These grafts could potentially be applied in the treatment of periodontal infrabony defects, in extraction sites to prevent ridge resorption, in alveolar ridge augmentation, and in bone reconstruction associated with dental implant placement and dental implant complications as well as in cysts or bone defects of the jaw (Caplanis et al. 1997; Becker et al. 1998; Campbell 1998; Kim et al. 1998; Kumta et al. 1998; Parashis et al. 1998; Rosenberg and Rose 1998; Wiesen and Kitzis 1997). If a greater volume of bone graft is required, such as in maxillary sinus augmentation prior to dental implant placement, then DFDBA may be used as a bone graft expander to reduce the volume of bone graft required to fill an osseous defect (Blomqvist et al. 1998; Goldberg and Baer 1997; Stevenson 1998). This reduced graft volume may allow the use of an intraoral harvest site, which may reduce patient morbidity by avoiding an extra-oral donor site. However, the major disadvantage of this technique is the cost of the DFDBA scaffold; moreover, donor variability also limits the predictability of DFDBA as an osteoinductive material (Boyan et al. 2006).

2.1.3 Xenografts

Xenografts consist of bone tissue that is harvested from one species and transferred to the recipient site of another species (Auchincloss and Sachs 1998; Hammer et al. 2009). These grafts can be derived from mammalian bones or coral exoskeletons.

Bovine derived bone grafts (Bio-Oss®) have been commonly used, although other sources, such as porcine or murine bone, are also available (Jensen et al. 1996; Sukumar and Drízhal 2008). This bone graft has only the structure of bone, making it osteoconductive without osteoinductive properties. Eventually, the xenograft should be replaced by host bone tissue, which would make it useful for defect or extraction site filling in the alveolus prior to dental implant placement or prosthetic rehabilitation (Skoglund et al. 1997; Valentini et al. 1998; Lang et al. 2007; Juodzbalys and Wang 2007; Bornstein et al. 2008). Resorption of bovine-derived bone has been observed in animal studies but not consistently in human clinical trials (Merkx et al. 2003; Skoglund et al. 1997; Valentini et al. 1998). Moreover, this bone graft is usually a powder, and it may be necessary to use a retentive structure, such as a membrane, to keep the graft in the desired location (Avera et al. 1997; Zitzmann et al. 1997). The main disadvantage is the concern regarding the possibility of future variant Creutzfeldt–Jakob disease due to potential prion transmission (Bons et al. 2002; Hunter 2002).

Biocoral, which is derived directly from the exoskeletons of corals from the Madrepora group of the genus *Acropora*, has been evaluated as a xenogeneic bone graft (Guillemin et al. 1987). Both solid blocks and particulated grafts fashioned from biocoral are osteoconductive due to its calcium carbonate composition (Piattelli et al. 1997). Moreover, this graft is simultaneously incorporated into the host bone and replaced by newly formed bone. The enzyme carbonic anhydrase liberated by osteoclasts is responsible for the degradation of this graft. Because the use of biocoral graft granules can induce new bone formation which, together with the material's eventual replacement, could decrease morbidity by avoiding the need for a bone graft harvest donor site (Sandor et al. 2003b).

2.2 Distraction Osteogenesis

Distraction osteogenesis (DO) is a method for either restoring atrophic jaws in the vertical dimension or for expanding congenital defected jaws in the maxillofacial region (Cheung et al. 2010). The DO technique is a two-stage surgical technique and can be used either when teeth are missing and the alveolar ridge needs to be vertically expanded with bone before dental implants or in the case of an open bite with good occlusion in the molar region of the jaws when conventional orthognathic surgery is not possible (Cano et al. 2006). A reliable patient may be needed who will expand the device each day, and DO also necessitates a long retaining period that includes orthodontic treatment. Moreover, the device needs to be

removed surgically after the retaining period (Saulacic et al. 2009). The benefits of DO are that donor site morbidity from the harvesting of bone grafts and dehiscence of the grafted bone are avoided. However, a second surgery to remove and perhaps replace hardware is needed. Moreover, the patient may suffer the inconvenience of wearing and tolerating potentially cumbersome hardware for longer periods of time (Sàndor et al. 2003a).

2.3 Guided Bone Regeneration

Guided bone regeneration (GBR) has been used for minor augmentation procedures in the maxillofacial region and prior to dental implant placement (Buser et al. 1990; Simion et al. 2001). GBR is a technique in which bone formation is enhanced by preventing soft tissue ingrowth into the desired area by utilizing either resorbable or nonresorbable membranes. Recently, metallic membranes or membranes supported by a titanium frame have also been tested (von Arx et al. 1996). In particular, an acellular dermal matrix has been used as a barrier membrane with DFDBA (Fowler et al. 2000). However, the use of nonresorbable membranes requires a second operation for their removal (von Arx et al. 1996), while resorbable membranes may be associated with inflammation (Yoshinari et al. 1998).

3 The Biomaterials Used for Maxillofacial Bone Regeneration

3.1 Biomaterials Clinically Used for Maxillofacial Regeneration

For the purpose of replacing natural bone, a variety of biomaterials consisting of inorganic and organic materials have been developed as alternatives to autogenous bone grafts. Biomaterials including ceramics, glasses, and polymers have been clinically used for maxillofacial bone regeneration.

Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, HA) bioceramics are common bone graft materials due to their good bioactive properties. HA bioceramic began commercial production in the early 1970s and then was tested for bone regeneration in 24 human patients by Weissman to augment frontal and ethmoid sinus regions and mastoid cavities (Weissman et al. 1996). To date, HA has been used in several clinical applications, including the filling of bony defects, the retention of alveolar ridge forms following tooth extraction and use as a bone expander when combined with autogenous bone during ridge augmentation and sinus grafting procedures (Sàndor et al. 2003a). However, the brittleness and low solubility of HA bioceramics may limit their further clinical application in bone regeneration (Fleming et al.2000).

Tricalcium phosphate ($Ca_3(PO_4)_2$, TCP) is similar to HA, being a calcium phosphate with a different stoichiometric profile (Mors and Kaminski 1975; Hollinger et al. 1989). Two products (Norian SRS®, Norian Corporation, Cupertino, California, USA and Bone Source®, Leibinger, Dallas, Texas, USA) have been used for the repair of cranial vault defects. Our group has also evaluated porous β-TCP combined with autologous osteoblasts to augment alveolar ridge and maxillary sinus and found that it could achieve repair effectiveness superior to that of autogenous bone for simultaneous implantation (Fig. 1) (Wang et al. 2009, 2010, 2011a, b). Importantly, our group has also successfully regenerated 30 mm segmental mandibles by combining autogenous bone marrow stem cells (BMSCs) with β-TCP in dogs (He et al. 2007). However, the major disadvantage of TCP for

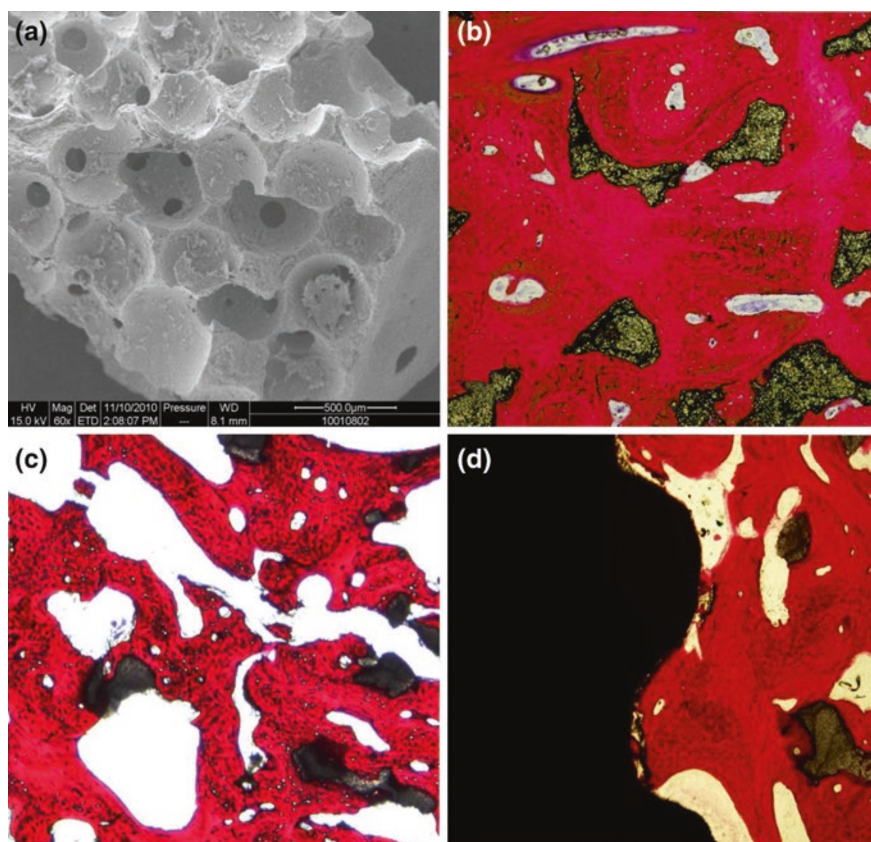

Fig. 1 Constructs of β-TCP and osteoblasts for maxillofacial bone regeneration. **a** Scanning electron microscopic evaluation of osteoblasts seeded on β-TCP.; **b** Vertical alveolar ridge augmentation with β-TCP and osteoblasts in a canine model.; **c** Maxillary sinus augmentation with β-TCP and osteoblasts in a canine model.; **d** Osseointegration of dental implants with tissue-engineered bone constructed with β-TCP and osteoblasts

clinic applications is its unpredictable rate of bioresorption. Its degradation has not always been associated with the concomitant deposition of bone (Ohgushi et al. 1990; Buser et al. 1998).

Bioactive glasses are silicophosphate chains used in dentistry as restorative materials, such as glass ionomer cement. The original Bioglass 45S5, which bonds with bone rapidly and promotes bone regeneration, has been applied in more than a million patients to repair bone defects (Cao and Hench 1996; Hench 2011; Mitchell et al. 2011; Andersson and Kangasniemi 1991). The first commercial product was perioglas, which was released in 1993 as a synthetic bone graft for the repair of defects in the jaw that result from periodontal disease. Moreover, the next generations of Bioglass 45S5 devices, such as ovabone®, Biogran®, and bonalive®, received European approval for orthopedic use as bone graft substitutes in 2006 (Jones 2013). However, clinical and in vivo studies on commercially available bioactive glass particulates showed that bioactive glasses might perform better than other bioceramic particles but not as well as autograft bone.

In recent years, polymeric biomaterials have been extensively studied for bone regeneration; in particular, more attention has been paid to synthetic biodegradable polymers because of their physicochemical properties and because they have already been successfully used in some clinical applications. The most widely used synthetic polyesters in oral and maxillofacial applications are poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and a combination of PGA and PLA (PLGA) (Sokolsky-Papkov et al. 2007). Resorbable membranes made of PLA and PLGA have been used as barriers in guided tissue regeneration (GTR) to treat periodontal defects (Christgau et al. 1998). Root replicates fabricated with PLA and bioglasses have been introduced to preserve the form of the alveolar ridge after tooth loss (Suhonen and Meyer 1996). However, giant cell reactions presented a problem with earlier combinations of PLGA (Brekke 1995). Moreover, the incomplete resorption of polymeric biomaterials can delay placement of dental implants (Suhonen and Meyer 1996).

3.2 Recent Advances in Biomaterials Used for Maxillofacial Regeneration

In maxillofacial bone regeneration, the ideal bone biomaterials should replace the function of the bone tissue by matching the mechanical properties of the material to those of native bone and by incorporating the ability to transmit mechanical cues, which can regulate cell and matrix biology and promote remodeling and regeneration. These biomaterials should possess innate osteoconductivity due to their porosity, permeability, and diffusivity and allow the integration of cells or bioactive factors. Moreover, a critical balance between the degradation rate of the implanted biomaterial and the rate of new matrix deposition should be matched (McMahon et al. 2013). To date, no bone substitute material that clinically satisfies all these conditions has been developed.

Various biomaterial design strategies have been developed to address the clinical need for maxillofacial bone repair, reconstruction, and regeneration. Both structural design and chemical composition (bioinorganics) are parameters that have been addressed to overcome issues with bone formation in vitro and in vivo (Christenson et al. 2007; James et al. 2011; Zhang 2011). Moreover, bone is highly vascularized; therefore, the performance of an ideal bone scaffold is dictated by its ability to induce angiogenesis (Rouwkema et al. 2008; Bramfeldt et al. 2010; Jain et al. 2005).

3.2.1 Structural Design

The latest approach to engineering biomaterial architectures is fabricating nanometer scale features within a macroscale defect size-specific design (McMahon et al. 2013). Moreover, computer-aided design/computer-aided manufacturing (CAD/CAM) techniques can be used to fabricate anatomically customized scaffolds (Jiang 2011).

Nanotechnology

The reconstruction of bone defects in the maxillofacial area caused by various factors requires both functional and esthetic maintenance and improvement (Jiang 2011). The current biomaterials used for bone tissue engineering, such as bioceramics, bioglasses and others, leave much room for modification to become true bone structure biomimetics, which could provide cells with native mechanical and physicochemical cues.

Bone is composed of organic (protein) and inorganic (mineral) phases. On the macroscale, cortical bone wraps around cancellous bone. The former, as a shell, protects the inner bone and provides support and protection, while the latter, as a sponge, contains bone marrow, transfers forces, and balances the weight of the integral organ (McMahon et al. 2013). However, the hierarchical organization, ranging from trabeculae (in cancellous bone) and osteon units (in cortical bone) to collagen fibers, collagen fibrils, collagen molecules, and the dispersion of HA crystals, gradually decreases in size, spanning several orders of magnitude, from the macro- to the nanoscale. Thus, it can be assumed that functional cells will be more accustomed to nanoscale structures and properties due to their interactions at the cell–matrix interface. Because the mimicking of natural structures is typically the most basic and effective route to artificial materials design, the consideration of the hierarchical structure of bone is of vital importance for developing a new generation of biomaterials. In fact, nanobiomaterials and nanocomposites have been developed using different materials and techniques to recapitulate native bone for years.

Nanospheres or nanoparticles can be dispersed throughout a continuous matrix to induce porosity, to improve the mechanical properties of a bulk scaffold

as a reinforcement phase or as a crosslinking agent, and to act as drug delivery vehicles (Habraken et al. 2006; Matsuno et al. 2008; Arimura et al. 2005; Gupta et al. 2009). They can also be used as building blocks to establish scaffolds by a bottom-up approach without a surrounding matrix material (Matsuno et al. 2008; Habraken et al. 2006; Arimura et al. 2005; Anderson et al. 2005; Kim and Fisher 2007; Wang et al. 2011). With random packing or directed assembly, an injectable gel, which acts as a scaffold upon implantation, can be developed for minimally invasive surgery. These can be mixtures of nano- and micro-particles or mixtures of different materials to prepare composite gels. The poor integrity of these injectable gel scaffolds, resulting from their weak interparticle interactions, leads to poor mechanical stability; therefore, glues or cross-linkers have been applied to preserve the agglomeration of micro/nanosphere formulations after implantation (Ahmed et al. 2008; Lemperle et al. 2004; Cho et al. 2008). Directing assembly by introducing interparticle forces (such as electrostatic forces or hydrophobic interactions) can produce injectable formulations with enhanced structural integrity and mechanical stability without the use of glues or cross-linkers.

Nanofiber scaffolds can be fabricated using phase separation, self-assembly, and electrospinning methods (Smith and Ma 2004; Whitesides and Boncheva 2002; Prabhakaran et al. 2011). For example, nanofibrous PLLA scaffolds, fabricated by either electrospinning or phase separation, have been widely studied in in vivo animal models. In a rat critical-size calvarial bone defect model, nanofibrous PLLA scaffolds could support substantially more new bone tissue formation than solid wall scaffolds in a control group. Moreover, abundant collagen deposition and strong immunostaining for Runx2 and BSP were observed in the nanofibrous group but not in the control group (Woo et al. 2009). It was also reported that nanofibrous PLLA scaffolds could be combined with collagenous guided bone regeneration membranes or with BMP-2 to enhance their performance in vivo (Cai et al. 2010; Schofer et al. 2011). These studies strongly suggest the advantages of applying nanofibrous scaffolds to enhance bone regeneration.

Carbon nanotubes (CNTs) are well-ordered, hollow structures with excellent mechanical strength. CNTs can exist as single-walled nanotubes or as concentric cylinders of carbon (multiwalled CNTs, MWCNT). A previous study incorporated CNTs into HA to improve its mechanical properties for load-bearing applications (Aryal et al. 2006). It was also reported that CNTs could promote the attachment, proliferation, and differentiation of osteoblasts and BMSCs (Nair et al. 2004; Balani et al. 2007; Price et al. 2003). Moreover, nanotube composites made of CNT homogenously distributed in microporous PPF/propylene fumarate diacrylate scaffolds have been demonstrated to repair subcutaneous and bilateral femoral defects in a New Zealand white rabbit model (Sitharaman et al. 2008). The results showed that nanocomposite scaffolds could achieve greater bone tissue ingrowth compared to that with control polymer scaffolds. Additionally, the 12-week samples showed reduced inflammatory cell density and increased connective tissue organization. However, some studies have indicated CNT cytotoxicity, while others have shown nanotubes to be excellent substrates for cell growth, and CNTs

have been incorporated into composites to enhance matrix mechanical problems with little to no in vivo cytotoxic effect (Harrison 2007).

Recently, the synthesis of materials with specific morphologies has attracted great interest because of the unique physical and biological properties of these materials and their potential applications in advanced functional materials (Lin et al. 2011a). The morphology of an HA bioceramic is of great importance to its performance. For example, fibrous and plate-like HA particles have stronger adsorption properties resulting from their increased surface area, while HA whiskers can be used for the mechanical reinforcement of biomaterials (Vasiliev et al. 2008; Roeder et al. 2003; Müller et al. 2007). Recently, it was reported that various nanostructure morphologies of HA, from single morphologies such as nanofibers, nanorods, nanosheet, etc. to multimorphologies, have been fabricated using the hydrothermal method (Lin et al. 2011a, b; Liu et al. 2011). More importantly, our group has obtained a macroporous HA bioceramic with highly interconnective pore structures and distinct nanostructure topographies comprising nanosheets, nanorods, and micro-nano-hybrids (hybrids of nanorods and microrods) from α-TCP ceramics as a precursor under hydrothermal reaction conditions (Lin et al. 2013a). Through the systematic analysis of rat BMMSC attachment, spreading, proliferation, and osteogenic differentiation, we proved that HA bioceramics with these nanostructures could promote the in vitro cell behaviors mentioned above, while the micro-nano-hybrid surface possessed the highest stimulatory effect (Fig. 2). The in vivo findings

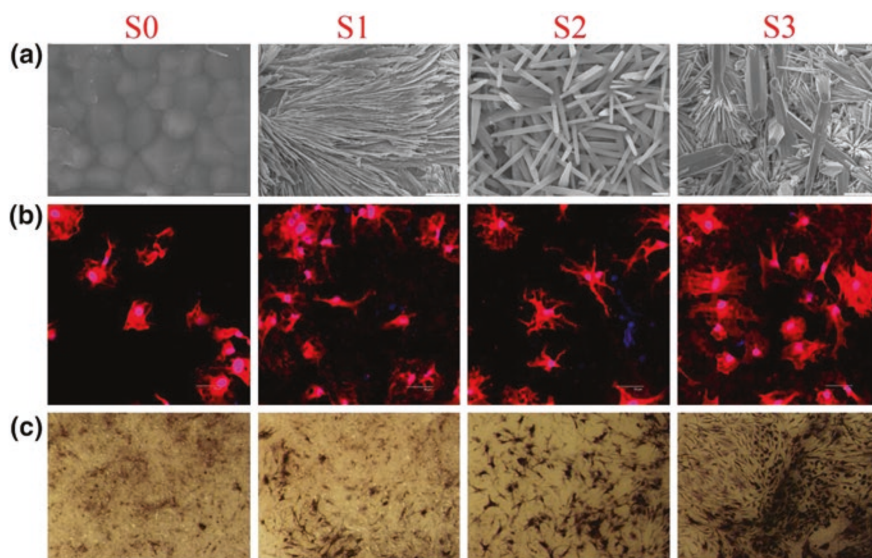

Fig. 2 Nanostructured surfaces of HA bioceramics for bone regeneration. **a** Scanning electron microscope images of the control sample S0 and of the fabricated HA bioceramics with different topographic surfaces: nanosheets (S1), nanorods (S2), and micro-nano-hybrids (S3).; **b** Confocal microscope images of the topographic effect of the HA nanostructures on BMSCs cell adhesion after 6 h of seeding.; **c** ALP staining of BMSCs cultured in samples S0–S3 for 10 days

further proved that HA bioceramic scaffolds with nano-topography surfaces promoted new bone formation and mineralization, with the micro-nano-hybrid surface exhibiting the best performance (Xia et al. 2013).

CAD/CAM Technique

Recently, the fabrication techniques used for scaffolds in bone regeneration have not only undergone a transformation from two-dimensional (2D) substrates to three-dimensional (3D) patterned scaffolds but have also been increasingly combined closely with computer-aided design, especially CAD/CAM techniques (Bose et al. 2012).

Traditional fabrication methods, including freeze-drying, emulsion freeze-drying, solution casting, salt leaching, electrospinning, gas foaming, melt molding, phase separation, and fiber deposition, can allow control over bulk physical properties, such as material stiffness and swelling. However, these methods may not precisely control porosity, pore size, pore geometry, pore distribution, and the internal channels between pores. This could lead to the uneven distribution of oxygen and nutrients, encouraging cells to migrate to the superficial layer of the scaffold. Through the application of digital technology, the scaffold can be designed according to the morphology of the defect, with the structure of cortical bone and cancellous bone inside. The pore size, pore geometry, pore distribution, and the internal channels between the pores can be designed individually, and the overall level of pore space can be controlled precisely (Chu and Liu 2008).

Computer-assisted direct-writing approaches, which could precisely dictate internal architecture as well as customize the appearance of the material, may facilitate greater control over seeded or recruited cells. Rapid prototyping (RP), also known as additive manufacturing, additive fabrication, solid freeform fabrication (SFF), and layered manufacturing, is the name of a host of technologies that are used to quickly fabricate physical objects from CAD data sources (Yeong et al. 2004). Generally, a required object is modeled by computer software and broken down into a series of image slices in a manner similar to the inverse process of 3D reconstruction from computer tomography (CT) or magnetic resonance images (MRI). Fabricating the slices in a layer-by-layer fashion, the 3D scaffold is obtained by stacking continuous solidified layers. The basis for the CAD data sets can be a CT or MRI scan of the defect region. In contrast to the conventional methods used for scaffold fabrication, the main advantage of RP techniques is their ability to design and control the external and internal architecture of a scaffold in a cost efficient, convenient, and customized manner (Peltola et al. 2008).

As mentioned above, RP fabrication includes dozens of techniques, such as stereolithography (SLA for stereolithography apparatus), selective laser sintering (SLS), fused deposition modeling (FDM), three-dimensional plotting, electron beam melting, inkjet-based systems, and three-dimensional printing (3DP) (Yeong et al. 2004). Each of these technologies handles different raw materials and boasts its own unique characteristics. In practice, the names of these specific processes are sometimes

recognized as synonyms for the entire field of RP. Several methods, such as SLA, FDM, modified thermal inkjet printing, and 3DP, have been applied within the field of bone regeneration and achieved promising results for new bone formation in vivo.

The SLA process applies an ultraviolet laser beam to initiate the polymerization and solidification of a liquid, photocurable monomer for each layer in an iterative manner. The UV beam is guided on each plane (2D) according to the CAD set. After the previous layer is built, the model elevator is lowered to allow the accumulation of the liquid photopolymer (3D) (Yang et al. 2002). This system requires support structures for unconnected parts to prevent any features from falling to the bottom of the vat, which would be manually removed after completion.

The FDM process uses a moving nozzle to selectively extrude a thermoplastic fiber (2D). The model is lowered, and the procedure repeats layer-by-layer (3D). Because the pore sizes are sufficiently small for tissue engineering scaffolds, no support structure is required, as the fiber can bridge across unconnected parts (Yeong et al. 2004; Chandramohan and Marimuthu 2011).

The inkjet printing process utilizes thermal, piezoelectric, and electromagnetic approaches to create tiny ink drops, with which a 2D section is reproduced via a moving nozzle in accordance with the digital pattern information. This process is repeated for every layer until the 3D structure is completed. Many materials, such as DNA molecules and mammalian cells, are currently available to be employed as ink drops (Singh et al. 2010).

The 3DP process incorporates conventional ink jet printing technology to extrude a binder fluid from a jet head onto a polymer powder bed. The fluid joins adjacent powder particles and thus forms part of the solid's cross section (2D). The piston chamber is lowered and refilled with another layer of powder. The solidification, displacement in the z-axis, and powder refilling process are repeated layer-by-layer to obtain a 3D object. The remaining powder must be removed after component completion. A crucial advantage of 3DP in bone regeneration is the wide range of materials that can be used. The sole requirement is the availability of the material in powder form. Theoretically, the combination of different powders is possible if suitable binders can be employed. To date, synthetic and natural polymers, ceramics, bioglass, and several composites have been used in powder-based 3DP for bone tissue engineering (Chandramohan and Marimuthu 2011). However, several shortcomings, such as low resolution, inadequate mechanical properties, unsatisfactory biocompatibility, and limited vascularization caused by the long-term viability of seeding cells, need to be mentioned. Some of these challenges and limiting factors are also faced by scaffolds fabricated by traditional methods; however, it is reasonable to assume that 3DP possesses clearer strategies to address these problems. The realization of functional materials with proper compositions, of fine powder particles with high flowability, of binder droplets with optimal sizes, and of high resolution radiographic imaging could largely improve the use of 3DP for scaffold engineering (Chua et al. 2010).

Aside from RP techniques for scaffold fabrication, surface-based technologies (for example, lithographic techniques) also account for an important approach in the field of bone tissue engineering. It has been demonstrated that specific surface

topologies can enhance material biocompatibility, lead to better cell attachment, improve cell–material interactions, and direct cell fate when implanted in vivo. In fact, a 3D scaffold can be built by stacking the fabricated membranes. Specific methods, such as micropatterning or microcontact printing, colloidal lithography, and electrically induced pattern transfer are widely applied in microelectronics, surface chemistry, and cell biology (Moroni et al. 2008).

3.2.2 Bioinorganics

Because both calcium and phosphorus are the main inorganic components of skeletal tissue, bioinorganic stents comprising calcium and phosphorus could provide a living environment similar to human skeletal tissue for the proliferation of osteoblasts. The field of bioinorganics has been well established in the development of therapies for bone defects. The effect of inorganic ions on health is largely known through the documented effects of deficiencies of essential micronutrients. In addition to the ions of calcium, phosphate, and silicate described previously, substantial evidence from the literature shows that magnesium, strontium, zinc, copper, and lithium ions play a vital role in osteogenesis.

Magnesium

Magnesium (Mg) is an essential element and the tenth most abundant element in the human body, with approximately 65 % of the total body magnesium contained in the bones and teeth (Rude et al. 2005). In vivo studies have noted that calcium phosphate cement (CPC) doped with magnesium phosphate in the maxillary sinus floor elevation showed greater biodegradability and excellent osteoconductivity when compared to control CPC. These studies also indicated that tissue-engineered bone constructed of CMPC and BMSCs might be a potential alterative graft for maxillofacial bone regeneration (Fig. 3) (Zeng et al. 2012). Magnesium has been used clinically in magnesium phosphate bone cements and in several different bioglass compositions.

Strontium

Strontium (Sr) is a nonessential element that accounts for 0.035 % of the calcium content in our skeleton system. As a bone seeker, it has been shown that Sr^{2+} can enhance bone regeneration when incorporated into synthetic bone grafts. Essentially, because it is similar to Ca^{2+} in size, it is thought to displace Ca^{2+} ions in osteoblast-mediated processes. Researchers have identified that strontium most likely stimulates bone formation by a dual mode of action. First, it activates the calcium sensing receptor (CaSR) in osteoblasts, which simultaneously increases osteoprotegerin (OPG) production and decreases the receptor activator of nuclear factor kappa beta ligand (RANKL) expression (Coulombe et al. 2004). Recently, we

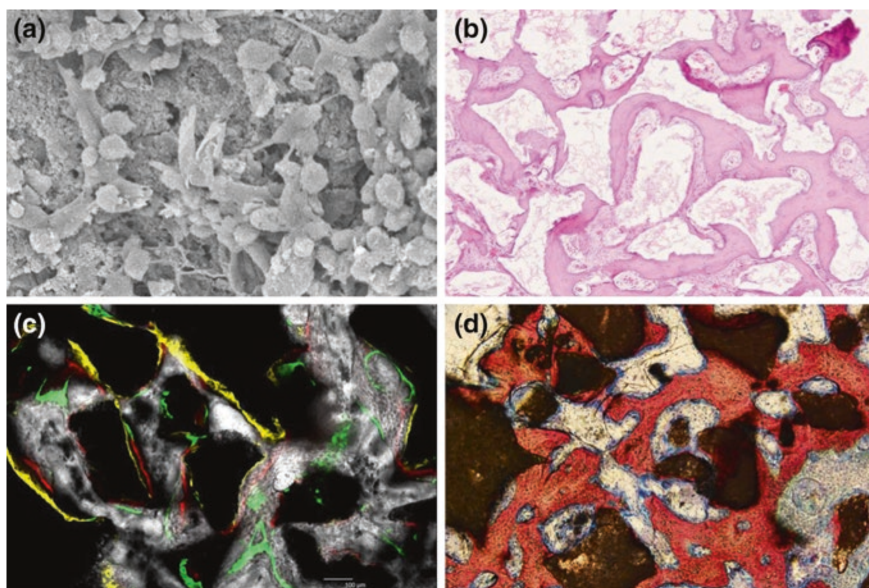

Fig. 3 Maxillary sinus floor elevation with CMPC and BMSCs in rabbits. **a** Scanning electron microscope images of BMSCs attached to CMPC.; **b** HE staining image of newly formed bone in the CMPC/BMSCs group.; **c** Sequential fluorescent labeling was used to determine the rate of bone formation and mineralization in the CMPC/BMSCs group at 2, 4, and 8 weeks after operation.; **d** Microscopic view of the bone formation in the maxillary sinus from a nondecalcified slide in the CMPC/BMSCs group

reported that Sr-substituted calcium silicate (SrCS) ceramic scaffolds could promote the osteogenic differentiation of BMSCs derived from ovariectomized rats (BMSCs-OVX). Moreover, in vivo results revealed that SrCS dramatically stimulated bone regeneration in a critical-sized OVX calvarial defect model (Lin et al. 2013b).

Zinc

Zinc (Zn) has been known to play an important role in various physiological processes because it is involved in the synthesis of a large number of proteins and is required for their stability. There are several important metalloenzymes that utilize zinc for structural, catalytic, or regulatory actions. One such enzyme, which is absolutely vital for the maturation of new bone formations, is alkaline phosphatase (ALP). Furthermore, zinc deficiency is associated with a number of skeletal anomalies in fetal and postnatal development, such as decreased bone age. As a recent review reported, zinc has been shown to have an inhibitory effect on bone resorption in tissue culture systems in vitro and to suppress osteoclastogenesis in osteoclast precursor cells derived from bone marrow. A number of studies have been performed in vitro to study the effect of the incorporation of zinc

into calcium phosphate bioceramics on the biological processes related to bone formation and turnover. One study investigated the osteogenic ability of rat and human BMSCs cultured in HA/TCP ceramics containing zinc in amounts varying between 0 and 1.3 wt%. Both rat and human BMSCs cultured in an osteogenic medium showed an increase in ALP expression with increasing zinc content in the HA/TCP ceramic (Ikeuchi et al. 2003). In another study, a positive effect on the proliferation of the MC3T3 osteoblastic cell line was observed in HA/TCP ceramics containing up to 1.3 wt% zinc, whereas higher concentrations caused cytotoxicity (Xue et al. 2008). The addition of zinc to brushite-forming β-TCP cement also showed a positive effect on adhesion, proliferation, ALP activity, and COL1 secretion of MC3T3-E1 cells (Pina et al. 2010).

Copper

Copper (Cu), which is an essential trace element with its highest abundance in the liver tissue, is known for its stimulatory effect on angiogenesis in endothelial cells. Cu functions as a cofactor and is an important component in the structural and catalytic properties of many enzymes, such as superoxide dismutase, which protects the body against the harmful effects of superoxide (O_2^{2-}) by decomposing it into hydrogen peroxide and oxygen. Copper deficiency is potentially life threatening. Mechanically, the bones of copper-deficient animals are brittle. This phenomenon has been attributed to an increase in collagen solubility (less cross-linking). Enhanced activity and proliferation of osteoblastic cells was observed when Cu^{2+} ions were loaded on CPC scaffolds. Mesoporous bioactive glass (MBG) scaffolds showed multifunctional characteristics, such as angiogenesis potential, osteostimulation, and antibacterial properties (Wu et al. 2013).

Lithium

Lithium (Li) is an ion of interest that received attention due to its role in osteogenesis and is now a fairly new bone substitute additive of interest. Traditionally, Li has been given as a medication to treat bipolar and other psychiatric disorders. Interestingly, hyperparathyroidism has been linked to bone loss, but an amazing result was found in a study in which 75 patients treated with lithium were found to exhibit significantly elevated bone mass (Zamani et al. 2009). Other research has noted lithium's ability to inhibit glycogen synthase kinase 3 (GSK3), which is a negative regulator of the Wnt signaling pathway. β-catenin is known for its central role as a signaling mediator in the canonical Wnt signaling pathway, which is one of the most important signal cascades in bone formation and the bone remodeling process, and it has been shown that lithium activates β-catenin-mediated T cell factor (TCF)-dependent transcription during bone and cartilage fracture healing. This suggests that lithium has the potential to be a candidate for inclusion in CaP bone substitutes for orthopedic implant applications. In a previous study, lithium

was deposited with calcium phosphate on titanium substrata, and then the attachment and initial proliferation of MG-63 osteoblasts were stimulated with a burst release of approximately 90 % of the lithium from the layers.

3.2.3 Angiogenesis

Angiogenesis is defined as the development of new vessels from preexisting vessels from the surrounding tissues and appears as a complex cascade. As early as 1763, Hunter suggested that blood vessels are key contributors to the process of osteogenesis, both in development and during bone repair (Hunter 1974). The newly generated blood supply to the callus and cortical bone appears to persist until the medullary blood supply is fully regenerated. The heterogeneity in vascularity after bone damage could help to explain local differences in bone formation in normal, delayed, and malunions.

Strategies to direct angiogenesis within nanomaterials for bone regeneration have been developed in recent years. It was reported that the addition of a limited concentration of nanosized bioactive glass particles (10 wt%) to collagen films could induce an early angiogenic response (Vargas et al. 2013). It was also reported that nano-hydroxyapatite–Pullulan/dextran polysaccharide composite macroporous materials could subcutaneously retain local growth factors, including BMP-2 and VEGF165, after heterotopic implantation in mice and goats (Fricain et al. 2013). Moreover, modifications to chemical composition (bioinorganics) have also been developed to direct angiogenesis. Previous studies have demonstrated that three Ca-Mg-Si-containing bioceramics (bredigite $Ca_7MgSi_4O_{16}$, akermanite $Ca_2MgSi_2O_7$, and diopside $CaMgSi_2O_6$) had osteogenic and angiogenic potential. The extracts from these three silicate bioceramics stimulated human aortic endothelial cell (HAEC) proliferation and in vitro angiogenesis with improved NO synthesis and angiogenic gene expression. Furthermore, the important role of Si ions in stimulating human umbilical vein endothelial cell (HUVEC) proliferation and angiogenesis has been demonstrated (Zhai et al. 2013). Our group also showed that Sr-substituted calcium silicate (SrCS) can stimulate HUVECs' proliferation, differentiation, and angiogenesis; moreover, in vivo experiments revealed that SrCS dramatically stimulated bone regeneration and angiogenesis in a rat critical-sized calvarial defect model (Lin et al. 2013b).

Strategies for constructing growth factor delivery systems tailored to promote angiogenesis and osteogenesis are also under evaluation. VEGF is a fundamental regulator of normal and abnormal angiogenesis predominantly produced in tissues that acquire new capillary networks. It plays a key role not only in bone angiogenesis but also in different aspects of bone development, including chondroblast differentiation, osteoblast differentiation, and osteoclast recruitment. It has been demonstrated that high frequency mechanical traction of maxillofacial bones can upregulate the gene expression of angiogenic mediators (e.g., VEGF), resulting in an increase in new vessel formation (Zhang et al. 2009). Kleinheinz et al.(2005) filled mandibular defects with collagen complexed with recombinant human

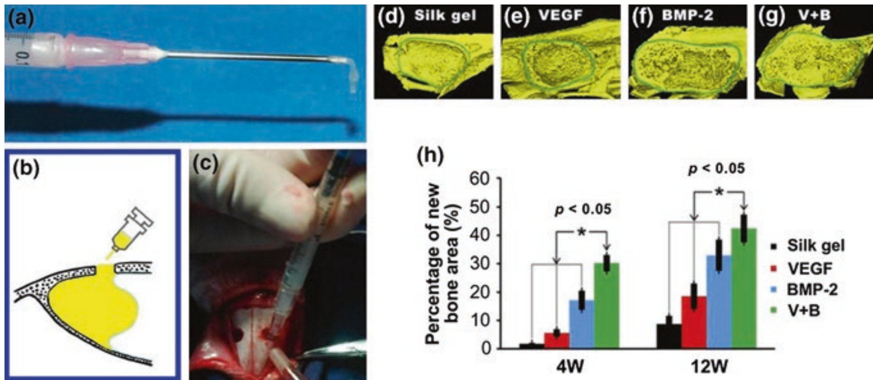

Fig. 4 Elevation of the maxillary sinus floor with the use of injectable sonication-induced silk hydrogel for $VEGF_{165}$ and BMP-2 delivery in rabbits. **a** Injectable property of the silk gel.; **b** Diagram of elevated maxillary sinus in sagittal plane sections. The *yellow area* represents the silk gel in the sinus cavity.; **c** The surgical process of silk gel injection into the rabbit sinus from the small bony window.; **d** Micro-CT 3D reconstructed images of the augmented sinus were taken 12 weeks after operation; **e** The new bone formation area assessed by histomorphometric analysis at weeks 4 and 12 after implantation

VEGF (rhVEGF). Finally, the activation of angiogenesis using rhVEGF leads to more intensive angiogenesis and bone regeneration. Our group combined VEGF and BMP2 proteins together in a silk hydrogel for the elevation of maxillary sinus in a rabbit model and demonstrated that the combination of angiogenic and osteogenic growth factors exerted additive effects on bone formation (Fig. 4). Thus, enhanced angiogenesis significantly contributes to accelerate bone regeneration in the maxillofacial region (Zhang et al. 2011).

4 Summary and Future Directions

Currently, the clinical repair and reconstruction of bone defects in the maxillofacial region are achieved using autografts and allografts, with limited success to meet both morphological and functional restoration. To circumvent the problems associated with current clinical treatments, many researchers have worked for many years to develop an ideal bone substitute material. In maxillofacial bone regeneration, the biomaterial scaffold can act as a GBR membrane, a temporary bone substitute, a drug delivery system for growth factors, or a 3D scaffold for cell seeding, cell proliferation, and cell differentiation, according to the circumstances of the bone defects. In many cases, the ideal biomaterial bone graft should possess adequate mechanical properties, biocompatibility, controlled bioresorbability, and bioactivity that leads to the formation of a bond between the host tissue and the implant material (Kinoshita and Maeda 2013). Although some biomaterial scaffolds have been exploited and applied to clinical cases, especially for maxillofacial

bone regeneration, these scaffolds may not clinically completely achieve both morphological and functional restoration. The development of a more functional biomaterial is needed so that it may be applied more widely.

The remaining challenges for developing an ideal biomaterial are as follows: (1) the development of a biomaterial that has adequate mechanical properties throughout the process of bone regeneration, (2) the development of a biomaterial for drug delivery systems that encapsulates growth factors and has closely controlled temporal–spatial long-term release profiles with efficacy and nontoxicity, (3) the development of a 3D biomaterial with a structure mimicking the ECM of natural bone, and (4) the development of a 3D biomaterial that promotes vascularization (Kinoshita and Maeda 2013). The increased development of highly functional composite scaffolds with architecturally elaborate structures mimicking natural ECM is eagerly awaited. The fabrication of scaffolds based on recent advances in nanotechnology and CAD/CAM technology will enable the realization of this goal. Overall, novel strategies that combine various compositions and nanoscale properties along with these other aspects could be developed for clinical applications in the near future.

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Advances and Applications of Nanomechanical Tools in Cartilage Tissue Engineering

Lin Han and Alan J. Grodzinsky

Abstract Native articular cartilage is a hydrated macromolecular composite with heterogeneous composition, structure, and mechanical properties at a hierarchy of length scales. Recently, with the advances of nanotechnology, nanomechanical tools have shown great promises in understanding the mechanistic origins of cartilage function and improving the design of tissue repair strategies. This chapter reviews the current state-of-the-art nanomechanical tools, with a special focus on atomic force microscopy (AFM)-based methods. With the aid of these tools, ultrastructure of individual molecules and spatially variant mechanical properties of tissue engineered products can be directly quantified. Novel scientific information was derived from recent studies via the nanomechanical methods, including choice of cell sources, cell differentiation and purification, as well as biochemical and biomechanical stimulations. It is hoped that further progress in nanomechanical techniques and their applications on engineered cartilage could provide molecular-level mechanistic insight necessary to improve current tissue engineering strategies and propel them toward a functional repair of damaged cartilage.

Keywords Cartilage · Tissueengineering · Nanomechanics · AFM · Nanoindentation

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1 Introduction

The avascular articular cartilage has poor intrinsic reparative capabilities. Aggravated by the large range of motion and mechanical stresses, local articular cartilage defects often lead to the development of osteoarthritis (OA) (Mankin 1982; Pearle et al. 2005). As of 2012, OA is reported to affect tens of millions of Americans, especially the elderly population (Barbour et al. 2013). In most cases, older patients progress to severe forms of OA, in which full articular cartilage loss occurs and a total joint replacement is required. Numerous experimental and clinical attempts have been made to repair defects within articular cartilage in order to establish a structurally and functionally repair tissue of an enduring nature (Hunziker 2002). Such attempts include surgical interventions, autogenic and allogeneic tissue transplantations, and tissue engineering. Despite these efforts, articular cartilage regeneration remains elusive (Huey et al. 2012). Techniques such as marrow stimulation, allografts, and autografts had limited success as they often result in the formation of mechanically inferior fibrocartilage and/or the lack of integration with native tissue (Huey et al. 2012).

In early 1990s, the concept of tissue engineering promised healing of damaged tissues through manipulation of cells and scaffolds and introduction of stimuli to regenerate and integrate native tissues (Langer and Vacanti 1993). Cell-based tissue engineering typically involves seeding cells within macromolecular scaffolds or in scaffold-free environments and subjecting them into stimulatory biochemical and/or mechanical factors to promote synthesis and growth of neo-tissues. The ultimate goal of this approach is to reconstruct articular cartilage both structurally and functionally through in vitro cell expansion and culture, followed by in vivo integration. Thus far, this particular approach has had very limited clinical success, mainly due to the complex biology, structure, and function associated with articular cartilage. The fact that repaired tissue does not possess the structural hierarchy identical to the native one and carries inferior functional mechanical properties, continues to present a major challenge (Huey et al. 2012).

This chapter focuses on the therapeutic potential of applying the emerging nanomechanical techniques to improve articular cartilage tissue engineering. As reviewed in Sect. 2, the mechanical function of articular cartilage is governed by its extracellular matrix (ECM) with structural heterogeneity at a cascade of length scales (Fig. 1). Metabolism and synthesis of chondrocytes in vivo are determined by a multitude of biochemical and biomechanical microenvironments. Regulatory biomolecules-governed ECM assembly is another critical factor necessary for the development of newly synthesized matrix molecules, mostly aggrecan and collagen, into the mechanically functional ECM. All these phenomena take place at the fundamental building blocks of matrix macromolecules at the nanometer scale. As cartilage primarily serves the biomechanical role of force distribution and joint lubrication during motion, the ability to monitor mechanical properties of engineered neo-tissues at the nanoscale is critical (Guilak et al. 2001). Nanomechanical approaches differ from conventional characterization tools such

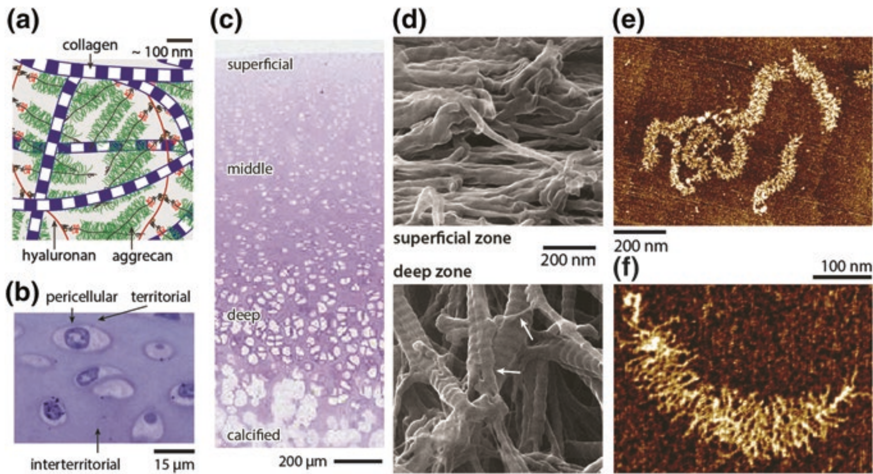

Fig. 1 Heterogeneous hierarchical structure of articular cartilage. **a** Schematic of articular cartilage extracellular matrix (ECM) constituents, including the type II/IX/XI fibrillar collagen network, aggrecan moiety, and hyaluronan that aggrecan binds to (Hardingham and Muir 1972), which is stabilized by the link protein (Buckwalter et al. 1984). Molecular density is reduced to increase clarity. **b**, **c** Toluidine blue histology images from cryosectioned 2-month-old New Zealand rabbits showing **b** cartilage ECM is organized into pericellular, territorial, and interterritorial matrices; **c** depth-dependent zonal heterogeneity of cartilage. **d** Helium ion microscopy images of trypsin and hyaluronidase digested, cryofractured cartilage cross-section from 16-week-old New Zealand female rabbits showing transversely aligned collagen fibrils in the superficial layer, randomly aligned fibrils in the deep layer, and heterogeneity in collagen fibril diameters (*arrows*). **e** Tapping mode AFM height images showing nanostructure of **e** aggregates of fetal bovine epiphyseal aggrecan noncovalently bound to hyaluronan in vitro, stabilized by link proteins (courtesy of Dr. H.-Y. Lee), **f** individual aggrecan monomer from newborn human knee cartilage. Panels are adapted from Rojas et al. (2014) **a**, Hunziker et al. (2007) **b** and **c**, Vanden Berg-Foels et al. (2012) **d** and Lee et al. (2013) **f**, with permissions

as biochemical (Farndale et al. 1986; Hollander et al. 1994), histological, immunohistochemical (Freed and Vunjak-Novakovic 1995), chromatographic (Brown et al. 2007), and electrophoretic (Calabro et al. 2001; Riesle et al. 1998) techniques, which only focus on the molecular composition of engineered products, and do not offer direct mechanical assessments. Nanomechanical tools are more advantageous than the listed assays as they provide direct information regarding the ultrastructure and corresponding nanomechanical properties of neo-tissues. As summarized in this chapter, recent studies employing nanomechanical tools together with conventional assays have provided a more comprehensive and quantitative understanding of the impact of various stimuli on the engineered products. This interdisciplinary approach represents a significant step forward in the optimization of tissue engineering protocols that may eventually lead to a successful functional repair of articular cartilage.

This chapter provides an up-to-date summary of current advances and applications of nanomechanical tools in articular cartilage tissue engineering. It begins

with the discussion of the heterogeneous structure and mechanical properties of native articular cartilage (Sect. 2), and is followed by a summary of current advances in nanomechanical tools (Sect. 3), as well as an up-to-date review of current applications of nanomechanical tools to articular cartilage tissue engineering (Sect. 4), and concludes with a summary and a future outlook (Sect. 5).

2 Heterogeneity of Native Cartilage at a Hierarchy of Length Scales

Articular cartilage is made of a highly hydrated, avascular ECM (~65–75 % wt. water), embedded with resident cells, i.e., chondrocytes (Fig. 1). The ECM is mainly composed of a type II/IX/XI fibrillar collagen network (~20–30 % wet wt.) and highly negatively charged brush-like proteoglycan (PG), aggrecan (~10 % wet wt.) (Fig. 1a) (Maroudas 1979). Other constituents include small leucine-rich proteoglycans, matrilins, cartilage oligomeric matrix protein (COMP), DNAs, and other nonfibrillar collagens, which accounts for <5 % wet wt. of the matrix in total. These quantitatively minor molecules do not directly contribute to articular cartilage mechanical function. Instead, they govern the ECM assembly and postnatal maintenance through regulating chondrocyte signaling and extracellular collagen fibrillogenesis (Heinegård 2009).

Compositional, structural, and mechanical heterogeneity of articular cartilage exists at a hierarchy of length scales. At the micro- to macroscale, articular cartilage properties are dependent on tissue depth (superficial, middle, deep, and calcified), and extracellular zones (pericellular, territorial, and interterritorial) (Fig. 1b, c) (Hunziker et al. 2007). In the territorial and interterritorial zones of the middle and deep layers, the ECM is filled with collagen fibrillar network (~30–80 nm in diameter and are spaced ~100 nm apart) (Fig. 1d) entrapping densely packed aggrecan and other ECM molecules (Meachim and Stockwell 1979). The collagen fibrils are aligned randomly in the middle layer and more perpendicularly in the deep layer (Maroudas 1979; Vanden Berg-Foels et al. 2012; Wong and Carter 2003) (Fig. 1d). Aggrecan monomers are end-attached to hyaluronan to form aggregates (Hardingham and Muir 1972), which are stabilized by link proteins (Buckwalter et al. 1984) (Fig. 1e, f). Each aggrecan monomer consists of a core protein (contour length ≈400 nm) with ≈100 covalently bound chondroitin sulfate glycosaminoglycan (CS-GAG) side chains (contour length ≈40 nm) that are closely spaced (2–4 nm) and negatively charged, along with smaller keratan sulfate GAGs and oligosaccharides (Hardingham and Fosang 1992; Ng et al. 2003). The collagen networks and the aggrecan aggregates work synergistically to determine the time-dependent mechanical properties of articular cartilage bulk (Han et al. 2011a). The topmost superficial layer (≈100–200 μm thick in human) has a distinctive transversely aligned collagen mesh (Fig. 1d), supplemented with lower concentration of aggrecan.

The mucin-like proteoglycan, lubricin (or PRG4) (Jay et al. 2000), is localized within this layer and in the synovial fluid (Jay 2004), which is suggested as a critical factor in articular cartilage surface lubrication (Chan et al. 2010; Jay et al. 2007; Jones et al. 2007; Schmidt et al. 2007). In addition, articular chondrocytes also transfer from a flattened morphology to larger and rounder with increasing depth (Hunziker et al. 2007). At the very bottom, articular cartilage integrates with bone through a calcified tidemark region with a "zig-zag," irregularly shaped boundary with the deep layer. The exact structure and mechanical design of the calcified layer is not well understood (Gupta et al. 2005). In contrast to the collagen fibril-reinforced ECM, the pericellular matrix (PCM), a ≈2 μm thick zone surrounding each chondrocyte (Fig. 1b) is enriched with nonfibrillar type VI collagen and aggrecan. These compositional and structural heterogeneities result in both depth- and zonal-dependent heterogeneity in the time-dependent mechanical properties of articular cartilage. The superficial layer has lower modulus than middle/deep layers, and the PCM is softer than the territorial/interterritorial zones. Despite their critical biological function, chondrocytes make up only 3–5 % of the volume of adult articular cartilage (Muir 1979). The stiffness of chondrocytes is two to three orders of magnitude less than that of the ECM, and therefore it has negligible direct contribution to the bulk mechanical properties of the tissue (Stockwell and Meachim 1979).

Taken together, these features underline the necessity of treating articular cartilage as a heterogeneous biomaterial composite. Nanomechanical tools are necessary to the understanding of the structural and mechanical design of native cartilage, documenting the local disease progression, as well as evaluating the quality of engineered tissues. As summarized in (Han et al. 2011b), recent nanomechanical studies have started to understand the mechanistic origins of articular cartilage function and dysfunction. The advancements include novel information about the molecular mechanics of single matrix molecules (Liu et al. 2004, 2005; Sun et al. 2004) or molecular assemblies (Dean et al. 2005, 2006; Han et al. 2007a, b; Seog et al. 2002, 2004, 2005), molecular origins of articular cartilage surface lubrication (Benz et al. 2004; Chang et al. 2008, 2009, 2014; Coles et al. 2010; Park et al. 2004; Seror et al. 2012; Zappone et al. 2007, 2008), interactions between articular cartilage ECM constituents (Chen et al. 2006; Han et al. 2008; Harder et al. 2010; Rojas et al. 2014), viscoelastic mechanics of individual chondrocytes (Darling et al. 2006; Shieh et al. 2006; Trickey et al. 2004), spatial and zonal heterogeneity of ECM and PCM mechanics (Darling et al. 2010; Loparic et al. 2010; McLeod et al. 2013; Stolz et al. 2004; Wilusz et al. 2012), nanoscale energy dissipation mechanisms of cartilage tissue (Han et al. 2011a; Nia et al. 2011, 2013), and degradation of articular cartilage during osteoarthritis (OA) (Chan et al. 2010; Desrochers et al. 2010; Stolz et al. 2009). In particular, as discussed in Sect. 4, nanomechanical tools have shown promises to provide quantitative information about the impacts of cell types, differentiation processes, and chemical and mechanical stimuli on the mechanical function of engineered tissues.

3 Current Advances in Nanomechanical Methods

Recent advances in microscopy opened the door for studying mechanistic origins of tissue function, documenting disease progression, and evaluating repair protocols at the nanometer level (the length scale corresponding to their fundamental molecular building blocks) (Stolz et al. 2007). Over the past two decades, researchers have developed and applied a variety of nanomechanical methods to understand the structure and mechanics of articular cartilage at nano- to micro-scales, as reviewed in (Han et al. 2011b). These methods include instrumented nano/microindentation, optical tweezers, micropipette, surface force apparatus, and most popularly, atomic force microscopy (AFM).

The AFM is set up with a nanosized sharp or microspherical tip attached at the end a reflective silicon/silicon nitride cantilever. The cantilever directs a laser beam onto a position-sensitive photodiode detector (Binnig et al. 1986). During the experiment, the probe tip is controlled by a piezo head to move perpendicularly or laterally with respect to the sample. In the meantime, cantilever bending and twisting are recorded by the photodiode. A photodiode-piezo signal feedback loop enables the AFM to measure sample topography and tip-sample interaction forces simultaneously. Due to a wide array of available cantilever spring constant (~0.01 to >300 N/m) and tip geometry (pyramidal, spherical, conical, and flat-punch, radius from <5 nm to ~100 μm), AFM can test topography/structure and mechanics of molecules, cells, and tissues at a cascade of spatial dimensions (~1 nm–100 μm) and forces (~10 pN to ~1 mN) in ambient and various fluidic environments. It, therefore, offers a versatile platform to study various structural and mechanical aspects of cartilage at a hierarchy of length scales in different deformation modes. In this section, we introduce the fundamental principles and methods of AFM-based nanomechanical tools, with a special focus on the most used tool, AFM-based nanoindentation (Fig. 2).

3.1 AFM-Based Nanoindentation

AFM-based nanoindentation is the most popular method used in evaluating engineered articular cartilage at the cellular and tissue levels (see Sect. 4). In this mode, a spherical or pyramidal tip is programed to indent into the sample (cell or tissue) at a constant piezo displacement rate, ranging from ~0.05 to 10 μm/s (approximately the indentation depth rate), up to a preset maximum indentation force or depth. The tip is then immediately retracted from the sample, or is held at the constant position for a given amount of time before doing so. Once the effective tip-sample contact point is determined, the indentation force versus depth (*F–D*) curves can be used to extract mechanical properties of tested samples. Two linear, isotropic elastic analytical models have been widely used to calculate the effective indentation moduli from the *F–D* curves.

The first approach uses the Hertz model (Hertz 1882) to compute the resistance to indentation from the loading portion of *F–D* curves, as exemplified in Fig. 2. In the

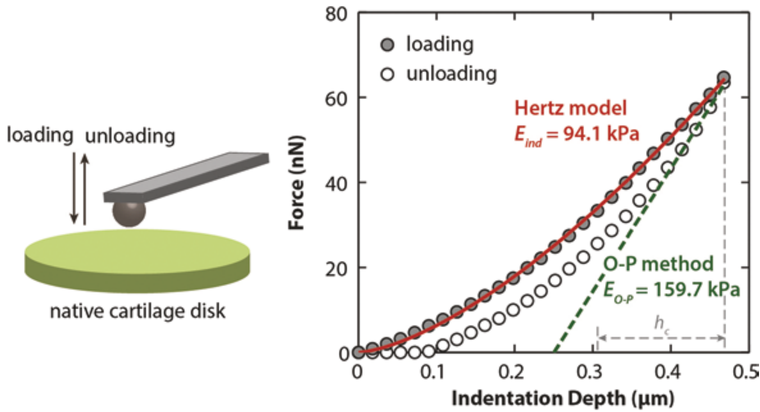

Fig. 2 Typical nanoindentation curve on native articular cartilage and corresponding data analysis. Experimental data [*circles*, raw data from Fig. 2a in Han et al. (2011a)] were obtained on native middle zone calf knee cartilage disk at 0.1 μm/s displacement rate with a borosilicate microspherical tip ($R \approx 2.5$ μm) in phosphate buffered saline (PBS). Indentation moduli were calculated by least squares linear regression with (1) the Hertz model on the whole loading curve, and (2) the Oliver–Pharr method on the top 25 % of the unloading curve (punch parameter $\varepsilon = 0.75$, h_c denotes the corresponding contact depth). Both fits yield $R^2 > 0.99$

Hertzian deformation framework, the samples are assumed to be linearly elastic and isotropic, and tip–sample contact is frictionless. For a spherical indenter in the limit of small deformation (maximum indentation depth <0.4R, where R is the indenter tip radius) (Mahaffy et al. 2000), the effective indentation modulus, E_{ind}, is calculated as,

$$F = \frac{4}{3}\frac{E_{\text{ind}}}{\left(1 - \nu^2\right)} R_r^{1/2} D^{3/2}, \tag{1}$$

where F is the indentation force, D the indentation depth, R_r the reduced contact radius, and ν the Poisson's ratio of the sample [$\nu = 0.1$ for young bovine articular cartilage (Buschmann et al. 1999)]. The reduced contact radius R_r is a function of the radii of curvature of both the probe tip and the tested sample:

$$\frac{1}{R_r} = \frac{1}{R_1} + \frac{1}{R_2}, \tag{2}$$

where R_1 is the tip radius R, and R_2 is the radius of curvature of the tested sample (equals the radius of a cell, or is infinity for flat tissues). We note that application of the Hertz model fit well to the calf knee cartilage indentation data of Fig. 2 over the entire ≈470 nm extent of the loading (approach) curve, confirming the validity of Hertz model to predict the effective indentation resistance within this range of deformation. Later on, this model was extended to evaluate microtomed or cryosectioned samples (Darling et al. 2010; Wilusz et al. 2012) with finite thicknesses correction (Dimitriadis et al. 2002). The Taylor expansion of this model was also

used to calculate cartilage dynamic moduli in the custom-built, AFM-based nanorheometer (Han et al. 2011a; Mahaffy et al. 2004). A more detailed review of analytical models within the Hertzian framework, including other contact geometries, is available in Lin and Horkay (2008).

Another frequently employed approach is the Oliver–Pharr method that uses the initial slope of the unloading portion of *F–D* curves (top 25–75 %) to estimate the modulus (Oliver and Pharr 1992; Stolz et al. 2004) (Fig. 2). This method was originally developed to calculate the elastic recovery modulus of ceramics and metals, e.g., materials that undergo elastic and plastic deformation during indentation. The loading curve is thought to represent elastic and irreversible plastic deformation, while the unloading curve is assumed to correspond to linear elastic recovery. The Oliver–Pharr method first calculates the slope of the initial portion of the unloading curve as the stiffness, $S = \mathrm{d}F/\mathrm{d}D$. From the stiffness, the elastic modulus is given by,

$$E_{O-P} = \frac{\sqrt{\pi}}{2}\left(1 - \nu^2\right)\frac{S}{\sqrt{A}}, \tag{3}$$

where A is the area function related to the effective cross-sectional or projecting area of the indenter. For example, for spherical indenter, $A = 2\pi R h_c$ ($R \gg h_c$), where h_c, the contact depth, is the distance between the unloading slope-indentation depth axis intercept and the maximum indentation depth, scaled by the punch factor ε ($\varepsilon = 0.75$ for spherical tip). It is interesting to note that the Oliver–Pharr method (using the unloading curve) predicts a value of the indentation modulus for the data of Fig. 2 that is $\approx 1.7 \times$ the value of that predicted by the Hertz model, which uses the loading curve. There may be several reasons why use of the unloading curve may give this result, as described below.

In both of the original models, linear elasticity is assumed, i.e., the modulus is the same under compression and tension (Young's modulus). Articular cartilage is known to exhibit time/rate-dependent mechanical properties, governed by both intrinsic macromolecular frictional viscoelasticity (June et al. 2009) and fluid-flow induced poroelasticity (Mow et al. 1980). Furthermore, the collagen-aggrecan composite ECM of cartilage behaves very differently under tension versus compression (Maroudas 1979). Therefore, while fitting data to the Hertz model or the Oliver–Pharr method can yield values of indentation moduli, these values do not represent the inherent Young's modulus of cartilage. Furthermore, the compressive behavior of articular cartilage can be linear for small enough deformations and nonlinear for larger deformations. Even for the case of linear compressive behavior at small indentation depth, neither of these methods incorporates time-dependent, poro-viscoelastic dissipation during deformation, as both models assume quasi-static (equilibrium) conditions. The values calculated from Hertz model from the loading curve thus represents an "effective indentation modulus" reflecting mostly the compressive resistance of cartilage at the given indentation rate. The unloading curve for cartilage most likely includes the combined effects of both elastic recovery and poro-viscoelastic force relaxation, and thus, the elastic–plastic deformation assumption in the Oliver–Pharr method does not hold. Importantly, the difference between the loading and unloading curves of cartilage (Fig. 2) is due primarily to poroviscoelastic (hysteretic) effects and not to

elastic–plastic deformation, as confirmed by the absence of permanent damage at the indentation site. Under a scenario that the poro-viscoelastic relaxation time constant is on the same order of the unloading time frame, a steep unloading slope simply due to relaxation may lead to falsified high E_{O-P} values.

To extract the precise time-dependent, nonlinear deformation mechanisms of cartilage from the *F–D* curves, complex experimental procedures and theoretical or finite element models are needed (Setton et al. 1993). For example, a recently developed AFM-based nanorheology system has demonstrated the frequency dependence of cartilage dynamic moduli and force–depth phase lag over four decades of frequencies (≈1 to 10 kHz). This approach can deconvolute the elastic, viscoelastic, and poroelastic properties of cartilage, and therefore differentiate the biomechanical function of cartilage relevant to joint activities at different time scales, e.g., walking, running, jumping, and traumatic impacts (Nia et al. 2011).

While it may be critical to identify the poroviscoelastic and elastic properties of engineered tissue, (as they are extremely important to native cartilage function), researchers have used both the Hertz model and the Oliver–Pharr method to study relative changes in the construct behavior (e.g., during culture or with depth). Under many circumstances, application of both these methods can identify relative differences in the quality of engineered products and extract necessary mechanics-related information (Ebenstein and Pruitt 2004; Tomkoria et al. 2007). In this chapter, to differentiate the results calculated from these two approaches, we indicate the moduli calculated from the Hertz model as E_{ind}, and those from the Oliver–Pharr method as E_{O-P}.

3.2 AFM-Based Force Spectroscopy and Imaging

Similar to nanoindentation, in high resolution force spectroscopy (HRFS), a probe tip attached to a soft cantilever (≈0.1 N/m or less) perpendicularly approaches to and retracts from the test sample surface. The method of using the probe tip end-functionalized with self-assembled monolayers of biomacromolecules (aggrecan or GAGs), enables the measurement of interaction of forces involving groups of densely packed macromolecules [e.g., aggrecan (Dean et al. 2006; Han et al. 2008; Rojas et al. 2014), GAGs (Seog et al. 2002, 2004, 2005) and collagen (Rojas et al. 2014)], or a pair of individual molecules (Harder et al. 2010).

In contact mode AFM imaging, the tip scans over the surface at constant applied compressive forces to measure sample surface topography in both ambient and fluidic conditions. This method, in combination with soft lithography (Wilbur et al. 1994), was used to quantify the electrostatics-governed compressive nanomechanics of aggrecan, as previously demonstrated (Dean et al. 2005, 2006). In a special contact mode, the lateral (friction) force microscopy (LFM), cantilever twisting due to tip-sample friction is recorded simultaneously with surface topography. Aided by the calibration of lateral sensitivity to quantify the lateral forces (Carpick et al. 1999; Han et al. 2007b; Varenberg et al. 2003), LFM has been used to record the compression and shear behaviors of aggrecan simultaneously (Han et al. 2007a, b), microscale surface friction of cartilage (Chan et al. 2010; Desrochers et al. 2010;

Park et al. 2004), as well as other physisorbed cartilage matrix biomacromolecules including lubricin and hyaluronan (Chang et al. 2009, 2014).

In tapping mode AFM, the cantilever is oscillated near its resonance frequency (>10 kHz to ≈1 MHz), while scanning the surface. As the tip–sample contact shear forces are minimized, tapping mode AFM results in images with a spatial resolution <2 nm. This method enabled direct visualization of the ultrastructure of individual aggrecan and hyaluronan molecules (Ng et al. 2003), and the collagen fibril structure. With this technique, effects of aging and enzymatic degradation can be directly visualized (Lee et al. 2013). These studies set a standard to evaluate the ultrastructural features of tissue-engineered aggrecan, as will be shown in Sect. 4.

3.3 Other Nanomechanical Techniques

Besides AFM, other nanomechanical techniques have also been used to study the nanomechanics of articular cartilage at the tissue, cellular, and molecular levels. Such techniques include instrumented nanoindentation, micropipette, optical tweezers, and surface force apparatus. Instrumented nanoindentation functions similarly as AFM nanoindentation, although it adapts a different instrumental design and probes forces at a larger scale (>10 μN) (Ebenstein et al. 2004; Franke et al. 2007). Micropipette is a powerful tool to study the time-dependent creep behavior of individual cells (Guilak 2000). Optical tweezers work well for single molecular force spectroscopy and offers the highest pN-level force resolution (Sun et al. 2004). Surface force apparatus measures repulsion and friction forces between physisorbed biopolymers, and thus has provided important insights into the lubrication mechanisms (Benz et al. 2004; Zappone et al. 2007, 2008). While the above-mentioned methods have not been widely applied to engineered cartilage, knowledge obtained from studies employing these techniques largely improves our understanding of the origins of native cartilage mechanical functions.

3.4 Multiscale Modeling

Due to the fact that heterogeneity in cartilage exists at a hierarchy of length scales, theoretical models accounting for structural and mechanical heterogeneity are necessary to quantitatively elucidate the origins of cartilage function. The fibril-reinforced finite element model (Soulhat et al. 1999) has successfully captured the fluid-flow governed energy dissipation of articular cartilage (Nia et al. 2011). The magnitude of energy dissipation in cartilage is markedly enhanced by the collagen fibril-induced tension-compression asymmetry as compared to a homogeneous continuum medium (Nia et al. 2011). In addition, the unit cell (Buschmann and Grodzinsky 1995) and charged rod (Dean et al. 2003) models account for the

heterogeneous electrical field distribution within cartilage at physiological ionic strength (0.15 M). These models were able to predict the compressive behaviors of aggrecan (Dean et al. 2006) and CS-GAGs (Seog et al. 2005), while a continuum Donnan model (Donnan 1911) drastically overestimated their compressive resistance. With further advances in nanomechanical experimental tools, multiscale modeling focusing on the heterogeneity of cartilage will be an indispensable, complementary tool to fully capture the mechanical function of cartilage both experimentally and theoretically.

4 Applications of Nanomechanics to Cartilage Tissue Engineering

Earlier studies using either instrumented microindentation (Ebenstein et al. 2004; Franke et al. 2007) or AFM-based nanoindentation (Tomkoria et al. 2007) have validated nanoindentation as a viable assay to differentiate mechanical functions between native and repaired articular cartilage. Recently, a number of pioneering studies have used various AFM-based methods as the major tool to optimize the repair protocol of cartilage. In this section, with the help of multiple examples, we elucidate how AFM methods can provide quantitative understanding of the effects of chemical and mechanical stimuli, the potential of using stem cells as alternative cell sources and the importance of in vitro predifferentiation during cell-based cartilage tissue engineering.

4.1 Effects of Cytokines on Chondrocyte Synthesis

In native articular cartilage, chondrocytes are surrounded by the 2–4 μm thick PCM. The PCM is enriched with PG and localized type VI collagen (Poole et al. 1992, 1988), with a modulus ≈60–70 kPa (Alexopoulos et al. 2003; Guilak et al. 1999). Synthesis and metabolism of chondrocytes are sensitive to mechanical loads transmitted by the PCM (Fitzgerald et al. 2004; Guilak et al. 1994; Kim et al. 1994; Valhmu et al. 1998). During in vitro culture, mechanical behaviors of the newly synthesized PCM can in turn affect chondrocyte activities (Graff et al. 2003). To improve cartilage repair, it is important to understand and control the mechanical behaviors of the PCM in vitro, by controlling the chemical and/or mechanical environments.

The combination of growth factors insulin-like growth factor-1 (IGF-1) and osteogenic protein-1 (OP-1) were previously shown to increase PG accumulation during in vitro culture (Flechtenmacher et al. 1996; Loeser et al. 2003; McQuillan et al. 1986; Nishida et al. 2000; van Osch et al. 1998). To further probe the mechanical significance of these two growth factors, Ng et al. (2007) monitored the temporal evolution of PCM-chondrocyte composites during a 28-day culture in the standard

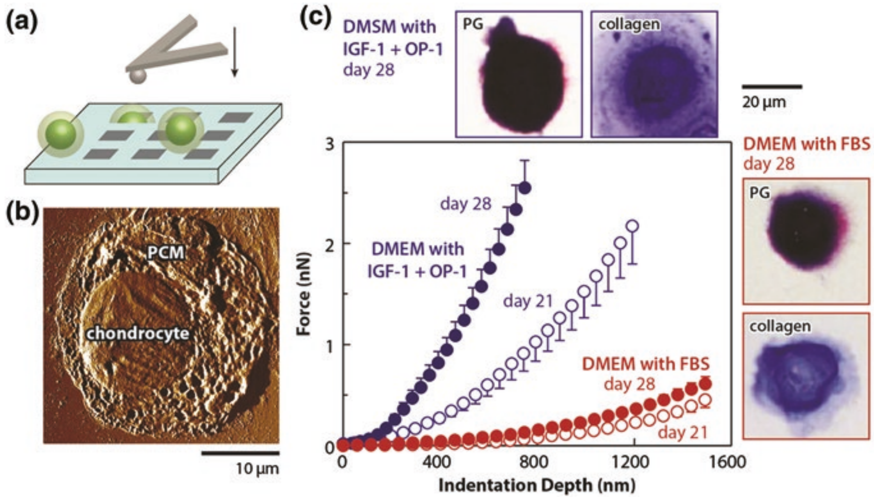

Fig. 3 Impacts of growth factors on chondrocyte-engineered pericellular matrix (PCM) composite nanomechanics. **a** Schematic of AFM nanoindentation ($R \approx 2.5$ µm) on individual chondrocytes-PCM composites immobilized within silicon pyramidal wells. **b** Tapping mode AFM amplitude image of the composite after 11-day culture in DMEM with 10 % fetal bovine serum (FBS). **c** Typical indentation force versus depth loading curves (mean ± SEM for $n \geq 5$ cells) on the on individual composite after 21-day and 28-day culture in DMEM with 10 % FBS and with insulin-like growth factor-1 (IGF-1) and osteogenic protein-1 (OP-1). Shown together are histology images of 28-day culture cells stained for proteoglycan (PG, by toluidine blue O) and collagen (phosphomolybdic acid followed by aniline blue). Adapted from Ng et al. (2007) with permission

high-glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with IGF-1 and OP-1, or without these factors [only 10 % fetal bovine serum—FBS] (Fig. 3). During culture, the development of the PCM was directly visualized by both histological staining and AFM imaging (Fig. 3b, c). Indentation moduli E_{ind} of the cell-PCM composites were measured at different culture days, while changes in matrix PG and collagen were simultaneously monitored. During indentation, individual cell-PCM composite was immobilized in a micro-fabricated pyramidal silicon well to prevent dedifferentiation (Fig. 3a). Increasing culture duration resulted in the accumulation of collagen and PG, and thus, higher E_{ind} of the cell-PCM composites. Adding IGF-1 and OP-1 significantly stiffened the neo-PCM compared to the culture with FBS (Fig. 3c). Since nanoindentation was performed at 0.2–10 µm/s rates, the measured mechanical properties reflected both poro-viscoelastic and elastic behaviors of the composite. In a follow-up study using the custom-built AFM-nanorheometer (Lee et al. 2010a), fluid-flow governed poroelastic mechanical properties of the composite were quantified through a ≈5 nm oscillation amplitude at 1–316 Hz frequencies superimposed onto a ≈1 µm static indentation depth. In this frequency domain, hardening of the composite became even more pronounced and the elastic response increased (lower energy loss).

In comparison to the salient biomechanical differences and expected increase in PG content (Loeser et al. 2003), the presence of IGF-1 and OP-1 did not have appreciable effects on the total amount of collagen. The discrepancies between biomechanical and biochemical results were attributed to the molecular assembly, such as collagen fibrillogenesis and cross-linking regulated by nonfibrillar collagens and small leucine-rich proteins (Chang and Poole 1997; Eyre et al. 1987; Poole et al. 1988). These processes could be enhanced by the presence of IGF-1 and OP-1. The importance of nanomechanical evaluation was thus highlighted here, as biochemical assays alone would not be able to provide information related to the nanoscale molecular organizations or the functional mechanical properties.

In addition to the positive impacts of IGF-1 and OP-1, negative impacts of inflammatory factor interleukin-1β (IL-1β), was also detected during in vitro culture of chondrocytes. A recent study by Peñuela et al. quantified the effects of IL-1β on the spatially variant indentation modulus of engineered cartilage, E_{O-P}, calculated by the Oliver–Pharr method. The tissue was generated by 17 days culture of 1-mm-radius pellet of expanded human nasal chondrocytes in the standard DMEM (Peñuela et al. 2014). The pellet was exposed to 1 ng/mL IL-1β in the last 3 days. The values of E_{O-P} were measured on the cross-sectional cut of individual pellets using the pyramidal, nanosized tip. Exposure to IL-1β was found to significantly decrease E_{O-P} by $\approx$2.6×, and total GAG content by $\approx$1.4×. This reduction was most pronounced in the central region of each pellet, which can be possibly contributed by the amplified effects of IL-1β under lower oxygen concentration environments (Scotti et al. 2012). This observation is consistent with previous reports that IL-1β exposure causes remodeling and degradation of tissue-engineered cartilage (Felka et al. 2009; Francioli et al. 2011; Lima et al. 2008; Scotti et al. 2012; Wehling et al. 2009). This study thus further confirmed the effectiveness of nanomechanical tools in the evaluation of cartilage neo-tissue development in response to specific biochemical conditions (Felka et al. 2009; Ongaro et al. 2012).

4.2 Effects of Mechanical Loading on Chondrocyte Synthesis

In additional to biochemical stimuli, biomechanical stimuli are also known to have a critical impact on chondrocyte activities (Schulz and Bader 2007). The study conducted by Grad et al. (2012) evaluated the effects of dynamic loading and lateral sliding on engineered tissue mechanics. Bovine articular chondrocytes, seeded in polyurethane scaffolds, were cultured for 3 weeks in the standard DMEM in three study groups: control without loading, loading group 1 (LG1) for one-hour-per-day dynamic compression, and loading group 2 (LG2) for one-hour-per-day dynamic compression and shear oscillation (Fig. 4). This study found the lowest surface friction coefficient μ for the group LG2 measured by LFM employing a microspherical tip ($R \approx 5$ μm) (Fig. 4a). In addition, this group also yielded

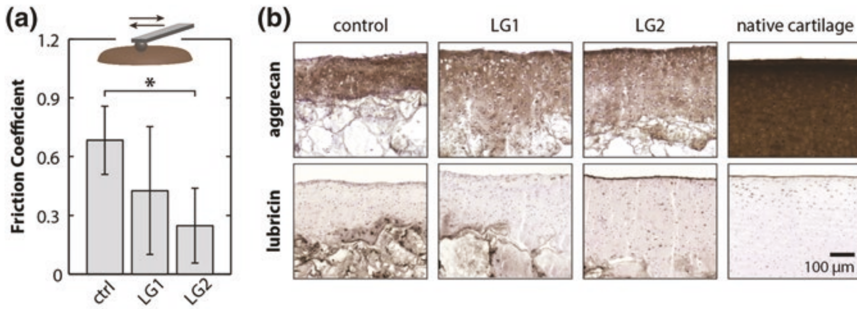

Fig. 4 Impacts of dynamic loading on the nanomechanics of engineered cartilage. **a** Surface friction coefficient of cell constructs measured via lateral force microscopy using a microspherical tip ($R \approx 5$ μm) (mean ±95 % confidence intervals, *: $p < 0.05$ via one-way analysis of variance with least significant difference (LSD) post hoc tests). **b** Immunolabeling for aggrecan and lubricin of the constructs and native bovine articular cartilage as the positive control. All polyurethane-based constructs containing chondrocytes from 4 to 8 old metacarpal calf joint cartilage were cultured for 4 weeks in standard DMEM, with mechanical stimuli applied in the later 3 weeks (6 days a week). Constructs of LG1 were stimulated by dynamic compression only; constructs of LG2 were stimulated by dynamic compression and sliding surface motion. Adapted from Grad et al. (2012) with permission

highest E_{O-P} by AFM nanoindentation. These biomechanical differences were consistent with immunohistochemistry staining, which found deeper penetration of type II collagen and PGs in the loaded groups, and a unique localization of lubricin, a cartilage surface lubricating proteoglycan, on the surface of LG2 scaffolds (Fig. 4b). On native articular cartilage surface, lubricin was suggested to play a vital role in the excellent surface lubrication (Chan et al. 2010; Jay et al. 2007; Jones et al. 2007; Schmidt et al. 2007). This study provided quantitative mechanical evidence to confirm that surface sliding benefits chondrocyte PG synthesis and/or increased PG-collagen and collagen–collagen cross-linking at the contact surface (Hedlund et al. 1999; Loparic et al. 2010). Furthermore, the lower moduli in all constructs compared to native cartilage (≈0.1–1 MPa) suggested the engineered matrix was at early stages of collagen fibrillogenesis, during which collagen microfibrils are very thin and without mature cross-link bonds (pyridinoline, deoxypyridinoline) (Ströbel et al. 2010).

4.3 Engineered Aggrecan by Bone Marrow Stromal Cells

While primary chondrocytes are a natural cell candidate for cartilage regeneration, their clinical application is limited by the decreased matrix synthesis potential which occurs with age (Barbero et al. 2004; Bolton et al. 1999; Plaas and Sandy 1984; Tran-Khanh et al. 2005), and by the invasive surgical procedures required

for its derivation (Lee et al. 2000). Adult stem cells are a promising alternative source due to their minimally invasive isolation procedures and chondrogenic potential (Guilak et al. 2010; Nöth et al. 2008). For example, bone marrow stromal cells (BMSCs) undergoing chondrogenesis in vitro showed better sustained tissue forming capacity with age than primary chondrocytes (Connelly et al. 2008; Im et al. 2006; Jiang et al. 2008; Scharstuhl et al. 2007). To evaluate the validity of BMSCs as an alternative cell candidate, Lee et al. studied the ultrastructure and compressive nanomechanics of aggrecan monomers synthesized by adult equine BMSCs undergoing chondrogenesis in peptide hydrogels. These properties were compared to aggrecan directly extracted from age-matched adult equine cartilage (Lee et al. 2010b). BMSCs were stimulated to undergo chondrogenesis in a self-assembling peptide hydrogel scaffold after 21 days of culture. Aggrecan synthesized by BMSCs were then extracted and deposited on an atomically flat mica surface for AFM tapping mode imaging. The BMSC aggrecan population showed a higher portion of full length monomers than the cartilage-extracted aggrecan population. This difference could be mostly attributed to extracellular enzymatic hydrolysis by aggrecanases (Nagase and Kashiwagi 2003) and calpain (Oshita et al. 2004), thus did not reflect their cellular synthetic activities. In the subpopulation of full length aggrecan, trace lengths of the core protein of each monomer were similar in both populations (Fig. 5a, b). However, the CS-GAG side chains of BMSC aggrecan were >2 × longer than those of cartilage aggrecan (Fig. 5a, b). The elongation of GAGs was also accompanied by different glycosylation patterns observed by fluorophore-assisted carbohydrate electrophoresis (FACE) analysis (Calabro et al. 2001).

To further elucidate the mechanical insights of BMSC aggrecan, following the previous protocol used for studying native aggrecan (Dean et al. 2006), Lee et al. used the thiol-functionalized BMSC aggrecan monomers, and chemically end-attached them onto a planar gold substrate with well-defined microscale boundaries (Fig. 5c) via soft lithography (Wilbur et al. 1994). HRFS was then applied with a neutral, hydrophilic microspherical tip ($R \approx 2.5$ μm) to quantify the electrostatics-governed compressive behaviors at various ionic strengths (Lee et al. 2010b). BMSC aggrecan showed superior compressive resistance than the cartilage-extracted aggrecan (Fig. 5c), including greater end-attached monolayer height and larger compressive stiffness in both electrostatics-dominating low ionic strength (0.001 M) and near physiological condition (0.1 M). This effect persisted even after normalizing at comparable GAG charge density (Lee et al. 2010b), which illustrated the importance of molecular structure and dimension of GAGs in overall aggrecan stiffness. This superior ultrastructure of BMSC aggrecan was also observed in a separate study, which studied the aggrecan synthesized by BMSCs from young and adult horses and compared it to those synthesized by age-matched primary chondrocytes in vitro (Kopesky et al. 2010). The more favorable nanostructure and greater stiffness of BMSC aggrecan are molecular-level evidences in support of BMSC as a promising cell candidate for cartilage tissue engineering.

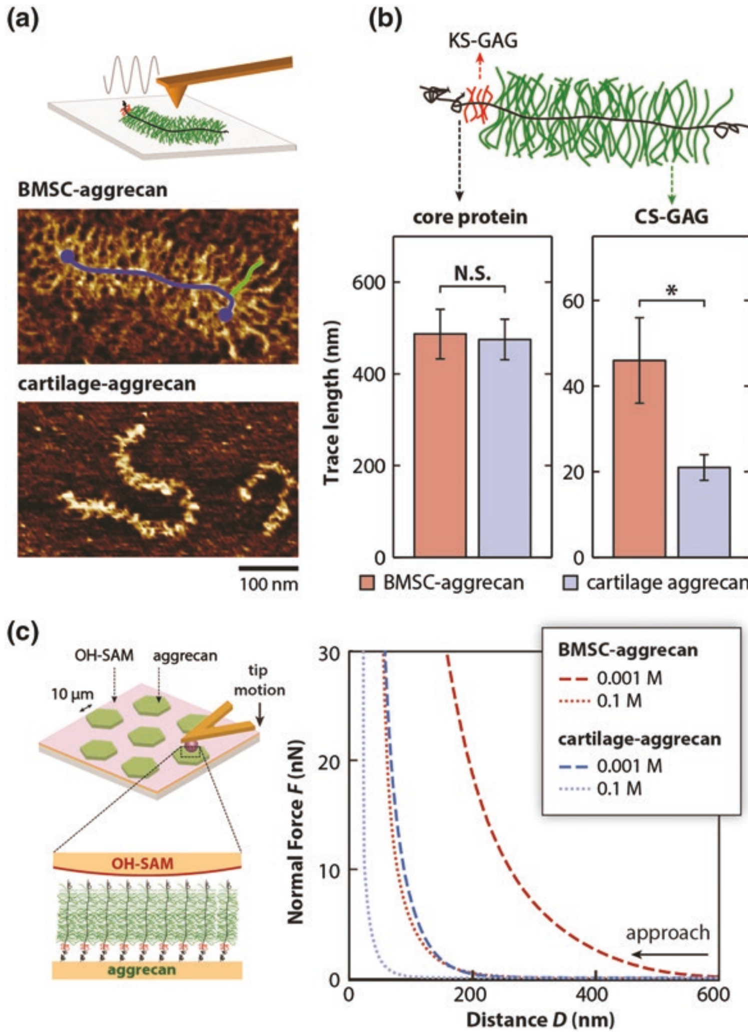

Fig. 5 Comparison of the ultrastructure and compressive nanomechanics of bone marrow stromal cells (BMSCs)-aggrecan and native cartilage aggrecan. **a** Tapping mode AFM height images of individual aggrecan monomers. *Blue* and *green lines* highlighted the traces of each core protein and chondroitin sulfate glycosaminoglycan (CS-GAG) chain, respectively. **b** Comparison of core protein and CS-GAG trace lengths measured on full length aggrecan monomers (mean ± SEM, $n = 119$ molecules for BMSC-aggrecan, $n = 20$ for cartilage aggrecan, *: $p < 0.0001$ via unpaired student's t-test). **c** Schematic of high resolution force spectroscopy measurement on micro-patterned, end-attached aggrecan by a microspherical, neutral tip ($R \approx 2.5$ μm), and corresponding force versus distance curves in 0.001 and 0.1 M NaCl solutions (pH ≈ 5.6). BMSC-aggrecan molecules were synthesized by adult equine BMSCs cultured in self-assembling peptide hydrogel scaffold. Native aggrecan molecules were directly extracted from articular cartilage of adult equine knee joint femoropatellar grooves. Adapted from Lee et al. (2010b) with permission

4.4 Engineered Tissue by Induced Pluripotent Stem Cells

The clinical application of BMSCs also has multiple limitations, including low percentage of BMSCs in bone marrow (Pittenger et al. 1999) and reduced chondrogenesis potential and lower proliferation rates if derived from older or OA patients (Dexheimer et al. 2011; Murphy et al. 2002). Another alternative cell source is, the more abundant and easy-to-isolate, induced pluripotent stem cells (iPSCs). iPSCs were recently shown to have appreciable expansion potential and the ability to provide patient-specific cell and tissue models (Israel et al. 2012; Park et al. 2008). One challenge of employing iPSC was the difficulty in achieving uniform cell population for chondrogenic differentiation (Yoshida and Yamanaka 2010), whereas a nonuniform cell population could limit the effectiveness of the therapy and increase the risk of teratoma formation (Blin et al. 2010).

A uniformly differentiated iPSC population is thus thought to be critical to predictably recapitulating the physiological characters of cartilage. In order to test the importance of uniform differentiation, Diekman et al. purified chondrogenically differentiated iPSCs from adult mouse fibroblasts by type II collagen (col2)-driven green fluorescent protein (GFP) expression (Diekman et al. 2012). The GFP+ population was successfully predifferentiated toward the chondrogenic lineage, characterized with upregulated col2 and aggrecan expression compared to the GFP− population that was not. Millimeter-sized spherical pellets (0.6–1.4 mm diameter) formed by centrifuging each cell population (Fig. 6a) were cultured in

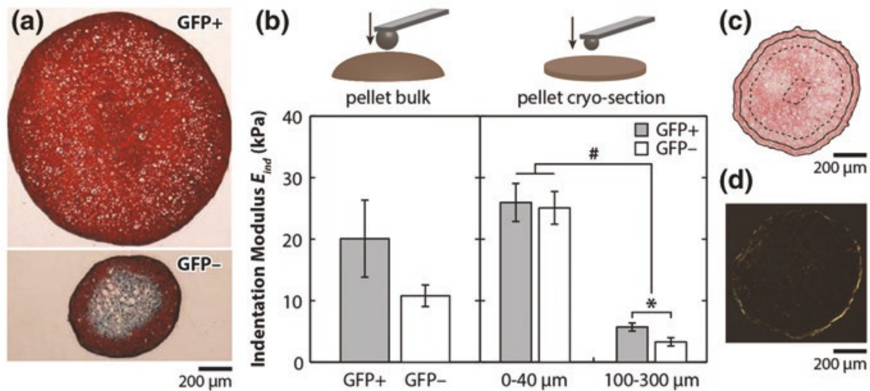

Fig. 6 Impacts of Predifferentiation on the chondrogenetic activities of induced pluripotent stem cells (iPSCs). **a** Safranin-O/Fast-Green/Hematoxylin-stained section from pellets of GFP+ or GFP− iPSC cells after two passages. **b** Indentation modulus E_{ind} of the pellet bulk (measured by a microspherical tip $R \approx 12.5$ μm) and cryosections ($R \approx 2.5$ μm), calculated by the Hertz model (mean ± SEM, *: $p < 0.05$ by region, #: $p < 0.05$ by cell type via unpaired student's t-test). **c** Picrosirius red stained section of GFP+ pellet depicting regions tested in panel (**b**): 0–40 μm (between *solid lines*) and 100–300 μm (between *dashed lines*). **d** Polarized light microscopy image of section in panel (**c**), where the brighter area corresponded to well-aligned collagen fibrils. Pellets with purified iPSCs derived from 8 to 10 week-old mice were cultured in standard DMEM for 21 days. Adapted from Diekman et al. (2012) with permission

chondrogenic differentiation medium with 10 ng/mL transforming growth factor (TGF)-β3 and 100 nM dexamethasome for 21 days. AFM-based nanoindentation and Hertz model were then used to quantify the E_{ind} of engineered tissue within the pellets. Indentation with a microsphere ($R \approx 12.5$ μm) on the pellet bulk did not yield statistical differences between these two populations (Fig. 6b). However, indentation on the cryosection with a smaller sphere ($R \approx 2.5$ μm) showed significantly higher E_{ind} in the central region (100–300 μm from the edge) for the GFP+ population, and E_{ind} in the peripheral region was similar for both populations (outer 0–40 μm) (Fig. 6b, c). This biomechanical difference is consistent with enhanced GAG (Fig. 6a), type II collagen synthesis, and better in vivo integrative strengths of the GFP+ cells. In addition, E_{ind} in the peripheral was found to be $\approx 5 \times$ higher than the central region for both pellets (Fig. 6b). This zonal variation also correlated well with the higher degree of collagen alignment in the outer layer (Fig. 6d). These nanomechanical results together provided biomechanical clues that illustrated the proof-of-concept of using predifferentiated and purified iPSCs as a viable cartilage tissue engineering candidate.

4.5 Chondrogenesis Differentiation of Adipose-Derived Stem Cells

In addition to directly evaluating the quality of tissue engineered products, nanomechanical tools were also used to reveal the chondrogenesis differentiation processes of adult adipose-derived stem cells (ASCs). Unlike the cases for BMSCs or iPSCs, the chondrogenesis protocol for ASCs is not well established. Optimization and identification of influential growth factors that can diminish hypertrophic dedifferentiation is still needed (Kuhbier et al. 2010). Jungmann et al. (2012) investigated the influences of small GTPases (Rac1 and RhoA) and bone morphogenetic protein-2 (BMP-2) on TGF-β1-mediated chondrogenic differentiation of ASCs. Groups of human ASCs were isolated, expanded, and cultured for 14 days under five different conditions: (1) the standard DMEM, (2) DMEM with BMP-2, (3) DMEM with Rac1-inhibitor, (4) DMEM with RhoA-inhibitor, all in 3D alginate beads, and (5) 2D expansion medium where no chondrogenesis was induced. AFM-based nanoindentation with a microspherical tip ($R \approx 5$ μm) were applied on individual ASCs, together with fluorescence staining, cell volume measurement, and mRNA analysis to evaluate the differentiation grade of ASCs into chondrocytes. Undifferentiated ASCs (group 5) were identified with higher E_{ind} and higher volume than primary chondrocytes, as expected (Darling et al. 2008). Standard chondrogenic stimulation (group 1) reduced the E_{ind} and volume of ASCs to the level of chondrocytes as a result of chondrogenesis differentiation. Furthermore, all additional stimuli (groups 2–4) resulted in higher cell E_{ind} than the standard culture (group 1), but at a level still lower than undifferentiated ASCs (group 5). Addition of BMP-2 (group 2) increased E_{ind}, actin fibers assembly

(Solursh 1989), and cell volume. This was a sign of previously known BMP-2 induced transdifferentiation of ASCs into stiffer osteoblasts (Docheva et al. 2008). Similar effects were observed for group 4 with RhoA-inhibitors, suggesting that RhoA-inhibitors also promoted osteogenic differentiation. For group 3, the presence of Rac1-inhibitor further reduced cell volume and actin fibers, but increased the type II collagen production when compared to the standard group 1. Rac1-inhibitor was thus found to promote chondrogenesis and reduce cell hypertrophy. When examined together, the biomechanical properties and the results from biochemical assays showed the impacts of GTPases and BMP-2 on TGF-β1-mediated chondrogenic differentiation of ASCs (Jungmann et al. 2012).

5 Summary and Future Outlook

As illustrated by multiple recent studies, nanomechanical measurements have been established as an indispensable tool in the process of evaluating articular chondrogenic differentiation potential of stem cells, differentiating impacts of growth factors and inflammatory cytokines, identifying beneficial biomechanical stimuli, and quantitatively assessing the mechanical function of neo-tissues. As previously summarized in Han et al. (2011b), spatial heterogeneity in the hierarchical structure, composition, and mechanics of cartilage is critical to its biomechanical and biophysical function. In order to achieve a functional cure for damaged native cartilage resulted from injury or OA, we need to fully understand, capture, and replicate these features in tissue-engineered cartilage. Nanomechanical research of both native and engineered cartilage is still in its early stage, and swift application of nanomechanical tools is likely to advance scientific findings and aid in addressing a multitude of cartilage tissue engineering problems, such as,

- *Improve* in vitro *formation of the collagen fibrillar network.* Engineered cartilage (<100 kPa) normally has inferior stiffness compared to native cartilage (≈1 MPa) (see examples in Sect. 4), largely owing to the lack of well-assembled collagen fibrillar network. While past approaches have been primarily focusing on stimulating cell synthetic activities, a microenvironment with proper stimuli to promote collagen extracellular fibrillogenesis and cross-linking could substantially increase the stiffness of neo-tissues.
- *Simulate the structural heterogeneity.* While the exact mechanical implications of the zonal and depth-variant cartilage structure is still under investigation, to fully recapitulate the function of native cartilage requires the engineered tissue to have at least similar heterogeneous hierarchy as the native one. This may involve different biomechanical and biochemical stimuli to regenerate tissue within different zones, as illustrated in Grad et al. (2012).
- *Capture the energy dissipation and lubrication mechanisms.* Limited information exists about the time-dependent poro-viscoelasticity and surface lubrication mechanisms of engineered tissue. As native cartilage sustains a wide

range of loading rates and stresses, recapitulation of these complex mechanical properties is necessary for engineered tissues to be mechanically functional. Multidisciplinary approaches that combine tissue engineering and biomechanics research is an essential step for future progress.

- *Integrate neo-tissue with native cartilage.* Engineered tissue needs to be integrated with native tissue to be fully functional. A mismatch in mechanics and structure may result in weak points that are susceptible to lesions or inflammation. In particular, at the cartilage–bone interface, there is a sharp transition in molecular composition, mineralization, and mechanical properties.

It is our hope that nanomechanical tools will help to address, advance, and/or solve the listed issues above. Further advances in this direction will provide important knowledge to the development and improvement of molecule and cell-based therapeutics for cartilage tissue engineering. As the trend of merging engineering and biological expertise grows, multidisciplinary teams, which combine knowledge of nanomechanics and cartilage biology, are likely to make significant advances in cartilage tissue engineering and progress toward a functional cure of OA.

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Signalling Pathways in Osteochondral Defect Regeneration

Henning Madry and Magali Cucchiarini

Abstract Osteochondral defects are difficult to treat because the subchondral bone and the articular cartilage are dissimilar tissues with divergent intrinsic healing capacities. Understanding the signalling pathways in osteochondral defect regeneration therefore holds great promise for translation into further improvements to restore the entire osteochondral unit. In osteochondral defects, mesenchymal stem cells (MSCs) from the subarticular spongiosa migrate into the lesion and subsequently initiate osseous and chondral repair. In the upper region of the defect, MSCs undergo differentiation processes to commit toward the chondrocyte phenotype in a defined sequence of cellular and molecular events. In the deeper regions, MSCs differentiate into osteoblasts to form bone in an attempt to reconstitute the subchondral bone plate via a process similar to endochondral ossification. These processes and their interplay are complex and involve several overlapping mechanical and biological pathways. Further research will lead to a better understanding on how the mechanisms of chondral and bone repair interact together over time in osteochondral repair.

1 Introduction

Osteochondral defects are cartilage lesions that extend deep into the subchondral bone presenting significant clinical problems in the repair and regeneration of cartilage often leading to osteoarthritis (OA) and related disorders (Michael et al.

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2008; Panseri et al. 2011). Osteochondral defects are difficult to treat because the subchondral bone and the articular cartilage are dissimilar tissues with divergent intrinsic healing capacities (Huey et al. 2012; Hunziker 2009). Articular cartilage tissue repair remains a key problem due to the degradation of articular cartilage over time combined with an intrinsic inability of repair processes to regenerate native hyaline cartilage within the defect in the articular cartilage. Several pathological features in the subchondral bone are associated with spontaneous osteochondral repair processes leading to suboptimal articular cartilage repair. These include the upward migration of the subchondral bone plate, formation of intralesional osteophytes, appearance of subchondral bone cysts and the impairment of the osseous microarchitecture as potential problems (Orth et al. 2013a). Such deterioration of the subchondral bone has long been an underestimated factor influencing the long-term outcome of osteochondral repair. Understanding the role of the signalling pathways in osteochondral defect regeneration therefore holds great promise for translation into further improvements in articular cartilage repair techniques for restoration of the entire osteochondral unit (Orth et al. 2013a).

2 Definition of the Osteochondral Unit and Osteochondral Defects

The osteochondral unit is composed of the articular cartilage connected to the underlying subchondral bone (Fig. 1a) (Lyons et al. 2006; Madry et al. 2010; Muller-Gerbl 1998; Radin et al. 1972). The articular cartilage provides the joint with a low friction environment and is characterised by a lack of vascularisation, low cellularity and the limited metabolic activity of mature chondrocytes—the unique cell type resident within cartilage (Newman 1998). Type-II collagen is the main collagen present in the articular cartilage (Bhosale and Richardson 2008). Proteoglycans are another fundamental component of the extracellular matrix (ECM). As they are able to retain water molecules (65–80 % of wet weight) within the cartilage, the load-dependent deformation of the cartilage is possible. The articular cartilage is connected with the subchondral bone plate through the calcified cartilage layer. The calcified cartilage contains type-X collagen besides the type-II collagen fibrils extending from the non-calcified articular cartilage into it. The junction of the calcified cartilage with the subchondral bone plate is called the cement line where there are no collagen fibrils extend from the cartilage into the subchondral bone which contains mainly type-I collagen). The subchondral bone is composed of the subchondral bone plate and the subarticular spongiosa.

The subchondral bone plate is made of cancellous bone plates joined together to enclose few narrow intervening spaces. The subchondral bone consists of a mineralised matrix containing embedded osteocytes and lined with osteoblasts and

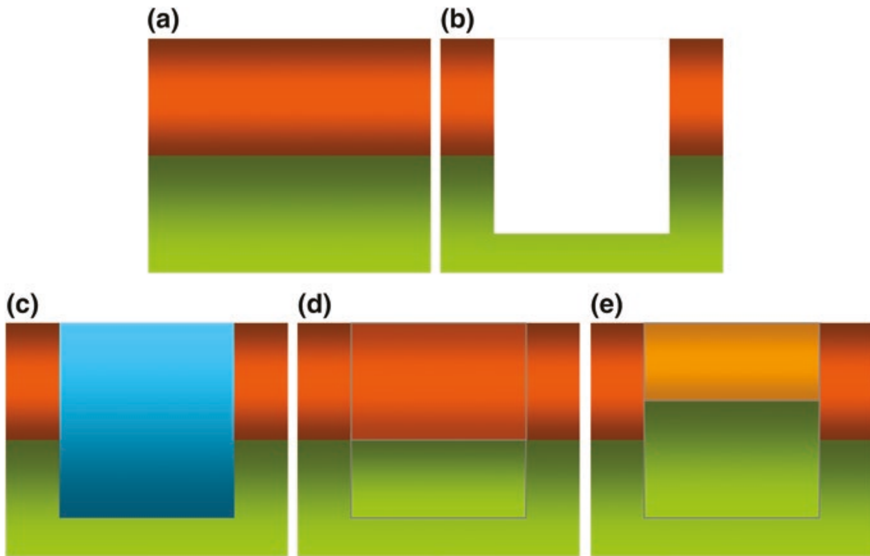

Fig. 1 **a** Scheme of the osteochondral unit, composed of the articular cartilage (*red colour*) and to the underlying subchondral bone (*green colour*). **b** Osteochondral defects are characterised by both a deficiency of articular cartilage and subchondral bone. **c–e** Spontaneous osteochondral repair involves the filling of the defect with a blood clot originating from the bone marrow. **c** Next, mesenchymal stem cells (MSCs) from the subarticular spongiosa migrate into and completely fill the defect. **d** Subsequently, they initiate osseous and chondral repair, leading to a re-establishment of the two components of the osteochondral unit. **e** Over time, however, the articular cartilage repair tissue degenerates (*orange colour*) and the subchondral bone plate advances into the cartilaginous part of the repair tissue

osteoclasts capable of remodelling the bone. Towards the metaphysis, the subarticular spongiosa follows, which is composed of cancellous bone.

Osteochondral defects are characterized by a deficiency of both articular cartilage and subchondral bone tissue, in contrast to chondral defects which are solely cartilaginous lesions, i.e., not penetrating the subchondral bone. Consequently, due to the nature of the defect, simultaneous regeneration of both cartilage and bone is required for defect repair (Fig. 1c–e).

Focal osteochondral defects often occur in the course of diseases of the subchondral bone. These mainly include osteochondritis dissecans and osteochondral fractures (Menetrey et al. 2010) and are usually well defined and are often deep lesions. In the context of this chapter, we will outline mechanical and biological signalling pathways in osteochondral defect regeneration based on data from animal models and clinical investigations of focal osteochondral defects, but will not address the diffuse involvement of the subchondral bone during end-stage OA (Pritzker et al. 2006).

3 Principles of Tissue Regeneration in Osteochondral Defects

Injuries that reach the subchondral bone cause haemorrhage and fibrin clot formation, activating inflammatory responses (Hunziker 2002). Soon after injury, blood from the damaged bone blood vessels form a hematoma and the osteochondral defect is spontaneously filled with a blood clot originating from the bone marrow compartment. Platelets within the clot release vasoactive mediators, growth factors and cytokines (transforming growth factor beta (TGF-β); platelet-derived growth factors (PDGF) exhibit key roles in cell migration, proliferation, differentiation and matrix production (Hunziker 2002). Bone matrix also contains such factors (TGF-β and PDGF but also the bone morphogenetic proteins—(BMPs), insulin-like growth factors I and II—(IGF-I and IGF-II), among others) that become accessible to the lesion where they play similar roles in initiating reparative responses (Hunziker 2002; Rosen and Thies 1992). Under such stimuli, mesenchymal stem cells (MSCs) from the subarticular spongiosa adjacent to the defect migrate into and completely fill the lesion within several weeks (Fig. 1c). Here, they can subsequently initiate osseous and chondral repair in their appropriate location while the fibrin clot has been almost completely resorbed (Fig. 1d).

3.1 Cartilage Regeneration in Osteochondral Defects

In the upper region of the lesion, MSCs undergo differentiation processes to commit towards the chondrocyte phenotype in a defined sequence of cellular and molecular events (Sekiya et al. 2002). Initially, cells that have migrated in the defect initiate chondrogenesis by condensation through cell–cell interactions mediated by expression of N-cadherin (Anraku et al. 2009). After some days, expression of this molecule is lost and the cells acquire a spindle-shaped, undifferentiated appearance with the presence of a fibrocartilaginous ECM consisting of type-I collagen. They express the PTH/PTHrP receptor that signals to slow down the progression of chondrogenic differentiation (Jackson et al. 2001; Shapiro et al. 1993; Anraku et al. 2009; Mizuta et al. 2006). Within two weeks, the PTH/PTHrP receptor is still detectable but the cells start to assume a typical rounded morphology, with the production of a matrix containing type-II collagen and expression of relatively high levels of proteoglycans (Anraku et al. 2009; Jackson et al. 2001; Shapiro et al. 1993). These changes are associated with increases in the expression of members of the SOX (sex-determining region Y-type high mobility group box) family of DNA binding proteins among which is SOX9 (Anraku et al. 2009), a critical transcription factor for chondrocyte differentiation. SOX9 activates cartilage-specific genes including the gene for type-II collagen (Fig. 2) (Bell et al. 1997; Bi et al. 1999; Lefebvre et al. 1997; Xie et al. 1999). By 1–2 months, the repair tissue in the chondral region of the defect still contains chondrocyte-like

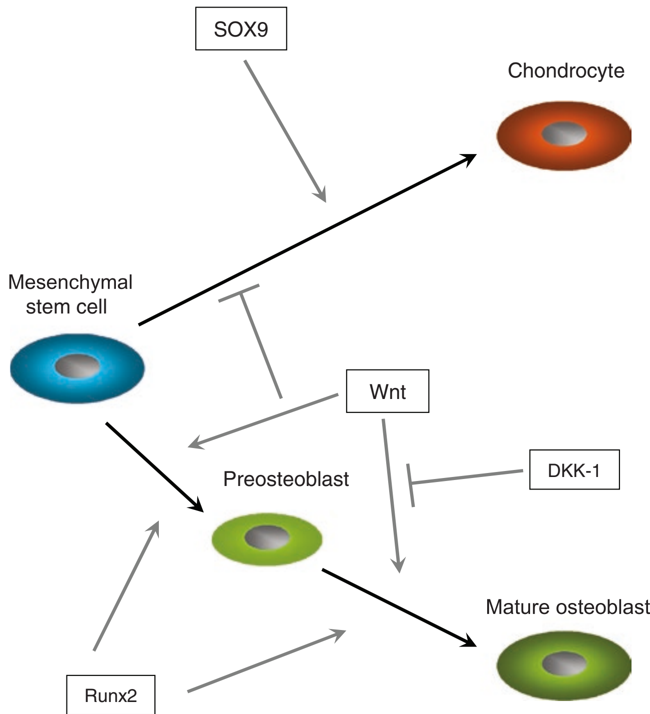

Fig. 2 Scheme showing the SOX9 and Wnt signalling effects on chondrocytic and osteoblastic differentiation of MSCs. SOX9, a member of the sex-determining region Y-type high mobility group box family of DNA binding proteins is a critical transcription factor for chondrocyte differentiation and cartilage formation that activates cartilage-specific genes, among which type-II collagen. Canonical, and in part non-canonical, Wnt pathways promote the commitment of MSCs towards the osteoblast differentiation program. At the same time, they inhibit the progression of precursors to the chondrogenic fate. DKK-1 blocks Wnt signalling by binding to the Wnt receptor complex on the surface of the osteoblast lineage cell, thus arresting osteoblast proliferation and differentiation. Blockade of DKK-1 (e.g. by tumour necrosis factor alpha—TNF-α) supports the progression of osteoblast differentiation. Modified from (Rodda and McMahon 2006) and from (Goldring and Goldring 2007)

cells in a matrix of proteoglycans and type-II collagen but also contains type-I collagen. This indicates the presence of an intermediate material, between hyaline and fibrocartilage that resembles but does not replicate, the normal articular cartilage. The repair tissue does not exhibit an arcade-like organisation of collagen fibres with zonal chondrocyte stratification typical of hyaline cartilage, but is rather a fibrous type of cartilage with lesser mechanical properties without complete integration and bonding of the collagen fibrils with the surrounding, unaffected cartilage. By 6–12 months, the cartilaginous repair tissue within the defect shows signs of degeneration with matrix depletion, fragmentation and fibrillation, showing even areas of exposed bone (Hunziker 2002) (Fig. 1e).

3.2 Bone Regeneration in Osteochondral Defects

In the deeper regions of the lesion, MSCs differentiate into osteoblasts to form bone in an attempt to reconstitute the subchondral bone plate (Jackson et al. 2001; Shapiro et al. 1993), probably via a process similar to endochondral ossification which occurs during fracture healing (Farrell et al. 2011; Scotti et al. 2010; Sheehy et al. 2013). This results in the formation of immature bone that usually restores the original level of the subchondral bone in a distinct pattern (Orth et al. 2012a). This is associated with an absence of SOX9 expression (Anraku et al. 2009) and enhanced expression of type-X collagen (Anraku et al. 2009) and of the Runt-related transcription factor 2 (Runx2) (Fig. 2) (Anraku et al. 2009), a factor required for osteoblast differentiation that also regulates chondrocyte maturation and terminal differentiation (Ducy et al. 1997; Inada et al. 1999). Over time, this new subchondral bone often advances toward the joint space and intralesional osteophytes may also form (Orth et al. 2012b) (Fig. 1e).

Several cell types present in an osteochondral defect are capable of sensing and of transducing mechanical stresses into a biological response: the MSCs themselves as osteoprogenitor cells, the MSC-derived osteoblasts that form the new subchondral bone, the osteocytes (terminally differentiated from osteoblasts and embedded within the subchondral bone) and the bone-resorbing osteoclasts (derived hematopoietic cells of the macrophage lineage) (Huang and Ogawa 2010; Thompson et al. 2012). Osteocytes located within the newly formed subchondral bone plate have an important role in sensing external loads and regulating the adaptation of the architecture of the subchondral bone and the overall remodelling process that results in an advancement of the subchondral bone into the articular cartilage repair tissue. Physical forces are translated into biochemical signals by intracellular ion channels (such as K^+ and Ca^{++} channels), intracellular signalling (Wingless-type (Wnt)/β-catenin), mechanically induced signalling molecules (prostaglandins, nitric oxide), transmembrane molecules (integrins), growth factors (IGF-I, BMPs) and systemic hormones (parathyroid hormone—PTH) (Allori et al. 2008; Epari et al. 2011; Huang and Ogawa 2010; Regard et al. 2012). For example, Wnt crosstalk and functional antagonism with the low-density lipoprotein receptor-related protein 5 (LRP5) co-receptor receptor plays an important and complex role (Goldring and Goldring 2007; Rodda and McMahon 2006) in the maintenance and osteogenic differentiation of MSCs (Baksh et al. 2007; de Boer et al. 2004; Rodda and McMahon 2006), suggesting a possible role of the Wnt pathway in this phenomenon.

3.3 Interplay Between Cartilage and Bone Regeneration in Osteochondral Defects

Translational studies reveal that the reconstitution of the subchondral bone within osteochondral defects proceeds in a defined chronological order, often resulting in an expansion of the subchondral bone plate into the cartilaginous repair tissue

at later stages (Orth et al. 2012a). At the same time, cartilage repair within such defects is initially improved but this cartilage is later degraded, providing the basis for the current inability to adequately repair osteochondral defects. Of note, while individual parameters of subchondral bone and cartilage repair are internally correlated, no correlation has been detected so far between the subchondral bone plate migration and the degradation of the repair cartilage (Orth et al. 2013a), suggesting that the advancement of the subchondral bone plate is not responsible for the degradation of the cartilaginous repair tissue, at least in the experimental model systems employed (Orth et al. 2012a, 2013b).

Importantly, mechanical signals influence the lineage allocation of MSCs and all cells involved in osteochondral repair are capable of responding both directly and indirectly to mechanical signals. The structure of the subchondral bone is possibly defined by regulated cellular interactions, including osteocyte control of anabolic and catabolic turnover, signalling between MSCs, chondrocytes, osteoblasts, osteocytes and osteoclasts (Thompson et al. 2012). The advancement of the subchondral plate may be due to as yet unknown anabolic signals generated in the repairing or degrading osteochondral tissues which act to recruit MSCs and induce their differentiation into osteoblasts.

Taken together, this process is likely to be complex and may involve several, currently unknown overlapping mechanical and biological pathways. It is likely regulated through an interaction between the cells within the articular cartilage repair tissue and possibly also involving regulatory systems that operate in physiological subchondral bone remodelling. Further research that aims at elucidating this pathological process is therefore of crucial importance to better understand the mechanisms of subchondral bone repair.

4 Conclusions

Clinical evidence shows that the subchondral bone plays an elementary role in dynamically modulating the effects of stresses applied to the joint, leading to its physical adaptation. Likewise, pathological remodelling patterns have been described that are associated both with spontaneous osteochondral repair following an acute injury and with the major articular cartilage repair procedures (Orth et al. 2013a). Much remains to be elucidated in osteochondral defect regeneration signalling pathways. Some questions of importance include: (1) which signalling cascade(s) play(s) the major role(s) in osteochondral regeneration and how do they interact, (2) which cell type is responsible for the advancement of the subchondral bone plate and (3) how is it possible to therapeutically inhibit this process? The study of the role of the subchondral bone in cartilage repair is a highly promising field and it is likely that a deeper understanding of the fundamental molecular signalling pathways involved in osteochondral defect regeneration will greatly facilitate the improvement of current therapies for such lesions, either via pharmaceutical treatments or through reconstructive surgical interventions, which address

this problem and thereby improve osteochondral repair. Lessons learnt from developmental biology (Lefebvre and Smits 2005; Onyekwelu et al. 2009; Rosen and Thies 1992) may help to better understand how the chondral and bone compartments interact together over time in osteochondral repair.

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Polymer-Assisted Cartilage and Tendon Repair

Gundula Schulze-Tanzil

Abstract Intrinsic repair of traumatic cartilage injuries is generally poor; in a similar manner, the repair of ruptured tendons can be associated with unwanted results such as scar formation and altered biomechanical tissue properties. Therefore, further research utilizing tissue engineering (TE) techniques should help to reduce healing times and to restore natural structure of cartilage and tendon in response to injury. Natural and synthetic polymers play a pivotal role as artificial matrices for cartilage and tendon tissue engineering. Some TE-based therapeutical approaches have already found entry in the clinical praxis. This chapter discusses which peculiarities of cartilage and tendon have to be addressed for the use of synthetic polymers for TE, which kinds of polymers have been tested so far, and which unmet medical needs remain for cartilage and tendon TE. The important issue of reestablishing the tendon-to-bone interface for stable polymer-based TE tendon reconstruction strategies will also be discussed. Future directions for TE-assisted cartilage and tendon reconstruction are to develop biomimetic polymer scaffolds, to fully restore tissue zonality and achieve implant integration, mechanocompetence, and last but not least, to establish one step strategies for clinical application. Additionally, polymers could be used to help achieve more rapid expansion of chondrocyte and tenocyte numbers in culture, and for preculturing procedures.

Keywords Cartilage repair · Tendon repair · Biomimetic polymers · Tenogenesis · Chondrogenesis

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Abbreviation List

2D	Two-dimensional
3D	Three-dimensional
BMP	Bone morphogenetic protein
COMP	Cartilage oligomeric protein
CS	Chondroitin sulfate
ECM	Extracellular matrix
FDA	Food and drug administration
FGF	Fibroblast growth factor
GAG	Glycosaminoglycans
HA	Hyaluronan
HD	High-density culture
IGF	Insulin-like growth factor
iPS	Induced pluripotent stem cells
MSC	Mesenchymal stem cells
PCL	Polycaprolacton
PDGF	Platelet-derived growth factor
PDS	Polydioxanone
PEG	Polyethylenglycol
PET	Polyethylene terephthalate
PGA	Polyglycolic acid
PLA	Polylactic acid
P(LLA-CL)	Poly(l-lactide-co-ε-caprolactone)
PTFE	Poly(tetrafluoro ethylene)
PUU	Polyurethran
PVA	Polyvinylalkohol
RGD	Arginin, glycin und asparaginsäure
SDF	Stromal cell-derived factor
TE	Tissue engineering
TGF	Transforming growth factor

1 Introduction

Articular cartilage covers the articulating bone surfaces whereas tendons connect muscles with bone and ligaments bridge two bones (Figs. 1 and 2). Despite the fact that tendons and ligaments possess some minor metabolic differences (e.g., elastin and pyridinoline cross-link content), they show no major differences in their architecture (Kuo et al. 2010). Therefore, the ongoing chapter refers solely to tendon. This chapter will present application of natural and synthetic polymers, which represent macromolecules consisting of many similar monomeric subunits, in cartilage and in tendon TE to improve healing response in these tissues.

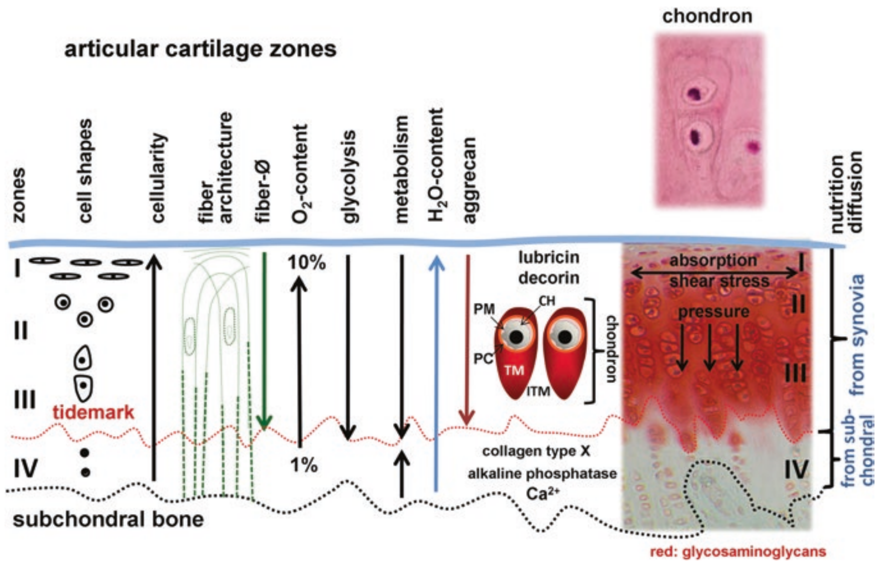

Fig. 1 Synopsis of articular cartilage zones. *I–IV* articular cartilage zones, *I* superficial, *II* transition, *III* deep zone, *IV* calcified cartilage layer. Chondrocyte (*CH*), pericellular capsule (*PC*), pericellular (*PM*), territorial (*TM*), interterritorial extracellular matrix (*ITM*), *on the right* a chondron and two isogen chondrocytes are depicted.

The aim of TE is to design a biomimetic implant with the help of biomaterials and cells which closely resembles the native tissue structurally, biochemically, and biomechanically. A great deal of research has been directed toward developing a three-dimensional (3D) scaffold that would induce neotissue formation mimicking the hierarchical architecture of native tissue extracellular matrix (ECM) (Deng et al. 2012). TE provides therefore a promising strategy to produce grafts to treat traumatically injured articular cartilage and tendons (Kuo et al. 2010; Mollon et al. 2013), since the healing processes in these tissues are of long duration and are often unsatisfactory.

Natural polymers such as silk and collagen or synthetic polymers, i.e., poly glycolic acid (PGA) have attracted much interest for cartilage and tendon reconstruction. Novel and future TE constructs will contain composites of two or more polymers to fulfill the many biomechanical requirements of the tissue to be reconstructed (Ko et al. 2010; Gloria et al. 2010).

1.1 Unmet Medical Need

Despite many similarities in their biology, cartilage and tendon TE need different strategies to be addressed. Nevertheless, there are so far no clinically available scaffolds that can regenerate cartilage and tendon to a satisfying degree

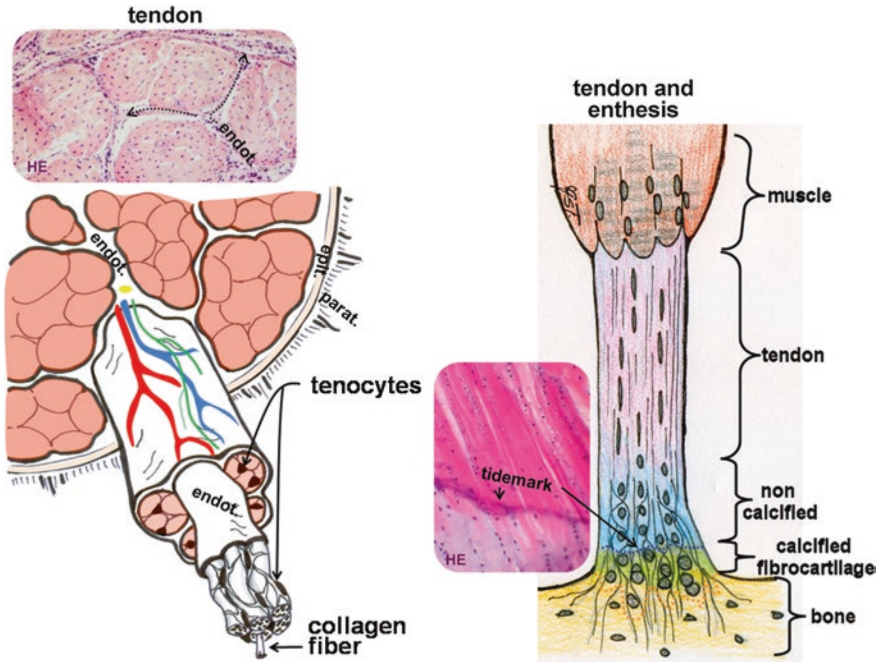

Fig. 2 Histological structure of tendon and enthesis. *On the left* a scheme of tendon architecture is shown, above (*left side*): a cross section of a rat tail tendon is depicted (Hematoxylin-Eosin staining). *On the right* tendon and enthesis are visualized schematically. endot., epit., parat.: connective tissue layers of endo-, epi- und paratenon

(Huey et al. 2012; Shearn et al. 2011). For this reason, there remains a pressing demand to develop novel strategies to combine and optimize existing polymeric compounds for cartilage and tendon TE that effectively consider the molecular architecture and biomechanical needs of these tissues.

2 Articular Cartilage

Articular cartilage contains only few cells, the chondrocytes, which are mechanosensitive. Particularly, the abundant ECM mediates the unique functionality of cartilage (Archer and Francis-West 2003). The chondrocytes have to organize, remodel, and repair an abundant ECM in response to mechanical stimuli. The joint cartilage ECM consists mainly of type II collagen and the large proteoglycan aggrecan which contains many glycosaminoglycan (GAG) side chains (Archer and Francis-West 2003). Type II collagen and aggrecan synthesis are indicators of a chondrocyte-differentiated phenotype (Schulze-Tanzil 2009). In aggrecan, chondroitin sulfate (CS) and keratan sulfate GAGs can be found which are responsible for abundant negative charges. Reversible binding of water triggered by GAGs provides the typical viscoelasticity

of cartilage and therefore one main aspect of its functionality. The ECM architecture and chondrocyte synthetic profile slightly vary depending on articular cartilage zones (Archer and Francis-West 2003) (Fig. 1).

Articular cartilage is nourished by diffusion of synovial fluid and by blood fluid from subchondral bone-derived blood vessels whereby the tidemark (Fig. 1) presents the limit between both nutrition regions (Madry et al. 2010). In contrast to other tissues, most of the chondrocytes do not directly communicate with each other via cellular processes but have to manage the exchange of paracrine mediators through the dense ECM. The cellularity in cartilage depends on the particular location of cartilage in the body, the species, and the articular cartilage zone (Fig. 1). It is generally lowest in human cartilage compared with that of small and large domestic animal species where cells represent ~1–10 % of cartilage volume (Stockwell 1971; Archer and Francis-West 2003; Bruckner and van der Rest 1994; Martinek 2003). The cell size also varies slightly dependent on the joint involved, the joint cartilage area, and the cartilage zones (Fig. 1), and is influenced by the grade of cartilage degeneration. Articular chondrocytes have a cell diameter between ~10 and 72 μm in situ (Muller et al. 2013; Bush and Hall 2003; Trippel et al. 1980). Moreover, we generally observe that the cell size of chondrocytes increases during culturing. The density of the ECM limits the diffusion of factors—the exclusion range is assumed to be 60 nm in diameter for particles (Rothenfluh et al. 2008).

The gliding, low friction properties of the cartilage surface are mandatory in cartilage for joint function, durability, and low generation of wear debris (Grad et al. 2012; Rhee et al. 2005). The concepts of balanced friction, wear, and lubrication of interacting surfaces are addressed by the emerging research discipline of biotribology which requires particular attention in view of the need for the restoration of low wearing joint surface characteristics with TE constructs (Neu et al. 2008). Therefore, it is important to achieve a smooth and stable tissue-engineered construct surface for optimal articular cartilage reconstruction. The glycoprotein lubricin and the GAG hyaluronan (HA) produced by superficial zone chondrocytes and synovial fibroblasts play a pivotal role in articular cartilage surface lubrication additional to that of other constituents of the synovial fluid. Lubricin synthesis could be induced in mesenchymal stromal cells (MSCs) seeded in an agarose hydrogel-based construct which was designed for articular cartilage engineering (Thorpe et al. 2013). According to these requirements in cartilage, low friction polymeric hydrogels have been recently designed for cartilage TE (Blum and Ovaert 2013).

3 Healing in Cartilage

The kind of repair tissue formed in response to traumatic defects depends on the localization of the defect in cartilage and its depth (partial or full thickness for chondral defects or osteochondral lesions). Chondral defects remain mostly

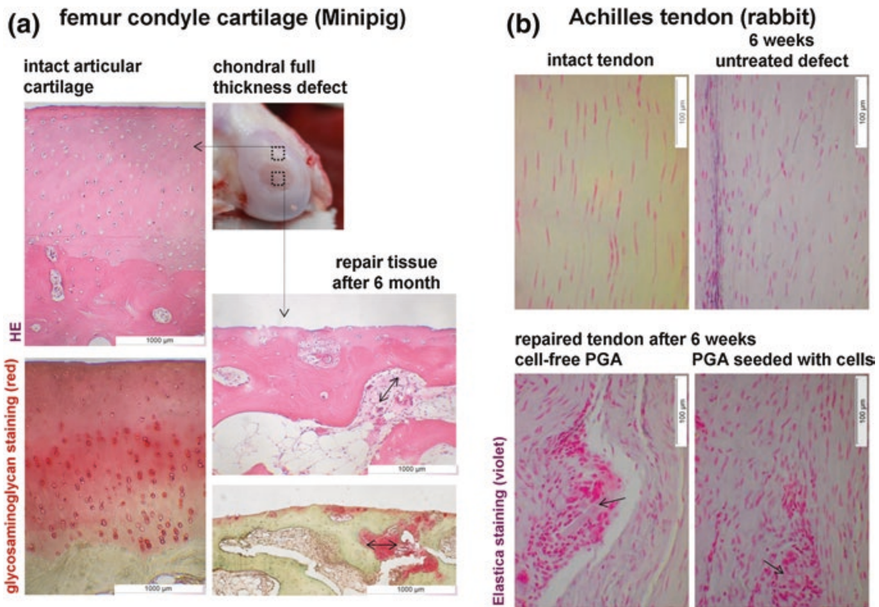

Fig. 3 Healing cartilage and tendon defect. **a** A full thickness cartilage defect is shown (Hematoxylin–Eosin and Safranin O [proteoglycan staining]) and compared with an intact cartilage area. Despite activation of mesenchymal stem cells from the subchondral bone marrow cavity (*double-head arrows*), the defect was not filled after 6 months. **b** A healing partial tendon defect in a rabbit Achilles tendon is shown after 6 weeks in comparison to a healthycontrol. The damaged tendon was either untreated, treated with an PGA felt without cells, or treated with a tenocyte-seeded PGA felt. *Arrow:* PGA fibers. Resorcin Fuchsin staining was used to depict elastic fibers

uncovered (Fig. 3) or are covered by a soft tissue produced by synovium-derived cells (mostly fibroblasts and MSC) colonizing the defect (Hunziker and Rosenberg 1996; Madry et al. 2010). Osteochondral defects can be settled by MSCs mobilized from the subchondral bone marrow cavities (Madry et al. 2010). The repair tissue in cartilage defects generally remains fibrocartilaginous in nature with low biomechanical resilience (Steinwachs et al. 2011).

4 Tendons and Healing Tendon

Tendon (Figs. 2 and 3) is a mesodermal tissue which shares many similarities with cartilage. Tendon tissue has poor blood supply resulting in slow and low exchange rates (Milz et al. 2009). The cells have to sustain viability under low oxygen tension and thus, undertake mostly anaerobic metabolism. For this reason both tissues were designated as bradytrophic (Tozer and Duprez 2005). Tendon ECM consists of parallel running type I collagen bundles and is associated with only

few cells (~5 % of tissue volume) associated in parallel rows with bundles (Tozer and Duprez 2005; Ippolito et al. 1980). Most of the tendon—derived cells (almost 95 %) are tenocytes and tenoblasts (Milz et al. 2009). Tendon tissue is highly hierarchically organized hereby providing optimized flexibility by several looser connective tissue layers of para-, epi-, peri-, and endotenon surrounding tendon and tendon fascicles (Al-Sadi et al. 2011; Milz et al. 2005) (Fig. 2). These connective tissue layers protect supplying structures such as blood, lymphatic vessels, and nerves during mechanical movements and flexibly bind tendon to the surrounding tissue. Tenocytes were characterized by scleraxis, tenomodulin, and tenascin C expression (Milz et al. 2009). Tendon meets cartilage or bone in several regions in the human body, particularly in the enthesis regions, areas which are exposed to specific biomechanical burdens (Benjamin et al. 2006). These contact areas provide a particular challenge (Benjamin et al. 2006) therefore, this interface will also be discussed in this chapter. In contrast to cartilage which sustains pressure loads, tendon is prone to unilateral tension. Moreover, tendon-derived cells can transdifferentiate into fibrochondrocytes, a fact which becomes evident in tendon regions which are prone to pressure because the tendon bends around a bony extension, a so-called hypomochlion (Benjamin et al. 2008; Milz et al. 2005; Benjamin and Ralphs 1998). Cells form mechanosensitive networks communicating via cellular extensions and gap junctions consisting of connexins such as connexin 32 and 43 (Maeda et al. 2012; Wall and Banes 2005). Lubricin has also been shown to fulfill important functions in tendon. It allows wear-less gliding of intrasynovial tendons within their synovial sheath and additionally facilitates interfascicular gliding in tendons and prevents adhesion formation (Hayashi et al. 2013; Funakoshi et al. 2008).

The healing process in tendon is triggered by resident (intrinsic) and extrinsic immigrating cells and can be divided into several steps such as bleeding, inflammation, proliferation, granulation, and remodeling phases (Aspenberg 2007; Sharma and Maffulli 2005). The healing success is influenced by the type of tendon and its local environment in the body (extra-/intrasynovial, extra-/intraarticular, local blood supply and loading, etc.).

Nevertheless, the healing of injured tendon and ligaments takes several months (Gross and Hoffmann 2013; Aspenberg 2007) often resulting in unwanted healing responses such as scar or adhesion formation, and tendon degeneration (Sharma and Maffulli 2006). In addition, impaired tendon functionality bears the risk of muscle atrophy. In the current clinical praxis, dependent on the location ruptured, tendons are either sutured, or substituted by autografts (Ekdahl et al. 2008; Rawson et al. 2013). These strategies were limited by restricted availability and donor site morbidity of autografts (Ekdahl et al. 2008). Cell-free synthetic materials have not shown sufficient longevity or have caused inflammation due to the generation of wear debris (Mascarenhas and MacDonald 2008).

Nevertheless, many factors contributing to the limited healing response in tendon remain to be determined. Without doubt, a rapid access to blood vessels and appropriate vascularization is necessary to achieve successful tendon repair using biological or synthetic polymers-based TE strategies. The question still remains whether tenocyte alignment, which is crucial for aligned fiber synthesis and

therefore tendon biomechanics, can be reestablished during healing together with the essential cell–cell communication which is mandatory for the functionality of this mechanosensitive tissue. Further, the gliding properties of tendon have to be reestablished in engineered tendon (Theobald et al. 2012).

5 Reconstruction of Articular Cartilage and Tendon-to-Bone Interface

5.1 Articular Cartilage-to-Bone Interface

The intimate interconnection of the articular cartilage with the subchondral bone presents an important interface (Gomoll et al. 2010; Madry et al. 2010). However, in the articular cartilage-to-bone interface, the articular cartilage-derived collagen type II arcades fiber bundles are not fixed in the bone ECM (Madry et al. 2010) (Fig. 1). An interdigitation between both surfaces (bone and calcified articular cartilage) allows stability by high contact surface. Nevertheless, this interface is a *locus minoris resistentiae*. Osteochondral cartilage defects include the bone-to-cartilage interface and provide a particular challenge in view of adequate reconstruction (Madry et al. 2010).

5.1.1 Fibrocartilaginous Enthesis

Tendon presents two interfaces particularly at the osteotendinous and the myotendinous junction (Fig. 2). The last one which connects muscles to tendon will not be further addressed here. The tendon-to-bone interface includes in the most cases a fibrocartilaginous tissue layer and is thus called a fibrocartilaginous enthesis (Benjamin et al. 2006). To achieve a stable reconstruction of tendons, a firm attachment of tendon to the bone interface is required (Dickerson et al. 2013). Biological interfaces are characterized by spatial gradients in composition, structure, and mechanical properties. These gradients minimize mechanical stress emergence and mediate load transfer and distribution between the tendon and bone. The transition phase in enthesis consisting of fibrocartilage should facilitate tension and pressure transmission and distribution between the two very dissimilar tissues; tendons and bone (Smith et al. 2012; Benjamin and Ralphs 1998). Altogether, a gradation realized by four zones appears in the fibrocartilaginous enthesis: (1) dense connective tendon zone, (2) noncalcified fibrocartilage, (3) calcified fibrocartilage (the latter two separated by the tidemark), and (4) the bone (Fig. 2). Attaching dissimilar materials without loosening and wear debris remains a major challenge in engineering and orthopedic surgery (Lu et al. 2010). An interdigitation between the bone and calcified fibrocartilage is realized at the enthesis and is suitable to achieve a large contact surface (Benjamin and Ralphs 1998).

Calcified fibrocartilage acts as a transitional zone. The attachment angle of tendon into bone is shallow at the fibrocartilage tissue layer region (Liu et al. 2011). In contrast to a fibrocartilaginous enthesis a fibrous (or indirect) enthesis consists only of a tendon and bone zone (Gross and Hoffmann 2013). A commonly used autograft-based anterior cruciate ligament reconstruction strategy utilizes a bone–tendon–bone technique to optimize graft integration (Maletis et al. 2013). MSCs co-cultured in the immediate vicinity of both ligament and bone cells on a hybrid silk polymer scaffold indeed differentiated into fibrocartilage (He et al. 2012). Co-cultures of rabbit ligament cells and MSCs revealed communication of both cell types via gap junction expression (Nayak et al. 2010). In view of TE-based reconstruction of the interface, a combination of particular polymeric biomaterials with spatially organized composition and structure is necessary (Seidi et al. 2011; Lu et al. 2010; Lu and Spalazzi 2009; Yang and Temenoff 2009). The delivery of osteogenic factors such as bone morphogenetic protein (BMP)-2 by an injectable hydrogel has also been used to achieve a fibrocartilaginous interface for enthesis reconstruction based on poly(L-lactide-co-ε-caprolactone) scaffolds (Lee et al. 2011).

6 Cell Sources and Properties

Mature chondrocytes, tenocytes, and various stem cells, particularly MSCs are the typically recruited cell sources for cartilage and tendon TE (Oldershaw 2012). Due to their plasticity, easy and abundant expansion potential as well as low donor site morbidity, MSCs have attracted increasing interest for cartilage (Gardner et al. 2013; Oldershaw 2012) and tendon TE (Yin et al. 2010a, b; Rodrigues et al. 2013; Sassoon et al. 2012; Tan et al. 2012). Moreover, versatile chondrogenic biomaterials combined with rapid intraoperative cell isolation/enrichment of the desired precursor cells would allow a one-step therapy for cartilage repair (Steinwachs et al. 2011). Meanwhile, diverse sources of MSCs have been recruited and studied for their influence on tendon or cartilage repair (Oldershaw 2012; Stoltz et al. 2012; Beane and Darling 2012). However, in several situations, ectopic bone formation or tumor growth has been reported in response to MSC or induced pluripotent stem cell (iPS) transplantation (Harris et al. 2004; Liu et al. 2013). Differentiation of MSCs in the tenogenic lineage has been performed using growth /differentiation factor (GDF)-5 as an inductive factor (Sassoon et al. 2012; Tan et al. 2012) but the tracking of the tenogenic differentiating of stem cells is still difficult due to the lack of definitive biomarkers for tendon (Lui et al. 2011). Although fibroblasts, e.g., derived from subcutaneous tissue reveal some plasticity and have therefore been implicated in therapeutic approaches, their differentiation capacity was obviously inferior compared to MSC (Tuan 2009). They are used as a basis for generation of iPS. The increasing research activity in establishing iPS for application in cartilage TE (Outani et al. 2013) is summarized in detail in another chapter. In view of the need for an interaction with a supporting polymeric biomaterial, the cells have to find attachment points on the scaffold surface. They should exert sufficient metabolic activity and ECM

synthesis, but should not mediate scaffold contraction (Caliari and Harley 2011). Co-culture techniques improved results of polymer-based reconstruction of cartilage (Sayed et al. 2012) and could also be valuable in tendon TE (Canseco et al. 2012).

7 Expansion of Chondrocytes and Tenocytes for TE

For TE, cells have to be expanded to sufficient cell numbers. Substantial proliferation of both chondrocytes and tenocytes can only be achieved in monolayer culture. However, it is well known that chondrocytes (Bobick et al. 2009; Schulze-Tanzil 2009) and tenocytes (Wagenhauser et al. 2012; Stoll et al. 2010) undergo a substantial phenotypic shift when cultured in two-dimensional (2D) monolayer culture. For chondrocytes, this shift in 2D culture has already been reported many years ago (Benya and Shaffer 1982). Some reversibility of chondrocyte dedifferentiation was found in various 3D cultures for chondrocytes such as high-density (HD) and hydrogel culture (Schulze-Tanzil et al. 2002, 2004b; Bobick et al. 2009).

The gene expression profile for typical tendon ECM components has previously been analyzed in tenocytes under 2D and 3D conditions and compared with that in tendon (Stoll et al. 2010). It was demonstrated that the expression profile in the analyzed tenocyte cultures (monolayer, and two different 3D culture systems: polylactic glycolic acid [PLGA] and HD cultures) differed substantially from that in tendon. When compared with the PLGA culture, tenocytes in the HD culture achieved a time-dependent higher gene expression level of all ECM genes included in the study and usually found in tendon (Stoll et al. 2010).

HD culture seems to be more favorable for cartilage and tendon TE probably mimicking cell conditions during cartilage (Bobick et al. 2009) and tendon development (de Wreede and Ralphs 2009; Schulze-Tanzil et al. 2004a; Schwarz et al. 2012). In embryogenesis, the tendon and cartilage precursor cell population arise from dense mesenchymal cell condensations (Hall and Miyake 2000).

Another nice approach to expand chondrocytes or tenocytes for TE is by microcarrier culture. This approach has been tested for chondrocytes and MSCs (Melero-Martin et al. 2006; dos Santos et al. 2011; Cetinkaya et al. 2011) and recently for tenocytes (Stich et al. 2013). Microcarriers present a high surface area in a small volume and can be manufactured from various degradable and nondegradable synthetic and biological polymers. They can be cultured under dynamic conditions without trypsinizing for cell passaging, e.g., in a bioreactor device. Analyzing the gene expression of tenocytes cultured on cytodex type 3 microcarriers in comparison to monolayer culture, the studied collagen types did not reveal major differences whereas downregulation of proteoglycan and cartilage oligomeric protein (COMP) gene expression was observed on the carriers (Stich et al. 2013). However, the microcarrier technique might be promising in the future to continuously expand cells for TE and to seed scaffolds with microspheres. There is still a challenging demand for the development of optimized cell expansion techniques which could reduce the risk of cell dedifferentiation.

Further, microcarriers could be utilized for other therapeutic approaches; for example in combination with hydrogels as slow release systems for inductive growth factor delivery (Spiller et al. 2012).

8 Polymers for Cartilage and Tendon TE

The large group of polymers can be divided into natural and synthetic members. Many scaffolds consist of composites of both types. Additionally, polymers can be separated into absorbable/biodegradable and nonabsorbable members. For cartilage and tendon TE, solely bioabsorbable polymers are very attractive. Therefore, in contrast to other tissues such as bone, only few ceramics have been tested (Dorozhkin 2010). Material scientists have contributed extensive effort to develop and optimize biodegradable biomaterials for cartilage and tendon TE. Appropriate biomaterials should be biocompatible, biomechanically stable, exert biofunctionality, and show slow degradability. They should also be processable and allow stable handling (Gross and Hoffmann 2013). Highly interconnective porosity is desired for cartilage and tendon TE. It is well known that pore diameter and distribution affect cell migration and also influence biomechanical properties (Raghunath et al. 2007). Synthetic and natural polymers are used to produce porous scaffolds, hydrogels, fibrous scaffolds, and microspheres (Dhandayuthapani et al. 2011).

8.1 Natural Polymers

Natural polymers, mostly proteins or polysaccharides, generally possess a higher biocompatibility than synthetic polymers (Lynn et al. 2004). However, biocompatibility can be affected by the chemicals used for extraction, preparation, cross-linking strategies for stabilization or sterilization procedures (Kew et al. 2011; Freeman 2009). On the contrary, higher batch-to-batch variability and lower reproducibility can often be observed in natural polymers when compared with synthetic polymers (Gross and Hoffmann 2013; Kuo et al. 2010).

8.1.1 Collagen

Collagen has been one of the most commonly used polymers in TE for decades. Due to the fact that cartilage ECM consists of a type II collagen framework and tendon ECM contains aligned type I collagen fibers, biomaterials based on collagen are promising candidates for tendon reconstruction (Kew et al. 2011). Collagen has been processed into films, membranes, foams, scaffolds, felts, sponges, and hydrogels. The collagen fibril diameters range between 50 and 100 nm (Kuo et al. 2010). Denaturation of proteins can occur, e.g., during

preparation of collagen. The manufacturing process for collagen is challenging. The biocompatibility and other properties of collagen for TE purposes depend on cross-linking patterns (Kew et al. 2011) and manufacturing processes may or may not allow sufficient fibril formation. The properties of collagen depend also on donor species and tissue of origin. Most collagen scaffolds consist of type I and III collagen which are abundantly available and which are represented in tendon but not in healthy cartilage under in vivo conditions. Therefore, for cartilage TE, the use of type II collagen is advisable as a scaffold component (Mafi et al. 2012; Levingstone et al. 2014). Gelatin has also been tested as a scaffold matrix for cartilage TE (Pettersson et al. 2011; Zehbe et al. 2010). It has to be stabilized to remain firm at body temperature. Collagen from diverse species has been tested for TE.

8.1.2 Silk and Chitosan

Silk, as a natural polymer derived from diverse invertebrates such as spiders, silkworms, scorpions, mites, and flies has attracted high interest for TE in cartilage, tendon, and bone as a versatile biomaterial (Kundu et al. 2013) showing good applicability in TE (Silva et al. 2013). Silk is characterized by easy processing, high biocompatibility, suitable biomechanical properties, and tailorable degradation rates (Kasoju and Bora 2012; Mirahmadi et al. 2013). It can be used for film, sponge, and hydrogel production. In particular, the fact that silk comprises both unique elasticity and strength is promising for its application to cartilage and tendon TE (Kundu et al. 2013). It is often selected as a key component of composite scaffolds (Kasoju and Bora 2012; Sahoo et al. 2010).

Chitosan, another polymer derived from chitin of invertebrates, has also been used for cartilage and tendon TE (Mirahmadi et al. 2013; Jayakumar et al. 2011). Chitosan is prepared from chitin by fully or partial deacetylation. It is biocompatible and degradable and easily processable in hydrogels and scaffolds. Further, it has some antibacterial properties and can be used as a release system for bioactive factors (Jayakumar et al. 2011).

Cellulose, as a plant-derived polymer, is so far of minor relevance for cartilage and tendon TE due to its limited degradability (Ko et al. 2010).

8.1.3 Decellularized Extracellular Matrices

To achieve chondrogenesis and tenogenesis of cultured cells, decellularized ECM currently attracts increasing interest. A freshly produced ECM derived from tendon stem cells can stimulate chondrogenesis in cartilage TE (Ni et al. 2013). ECM extracts from mature tissue were able to stimulate proliferation and differentiation of cultured tenocytes (Yang et al. 2013).

For cartilage and tendon TE, natural decellularized ECM which comprises a natural composite of various polymers meets many biomechanical and biochemical requirements for these tissues (Schwarz et al. 2012; Whitlock et al. 2007; Lohan et al. 2013). For tendon TE, a high tensile strength is required which is difficult to

achieve by engineered materials. Therefore, from the biomechanical and biochemical point of view, the most suitable biomaterial for tendon TE could be a natural tendon ECM. Xenogenic ECMs such as porcine tendon are abundantly available in contrast to human tissues. Decellularization is required to remove the mostly cell-associated antigenic properties (Schulze-Tanzil et al. 2012). There exist various combined physical and enzymatic or mixed protocols for decellularization (Lohan et al. 2013; Schulze-Tanzil et al. 2012) which have to be individually adapted to each tissue. ECM can be recolonized by chondrocytes, tenocytes, and MSCs. However, some critical points in using xenogenic decellularized ECM should be kept in mind. Comparing, for example, the porcine tendon used as an ECM with the human tendon chosen as a cell donor, differences in cellularity, cell size, ECM density, and GAG deposition were detectable. Therefore, species differences could influence recellularization efficacy when using a xenogeneic ECM (Lohan et al. 2013). During recellularization, novel GAGs are synthesized by the immigrating cells (Lohan et al. 2013). Nevertheless, the cell distribution in recellularized tendon remained so far inhomogenous and therefore, still presents a challenge. As some growth factors and bioactive factors are generally bound to GAGs, one can also hypothesize a wash out of these bioactive mediators during decellularization. Finally, the remodeling of natural ECM by autologous cells remains unclear. Cartilage decellularization is much more challenging than that of tendon due to the homogenous and dense cartilage ECM. The recellularization success depends on interconnectivity of pores which is not present in the natural cartilage tissue and so has to be achieved by some loosening of the ECM structure (Schwarz et al. 2012).

9 Synthetic Polymers

Synthetic polymers are often cheap, well processable, and show a higher batch-to-batch reproducibility than natural polymers, however, they can lack functional biochemical groups required for sufficient cell attachment (Kuo et al. 2010; Dhandayuthapani et al. 2011). A large bulk of synthetic polymers has been developed and tested for cartilage and tendon TE, among them are PGA (Stoll et al. 2011), polylactic acid (PLA) (Yin et al. 2010b), PLGA (Stoll et al. 2010), polyurethrane urea (PUU), poly DTE carbonate (Freeman 2009), polydioxanone (PDS), polycaprolacton (PCL) (Ouyang et al. 2002), and poly(l-lactide-co-ε-caprolactone) [P(LLA-CL)] (Xu et al. 2013). Biocompatible biomaterials with higher mechanical strength and a slow degradation profile could be PCL and PDS (Hoyer et al. 2014).

Biomaterials should be degradable (Kuo et al. 2010; Oryan et al. 2013). For tendon and cartilage, an especially slow degradation rate is desired. Many materials are in use as commercially available suture materials or for other medical purposes so having well-established biocompatibility. Polymers should provide sufficient cell adhesion exposing binding sites for integrin receptors and subsequent focal adhesion site formation (Paxton et al. 2009). There are also nondegradable synthetic polymers tested for tendon repair which received already FDA approval including polyethylene terephthalate (PET), polypropylene, and poly(tetrafluoro ethylene)

(PTFE) (Thaker and Sharma 2012). Other biodegradable synthetic polymers are also approved by the FDA. Among them are PLA and PGA, PCL and PDS (Thaker and Sharma 2012). Although results from studies have been promising (Stoll et al. 2011), scaffolds made of PGA, have a limited applicability due to their mechanical weakness and their lack of functional groups for cell attachment. Most synthetic polymers are less immunogenic than natural polymers and last but not least, are more tailorable in regard to chemical, physical, and structural properties (Thaker and Sharma 2012). A major restriction for synthetic polymers use is that some of them release harmful degradation products (Freeman 2009). In contrast to surface degradation, the bulk degradation mode is associated with the abundant release of monomers (Sung et al. 2004). This affects the micro-milieu, and therefore can reduce cell viability and induce inflammation in vivo (Sung et al. 2004).

10 Polymer-Based Hydrogels for TE

10.1 Cartilage

For decades, hydrogels have been commonly applied, especially for chondrocyte 3D cultures and TE (Benya and Shaffer 1982) because they closely mimic the properties of the cartilage ECM. For example, natural hydrogels such as agarose, alginate (consisting of β-D-mannuric acid/α-L-glucuronic acid), collagen gels, fibrin, HA, and others have been described (Benya and Shaffer 1982; Schulze-Tanzil et al. 2004b; Kim et al. 2011; Chung and Burdick 2008).

Many of these materials can be polymerized in vivo. Synthetic hydrogels such as polyvinyl alcohol (PVA), PLA, or polyethylene glycol (PEG) are in use for cartilage TE (Blum and Ovaert 2013; Drira and Yadavalli 2013; Dhandayuthapani et al. 2011). A risk is that the polymerization/gelation process can affect the viability of the encapsulated cells by compressing them. Dependent on the gel density, some cells embedded in these gels cannot acquire an elongated cell shape and therefore are constrained to maintain a rounded phenotype. It is well known that cell shape is associated with a particular cytoskeletal architecture which influences the expression profile for differentiation-specific genes. Further, the nutrients can freely perfuse through the complete hydrogel construct due to the generally high water content (Sontjens et al. 2006). The density of hydrogels can in the most cases be varied by the concentration ratio of constituents.

10.2 Tendon

In contrast to cartilage, hydrogels remained only of limited and mostly experimental interest in tendon TE due to their limited tensile strength (Sun et al. 2012) but they are utilized as supplemental tools to achieve homogenous cell distribution

within a scaffold and to fix scaffolds at injury sites (Stoll et al. 2011). However, novel synthetic hydrogels have been developed recently with significant higher elasticity and toughness than those hitherto existing (Sun et al. 2012).

11 Hybrid/Biphasic Scaffolds

11.1 Cartilage

The generation of hybrid or multiphasic scaffolds allows the combination of suitable properties of biomaterials and mimics more closely in vivo conditions where multiple ECM components interact with cells. Such stratified composites are prepared, e.g., for osteochondral cartilage defect repair (Nooeaid et al. 2012) or to restore articular cartilage zonal architecture (Steele et al. 2013).

11.2 Tendon

In the ECM, collagen fibers are intimately associated with GAG. Therefore, collagen-GAG based scaffolds have been thoroughly tested for tendon TE (Caliari and Harley 2011, 2013). Natural and synthetic polymers are sometimes mixed in hybrid scaffolds to optimize mechanical properties for tendon TE (Sahoo et al. 2010). Multiphasic scaffolds are particularly interesting for tendon enthesis reconstruction (Smith et al. 2012).

12 Tools to Optimize Culture Conditions for Cartilage and Tendon TE

12.1 Cell Numbers

Initial high cell numbers are generally used for cartilage and tendon TE. High cellularity and intimate cell–cell contacts are also observed during early embryological development of cartilage and tendon (Hall and Miyake 2000; Schiele et al. 2013)—while during tissue maturation the ECM to cell ratio continuously increases in cartilage and tendon (Ippolito et al. 1980; Meller et al. 2009). Finally, the goal is to achieve tissue-specific cell numbers in the TE constructs. In both tissues, cellularity depends on species (Stockwell 1971), tissue subtype, localization, and donor age (Vignon et al. 1976; Meller et al. 2009). HD cultures favor the differentiated phenotype and ECM production of both chondrocytes and tenocytes. During HD culture the ECM to cell ratio continuously increases with time (Schulze-Tanzil et al. 2002; Stoll et al. 2010; Lin et al. 2006) mimicking the natural maturation process during connective tissue development.

12.2 Seeding Conditions

Various dynamic and static seeding techniques have been used to achieve optimal cell retention and homogeneous distribution in the biomaterial for cartilage and tendon TE (Yeatts et al. 2013; Stich et al. 2013). Novel approaches allow 3D bio-printing of viable cells into polymer scaffolds. Dynamic strategies, e.g., by using rotator devices help to achieve homogenous cell distribution, can stimulate cell differentiation due to the mechanical impulses (Smith et al. 2012). Bioreactor culture can provide a precise adjustment of the microenvironment and mimics the in vivo conditions more closely than conventional culturing (Forsey et al. 2012; Mabvuure et al. 2012). These facts will be extended in another chapter. One parameter which can be well controlled in bioreactors is the oxygen tension.

Low oxygen (2 %) allows better expansion of tendon-derived stem cells and tenocytes (Zhang et al. 2010; Lee et al. 2012). Many studies have demonstrated the chondrogenic and tenogenic effects of low oxygen tension (Hansen et al. 2001; Schrobback et al. 2012; Zhang et al. 2010). Reduced oxygen tension allows also the redifferentiation of dedifferentiated chondrocytes (Domm et al. 2002; Schrobback et al. 2012). The natural low oxygen tension in cartilage and tendon suggests that hypoxic conditions are adequate for cartilage and tendon TE (Schrobback et al. 2012; Zhang et al. 2010) and for expansion of tendon-derived stem cells (Lee et al. 2012).

Some bioreactors also allow mechanostimulation of cultured, polymer-assisted cell constructs. For mechanosensitive tissues such as cartilage and tendon, this treatment is of high benefit during 3D culturing (Abousleiman et al. 2009; Lujan et al. 2011; Brady et al. 2013; Thorpe et al. 2013). It is well known that tenocytes die when mechanical stimuli are lacking (Egerbacher et al. 2008). However, the stimulation profile has to be carefully adapted for each tissue and this issue will be extended in another chapter for cartilage TE. Tenogenesis in TE constructs benefits from cyclic strain which has been suggested should last for 48 h, in 8-h intervals, with tensile strain increasing from 0.7 to 1.0 %, and at a frequency of 0.5 Hz (Schiele et al. 2013).

12.3 Growth Factors in TE

It is well known that a supplementation with tenogenic and chondrogenic growth factors can improve chondrogenesis and tenogenesis (Longo et al. 2011) in TE constructs and support tendon healing (Aspenberg 2007). The release profile concerning time course and concentrations is important for chondrogenesis and tenogenesis, therefore, e.g., microcarrier-mediated slow release systems could be attractive in future.

12.3.1 Growth Factors for Tendon TE

Promising candidates for tendon TE are: stromal cell-derived factor (SDF)-1α, platelet-derived growth factor (PDGF), insulin-like growth factor (IGF)-1, basic

fibroblast growth factor (bFGF), and particularly GDF-5 (Aspenberg 2007; Shearn et al. 2011). Studies revealed that one factor was not sufficient to exert stimulatory effects, factor pairs (e.g., IGF-1 and GDF-5) were found to be effective to stabilize the tenocyte phenotype (Caliari and Harley 2013). This observation implies that the application of cocktails consisting of multiple bioactive factors is required to control cell proliferation and phenotype (Caliari and Harley 2013). The supplementation with the growth factors PDGF-BB and IGF-1 in aligned collagen-GAG scaffolds could dose-dependently enhance tendon cell migration, increase viability, and stimulate metabolism (Caliari and Harley 2011).

12.3.2 Growth Factors for Cartilage TE

Promising candidates for cartilage TE are among many others IGF-I, FGF, several BMPs, and transforming growth factor (TGF)-β (Madry et al. 2013; Kim et al. 2013; Chung and Burdick 2008). ECM extracts which contain a natural mixture of several growth factors and other mediators are effective tools to facilitate chondrogenesis and tenogenesis (Ni et al. 2013; Yang et al. 2013).

13 Polymer Topology

In addition to the chemical properties, the micro- and nanotopology of scaffolds is of high importance. Multiple techniques for scaffold production allow the design of particular topologies and biomechanical attributes. These technologies include electrospinning, weaving, felting, braiding, embroidering, 3D printing, and others which are outside the scope of this chapter.

Nanotopological features of the biomaterial, such as pores, ridges, groves, fibers, nodes, and their combinations, could influence cellular signaling and therefore cellular behavior (Deng et al. 2012). Scaffold topology could be chondro-/tendoconductive but the particular topological requirements for cartilage and tendon-derived cells remain mostly unclear.

Biodegradable polymers, e.g., polyesters, polyphosphazenes, polymer blends, and several composites can be electrospun into nanofibers mimicking the nanoscale of the natural ECM structure, thereby providing a high surface area for cell adhesion, and proliferation, and inductive stimuli for differentiation (Deng et al. 2012). Aligned scaffold fibers induced fibroblast alignment in rows (which are typical in tendon), supported tenogenic differentiation of MSC, and led to increased ECM synthesis by the cells (Xu et al. 2013; Hwang et al. 2009; Lee et al. 2005; Yin et al. 2010b). This ECM was also aligned (Cao et al. 2006; Kapoor et al. 2010). Moreover, the fiber diameter directly influenced the cell orientation whereby 30 μm fiber calibers induced a higher grade of alignment than did fibrils with diameters of 10 or 242 μm (Bosworth et al. 2013; Lee et al. 2005; Hwang et al. 2009). Electrospinning allows thin (nano-)fiber dimensions mimicking

collagen fibers in the natural ECM (Szentivanyi et al. 2011; Wan and Ying 2010). A typical property of natural collagen, anisotropy, when applied to scaffolds facilitates tenocyte alignment (Caliari and Harley 2011). Cell sheet engineering based on the use of thermoresponsive polymers such as poly(N-isopropylacrylamide), could present a novel perspective for cartilage and tendon TE (Elloumi-Hannachi et al. 2010). Further, more complex fiber structures such as core-shell like were also recommended for tendon TE (Caliari and Harley 2011).

Scaffold pore sizes, volume, and distribution influence not only cell viability, proliferation, and penetration into the scaffold, but also cell retention and metabolic activity as reported for tendon (Caliari and Harley 2011) and cartilage (Zhang et al. 2013). A high interconnective porosity is needed for cartilage and tendon scaffolds (Zhang et al. 2013). 150–250 μm was suggested as suitable pore size range for scaffolds for chondrocytes (Zhang et al. 2013) and 50–250 μm for tenocytes (Caliari and Harley 2011). An ellipsoid pore form is preferentially used in most studies (Caliari and Harley 2011; Zhang et al. 2013). In vivo cells should reside <200 μm distance from a blood vessel to receive sufficient nutrients via diffusion. This limit might have relevance in dense 3D scaffold cultures.

Scaffold functionalization, induced using various strategies, is often mandatory to improve cell adhesion, survival, spreading, and differentiation (Rossi and van Griensven 2013). For this reason, scaffolds are often supplemented with natural ECM compounds such as collagen and CS (Caliari and Harley 2011; Villanueva et al. 2010). Chemical surface alterations such as treatment with NaOH, ethylendiamine, or other agents can also be applied to optimize surface charge, provide hydrophilic properties and modify binding motifs (Chawla et al. 2012; Kwon et al. 2010). RGD motifs which represent the so-called cell adhesion peptide sequence (Arg-Gly-Asp) have been selected to improve cell adhesion to biomaterials. They facilitate integrin binding and therefore, focal adhesion formation by the cells (Paxton et al. 2009; Chen et al. 2003). Last but not least, many bioactive factors have been tested with the intention of stimulating cell growth and differentiation within artificial matrices, including the already mentioned growth factors (Khan and Ahmad 2013).

14 Conclusion: Future Challenges

Further efforts should be undertaken to create truly biomimetic composite scaffolds inspired by nature which can guide cell alignment and allow formation of tissue zonality. By surface modification of biomaterials, the immobilization of biomolecules can be achieved. Future challenges are to fully restore mechanocompetence of tissue engineered constructs. This requires restoring 3D communicating cell networks whereby the first efforts in this regard have been commenced with cartilage (Thorpe et al. 2013).

Surface characteristics of TE constructs with respect to adequate gliding and tribological properties are of high relevance in cartilage and tendon reconstruction.

Poor integration and attachment to the bone remains a problem in tendon reconstruction and could be improved by multiphase TE constructs. A further goal is to shorten and improve the time consuming cell expansion and construct preculturing procedures.

Chondro- or tenoinductive co-cultures could be valuable approaches to improve TE construct preparation (He et al. 2012; Canseco et al. 2012). Last but not least, it is necessary to understand the discrepancy between in vitro and in vivo results. Both, the in vitro models and the current in vivo models require improvement and conformity, and progression to testing in large animal models should be preferred (Shearn et al. 2011).

For the clinic, one-step TE-based strategies comprising cell recruitment, enrichment, and seeding on a polymer for implantation in one procedure should be therapeutically established in the future (Steinwachs et al. 2011). Another matter of debate would be to develop cryo-conservation strategies for TE implants. In several cases, patient refuses implantation, but desire it later. However, the cryo-conservation technologies for tendon and cartilage constructs are not yet satisfactory.

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