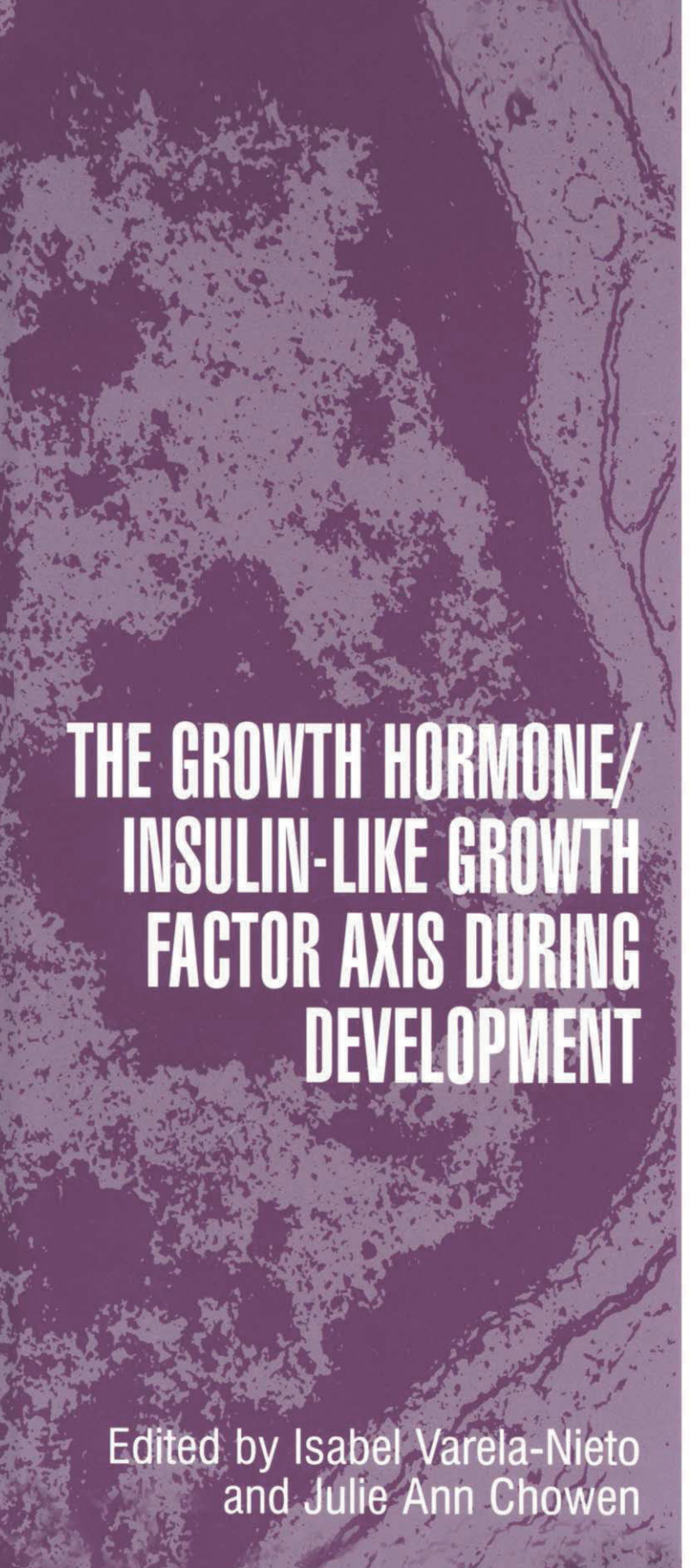

THE GROWTH HORMONE/ INSULIN-LIKE GROWTH FACTOR AXIS DURING DEVELOPMENT

Edited by Isabel Varela-Nieto and Julie Ann Chowen

ADVANCES IN EXPERIMENTAL MEDICINE AND BIOLOGY

Volume 567

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CONTENTS

INTRODUCTION

Although growth hormone (GH) was discovered in 1921, it was not until the 1950s that investigators began to suspect that some of its effects on growth were not direct but involved a mediating factor. Indeed, after the discovery of insulin-like growth factor (IGF) it was thought that most, if not all, GH effects involved stimulation of IGF-I production. However, it is now accepted that GH and IGF have both common and individual roles in growth, development and metabolism.

The IGF family, comprised of insulin and two factors similar to insulin termed IGF-I and IGF-II, has pleiotropic functions in mammals. The first member of this family to be identified was insulin, with subsequent investigation resulting in the elucidation of its role in glucose metabolism and its implication in the etiology of diabetes mellitus. This discovery effected an explosion in the investigation of the structure, function, and mechanisms of action of insulin. The enormous interest in this molecule resulted in the concession of three Nobel Prizes for the investigation of insulin: in 1923 for the discovery of its capacity to treat diabetes, in 1958 for the first sequence of a protein and in 1963 for the first determination of the three-dimensional structure of a protein. Hence, the investigation of insulin has been a pioneer in many scientific fields. Likewise, the insulin receptor was the first receptor with tyrosine kinase activity to be identified and cloned, and is the prototype of membrane receptors with enzymatic activity. Consequently, the mechanisms of action of this receptor have been the focus of intense study leading to the identification of intracellular signaling pathways in response to members of this protein family and has lead, once again, to the discovery of new molecules, pathways and signaling cascades that has had important reverberations in other scientific fields.

Later, the IGFs were discovered and found to be intricately involved in embryonic development and postnatal growth. This was followed by the discovery of a family of binding proteins (IGFBPs) that not only transport the IGFs in the circulation, but alter their function and biological activity. Such widespread and intricate regulation further emphasized the importance of these growth factors in normal physiology. The enormous interest in the insulin-IGF family of proteins lead to the generation of the first double and triple knock-out animals. These knock-out animals, null for a combination of the IGFs, their receptors and the BPs, has helped to shed light on the differential roles of these molecules and their importance in growth and embryonic development. These studies also demonstrated new and diverse functions

for these factors, amongst which their activities in the nervous system is noteworthy. Advances in the understanding of the expression, function, and structure of these factors has not only helped us to further our knowledge of embryonic and postnatal growth, but has also opened the door to new possibilities for their therapeutic use in the treatment of growth alterations, in neurodegeneration, and regenerative medicine.

The objective of this volume is to explain our current understanding regarding the cellular actions of these factors and how this basic knowledge has contributed to our comprehension of their implications in distinct illnesses, as well as the possibility for the development of new therapies.

Chapter 1 covers the basic physiology of the GH/IGF axis, while Chapter 2 discusses some of the most widely used experimental models that have helped to determine the roles of the various members of this axis. The role of IGFBPs in brain and other tissues is discussed in Chapter 3. The importance of IGFs in brain development and function is dealt with in various chapters, all with an individual focus. Chapter 8 covers overall brain development and the effect that each component of the GH/IGF-I system exerts in this process. The trophic effects that IGFs have on neurons in the mature brain and the protection that they exert during neurodegenerative processes or assaults by noxious substances is addressed in Chapter 10 and the interaction of IGFs with other trophic factors in Chapter 12. Chapter 9 focuses specifically on IGFs in neurosensory systems. The effect of the GH/IGF axis in the reproductive tract, skeletal muscle, cardiovascular system and hematopoeisis are addressed in Chapters 4-7. Two areas of current interest include the influence of IGF-I on aging and its possible role in cancer. These subjects are affronted in Chapters 11 and 13, respectively. In Chapter 14 the growing information regarding genetic implications in human growth, involving almost all members of the GH/IGF axis, is presented. The final chapter deals with a new and exciting area of investigation: the use of stem cells in investigation and the possible treatment of a variety of illnesses. This is a rapidly advancing field and future developments will necessitate the need for a second edition.

We would like to express our sincere gratitude to all of the authors for taking time out of their busy schedules to participate in this project. In addition, we would like to acknowledge and thank our colleagues that have generously donated their time and experience to act as reviewers in order to improve the scope and focus of this book. We are in debt to all.

Isabel Varela-Nieto and Julie A. Chowen

Chapter 1

BASIC PHYSIOLOGY OF THE GROWTH HORMONE/INSULIN-LIKE GROWTH FACTOR AXIS

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Key words: Binding proteins; growth hormone-releasing hormone; intracellular signaling; receptor; somatostatin.

1. INTRODUCTION

The growth hormone (GH)/insulin-like growth factor (IGF) axis is intimately involved in the integration of a multitude of signals that regulate systemic growth and metabolism throughout fetal and postnatal development. In the adult animal, maintenance of metabolic balance and cellular integrity, as well as involvement in normal tissue regeneration and in response to injury, can also be attributed to GH and the IGFs.

The classical components of this axis, including GH, the GH receptor (GHR), the GH binding proteins (GHBPs), IGF-I, IGF-II, IGF receptors and the six IGF binding proteins (IGFBPs), will be described briefly in this chapter. Although GH is produced mainly by the anterior pituitary, its receptor is expressed in many tissues throughout the organism[1-3], indicating that this hormone can have widespread effects. In contrast, the IGFs are produced by most tissues during both fetal and postnatal life, with both the circulating and locally produced factors having physiological functions[4,5].

Most tissues also express the IGF-I receptor, during at least one period of development. Hence, both GH and IGF-I have important widespread effects on the development and physiology of the organism.

The involvement of both GH and the IGFs in the development and functioning of distinct physiological processes and systems is addressed in greater depth in subsequent chapters.

2. GH PHYSIOLOGY

2.1 GH

GH was discovered in 1921 by Evans and Long when they demonstrated that extracts of the anterior pituitary administered intraperitoneally promoted growth and maturation[6]. Growth hormone was subsequently isolated from bovine pituitary in 1944[7]. Indeed, GH is produced primarily, or almost exclusively, in the somatotroph of the anterior pituitary. This hormone, belonging to the family of cytokine peptides, contains 191 amino acids, and the most common circulating form has a molecular mass of 22 kDa, while a 20-kDa form has also been detected.[8] Rat GH was one of the earliest cDNAs cloned[9], and human GH was one of the first loci to be assigned to a human chromosome[10]. The human GH gene cluster is located on the long arm of chromosome 17 (17q23) and spans 66.5 kilobases[11]. This cluster is comprised of five genes that from 5′ to 3′ include pituitary GH (GH1), the chorionic somatomammotrophin pseudogene 1 (*CSHP1*), the chorionic somatomammotriphin gene 1 (*CSH1*), the placental GH gene (*GH2*) and gene 2 for chorionic somatomammotrophin (*CSH2*). Each of these genes is comprised of five exons and four introns and all share very high homology throughout their entire sequence (92% to 98%).

In the human fetus GH can be detected in the anterior pituitary as early as the 6th week of gestation[12] and its levels gradually increase during gestation. In serum, GH is detected after the 8th week of gestation and reaches peak levels between 20 and 24 weeks of gestation, after which they begin to decrease. At birth, however, GH levels continue to be elevated and then gradually decline until prepubertal levels are reached at approximately 3 months of age[13]. These high circulating GH levels are somewhat of a paradox, as this hormone is apparently not implicated in fetal growth.

In rats, GH is first detectable on fetal day 18 or 19 and its level increases progressively until birth[14,15]. The total GH content of the pituitary is low at birth and increases gradually during prepubertal life. At the time of pubertal

onset, there is a significant increase in pituitary GH content and during adult life it continues to increase gradually[16]. These changes in pituitary GH content are similar in male and female rats although in postpubertal males, pituitary GH content is significantly higher than in females.

GH is secreted from the anterior pituitary in a pulsatile fashion and this pattern is affected by both age and sex. The physiological importance of this secretory pattern is exemplified by the correlation with its ability to promote growth and stimulate the production of growth factors and liver enzymes.[17,18] In the laboratory rat, the postpubertal GH secretory pattern is strikingly sexually dimorphic[19]. The male rat exhibits high- amplitude pulses with a periodicity of approximately 3 hours, superimposed on a low interpulse baseline. In contrast, the secretory pattern in female rats is comprised of more irregular, low-amplitude pulses with an elevated baseline level. In humans, this sexual dimorphism is less striking, with some discrepancy as to its existence[20]. However, in both instances, the pulsatile secretion of this hormone is fundamental for its physiological effect.

2.2 Hypothalamic control of GH secretion

The first evidence for central nervous system (CNS) control of GH secretion was introduced in 1940 by Heatherington and Ranson when they noted that lesions of the entire ventral hypothalamus retarded longitudinal bone growth[21]. It was later demonstrated that such lesions result in the depletion of pituitary GH content[22] and that electrical stimulation of the ventral medial hypothalamus (VMH) increases plasma GH levels[23]. The race to isolate and identify the hypothalamic factors involved in the control of pituitary hormones began in the 1950s, but the identity of many of these remained elusive for many years.

We now know that GH synthesis and secretion is under the control of the hypothalamus, with the neuropeptides growth hormone-releasing hormone (GHRH) stimulating and somatostatin (SS) inhibiting GH production and release from the anterior pituitary. In 1973, somatostatin, the 14-amino acid GH-inhibiting peptide, was isolated and sequenced[24]. The N-terminal-extended form of the tetradecapeptide composed of 28 amino acids was later isolated[25-27]. A common 15-kDa polypeptide appears to serve as the precursor for both the SS-14 and SS-28 isoforms[28]. It is believed that SS plays an important role in determining the basal or interpulse level of GH secretion[29,30]. Somatostatin is widely distributed throughout the brain and in peripheral tissues. The majority of the SS projections to the median eminence arise from neurons of the periventricular nucleus (PeN)[31] and these neurons are thought to play a key role in the neuroendocrine control of pituitary function[32].

The isolation of GHRH proved to be a formidable task, which is now attributed to its minuscule concentration in the CNS and its susceptibility to biological inactivation during purification procedures. In 1982 GHRH was ultimately purified and sequenced independently by two different groups[33,34]. Growth hormone-releasing hormone is produced by neurons located in the hypothalamic arcuate nucleus and ventromedial hypothalamic area, but those that project to the median eminence are primarily located in the arcuate nucleus[35,36] and are present in three molecular forms: GHRH(1-44), GHRH(1-40), and GHRH(1-37)[33,34,37]. The rat GHRH gene spans nearly 10 kilobases of rat genomic DNA, contains 5 exons, and encodes the 104-amino acid GHRH precursor[38].

The pulsatility of GH release is the result of an interplay between these two hormones, with the release of GHRH increasing and that of SS decreasing into the portal system during a GH burst and the inverse occurring during a nadir[39]. At the level of the pituitary, GHRH and SS have noncompetitive antagonistic effects[40]. GHRH stimulates GH synthesis and secretion through activation of the adenylate cyclase system[41] and SS suppresses cAMP levels in the pituitary. However, numerous other factors are also involved in this process via modulation of GHRH or SS secretion or a direct action on the somatotroph.

GH itself has a negative feedback action at the hypothalamus, stimulating SS and inhibiting GHRH synthesis[42]. Somatostatin neurons in the periventricular nucleus express the GH receptor, suggesting a direct action on this neuropeptide system[43,44]. In contrast, GHRH neurons do not appear to express this receptor[44,45]. The negative feedback effect on this neuropeptide system is thought to occur, at least in part, via modulation of neuropeptide Y (NPY) neurons in the arcuate nucleus[44,45]. As NPY is intimately involved in metabolic control, this is one possible vinculum between the control of growth and metabolism. Other circulating factors involved in growth and metabolism, such as IGF-I, leptin, ghrelin, free fatty acids, and insulin, also modulate GH secretion at the level of the hypothalamus.

2.3 The GH receptor

The growth hormone receptor (GHR) is a 620-amino-acid single-transmembrane glycoprotein that belongs to the class I cytokine receptor superfamily (M 130 000) [46]. This family includes receptors for prolactin, erythropoietin, thrombopoietin, leptin, ciliary neurotrophic factor, leukemia inhibitory factor, granulocyte-colony stimulating factor, granulocyte macrophage-colony stimulating factor, and several interleukins (ILs) [47]. The class I cytokine receptors span the membrane once and contain an

extracellular region, a single hydrophobic transmembrane domain of 24 amino acids and an intracellular region. In their extracellular ligand binding domain, four conserved cysteine residues are found in the N-terminal portion and a conserved WSXWS (Trp-Ser-X-Trp-Ser) motif is present in the C-terminal and predicted to function as a ligand interaction site. In the GHR, this motif is altered to YGEFS. It does not contact the ligand, but upon mutation of the last serine both binding affinity and trafficking of the receptor are impaired[48]. Because this motif does not make direct contact with the ligand, the YGEFS motif is likely to serve a structural role critical for ligand binding[49]. In the intracellular domains of cytokine receptors, no consensus catalytic site has been identified and limited amino acid homology has been recognized in only two motifs: box-1 and box-2. These motifs are located in the cytoplasmic membrane-proximal region of several members of the cytokine family and are known to be involved in signal transduction[50]. Box-1, a proline-rich motif present in all members, consists of a P-X-P sequence and a preceding cluster of hydrophobic amino acids. The other motif, box-2, is present in most of these receptors and is only 50% conserved in the members of the receptor family. It is characterized as a cluster of hydrophobic amino acids, is followed by negatively charged residues, and ends with one or two basic amino acids.

The GHR was initially cloned from rabbit and human cDNA libraries and subsequently from other species and has an amino acid sequence homology of 70% between the different species[51]. Expression of GHR mRNA is detectable in all tissues studied to date with liver, fat, muscle and kidney showing the highest levels of expression[3].

The GH receptor, which forms homodimers when ligand is bound, does not have tyrosine kinase activity, but is closely associated with protein kinases, in particular Janus kinase 2 (JAK2). When the GHR is bound by its ligand, JAK2 is autophosphorylated and then the receptor is phosphorylated[52]. This then triggers specific intracellular signaling cascades including mitogen-activated protein kinase (MAPK), the phosphatidyl-inositol-3´-kinase (PI3-kinase)-Akt, and latent transcription factors known as signal transducers and activators of transcription (STAT).

In addition to the membrane-bound form of GHR, a soluble circulating form of the receptor, named growth hormone binding protein (GHBP), has been characterized. The mechanism by which GHBPs are generated varies among species. In some species, including human, the GHBP is generated by proteolytic cleavage at the cell surface (shedding) of the membrane-bound form of the receptor by a recently identified metalloprotease called tumor necrosis factor (TNF)-α converting enzyme (TACE or ADAM-17) [53]. However, in rodents, the high-affinity GHBP and GH receptor are coded by

the same gene and produced by alternate RNA splicing to generate a truncated form[54].

Diverse biological functions have been attributed to GHBP. In plasma, complex formation of GHBP with GH creates a circulating reservoir, protects GH from degradation and excretion, prolongs its half-life, and may enhance its bioactivity *in vivo* through these mechanisms. The high-affinity, low-capacity GHBP has a molecular mass of 61 kDa and preferentially binds the 22-kDa form of GH. This protein is structurally identical to the extracellular portion of the GH receptor. The low-affinity, high-capacity GHBP has a molecular mass of 100 kDa and preferentially binds to the 20-kDa form of GH. Some authors suggest that serum GHBP measurement is a useful tool for measuring GHR abundance in the body[55].

Studies on the crystallographic structure of GH-GHR complexes revealed a trimeric complex of two receptors and a single molecule of hormone[49]. Recent studies suggest that the unliganded GHR may exist as a preformed dimer that undergoes a GH-induced conformational change to achieve activation[56]. Further analysis of the complex showed two binding sites on hGH on opposite sides with slightly different binding affinities[57]. The ligand binding domain and dimerization interface at the extracellular part of the GHR lie on its cysteine pairs and YGEFS motif. The binding of GH to the two receptors is considered the first step in the action of GH. This process is initiated with GH binding to one molecule of receptor through its site 1, a functional epitope of 11 amino acids, followed by association of this complex to a second receptor molecule through GH's site 2, a 31-amino-acid region that stabilizes and, together with a 500 Å contact region between the two extracellular domains of GHR, defines the binding of the second GHR[58]. Receptor dimerization is crucial for signal transduction since the hGH antagonist, G120R, capable of binding to the first GHR via its site 1, but defective at site 2 for inducing dimerization, cannot transduce signals. However, this antagonist is internalized as efficiently as the wild-type ligand[59,60].

Although the GHR and other cytokine receptors do not possess intrinsic tyrosine kinase activity, GH binding to its receptor results in rapid tyrosine phosphorylation of multiple cellular proteins because of the activation of GHR-associated tyrosine kinases of the JAK family. The ability to associate with and activate tyrosine kinases is essential for propagation of most intracellular signals. Several intracellular pathways are activated in response to GH (Fig. 1). These include the activation of STAT1, STAT3, STAT5, MAPKs, and PI3'-kinase[61,62]. Activation of JAK2 kinase activity is believed to be critical for the initiation of most, if not all, of these signaling pathways. Yet the mechanism(s) by which GH binding leads to JAK2 activation is as yet uncertain.

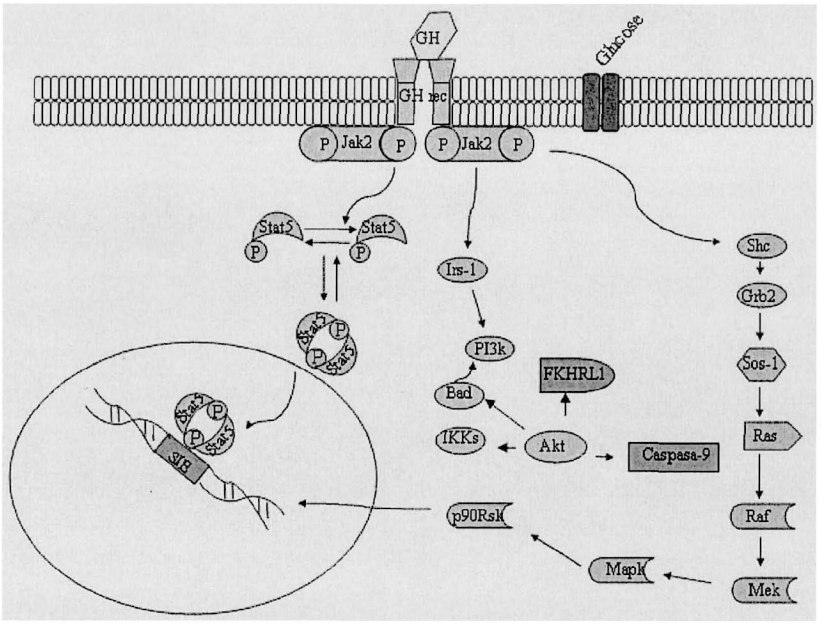

Figure 1. Intracellular signaling cascade for the growth hormone receptor.

The JAK kinases represent a distinct family of soluble tyrosine kinases that have been strongly implicated in signal transduction of many members of the cytokine family[63]. Among the known members of the JAK family, JAK2 is the main kinase activated by the GH-GHR complex and this activation is considered to be the initiating step in GHR signal transduction[64]. JAK2 binds to the GHR via box-1[65]. No specific amino acid within box-1 appears to be essential for the association between the receptor and JAK2. Mutation of each individual proline residue in box-1 or the simultaneous mutation of the first two proline residues does not impair association of the kinase. However, a specific secondary structure of the receptor is required as simultaneous mutation of the last two proline residues or of the three hydrophobic residues (isoleucine; leucine and valine into threonine) abolishes the capacity of the receptor to interact with and to activate JAK2[66]. The box-1 sequence of the GHR is very similar to proline-rich SH3 domains. However, no SH3 binding domain has been identified in JAK2 and the existence of an SH3-containing adapter protein could mediate the association. The first 46 residues of the GHR cytosolic tail, which

contain the box-1, are sufficient to induce some JAK2 activation, but maximal activity of this kinase requires downstream residues in the proximal transmembrane part of the GHR cytoplasmic domain. In resting cells, JAK kinases are thought to be associated with the receptor cytoplasmic domains, but catalytically inactive. Upon ligand stimulation, the GHR becomes firmly dimerized, initiating enzyme activity in two JAK2 molecules, which trans-phosphorylate each other on one or more tyrosine residues within the kinase domain of the paired JAK2.

Upon GH binding to its receptor, activation of JAK2 is rapid and transient, reaching maximal activity at 5 to 20 min, and returns to basal level by 60 min. Once activated, JAK2 phosphorylates the GHR on multiple tyrosine residues providing docking sites for other signaling molecules[67]. Some of the effects of GH are mediated directly through JAK2, such as stimulation of cell proliferation[65]. JAK2 contains 48 tyrosine residues, all of which may become phosphorylated upon GH binding, suggesting that this kinase can interact with multiple signaling molecules[68]. Among the direct substrates of JAK2 are several docking proteins involved in the Ras/MAPK pathway, the STAT proteins (signal transducers and activators of transcription), as well as the insulin receptor substrate proteins IRS-1 and IRS-2, which initiate the PI-3' kinase pathway[69]. To date, only GH-dependent calcium transport appears to involve a pathway that is independent of JAK2 activation[70]. Hence, regulation of JAK2 phosphorylation could regulate GH actions. Indeed, protein tyrosine phosphatase-1B (PTP-1B) has been shown to regulate GH signaling by reducing JAK2 phosphorylation, and this may be an important mechanism of limiting GH's actions during metabolic stress[71]. Other GH effects, such as GH-dependent expression of the insulin gene or the serine protease inhibitor (*Spi*) *2.1* gene require additional sequences in the GHR cytoplasmic domain[72,73]. In a more distal C-terminal portion of the GHR cytoplasmic domain, phosphorylation of Y487, Y534, Y566, or Y627 is required for tyrosine phosphorylation of STAT5, while phosphorylation of Y333 and/or Y338 GHR appears to be required for GH-stimulated lipid and protein synthesis[74,75].

2.4 GH Actions

The most well studied actions of GH are those on longitudinal growth and metabolism, although this hormone has more recently been shown to have neuromodulatory actions affecting neuronal survival and processes such as memory and cognition, which may or may not involve stimulation of

IGF-I production[76,77]. The growth and metabolic effects of GH occur in multiple tissues, either directly or via IGF-I. These actions include stimulation of protein synthesis and carbohydrate and lipid metabolism; hence, it could be classified as an anabolic, lipolytic, and diabetogenic hormone.

GH has both insulin-like and anti-insulin-like effects on cells and tissues. The insulin-like actions include stimulation of transient increases in glucose and amino acid transport, lipogenesis, and protein synthesis. Hence, GH and insulin may activate some common signaling pathways. Indeed, GH stimulates phosphorylation of the insulin receptor substrates 1, 2, and 3 (IRS-1, -2 and -3). Tyrosine phosphorylation of IRS proteins by JAK2 provides a binding site for the SH2 domain of the 85-kDa regulatory subunit of PI-3' kinase[78]. Although PI-3' kinase is required for insulin stimulated glucose transport, GH-induced glucose transport has been reported to not require PI-3' kinase[79]. However, inhibition of PI-3' kinase blocks GH-stimulated lipid synthesis[80] and the anti-lipolytic action of GH[81], suggesting that PI-3' kinase activity is important for the insulin-like actions of GH.

The reduction of body fat induced by GH involves both decreased lipogenesis and increased lipolysis. The signaling mechanisms by which GH regulates these processes are an active area of investigation. GH appears to inhibit adipocyte differentiation at a step preceding the induction of genes required for terminal differentiation, such as the gene encoding peroxisome proliferator activated receptor γ (PPARγ)[82]. One signaling event that may be important in this process is GH activation of the cAMP-specific phosphodiesterase PDE4A5 by a PI-3' kinase-dependent mechanism[83].

The signaling pathways involved in GH stimulation of protein synthesis remain largely unknown. Specific tyrosines in the cytoplasmic domain of the GH receptor are required for GH stimulation of protein synthesis, suggesting that recruitment of molecules to the GH receptor is involved[74]. GH is known to stimulate the activity of $p70^{S6k}$, an enzyme thought to regulate translational activity[83].

Chronically elevated GH is "anti-insulin-like," promoting insulin resistance and possibly type 2 diabetes. GH is thought to have this effect, at least in part, by interfering with the ability of insulin to stimulate carbohydrate metabolism. Potential underlying mechanisms for this action of GH are emerging. Decreased insulin receptor, IRS-1 and IRS-2, tyrosyl phosphorylation in response to insulin has been reported in rodent models of chronic GH excess[84]. In contrast, GH excess can lead to chronic activation of the IRS-PI-3' kinase pathway in liver, reducing the degree of insulin induced activation[85]. In addition, GH inhibits the expression of the gene encoding glucose transporter 1 (*GLUT1*), also potentially modulating glucose metabolism[86].

For many years it was believed that GH had no role in controlling fetal growth, because expression of the GH receptor is relatively low in fetal tissue. However, congenital GH deficiency can be associated with a reduction in birth length[87]. Furthermore, the GH-IGF-I axis is functional and responds normally to stimuli, such as undernutrition[88]. However, fetal growth is dependent mainly on the IGFs as discussed below.

3. IGF PHYSIOLOGY

3.1 The IGFs

The existence of the IGFs was first proposed in 1957 on the basis of studies indicating that GH did not directly stimulate the incorporation of sulfate into cartilage, but rather acted through a serum factor[89]. This factor was originally termed sulfation factor, then somatomedin and ultimately, insulin-like growth factor-I and II. IGF-I was not purified and characterized until more than two decades later[90]. The terminology "insulin-like" was used because these factors are able to stimulate glucose uptake into fat cells and muscle, and indeed, both IGF-I and IGF-II show approximately 50% homology with insulin[90,91].

Subsequent investigation demonstrated that GH, after binding to its transmembrane receptor, initiates a signaling cascade leading to transcriptional regulation of IGF-I and related genes. It was originally thought that systemic growth was promoted by GH acting mainly on the liver to stimulate IGF-I production, which then reached target tissues via the circulation to activate mechanisms involved in tissue proliferation, growth, and metabolism. It is now evident that not only does GH have independent actions that do not involve IGF-I production[92], but IGF-I synthesis occurs in many tissues under the control of a variety of local and circulating factors, which may or may not include GH[93-96]. Furthermore, this local production of IGF-I may be directly responsible for the growth promoting effects of GH, rather than the circulating growth factor[97].

The initial concept that IGF-I exerts endocrine actions was well-founded. It was identified and purified from blood, and the best data at the time indicated that the liver was the major, if not sole, site of synthesis. In the early 1980s it was observed that explants from multiple fetal mouse tissues released immunoreactive IGF-I into the cultured media and that a large portion of this material was recognized by cell surface receptors specific for IGF-I[93]. It was later shown that multiple human fetal tissues synthesize IGF-I, including intestine, muscle, kidney, placenta, stomach,

heart, skin, pancreas, hypothalamus, brain stem, spleen, and adrenal.[95] Later it was demonstrated that multiple adult rat tissues, including kidney, liver, lung, heart, and testes had IGF-I concentrations much higher than could be explained by the contribution from the blood and that IGF-I levels in many tissues were regulated by GH[98]. Hence, it was postulated that actions of IGF-I were predominately local, being exerted either on the cells of origin (autocrine actions) or on nearby cells (paracrine actions). Northern blot analyses and *in situ* hybridization histochemistry[95,98] indisputably showed that IGF-I expression was widespread in rodent and human tissues and, to a significant extent, dependent on GH, at least postnatally. Thus, ubiquitous IGF-I expression, accompanied by similarly widespread expression of type 1 IGF receptors and IGFBPs, make cellular signaling and regulation of IGF-I availability possible in the local milieu.

GH deficient and GH receptor deficient rodents and humans have given much insight into the GH-independent actions of IGF-I. Normal, or only slightly reduced, birth weights and lengths in GH-deficient or -resistant humans and mice supports the theory of GH-independent actions of IGF-I during embryonic and fetal development[99,100]. Although, their post-natal growth rate is greatly reduced and if untreated their final stature will be well below normal, these subjects have relatively normal tissue development, indicating that GH is not essential for the differentiation of these cell types.

The IGFs are mitogenic peptides that potently affect cell growth and differentiation[101]. As mentioned above, IGF-I is widely expressed both prenatally and postnatally in many tissues, including the brain[95,96,101,102]. Various components of the IGF-I system are highly expressed in regions of the postnatal brain undergoing remodeling or plasticity such as the cerebellum, hippocampus and olfactory bulb[4,103,104]. Using *in situ* hybridization, it has been demonstrated that IGF-IR colocalizes to or near sites of IGF-I expression in the olfactory bulb, the hippocampus and the cerebellum[4,105,106]. It has therefore been suggested that autocrine or paracrine modes of action are used by the IGF system in these brain regions.

3.2 The IGF-I receptor

The IGF-I receptor (IGF-IR) is ubiquitous and contributes to the regulation of biological functions of many cell types[107,108]. Only mature B lymphocytes and hepatocytes do not express this receptor[109]. The IGF-I receptor has approximately 70% homology with the insulin receptor, with both receptors having tyrosine kinase activity. IGF-IR is initially transcribed in the cell cytoplasm as a single protein, which is processed by cellular proteases to form a heterodimer having a $\alpha_2\beta_2$ configuration. The α and β

subunits are joined together by disulfide bonds, as both subunits contain several cysteine amino acid residues. Most of the extracellular domain of the IGF-IR, hence the ligand binding domain, is constituted by the α-subunit, which is heavily glycosylated[107-109]. The β-subunit contains a hydrophobic moiety, which functions as the transmembrane domain of the receptor. As expected, the tyrosine kinase domain of IGF-IR is in the cell cytoplasm.

There are three known ligands for the IGF-IR: IGF-I, IGF-II and insulin at supraphysiological concentrations. Binding of the ligand to the α-subunit of the IGF-I receptor triggers a series of events that lead to autophosphorylation of the receptor via its tyrosine kinase activity[107-109]. This, in turn, causes the interaction with certain cellular substrates that mediate the transmission of mitogenic and antiapoptotic signals to the cell nucleus (Fig. 2). The major intracellular substrates for IGF-IR are insulin receptor substrate-1 (IRS-1), Shc, and 14-3-3[110]. On these grounds, it is clear that IGF-IR has a relevant role in promoting cell growth, size and proliferation and in protecting cells from a variety of apoptotic stimuli[107-109]. It has also been demonstrated that an over-expressed and activated IGF-IR is quasi-obligatory for the establishment of a transformed cell phenotype[111]. Interestingly, the targeting of IGF-IR can reverse the malignant cell phenotype, without significant effects on the biology of normal cells[112-115]. The possible roles of IGFs in cancer are discussed in Chapter 13.

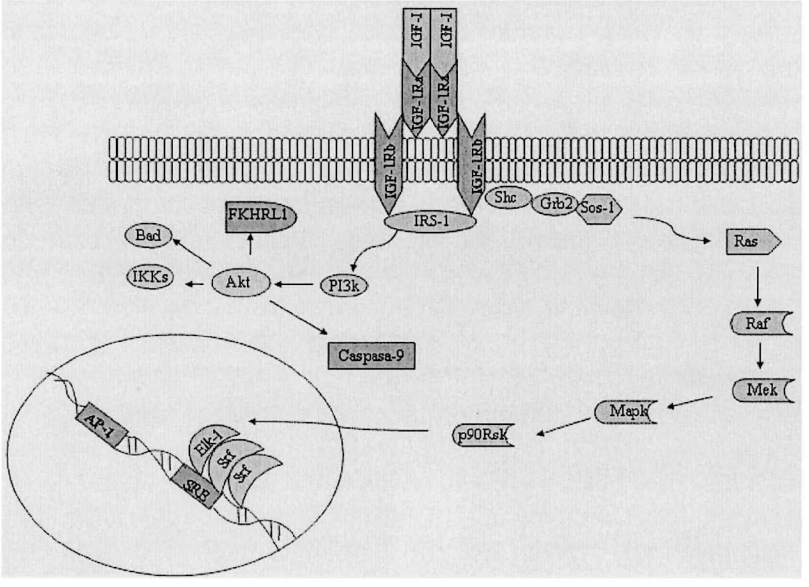

Figure 2. Intracellular signaling cascade of the IGF receptor.

A number of more recent studies have indicated that the biology of the IGF-I receptor is more complex than previously thought. In fact, in certain cellular contexts, activation of the IGF-I receptor can also promote cell differentiation in myoblasts[116,117], adipocytes[118,119], osteoblasts[120], and in cells of the CNS[121-125]. Interestingly, *in vitro* studies have shown that the IGF-IR system can induce differentiation in murine 32D hematopoietic cells along the granulocytic pathway[125]. Involvement of IGF-IR in cell differentiation appears to be somewhat of a paradox, as its main function consists in promoting cell proliferation and growth[108] as these cellular processes oppose the cell differentiation programs. However, it must be kept in consideration that the cellular context in any given paradigm also comes into play in mediating the signaling of the IGF-IR system. For example, the murine 32D hematopoietic culture system lacks IRS-I[126], which is the main intracellular mediator for mitogenic signals induced by the IGF-I receptor[127,128]. In the absence of IRS-I, the Shc-mediated signaling system leads to cell differentiation[129].

Another important property of the IGF-IR is the control of cell adhesion and motility[115,130]. The IGF-I receptor system also has a dual life in these processes depending on the cellular context[108]. There are three main intracellular substrates that interact with the activated IGF-IR: IRS-1, Shc and 14-3-3, each of which targets a distinct domain of IGF-IR and gives rise to a specific signal transduction pathway. The tyrosine kinase domain of IGF-I receptor triggers the activation of the IRS-1/PI-3´ kinase/Akt/p70^{S6k} pathway. Tyrosine 950 of the IGF-I receptor is mainly responsible for activation of the Shc/MAPK (mitogen-activated protein kinase) pathway. The third domain of the IGF-IR is composed of a serine quartet at position 1280-1283, which interacts with 14-3-3 proteins and consequently activates Raf by causing its mitochondrial translocation. All pathways lead to the neutralization of the BAD-induced apoptotic program[110,131], with the inhibition of apoptosis occurring via phosphorylation of BAD. However, just two of the three pathways can be sufficient for cell survival[130].

The tyrosine kinase domain of the IGF-IR is a necessary requirement for mitogenesis that is mainly controlled by the IRS-1/PI-3'-kinase/Akt/p70^{S6k} pathway. Activation of Shc and 14-3-3 can also contribute to some extent in delivering a mitogenic signal. The IRS-1/PI-3' kinase/Akt/p70^{S6k} pathway is also essential for the regulation of cell size increase, which is required for the predisposition of cells to mitosis[108].

The protective effect of the activated IGF-IR on cell survival has been known for some time. The variety of experimental procedures used to induce apoptosis suggests that the wild-type IGF-I receptor and/or its ligands have a widespread antiapoptotic effect on many death signals. Indeed, McCarthy

and colleagues[132] have suggested that only IGF-I and Bcl-2 truly suppress the initiation of the apoptotic program, while inhibitors of caspases can arrest the completion of the program, but have no effect on its initiation. The mechanism by which the IGF-IR protects cells from apoptosis has been the object of much investigation, culminating in a reasonable elucidation of the main pathway used by this receptor for protection against apoptotic injuries. This pathway originates with the interaction of the IGF-IR with one of its major substrates, IRS-1[133], which activates PI-3´ kinase, which in turn activates Akt/PKB. The concluding step is the phosphorylation of BAD, one of the members of the Bcl-2 family of proteins, by Akt. However, some investigators have suggested that this receptor must have alternative pathways[134]. One alternative pathway could involve MAPK, which originates, at least in part, from another major substrate of the IGF-IR, the Shc proteins[135,136]. Another IGF-IR mediated anti-apoptotic pathway is the activation of Raf-1 and its translocation to the mitochondria. All three pathways result in the maintenance of BAD phosphorylation.

3.3 The IGF Binding Proteins

Six high-affinity IGFBPs have been characterized to date. Circulating IGFBPs act as carrier proteins to transport the IGFs out of the circulation to their target tissues. The union of the IGFs to these binding proteins also prolongs their half-life by protecting them from proteolysis. A majority of circulating IGF-I is bound in a ternary complex composed of IGFBP-3, acid labile subunit (ALS), and IGF itself. More recently it has been reported that IGFBP-5 can also form a complex with IGF and ALS[137]. Most target tissues also express IGFBPs, with this expression being both tissue and developmental stage dependent. These binding proteins modulate the local actions of the IGFs at the cellular level and can be either stimulatory or inhibitory. In addition, independent effects of IGFBPs are becoming more apparent[138,139].

The serum profile of the IGFBPs differs in the fetus from the postnatal profile; IGFBP-1 and -2 levels are higher whereas IGFBP-3 levels are lower[140]. Serum IGFBP-1 is the most sensitive indicator of fetal nutrition. There is an inverse correlation between fetal IGFBP-1 concentrations and birth weight from as early as 16 weeks of gestation.

3.4 IGF Actions

The most important determinant of fetal growth in both humans and other mammals is the IGF-I system[88,101]. The IGFs are detectable in many fetal tissues from the first trimester and IGF concentrations in the fetal circulation increase during pregnancy[141]. It is therefore interesting that the most important regulator of fetal IGF-I concentrations is the availability of adequate glucose across the placenta. Glucose increases IGF-I concentrations through an increase in insulin secretion, which in turn regulates fetal IGF-I secretion[142].

Postnatal growth is also correlated with plasma IGF-I levels in humans[140,143] and IGF-I treatment increases both body weight and size, indicating a role for circulating IGF-I in body composition. Indeed, as mentioned earlier, IGF-I is the mediator of many of the growth-promoting actions of GH. This is exemplified by the fact that children with GH resistance, with high levels of GH and very low levels of circulating IGF-I, have a pathologically short stature.

IGF-I has many important physiological roles[107-127,131,135,136]. It is involved in cell division and differentiation and throughout postnatal life it functions as a tropic factor maintaining the integrity of many tissues. In addition, evidence continues to accumulate suggesting that IGF-I is also an important factor in the defense against noxious substances and degenerative processes. The role of IGF-I in the development of specific tissues and in preventing neurodegenerative diseases, as well as its possible role in cancer is address in depth in the subsequent chapters.

4. FUTURE PERSPECTIVES

Our understanding of the physiology of the GH/IGF-I axis will continue to expand as more hormones, receptors, and factors involved in this process are identified. Of course advances in genetic and molecular techniques will play a very important role in these new discoveries. Some of the most important advances will most likely involve the intracellular signaling mechanisms employed by these factors. Not only will new proteins be identified, but elucidation of how the numerous intracellular pathways interact will allow us to further understand how the same hormone or factor can cause different effects in different tissues, how different hormones can have the same effect in a given tissue, or how various factors interact at the cellular level to produce a physiologically significant outcome.

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Chapter 2

EXPERIMENTAL MODELS FOR UNDERSTANDING THE ROLE OF INSULIN-LIKE GROWTH FACTOR-I AND ITS RECEPTOR DURING DEVELOPMENT

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1. INTRODUCTION

During the last two decades, *in vivo* models have been employed extensively to elucidate key molecular mechanisms governing embryonic growth and development. Genetic manipulations of worms, flies, and mice provide powerful tools to unravel the complex program of development, and thus have revolutionized the study of factors and signaling pathways that regulate cellular differentiation, proliferation, and survival during embryogenesis and postnatal growth. The pioneering deletions of insulin-like growth factor (IGF)-I, IGF-II and/or the IGF-I receptor in mice have demonstrated definitively that this growth factor system provides essential signals for the regulation of embryonic and postnatal development in vertebrate species[1,2]. More recently, genetic ablations of signaling molecules have reinforced the role of IGF-I in development, whereas the advent of conditional gene targeting has challenged historical dogma regarding the

importance of circulating IGF-I for postnatal growth. Given the critical contributions of animal models to our current knowledge of IGF-I function, this chapter reviews the phenotypes of various mice bearing genetic modifications of the IGF system. Interestingly, the use of divergent model systems has revealed that many aspects of development are evolutionarily conserved, particularly those events regulated by signals from the insulin/IGF-I receptor. Therefore, this chapter also includes relevant findings from *Caenorhabditis elegans* and *Drosophila*, with the aim of illustrating how model organisms represent indispensable scientific tools for understanding developmental pathways and ultimately, for obtaining insights into the molecular mechanisms of human disease such as cancer.

2. THE TECHNOLOGY AND TERMINOLOGY OF CONTEMPORARY EXPERIMENTAL MODELS

Model systems have been used since the era of Mendel to understand basic, conserved biological processes. However, prior to the development of gene targeting, analysis of the inactivation or modification of a gene in mammals was limited to rare, spontaneous mutations that produced obvious phenotypes such as an inheritable human disease. Thus, the ability to generate genetically modified mice represents one of the major milestones of modern science as it has empowered researchers with very precise tools to determine the physiological function(s) of a gene product. Recognizing that techniques within this field progress rapidly, we will discuss superficially the more common methods for genetic manipulation of model systems.

2.1 Conventional knockouts

Deletion or knockout of a gene is the definitive way to abolish its expression. Knockouts in mice have been typically accomplished by deleting or replacing one or more crucial exons with a neomycin resistance gene using *in vitro* recombination in embryonic stem (ES) cells, followed by selection for the recombined (knockout) cells[3]. These cells are then injected into host blastocysts to produce chimeric embryos. Chimeric adults in which the knockout cells have "gone germline" are then bred in stages to obtain homozygous knockout mice. Although the success of this technique was somewhat tentative with the onset of its use in the late 1980s, the establishment of core facilities and companies dedicated solely to the production of knockout mice has perfected the technology, and thus these methods are now considered routine. However, the basic knockout technique still represents a major investment of time and money. In addition, some

gene ablations are incompatible with development or postnatal survival. The later complication is illustrated by the conventional knockouts of IGF-I and its receptor; IGF-I null mice display extremely low survival rates during the early postnatal period and *IGF-IR*$^{-/-}$ animals die at birth as a result of respiratory problems[1,2].

2.2 Conditional knockouts

As mentioned previously, conventional germline mutations can be lethal, in which case there is no mouse for the study of gene function. This biological reality prompted the development of methods for conditional gene targeting including tissue-specific and/or inducible deletion of a gene[4,5]. In addition, if a gene exerts its function at several stages of development and/or in multiple cell types, a whole-body conventional knockout may produce various phenotypes, making it difficult to discern the authentic physiological role(s) of the gene product. Here again conditional knockouts offer advantages because the deletion can be restricted to a particular point of development or to a cell-type of interest.

The most typical strategy for production of conditional knockouts exploits the bacteriophage-derived *Cre-loxP* recombination system[4]. Cre recombinase recognizes a sequence motif of 34 bp, known as loxP sites[6]. Mice with loxP-flanked (floxed) target genes are generated in several steps[7]. Conditional targeting of endogenous genes consists of flanking the gene sequence with loxP sites in ES cells via classical homologous recombination and subsequently deleting the neomycin resistance marker by transient transfection with Cre-encoding plasmid. This approach yields ES cell mutants in which the gene of interest is either flanked by loxP sites or deleted[6]. Either mutation can be transmitted into the germline. The conditional deletion of loxP-flanked genes is finally achieved by crossing the mutant line with a *Cre*-transgene in which the Cre enzyme is expressed under the control of cell-type specific or inducible promoter. (Collections of Cre-mice are maintained by various sources and include the expansive list of Andras Nagy at *www.mshri.on.ca/nagy*.) Liver-specific deletion of the IGF-I gene has been accomplished by crossing floxed IGF-I mice with transgenics expressing Cre via the albumin promoter which is highly active in liver[8,9]. Note that Cre-based conditional targeting can also be used for the introduction of subtle gene mutations for example, knockins of gain of function or loss of function mutants.

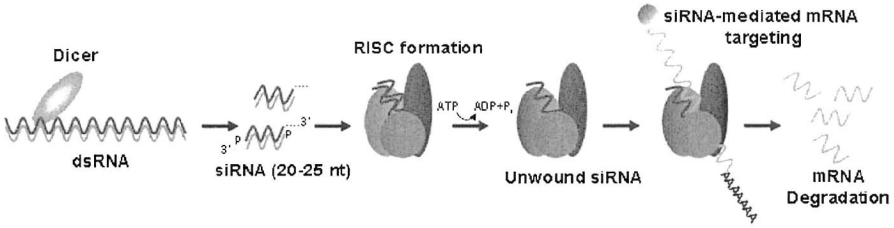

Figure 1. The RNA interference pathway. In the initiation step, double-stranded DNA (dsDNA) is cleaved by the RNAse-III-like enzyme Dicer into small interference RNAs (siRNA). These siRNAs contain characteristic 5' phosphorylated ends and 3' overhangs of 2 nt. Next, the siRNAs are incorporated into the RNA-induced silencing complex (RISC). Unwinding of the siRNA duplex requires ATP. Once unwound, the single-stranded antisense directs RISC to mRNA bearing a complementary sequence. As a consequence of this base-pairing, the mRNA is degraded, effectively silencing the target gene.

2.3 RNA interference/knockdowns

RNA interference (RNAi), also termed RNA silencing or post-transcriptional gene silencing, is a biological phenomenon by which small, double-stranded RNA (dsRNA) molecules induce sequence-specific degradation of homologous single-stranded RNA[11,12]. From a practical perspective, RNAi can be exploited to target gene expression and has already proven itself as a very powerful technique to knock down specific genes for the assessment of their physiological roles. Interest in gene silencing expanded when it was discovered that the introduction of dsRNA into *Caenorhabditis elegans* silences gene expression in a homology-dependent manner much more efficiently than does either sense or antisense RNA[13]. The technique is rapid and can inhibit the expression of most genes by more than 90%. Illustrating these advantages of RNAi technology, two groups recently reported the screen of *C. elegans* chromosomes I and III (a total of a third of the genome) for RNAi phenotypes[14,15]. Using RNAi in developing *Drosophila* embryos, Kim *et al* recently analyzed more than 5,800 genes (40% of all *Drosophila* genes) to identify transcription factors and cell signaling proteins required for heart development[16]. Thus, not surprisingly, RNAi is being proclaimed as a major scientific advance of the 2000s.

RNAi occurs in several steps that can be induced through transfection or microinjection of long dsRNA (Fig. 1). First, the dsRNA is cleaved into 19- to 23-nt RNA fragments known as small interfering RNAs (siRNA) by an evolutionarily conserved ribonuclease III-like protein Dicer[17]. siRNAs act as intermediates in the RNAi pathway, which is thought to protect cells from harmful transposons and highly repetitive sequences by targeting their RNA

transcripts for endonucleolytic cleavage and subsequent exonucleolytic degradation[18]. SiRNA is incorporated into the RNA-induced gene complex (RISC) that targets and cleaves mRNA complementary to the siRNA[12]. The siRNA must be 5' phosphorylated in order to enter the RISC. The duplex siRNA is then unwound, allowing the antisense strand to direct the RISC to homologous target for cleavage by endonucleases. Cleavage occurs at a single site in the center of the duplex region, 10 nt from the 5' end of the siRNA.

Until very recently, only researchers working with plants or *C. elegans* could utilize RNAi technology to obtain insight into gene function. This limitation was largely attributable to the fact that only RNA molecules <30 bp can be used to induce RNAi; longer nucleotide sequences in vertebrate cells induce a nonspecific, type I interferon response that triggers mRNA degradation[19]. However, the groundbreaking discovery that chemically synthesized, 21- to 23-nucleotide, double-stranded RNAs can function as siRNAs to silence genes in mammalian cells makes it possible now to contemplate RNAi in mammalian systems[20].

Other characteristics of RNAi that have delayed its application to the manipulation of vertebrate genes are amplification activity and the duration of gene silencing. In plants and nematodes, RNAi activity is long term and disseminates throughout the organism via an unknown amplification mechanism[21]. However, in mammalian cells, amplification activity seems absent, and interference activity is transient, lasting for only 3 to 5 days[22]. To circumvent these problems with RNAi technology in mammalian systems, DNA expression vectors have been developed to express hairpin or duplex siRNA, which employ the type III class of RNA polymerase promoters to drive the expression of siRNA molecules[12]. The majority of these systems take advantage of the ability of Dicer to process short hairpin RNAs into siRNAs and thereby silence the target gene. These technologies make it now possible to perform gene-silencing experiments in various mammalian cell types and cell lines, as well as to create transgenic animals that stably "knock down" a target gene.

3. MOUSE MODELS OF IGF-I DEFICIENCY

The insulin/IGF-I family of growth factors and their receptors regulates major aspects of mammalian development including systems of growth, metabolism, and reproduction[23]. At present, there are nine genes that encode insulin-like ligands in vertebrates: the two non-allelic insulin genes *ins1* and *ins2*, *igf1*, *igf2*, *relaxin*, and four insulin-like molecules of undetermined function *ins3,4,5* and *6*[24-26]. Mutant models of IGF action have provided

unique opportunities for *in vivo* studies of the growth-promoting actions of IGF-I in the developing mouse. Moreover, by targeting the genes encoding individual ligands of the IGF family and/or their receptors, knockout strategies have enabled researchers to distinguish the developmental function of IGF-I from those of IGF-II and insulin.

3.1 Null mutants of *igf1*

Homozygous disruption of the *igf1* gene (*IGF-I*$^{-/-}$ or IGF-I KO) produces marked intrauterine and postnatal growth retardation, and, depending upon genetic background, reduced postnatal survival[1,2,27]. At birth, IGF-I-deficient mice are smaller in size by 40% in comparison to their wild-type littermates but by 2 months of age, the size of the surviving IGF-I KO is only 30% of normal owing to the role of IGF-I during the postnatal period (Fig. 2). However, the hemizygous disruption of only a single IGF-I gene (*IGF-I*$^{+/-}$) has little impact on embryonic development and postnatal growth. Knockouts of IGF-I display generalized organ hypoplasia with developmental defects in bone, muscle, and reproductive systems. IGF-I deficiency also produces a central nervous system phenotype that includes reduced brain size, hypomyelination, reduced axonal diameters, and loss of selective neuronal populations[28-30]. A more detailed description of these phenotypes can be found in subsequent chapters which are dedicated to the discussion of specific organ systems.

Ablation of the *igf2* gene causes impairments of *in utero* growth (Fig. 2); similar to IGF-I-deficient mice, *IGF-II*$^{-/-}$ animals are 60% of normal size at birth[2,31]. However, in contrast to the effects of disrupting *igf1*, postnatal growth of IGF-II knockouts is normal, consistent with the fact that this factor in mice is expressed only during embryogenesis and has little if any influence on postnatal development[32]. Thus, the conventional whole-body deletions of *igf1* and *igf2* have been instrumental in distinguishing the roles of these growth factors in prenatal and postnatal mouse development. These original models confirmed that the development of most, if not all, tissues and organs is regulated to some degree by IGF-I.

Interestingly, the null *igf1* phenotype in mice is remarkably similar to that observed in a single individual carrying a homozygous partial deletion of the *IGF-I* gene[33]. This patient presented with severely impaired pre- and postnatal growth, microcephaly, mental retardation, insulin resistance, and sensorineural deafness. These defects are a direct consequence of the inability to produce IGF-I either locally or systemically. Treatment of this 17 year-old patient with recombinant human IGF-I improved linear growth and insulin sensitivity by restoring IGF-I levels and by normalizing the levels of circulating GH and insulin[34].

3.2 Conditional knockouts of IGF-I: Assessing local vs. systemic hormonal effects

Postnatal growth and development are coordinated by genetic and environmental influences and numerous growth factors. LeRoith and colleagues have reviewed extensively the critical role of the GH/IGF-I axis in these processes[35-37]. The basic assumption of the somatomedin hypothesis is that circulating IGF-I is produced by the liver in response to GH and mediates the effects of GH in peripheral tissues. Although the GH/IGF-I axis is a closely coordinated system, the biological distinctions between these factors have become apparent only in recent years through the characterization of clinical syndromes and the genetic manipulation of mice which has enabled investigators to re-examine hypotheses regarding the GH/IGF-I axis. Mice lacking both IGF-I and the receptor for GH have demonstrated that these two factors act independently and synergistically; IGF-I promotes both pre- and postnatal growth while GH is required only during postnatal development[38]. Moreover, the fact that IGF-I is produced locally by many tissues has suggested the importance of autocrine/paracrine actions of this growth factor.

The low survival rate of *igf1* null neonates has greatly limited their use in analyzing the function of circulating IGF-I during the postnatal period and in adult animals[2]. Thus, to assess the importance of endocrine vs. autocrine/paracrine IGF-1 in development and growth, conditional knockouts of this hormone in mice have been generated. Using a floxed exon of *igf-1* and transgenics expressing Cre under control of the albumin promoter, Yarkar *et al.* accomplished liver-specific deletion of *igf1*[8]. Another liver-specific ablation of *igf1* was produced via an inducible interferon promoter to drive the tissue-specific expression of Cre[39]. In both liver-deficient (LID) models, circulating levels of IGF-I were reduced by 75%. However, the animals were comparable in body weight, body length, and femoral length to wild-type littermates (Fig. 2). Therefore, these models demonstrate that hepatic IGF-I as a source of circulating IGF-I is not as crucial as previously believed for normal postnatal growth, indicating that kidney, spleen, fat, muscle. and bone may supply sufficient systemic IGF-I to promote normal development. Furthermore, these results suggest that the local production of IGF-I is critical for postnatal growth and that the growth retardation observed in the whole-body *igf1* knockout reflects the absence of autocrine/paracrine actions of IGF-I. Thus, the function of circulating IGF-I in adult animals may be compensated for by other hormones and/or by locally produced IGF-I.

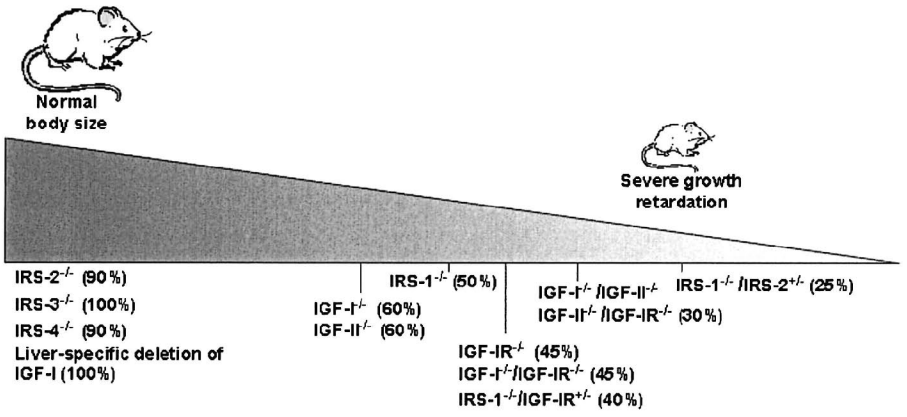

Figure 2. Alterations in mouse growth resulting from various genetic manipulations of the insulin/IGF-I system in mice. Growth impairment is expressed as the percentage of wild-type control body weight.

However, the significant reduction in circulating IGF-I levels is not totally without physiological consequence as defects have been noted in discrete brain regions of LID mice. Peripheral IGF-I is known to regulate adult dentate neurogenesis in rodents[40,41]. Interestingly, LID knockout mice display a 40% reduction in granule neuron density within the dentate gyrus of hippocampus compared with wild-type littermates. However, no differences in the number of CA3 or cerebral cortex neurons were noted in LID mice. In addition, neurogenesis within the granule cell layer of the dentate gyrus is reduced 33% in LID mice. These IGF-I-deficient animals also display impaired spatial learning and synaptic plasticity. The reduced neurogenesis in adult hippocampus correlates with the impairment of spatial learning, as assessed by the Morris water maze test[42].

Recent evidence from experiments in IGF-I mutant mice indicates that IGF-I has an important role in recovery from neural injury and neurodegenerative diseases[43], demonstrating that serum IGF-I is required for neuroprotection[42,44,45]. IGF-I levels decline with age and recently this hormone has been implicated in the pathogenesis of Alzheimer's disease (AD). Interestingly, brains of the LID transgenic mice contain a premature increase in levels of Aβ; this abnormality is present at 3 months of age and becomes more severe with aging[46]. These studies demonstrate that low levels of IGF-I are linked to premature brain amyloidosis and low brain levels of transthyretin and albumin.

These findings in LID mice suggest that although liver-derived IGF-I is not crucial for normal growth and development during pre- and postnatal stages, circulating IGF-I may be required to maintain normal brain function

in adults and to avoid premature aging and/or pathologic processes such as the deposition of amyloid plaques. Serum IGF-I levels may reflect a mechanism to coordinate peripheral metabolism with central processes such as the control of adult neurogenesis. Thus, IGF-I represents a potential therapeutic target in the treatment and prevention of neurodegenerative diseases and supplies a model for understanding the pathogenesis of some disorders, such as depression which have recently been associated with changes in IGF-I levels[47].

3.3 Classical IGF-I receptor knockout mice

The receptors for insulin (IR), IGF-I (IGF-IR), and the orphan insulin-like receptor (IRR) are plasma membrane proteins that belong to the family of ligand-activated tyrosine kinases[25,48,49]. However, unlike other receptor tyrosine kinases, the IR and IGF-IR exist as homodimers composed of two identical α/β monomers or as heterodimers consisting of two different receptor monomers (*e.g.*, $IR_{\alpha\beta}$/IGF-$IR_{\alpha\beta}$). Ligand binding induces a conformational change that enables the intracellular domain of these receptors to bind ATP and undergo autophosphorylation. Once activated, the intrinsic tyrosine kinase activity of the IR and IGF-IR phosphorylates intracellular substrates[50,51]. In contrast to IR and IGF-IR, the receptor for IGF-II (also known as the mannose-6-phosphate receptor) is not a tyrosine kinase but rather consists of a large extracellular domain with 15 repeats and a small domain with homology to fibronectin[52,53]. Insulin and IGF-I bind to their own receptors with high-affinity, but at high hormone concentrations, may bind with low affinity to the cognate receptors. IGF-II, however, exhibits high affinity binding to both IR and IGF-IR, and thus may activate signaling cascades via either receptor. The receptor for IGF-II has no signaling capacity; the binding of IGF-II to its receptor serves to clear the hormone from circulation[25].

To test the role of IGF-IR in development and postnatal growth, the laboratory of Efstratiadis generated the classical knockouts of *igf1r*[1]. Growth retardation was observed in IGF-IR-deficient mice as early as E10.5 and at birth these animals were 45% to 55 % smaller than normal. However, the *IGF-IR*$^{-/-}$ mice die at birth as a result of irreversible respiratory failure. Interestingly, double knockouts of *igf1* and *igf1r* display the same degree of growth retardation as the simple *igf1* null mice[1,2], demonstrating that all physiological effects of IGF-I are mediated by its own receptor. Moreover, *IGF-IR*$^{-/-}$ mice exhibit more profound intrauterine growth retardation than knockouts of either IGF-I or IGF-II, suggesting that these factors via activation of the IGF-IR to compensate the absence of one another during embryonic development[54].

The perinatal lethality caused by IGF-IR deficiency has restricted detailed analysis of this model to embryonic development. The brains of *igf1r* null mice are small relative to controls, although this defect is not so obvious as the reduced body weight. Moreover, the mutant mice display a striking increment in neuronal density[1]. Impaired brain growth is apparent from E14 to E18, as *IGF1R*$^{-/-}$ embryos exhibit reduced volume and increased density of cells in the mantle zone of the spinal cord and brainstem.[1] Interestingly, the absence of the IGF-IR appears specifically to alter certain populations of neurons and brain regions[28], for example, the number of parvalbumin^{+} neurons of the hippocampus in *IGF-IR*$^{-/-}$ embryos compared with wild-type littermates as determined by stereological counting. These observations reveal that, in addition to its global role in brain developmental, the IGF-IR exerts additional or more specialized functions in certain neuronal populations. In sharp contrast, brain-specific deletion of the insulin receptor via the use of nestin-*Cre* mice has no effect on brain development; the size and morphology of the brain from NIRKO mice is absolutely normal, further demonstrating that brain growth and development are regulated by the IGF-I/IGF-IR pathway[55].

Recently, another line of whole-body IGF-IR knockouts has been generated by deleting exon 3 of *igf1r* using *Cre/lox* recombination[56]. This model has been used to study the role of IGF-IR in aging where the authors report that *IGF1R*$^{+/-}$ females live 33% longer than wild-type females. Confirming the earlier studies of Liu *et al.* [1], *IGF-IR*$^{+/-}$ mice are apparently normal with respect to development, energy metabolism, nutrient uptake, physical activity, fertility, and reproduction. Interestingly, female heterozygous mice display greater resistance to oxidative stress, a known determinant of ageing. These results are both interesting and promising; on the one hand, IGF-IR signaling is required for protective functions such as the survival of neurons but this recent publication suggests that reduced IGF-IR signals may increase vertebrate lifespan, analogous to effects of mutations in the insulin-like receptor system in *Caenorhabditis elegans* and *Drosophila*. However, the significance of the longer lifespan of IGF-IR heterozygous females in this study must be carefully considered since the female wild-type controls died slightly sooner than controls of similar aging studies in mice[56]. Nevertheless, these observations imply that reducing oxidative stress may represent an effective way to diminish aging in all tissues and suggest a novel role for the IGF-IR in regulation of lifespan.

3.4 Conditional gene targeting of the IGF-IR

Although the characterization of the classical knockout of the *igf1r* suggested that embryonic development of brain, skin, muscle, and lung are more dependent on IGF-I than other organs[1], the perinatal lethality associated with this model has hampered extensions of these studies to postnatal development. As discussed earlier, this is frequently a problem that accompanies the production of whole-body knockouts. Moreover, compensatory mechanisms and/or selective feedback mechanisms specifically related to the pleiotropic effects of the insulin/IGF-I system may complicate analysis of the phenotype produced by gene targeting. One way to circumvent these problems is to create conditional knockouts. Recently, several types of conditional IGF-IR receptor knockouts have been produced to address various biological questions.

Holzenberger and colleagues devised a very clever Cre/*lox*-based strategy to achieve gene dosage of the *igf1r* in mice[58]. By crossing a line of *Cre* transgenics that produce a pattern of mosaic-early embryonic-ubiquitous gene deletion *(MeuCre)* with mice bearing the floxed IGF-IR allele, conditional mutants expressing various levels (5% to 82% of normal) of the IGF-I receptor were obtained. Animals were divided into groups of either strong (>50%, XS mice) or moderate (<50%, M mice) IGF-IR deficiency. Growth impairment in XS mice was noted by 3 weeks of age and persisted throughout the lifespan of the animals as compared to the relatively normal growth of M control littermates. The defect in postnatal growth was more pronounced in males than in females; at 9 weeks of age XS males weighed about 30% less than wild-type controls whereas XS females displayed a 20% reduction in body weight. By 10 to12 months of age, most organs and tissues displayed specific weight defects, with skin, bone and connective tissue, muscle, spleen, heart, lung, and brain being the most susceptible to the reduction in IGF-IR expression.

The generation of tissue-specific knockouts of IGF-IR has served to confirm requirements for the local actions of IGF-I in some cases, while in others the phenotype resulting from the cell-type specific ablation of *igf1r* appears at odds with findings from the whole-body deletion of this receptor. Mice harboring the osteoblast-specific deletion of IGF-IR display normal size and weight but display a striking decrease in cancellous bone volume, connectivity, and trabecular number, and an increase in trabecular spacing[59]. These bone abnormalities correlate with a marked decrease in the rate of mineralization of osteoid, confirming that IGF-I is essential for coupling matrix biosynthesis to sustained mineralization. However, ablation of IGF-IR specifically in pancreatic beta cells has little or no effect on the growth and development of insulin-producing cells but reduces expression of Glut2

and glucokinase leading to defective glucose-stimulated insulin secretion and impaired glucose tolerance[60]. These observations contrast findings from the classical IGF-IR knockout in which a reduction in beta cell number and morphological abnormalities have been reported[57]. These discrepancies between the effects of totally deleting the IGF-IR or of specifically targeting the deletion to β-cells may reflect the fact that (1) the IGF-IR may form a part of some systemic coordination during the course of embryonic development or (2) tissue-specific deletion may engage compensatory mechanisms.

4. GENETIC ABLATION OF IRS SIGNALING PROTEINS

Although a better understanding of insulin/insulin-like growth factor (IGF) action has evolved with the use of animal models, the discovery of insulin receptor substrate (IRS) proteins and their capacity to link cell surface receptors to the intracellular signaling cascades has provided an important advance in this field. The binding of insulin and IGF-I to their cell surface receptors stimulates receptor autophosphorylation and activates their intrinsic tyrosine kinase activity, which leads to the phosphorylation of intracellular substrates[49,61]. The IRS proteins were initially identified as large molecular weight, tyrosine-phosphorylated proteins that occur in cells during insulin or IGF-1 stimulation[50,62]. cDNA cloning[62-64] revealed that IRS-proteins contain multiple tyrosine phosphorylation sites that bind and activate SH2-domain containing effector proteins[65], including the regulatory subunit of the lipid kinase phosphatidylinositol-3 kinase (PI-3 kinase), Grb2, nck, and SHP2 (Fig. 3). Of these, activation of PI-3 kinase has been implicated in the action of insulin/IGF-I upon glucose transport, glycogen synthesis, protein synthesis, cell growth, and differentiation[50]. The lipid products of PI-3 kinase are necessary for the activation of Akt, which in turn regulates other downstream signaling molecules by phosphorylation[66]. The FoxO subfamily of forkhead transcription factors are targets of Akt; phosphorylation of FoxO1 by insulin/IGF-I signaling excludes this factor from the nucleus, thereby down-regulating its antiproliferative effect in cells[67-69]. Thus, the IRS protein signaling network mediates the pleiotropic effects of insulin and IGF-1 upon cellular function.

The first member of this family to be identified was IRS-1, which provided the initial example of a cytoplasmic docking protein coupling an activated receptor tyrosine kinase to various signaling proteins[62]. However, when knockouts of IRS-1 were stimulated with insulin, a large tyrosine phosphorylated protein was observed in tissue lysates, demonstrating the

existence of additional IRS proteins[63,64]. Indeed, currently there are four members of the IRS-protein family: IRS-1 and IRS-2 are ubiquitously expressed; IRS-3 is predominantly expressed in adipose tissue[70]; IRS-4 is detected mainly in kidney, pituitary and thymus[71,72]. Given the highly conserved structure of the four IRS proteins, it was considered during the initial stages of their discovery whether these molecules might represent a system of signaling redundancy. However, generation of mouse knockout models for each IRS protein clearly reveals specific physiological functions.

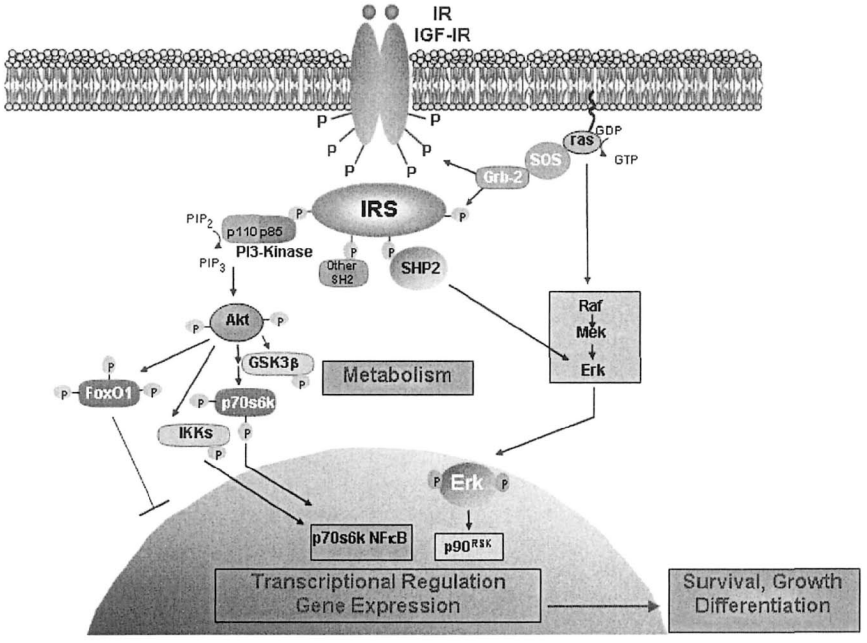

Figure 3. IRS-mediated signal transduction.

4.1 Knockouts demonstrate that IRS-1 is the major mediator of IGF-I growth signals

Deletion of IRS-1 reduces animal size by 40% to 50%, suggesting that IRS-1 mediates the IGF-I signaling required for embryonic growth and development[57,63]. By contrast, IRS-2 deficient mice are only 10% smaller than control littermates but deletion of IRS-2 produces a severe diabetic phenotype owing to peripheral insulin resistance and reduced beta cell mass

and function[73,74]. *IRS-1*$^{-/-}$ mice display mild peripheral insulin resistance; however, diabetes does not occur in this model due to hyperplasia of pancreatic beta cells[63,73]. Deletion of *IRS-3* is without effect on growth, glucose metabolism, or other physiological parameters[75]. Mice deficient for IRS-4 display very mild reduction in body growth and the females are slightly less fertile than controls[76]. Therefore, based on the analysis to date, IRS-3 and IRS-4 do not appear to play major roles in physiological insulin/IGF-I action. However, the differences in the phenotypes of *IRS-1*$^{-/-}$ and *IRS-2*$^{-/-}$ mice strongly suggest that these proteins have unique and critical physiological roles, despite the similarity of their tissue distribution.

To further distinguish the roles of IRS-1 and IRS-2 in development and growth, carbohydrate metabolism, and β-cell function, knockouts of these signaling proteins were intercrossed to generate animals with various states of IRS deficiency[57]. Growth curves were generated based on daily weights from birth to 30 days of age. Compound heterozygous mice (*IRS1*$^{+/-}$/*IRS2*$^{+/-}$) weighed 25% less than wild-type control littermates. *IRS1*$^{+/-}$/*IRS2*$^{-/-}$ mice were of similar size to *IRS1*$^{-/-}$ animals, whereas *IRS1*$^{-/-}$/*IRS2*$^{+/-}$ were 70% to 75% smaller than normal littermates. *IRS1*$^{-/-}$/*IRS2*$^{+/-}$ are amongst the smallest viable mice that have been generated by genetic manipulations. Double mutants null for both *irs1* and *irs2* were not observed in this study; however, Kubota *et al.* report the low viability of these double knockouts in lines from a different genetic background[74]. The findings of these intercrosses further implicate IRS-1 as the principal mediator of IGF-I-regulated somatic growth and suggest that although IRS-2 participates in development and postnatal growth, it cannot replace the function of IRS-1.

These *in vivo* observations are consistent with results from *in vitro* studies of studies of *IRS1*$^{-/-}$ cells. Embryonic fibroblasts derived from IRS-1 deficient mice display a 70% to 80% reduction in IGF-1-stimulated cell growth and parallel decreases in IGF-1-stimulated S-phase entry, PI-3 kinase activity, and induction of the immediate-early genes *c-fos* and *egr-1*[77]. While recombinant expression of IRS-1 in this cell system fully reconstituted the IGF-I signals required for cell growth, overexpression of IRS-2 had only slight effects on cell-cycle progression. Thus, IRS-1 and IRS-2 are not functionally interchangeable despite the high degree of structural similarity and a common tissue distribution.

4.2 IRS-2 knockouts: IGF-I signals in the development and function of neuroendocrine tissues

Given that insulin and IGF-I have a wide range of physiological effects and use common signaling pathways to accomplish these tasks, the IRS proteins add a unique layer of specificity and control to the system. This is

nicely illustrated by the neuroendocrine phenotypes produced specifically by the deletion of *irs2*. IRS-2-deficient mice develop diabetes because of defects in both insulin action and insulin production[73]. As early as 3 days postpartum male, *IRS2*$^{-/-}$ animals have elevated random blood sugars and by 6 weeks of age male animals, display hyperglycemia and markedly abnormal glucose tolerance tests. Untreated male animals die of nonketotic hyperosmolar coma between 12 and 20 weeks of age. IRS-2 knockout mice are also resistant to the peripheral actions of insulin. However, this diabetic phenotype displays a sexual dimorphism: female *IRS2*$^{-/-}$ mice are only mildly glucose intolerant and do not develop diabetes until 6 months of age[78]. Morphometric analysis of pancreatic islets from 4-week-old IRS-2 knockouts shows that they have 50% reduction in beta cell mass compared to control mice, demonstrating that IRS-2 signals are required for the normal development, growth, and/or differentiation of insulin-producing cells[73]. Indeed, more recently it was been shown the IGF-1-receptor/IRS-2 pathway is essential for normal β-cell development and compensation to insulin resistance[57]. Embryos homozygous for IGF-1-receptor-null alleles show a marked impairment of β-cell development, and animals lacking IRS-2 and heterozygous for IGF-1-receptor-null alleles display impaired β-cell development, proliferation, and survival[57]. Thus, the observation that deletion of IRS-2 impairs beta cell function suggests that IGF-I/IRS-2-dependent signaling pathways are critical for regulation of the proliferative and neogenic responses of the beta cell.

Analysis of female *irs2* null mice has revealed that these animals are infertile and have disorders of energy homeostasis that are independent of the diabetic phenotype[78]. IRS-2-null females have small, anovulatory ovaries with reduced numbers of follicles and an absence of corpora lutea. Ovaries are resistant to superovulation, suggesting intrinsic defects in ovarian function. However, plasma levels of luteinizing hormone, prolactin, and sex steroids are low, which suggests defects at the hypothalamic level as well as in the gonads and pituitary. Pituitaries of IRS-2-deficient mice are decreased in size and contain reduced numbers of gonadotrophs. Interestingly, food intake, body weight, and fat deposition are also dysregulated in IRS-2 knock-out females. Taken together, these observations suggest that IRS-2 pathways might integrate reproductive function and energy homeostasis in mammalian systems. Several lines of evidence have suggested that insulin and IGFs may function in the hypothalamo-pituitary-gonadal axis. Insulin, IGF-1 receptors, and IRS proteins are present in the hypothalamus and pituitary gland[26,79]. Furthermore, as discussed more extensively in the text that follows, the insulin/IGF-1 receptor homolog in *Caenorhabditis elegans* regulates development, reproduction, and longevity in response to environmental signals such as food. Mutations in these pathways can induce

developmental arrest at the dauer stage and reduce fertility[80]. Similarly, deletion of CHICO, the *Drosophila* IRS protein, causes female sterility as well as reduced somatic growth and increased lipid storage[81].

During development and regeneration of the mammalian brain, insulin and IGF-I have important functions in brain including metabolic, neurotrophic, and neuroendocrine actions. Interestingly, the brains of IRS-2 knockouts are significantly reduced in size (40%) compared to age-matched controls[82]. IRS-2-deficient mice are of normal body size, and thus display a significant reduction in body:brain ratio. Reduced brain size is apparent during early development; at e16, the brain weight of *IRS2*$^{-/-}$ mice is already significantly reduced in comparison to control embryos[82]. Analysis of neuronal proliferation and survival during embryogenesis and postnatal development has revealed that the small brain phenotype reflects impaired IGF-I-mediated proliferation rather than enhanced apoptosis. Brain-specific deletion of the insulin receptor has no effect on brain size[55]. Consistent with the role of IGF-I/IRS-2 signals in brain development, deletion of *chico* produces a pin-head phenotype, flies with reduced head size[81]. Thus, while IRS-1 has evolved to regulate global somatic growth in mammals, IRS-2 has acquired a more specialized function in mediating the local effects of insulin/IGF-I during the development and function of various neuroendocrine tissues.

	Development and growth	**Metabolism**	**Neuroendocrine system**
IRS-1	Severely impaired intrauterine and postnatal growth (40-50% reduction in size). Enhanced pancreatic β-cell mass.	Mild insulin resistance in skeletal muscle, hyperinsulinemia, impaired adipocyte differentiation.	Reduced fertility.
IRS-2	Slight impairment of intrauterine growth (10%). Reduced pancreatic β-cell development and growth. Small brain. Reduced pituitary size. Abnormal development of testes and ovary.	Profound hepatic insulin resistance, hyperinsulinemia, abnormal glucose intolerance and hyperglycemia, mild obesity, dyslipidemia, hypertension.	Female infertility, abnormal reproductive hormone levels. Mild obesity, hyperphagia, hyperleptinemia. Dysregulated Tau phosphorylation.
IRS-3	None	None	None
IRS-4	Mild growth retardation (10%) of males.	Mild glucose intolerance.	Reduced female fertility.

Table 1. Summary of phenotypes resulting from deletions of IRS signaling proteins.

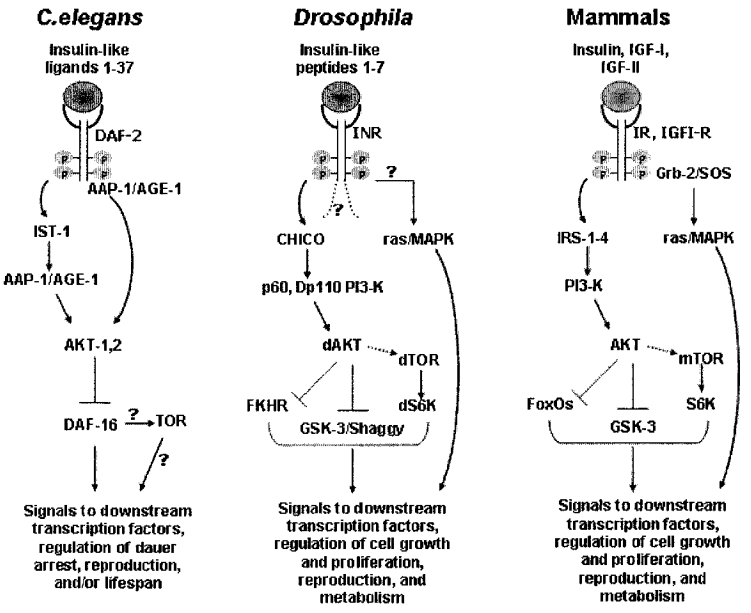

Figure 4. Evolutionarily conserved elements of the insulin/IGF-I signaling system.

5. THE INSULIN/IGF-I PATHWAY IN THE *CAENORHABDITIS ELEGANS* MODEL

The extensive knowledge of the biology of the nematode *Caenorhabditis elegans* biology together with the complete and well-annotated genome sequence make these worms a very attractive experimental model[83]. Moreover, the ability to use forward and/or reverse genetic screens in this model provides powerful tools to identify genes required for specific cell functions. As discussed earlier in this chapter, the recent application of RNA interference technology to *C. elegans* has expanded the advantages of this model system to a new level; this simple method allows for rapid, efficient gene inactivation, and thus facilitates identification of loss-of-function phenotypes of genes. Indeed, based on this new technique, a systematic study of the function of every *C. elegans* gene is currently in progress.

The identification of an insulin-like signaling cascade in *C. elegans* has provided novel insights into the molecular mechanisms of insulin action in mammals. A neurosecretory system regulates whether these animals enter the reproductive life cycle or arrest developmentally at the dauer diapause stage[84]. When food is limited, young animals become dauers instead of developing to adulthood[85]. *daf-2*, a key gene in the genetic pathway that

mediates this endocrine signaling, encodes a homolog of the insulin/IGF-I receptor[86-88]. Unlike vertebrates, these organisms contain a single insulin/IGF receptor gene and many developmentally regulated insulin-like genes. However, similar to insulin signaling in mammals, DAF-2 action requires *age-1,* which encodes a PI-3 kinase[80,89-91] and *akt-1* encoding Akt/PKB-like proteins which are activated by the lipid products of PI3-kinase[92]. The DAF-2 pathway controls both reproductive development and normal senescence; loss of function mutants of *daf-2, age-1* or *akt-1* arrest development at the dauer stage and convey an increase in life-span[80,87,89,90]. Recently, orthologs of the p50/55 regulatory subunit of PI-3 kinase and IRS proteins have been identified in *C. elegans* and genetic analysis has demonstrated that *aap-1* and *ist-1* function in the *daf-2* pathway[93]. Mutants of *aap-1* arrest in dauer larval stage and display an increase in lifespan. IST-1 appears to be required for a pathway that may act in parallel to AGE-1[93].

Some mutations suppress, to varying degrees, the effect of *daf-2* loss-of-function mutations, presumably because these genes counteract the effect of insulin signaling. Two of these genes, *daf-16* and *daf-18*, have been implicated in PI-3 kinase signaling[94-97]. Mutations in *daf-16*, which encodes the *C. elegans* homolog of the mammalian FOXO forkhead transcription factors, completely suppress the *dauer* phenotype produced by d*af-2* mutants[80,89]. Several lines of evidence indicate that FOXO1 is a transcriptional promoter and that its activity is inhibited by Akt and other lipid-dependent kinases; phosphorylation by these kinases excludes FOXO1 from the nucleus[69,98,99]. FOXO1 has been proposed to induce apoptosis, inhibit entry into the cell cycle, and stimulate glucose production[67,68]. Similar to observations in *C. elegans,* loss-of-function mutants of *Foxo1* rescues diabetes in the IRS-2 knockout model; β-cell mass is restored to almost normal in $Irs2^{-/-}Foxo1^{+/-}$ mice, demonstrating that IRS-2 signaling is required to regulate the negative impact of Foxo1 function on β-cell development, growth and/or survival[100].

Dauer arrest coincides with major metabolic changes. DAF-2 signaling normally allows non-dauer reproductive growth which is associated with the utilization of food for growth in cell number and size and with small stores of fat[87]. In *daf-2* mutants which dauer arrest, metabolism is shifted to the production of fat and glycogen in intestinal cells[89]. Thus, signals from the DAF-2 pathway regulate metabolism, growth, reproductive development, and longevity in this invertebrate system[101]. The structural and functional homology of DAF-2 to the mammalian insulin receptor provides a basis for a conserved mechanism that integrates metabolic signals with neuroendocrine regulation of reproduction and perhaps even aging. The increase in longevity associated with decreased DAF-2 signaling is

analogous to enhanced mammalian longevity associated with caloric restriction[97].

6. INSULIN RECEPTOR SIGNALING IN *DROSOPHILA*

As a model system, *Drosophila* enjoys many of the same advantages as *C. elegans* including ease of genetic manipulation and a known genome. The evolutionary conservation of the insulin signaling system and its role in the coordination of multiple systems is further illustrated by recent findings in *Drosophila*. The *Drosophila* insulin receptor homolog *inr* encodes a protein remarkably similar to its mammalian counterparts, including two α and two β subunits, with a cytoplasmic tyrosine kinase that is activated by insulin binding.[102] The overall level of identity between INR and human IR and IGF1R is 32.5% and 33.3%, respectively[103,104]. However, one structural difference between the *Drosophila* and human insulin receptors is the presence of a C-terminal tail of ~400 amino acids in the dINR[105]. This extension contains several potential tyrosine phosphorylation sites and three NPXYXXM motifs, suggesting that it may participate in signal transduction. Indeed, the tail of INR binds IRS-1 and PI-3 kinase when the *Drosophila* receptor is expressed in mammalian cells[106,107], but its precise function remains to be determined. Similar to results in worms and humans, partial loss of INR function reduces cell, organ and body size by decreasing both growth and proliferation[108-111].

CHICO, the *Drosophila* homolog of vertebrate IRS1-4, serves as a substrate of the INR and exerts a critical role in the control of cell growth, reproduction, metabolism, and lifespan[81,112,113]. Animals mutant for *chico* are half the size of wild-type animals owing to a reduction in cell size and number[81] and null *chico* mutants live up to 48% longer than controls[113]. Interestingly, CHICO also regulates metabolism; even though reduced in overall size, chico mutants display a 2-fold increase in lipid levels. Moreover, female *chico* mutants are sterile, suggesting that even flies possess a mechanism for coordinating metabolism and reproduction[81]. Thus, in both *C. elegans* and *Drosophila*, homologs of the insulin/IGF-I/IRS signaling network are implicated not only in growth, but also in the maintenance of fuel homeostasis and in the regulation of reproduction and longevity.

Glossary

AAP-1: *C.elegans* homolog of p50/55 regulatory subunit of PI3-kinase
AGE-1: *C.elegans* homolog of p110 catalytic subunit of PI3-kinase
Akt: protein kinase B
DAF-2: *C.elegans* homolog of insulin/IGF-I receptor
Dicer: RNAse-III equivalent
FKHR: forkhead family of transcription factors
Foxo: subfamily of forkhead transcription factors
Grb-2: growth-factor receptor binding protein-2
GSK-3: glycogen-synthase kinase
IGF-I: insulin-like growth factor
INR: *Drosophila* insulin receptor
IR: mammalian insulin receptor
IRS: insulin receptor substrate proteins
IST-1: C.elegans homolog of IRS
MAPK: mitogen-activated protein kinase
p60: Drosophila homolog of p85/PI3-K
PI3-Kinase: phosphatidyl-inositol kinase
RNAi: RNA interference to silence genes
S6K: p70 ribosomal 6 kinase
Shaggy: Drosophila homolog of GSK-3
SH2: *src*-homology domain 2
TOR: target of rapamycin

7. THE FUTURE OF EXPERIMENTAL MODELS

During the last two decades, many functions of the insulin/IGF-I system in development and postnatal growth have been revealed, chiefly by genetic modifications of various experimental model systems. However, as noted throughout this chapter, many questions remain regarding the role of this growth factor in the developmental events of mammals. The recent publication of the sequences of entire genomes[114-116] and the development of RNA interference will now greatly facilitate the production of new, more refined model systems for the study of IGF-I in development. One great challenge for the future is the development of technology that will enable us to apply what we learn about IGF-I in animal models to the diagnosis, treatment and prevention of human diseases such as cancer and diabetes.

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Chapter 3

INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS IN DEVELOPMENT

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1. OVERVIEW OF THE IGFBP FAMILY

Insulin-like growth factor-I (IGF-I) and IGF-II are growth-promoting polypeptides essential for normal growth and development[1]. Unlike other growth factors, both IGFs and their cognate membrane receptors are expressed in variety of tissues and cell types[1]. In both humans and rodents, IGFs share approximately 50% homology with proinsulin[2]. Close homology to insulin gives IGFs a unique characteristic among other growth factors as they not only regulate cell proliferation, differentiation, and apoptosis but also exert insulin mimetic metabolic effects in tissues expressing high levels of type I IGF receptor, such as skeletal muscle and pancreatic β-cells[3,4].

IGFs are present in plasma and other biological fluids predominantly in a complex with IGFBPs. IGFBPs comprise a family of six homologous proteins that bind IGFs with a high affinity in the range of 10^9 to 10^{11} l/mol, equal or higher than the affinity of IGFs for IGF type I membrane receptors[5,6]. IGFBPs control bioavailability of IGFs by regulating their transport, deposition in extracellular matrix, binding to membrane receptors, and degradation[5]. Besides acting through IGF-dependent mechanisms,

various IGF-independent effects of IGFBPs are increasingly described *in vitro*[7].

The *IGFBP* genes, like those of the IGFs themselves, are believed to have emerged early in vertebrate evolution[8,9]. Detailed examination of *IGFBP* gene family in the human genome revealed close linkage to the homeobox (*HOX*) gene family, which encodes transcription factors important in early embryogenesis. *IGFBP* genes are co-localized to four chromosomal regions with the *HOX* gene clusters. These observations suggest that *IGFBP* and *HOX* gene clusters duplicated together from a common ancestral chromosomal region.

The *IGFBP* genes share a common structural organization being coded by four conserved exons with the exception of IGFBP-3 gene which has a fifth exon in the 3'-untranslated region[5]. Exon 1 of the *IGFBP* gene family is shared by several other genes encoding a variety of IGFBP-related proteins, leading to the proposal that the IGFBPs belong to a larger superfamily of cysteine-rich proteins[9].

IGFBPs share a conserved structure consisting of three domains of approximately equal size: N-terminal, central, and C-terminal domains. Both N- and C-terminals contain 12 and 6 conserved cysteine residues, respectively with the exception of IGFBP-6 with 10 cysteine residues in N-terminal domain[9]. These cysteine residues form intermolecular cystine bridges by the pairing of adjacent cysteines within the domain[9]. Amino acid residues important in IGF-binding are found in the N-terminal and also in C-terminal domain[10]. The observation suggests the existence of an IGF-binding pocket formed by both domains. Other important subdomains have also been identified within the C-terminal region of various IGFBPs, such as Arg-Gly-Asp (RGD) integrin-binding motifs are located at residues 221–223 of IGFBP-1[11] and residues 265–267 of IGFBP-2[12]. Multifunctional 18-residue basic motifs with heparin-binding activity and nuclear localization signal (NLS), described at residues 215–232 of IGFBP-3 and residues 201–218 of IGFBP-5, are involved in interaction with the serum glycoprotein ALS (acid-labile subunit)[10,13,14] and other ligands such as plasminogen activator inhibitor-1 (PAI-1)[15] and transferrin[16], vitronectin[17], cell and extracellular matrix binding[10,18], and nuclear transport[19]. The C-terminal domain is homologous to the thyroglobulin type I repeat unit.

The central domain of the IGFBPs shows no structural similarity among any members of the family. Secondary IGFBP-5 binding sites for ALS[20] and heparin[21], and a potential cell-association domain of IGFBP-3[7,10,22], are also found in this region. Sites of *N*-linked glycosylation in IGFBP-3 and IGFBP-4 and *O*-glycosylation in IGFBP-5 and -6 are found in this region[23-26]. The extent of glycosylation of individual IGFBPs may differ between tissues, as has been shown for IGFBP-6[26]. Glycosylation does not appears to alter

IGFBP affinity for IGFs[23] but it may have an effect on proteolysis, cell membrane association, and clearance from circulation and thereby secondarily influence IGFBP affinity for IGFs[23,26-28].

Other sites of posttranslational modification are also found in the central domain. These include potential phosphoacceptor sites on all IGFBPs, some of which are phosphorylated in IGFBP-1, -3, and -5[29], and proteolytic cleavage sites in some of the proteins[26,30-32]. Posttranslational modifications modulate affinity of IGFBPs to IGFs. For example, it has been shown that serine phosphorylation of IGFBP-1 enhances its affinity for IGF-1 by six- to eightfold and also increases its capacity to inhibit IGF-I actions[33]. Although IGFBP-3 could also be phosphorylated on serine residues, it is present in circulation predominantly in an unphosphorylated form[29]. Interestingly, it has been recently shown that binding of IGFBP-3 to breast cancer cells is accompanied by phosphorylation at the plasma membrane level and that both processes are inhibited by IGF-I. However, once phosphorylated, the ability of IGFBP-3 to bind IGF-I is enhanced, possibly resulting in increased association of the IGF-I with the cell membrane[34].

Each of the six IGFBPs can undergo proteolysis, which decreases their affinity for IGFs. Therefore, proteolysis has a critical role in regulation of IGFs release from their binary and ternary complexes with IGFBPs. While some proteases are specific for a single IGFBP, others can use multiple IGFBP as substrates. IGFBP proteolysis has been recently reviewed extensively elsewhere[35,36].

In the circulation, IGFBP-3 and to a lesser extent IGFBP-5, form a ternary complex of IGF, IGFBP, and ALS. The ternary complex is responsible for transport of the majority of the IGFs in the circulation[37]. Under the stimulatory effect of growth hormone (GH), ALS and IGF-I are secreted from hepatocytes and IGF-I induces IGFBP-3 expression in hepatic endothelial cells[37-40]. This leads to formation of the ternary complex of IGF, IGFBP-3 or IGFBP-5, and ALS in 1:1:1 molar ratio[41]. This mechanism of the ternary complex formation has been confirmed in mouse models of selective IGF-I nullification in hepatocytes. In these mice with minimal production of IGF-I in the liver, serum levels of IGFBP-3 are decreased to ~30% of normal controls in the presence of low serum IGF-I and elevated GH[42]. See Table 1 for some of the structural features of the IGFBPs and ALS.

	MM (kDa)	IGF affinity	Posttranslational modification	Functional subdomains	Other interacting proteins
IGFBP-1	25.3	I = II	phosphorylation	RGD	A5β1 integrin receptor [11]
IGFBP-2	31.4	I < II		RGD	Heparin, extracellular matrix and proteoglycans [74,75]
IGFBP-3	28.7 (40-42)	I = II	Phosphorylation *N*-glycosylation	NLS	ALS[108], transferrin [16], fibrinonectin [187], importinβ[19], RXRα[79], PGI/AMF[188], humanin[189]
IGFBP-4	25.9 (29)	I = II	*N*-glycosylation		
IGFBP-5	28.6 (34)	I < II	phosphorylation *O*-glycosylation	NLS	ALS[13], PAI-1[15], vitronectin[17], importinβ[19], FHL2[93], αs_2-casein[94], collagen III and IV, laminin, fibronectin[186]
IGFBP-6	22.8 (32)	I << II	*O*-glycosylation		
ALS	63.3 (85)		glycosylation		IGFBP-3[108], IGFBP-5[13]

Table 1. Structural aspects of human IGFBPs

MM = molecular mass, *Numbers in parentheses indicate MM of glycosylated protein

2. IGFBPS: EXPRESSION AND REGULATION

In this section, individual IGFBPs are reviewed briefly. For a more detailed description of the individual IGFBPs, comprehensive reviews are available[5,7,9,26,43]. A summary of tissue expression in embryonic and postanatal life in humans and mice is given in Table 2.

2.1 IGFBP-1

IGFBP-1 was first isolated from midterm human amniotic fluid[44]. Brewer et al. isolated its cDNA from a human decidua library and showed that it can potentiate the effects of IGF-I *in vitro*[45].

	Human embryo	Mouse embryo
IGFBP-1	Liver (a), kidney (b)	After e12, restricted to the liver (c,d)
IGFBP-2	Moderate abundance in every tissue with the highest level observed in the liver (a); UD, committed glomerular and epithelial cells, mature glomerular epithelial cells, decreases in proximal tubule with maturation (e)	Widely distributed mainly in the ectodermal layer and also in the mesoderm derived part of the tongue (c); lung, liver, kidney, choroid plexus, floor plate (d)
IGFBP-3	Most abundant in the skin, muscle, and heart (a); mature UD, epithelium of the collecting ducts and pelvicalyceal system (e)	Only weakly in late gestational embryos (c,d) liver, lung, tongue, and nasal epithelium (d), choroids plexus, kidney (da), heart (db)
IGFBP-4	Moderate abundance equally in all tissues (a); kidney, stomach, intestine, and lung and least in the liver (14-18 w) (f); UD glomerular epithelial cells, increases in proximal tubule with maturation (e)	Widely distributed in the mesoderm derived tissues (c); mesencephalon, telencephalon, heart, liver, lung, tongue, blood vessels (d), kidney (da)
IGFBP-5	Muscle, skin, stomach, and intestine (14-18w) (f), most abundantly in the skin, muscle and stomach (a), mesenchyme of the vascular cleft, mature mesangium and supporting mesenchyme (e)	Unlike other BPs only after implantation in mouse embryo, widely distributed, predominantly in the meninges, vertebrae, lung, kidney (c); liver, gut endothelium, meninges, lung mesenchyme, tongue (d), notochord, floor plate, muscle (da)
IGFBP-6	Low abundance in all tissues (a), heart, muscle, gut, lung, brain and skin and least in the liver (f)	Weakly expressed in late gestational embryos (c,d), liver, nasal epithelium (d), hindbrain, heart (da), perichondral layers of cartilage (db)

Table 2. Tissue expression of IGFBPs during embryonic development in human and mouse tissues. E = mouse embryonic day, W = human fetal week, UD = ureteric duct. (a) W10-16 mRNA[190], (b) W15-23 mRNA[191], (c) E13.5 mRNA[192], (da) E13.5 protein and (db) E13.5 mRNA[193], € mRNA[194], (f) W14-18 mRNA[195]

IGFBP-1 is primarily expressed in liver, decidualized uterine endometrium, ovarian granulosa cells, and kidney[43]. Insulin inhibits IGFBP-1 expression in hepatocytes at the level of gene transcription[46]. IGFBP-1 expression is inhibited by GH[47] and also by IGF-I and -II in cells with type I IGF receptor[48, 49]. In these cells IGF-I may be a more potent inhibitor of IGFBP-1 expression than insulin. IGFBP-1 expression is stimulated by glucagon, glucocorticoids, T4, somatostatin, epidermal growth factor, interleukin (IL) IL-6, IL-1, and tumor necrosis factor (TNF)-α[43,50]. Comparison of human, rat and mouse promoters indicates high sequence similarity in the proximal ~300 bp, where IGFBP-1 promoter activity has been shown to be regulated by HNF1, HNF3, glucocorticoids, insulin, and cAMP *in vitro*. IGFBP-1 promoter is highly homologous to a promoter of PEPCK, a key gluconeogenetic enzyme, suggesting a role for IGFBP-1 in regulation of glucose metabolism[43]. Levels in the circulation are regulated predominantly by insulin and IGFBP-1 levels in the circulation reflect

hepatic portal insulin concentration and hepatic insulin sensitivity rather than whole body insulin sensitivity[51]. Human IGFBP-1 is secreted as a phosphoprotein with phosphorylated serine residues 101, 119, or 169. Phosphorylation of IGFBP-1 is associated with increased affinity for IGF peptides. It has been shown that site-directed mutagenesis of Ser-101 prevents phosphorylation of this residue and results in a three-fold decrease in affinity for IGF-I[29,33]. IGFBP-1 phosphorylation is developmentally and hormonally regulated[52,53].

The majority of *in vitro* and *in vivo* studies support an inhibitory role of IGFBP-1 on IGF-stimulated growth, differentiation, and metabolic activity. There is evidence for an inverse relationship between IGFBP-1 and bioactive free IGF-I[54]. During fasting low insulin levels lead to activation of IGFBP-1 transcription in hepatocytes, and a rise in IGFBP-1 in circulation is accompanied by a decline in free IGF-I[55]. IGFBP-1 is reduced in obese humans, which reflects elevated insulinemia in response to insulin resistance[56]. Further progression of insulin resistance with onset of type 2 diabetes mellitus (T2DM) is associated with an increase of IGFBP-1 back to values observed in lean individuals[56]. This is in a good agreement with the increased free IGF-I and IGF-II in obese subjects. Even more profound changes in IGFBP-1 are present in type 1 diabetes mellitus (T1DM), where insufficient insulin inhibitory effect on IGFBP-1 transcription in hepatocytes leads to increased IGFBP-1 production in the presence of low IGF-I, free IGF-I, and free IGF-II levels in circulation[56].

In vivo studies by Lewitt *et al.* demonstrated that human IGFBP-1 blocks the hypoglycemic response to intravenous IGF-I and may even cause a mild increase in glycemia when administered alone[57]. In a more recent study, Lang *et al.* demonstrated that acute *in vivo* elevation in IGFBP-1, of the magnitude observed in various catabolic conditions, is capable of selectively decreasing protein synthesis in fast-twitch skeletal muscle while it had no effect on glycemia in rats[58]. Since IGFBP-1 is upregulated by pro-inflammatory and stress-related cytokines, elevations in circulating and tissue levels of IGFBP-1 may be an important mediator of the muscle catabolism observed in these conditions.

2.2 IGFBP-2

Binkert first cloned the cDNA and determined primary structure of human IGFBP-2[12]. IGFBP-2 is highly expressed in fetal tissues, particularly in the CNS[59]. High levels of IGFBP-2 decrease after birth until puberty after which it gradually increases again, particularly after age of 60[60]. IGFBP-2 remains the most abundant protein in the CNS postnatally[61] and it is the predominant IGFBP in the lymph, bronchoalveolar lavage, prostate and seminal fluid of normal human subjects[62,63]. It is also expressed at high levels in liver,

kidney, and secretory endometrium. GH and insulin inhibit hepatic synthesis and thereby control levels in circulation[64-66]. Increased IGFBP-2 has been reported during prolonged fasting, and protein restriction[67,68], in GH deficiency[66] and after IGF-I administration possibly via suppression of GH[65,69]. IGFBP-2 is elevated in a number of pathological conditions, such as chronic renal failure, liver cirrhosis, non-islet-cell tumor hypoglycemia, whereas decreased IGFBP-2 has been observed in response to GH therapy in GH deficient subjects[65,66,70]. IGFBP-2 is lower in obese than lean subjects and unlike IGFBP-1, it further declines in T2DM subjects[56]. Marked reduction in IGFBP-2 has been observed in stromal cells of patients with benign prostatic hyperplasia, suggesting inhibitory effect of IGFBP-2 on stromal growth[71]. However, in prostate cancer IGFBP-2 may also have stimulatory effects on cell growth[72] and IGFBP-2 gene has been recently found to be highly overexpressed in the human glioblastoma, but not low-grade gliomas[73]. Similar observations have also been made for other IGFBPs and suggest an antagonistic role in modulation of IGF action, depending on the cell status.

IGFBP-2 can bind to heparin, extracellular matrix and proteoglycans in cooperation with IGFs[74,75]. This binding is enhanced by the IGFs, but does not change affinity for IGFs and it may inhibit IGF-II action in some cell types[76].

2.3 IGFBP-3

Based on sequence data derived from GH-dependent insulin-like growth factor (IGF) binding protein, purified from human plasma, Wood *et al.* isolated full-length IGFBP-3 cDNA clones[78]. As the most abundant IGFBP in circulation and a key component of the ternary complex, IGFBP-3 has a central role in regulation of IGF transport to tissues[37]. IGFBP-3 can be also transported into the nucleus by an importinβ-mediated mechanism, which requires intact nuclear localization signal in heparin binding subdomain of IGFBP-3 C-terminal[19]. In the nucleus, it has been shown to interact with the RXRα and possibly other nuclear elements[79]. The ability of IGFBP-3 to bind to a variety of other serum proteins, including ALS, transferrin, plasminogen, fibrinogen, vitronectin, and fibrin, has been reported[10,13-19]. Cell surface binding sites for IGFBP-3 have also been identified in some cell types[10,18,34].

The principal regulators of serum IGFBP-3 are its hepatic synthesis, which is regulated by IGF-I produced locally by hepatocytes and to a lesser extent direct regulation by GH and insulin[39-41]. Hepatic IGFBP-3 production is responsive to alterations in dietary intake and it parallels changes in hepatic production of IGF-I with IGFBP-3 being reduced during protein restriction or malnutrition[79,80]. Serum IGFBP-3 is relatively constant throughout the day[80]. Decreased IGFBP-3 is accompanied by increased IGF

clearance from circulation in conditions characterized by low IGFBP-3, such as hypopituitarism[81]. In acromegaly and GH deficient subjects, IGFBP-3 parallels changes in IGF-I in circulation. Measurement of IGFBP-3 and IGF-I in addition to GH dynamic testing is clinically useful in evaluation of GH functional status[82]. IGFBP-3 is also mildly elevated in obese and type II diabetic subjects[56]. While liver production is the most important IGFBP-3 source in circulation, IGFBP-3 is expressed in variety of other tissues such as skin, skeletal muscle, reproductive organs, bone, and endothelium[5]. Endothelial cells are an important source of IGFBP-3 as documented by *in situ* hybridization studies in the liver and ovary[40,41,83]. These cells also have binding sites for IGFBP-3[18]. Since IGF-I has been shown to regulate endothelial cell proliferation via direct effect as well as through modulation of the vascular endothelial growth factor (VEGF) system, IGFBP-3 may play a significant role in control of angiogenesis. Fraser *et al.* found that IGFBP-3 mRNA was expressed in the endothelium of the human corpus luteum[83]. The mRNA expression is high during the early luteal phase and its subsequent decline is prevented by administration of human chorionic gonadotropin (CG) and IGFBP-3 may play a role in controlling angiogenesis involution of the human corpus luteum by autocrine and paracrine mechanisms[83].

2.4 IGFBP-4

Shimasaki *et al.* cloned human IGFBP4 from human placenta, liver, and ovary cDNA libraries and they demonstrated tissue expression in all tissues examined, with highest expression in liver[84]. The central domain of IGFBP-4 has two extra Cys residues that form a cystine bridge and this may contribute to the distinctive biological behavior of IGFBP-4, which has only inhibitory actions on IGF action *in vitro*. IGFBP-4 does not associate with cell surface[85]. IGFBP-4 can be glycosylated at Asn104, but this does not affect its binding to IGF-I[85]. IGFBP-4 is expressed in a variety of cell types, including hepatocytes, vascular endothelium and smooth muscle, granulosa cells, osteoblasts, fibroblasts, and articular chondrocytes[86]. The regulation of IGFBP-4 expression is cell type specific with IGF-I having predominantly an inhibitory effect on IGFBP-4 expression. IGFBP-4 is detected in high levels in small androgen-dominant, growth-arrested, and atretic follicles, but in low levels in estrogen-dominant growing follicles[87]. Loss of IGFBP-4 inhibitory action in the dominant follicle, possibly the result of increased IGFBP-4 proteolysis, increases bioavailable IGFs in follicular fluid and may be involved in selection of the dominant follicle[87]. IGFs are the most abundant growth factors in bone and IGFBP-4 is the major IGFBP produced in bone microenvironment[88]. Parathyroid hormone and 1,25-dihydroxyvitamin-D3 stimulate local IGFBP-4 production in bone and this may inhibit bone formation[88]. Local IGFBP-4 administration inhibits bone

formation whereas systemic IGFBP-4 may increase bone formation in mice, probably by increased IGF delivery into bone[89]. IGFBP-4 is regulated developmentally and its levels in circulation increase with age[88].

2.5 IGFBP-5

IGFBP-5 was purified from human serum and subsequently cloned by Kiefer *et al*[90]. IGFBP-5 is homologous to IGFBP-3 and ~58% is present in the circulation in the ternary complex with ALS[91]. It shares several other binding partners with IGFBP-3 and is also transported to the nucleus[19]. An IGFBP-5 membrane receptor has been described[92], and using a yeast 2-hybrid screen, Amaar *et al.* found strong and specific interaction between IGFBP-5 and four and a half LIM 2 (FHL-2), a nuclear coactivator of the androgen receptor[93]. Purified FHL-2 did not interact with any other IGFBPs tested[93]. These data support the claim for IGF-independent effects of IGFBP-5. IGFBP-5 also binds to αs_2-casein, types III and IV collagen, laminin, and fibronectin and IGFBP-5 bound to ECM may potentiate the IGF effect[94,95]. Augmentation of IGF action typically involves proteolysis or reduction in the affinity of IGFBP-5 for IGF-1 after interaction with components of the extracellular matrix[95]. IGFBP-5 expression is not acutely altered by nutrients or insulin, and the most important determinants of levels in the circulation are protection by IGF-I from proteolysis and stimulation of transcription by IGF-I and GH, whereas IGFBP-5 expression is inhibited by dexamethasone[88,96,97]. Levels in the circulation increase during puberty and decline with aging[88,98].

IGFBP-5 plays a role in bone, ovary, kidney, and skeletal muscle homeostasis. Increased expression has been observed during involution in mammary gland[99] and prostate[100], and in ovarian follicles undergoing atresia, implying proapoptotic, antiproliferative effects[87]. IGFBP-5 concentrations in milk may reach 50 mg/l during rat mammary gland involution, the highest level of IGFBP reported in any biological fluid[99]. Prolactin may act by suppressing the production of IGFBP-5 from the mammary epithelium, thereby preventing the involution[99].

2.6 IGFBP-6

Martin *et al.* first purified IGFBP-6 from transformed human fibroblasts and Shimasaki *et al.* determined the human cDNA sequence[101,102]. IGFBP-6 binds IGF-II with marked preferential affinity over IGF-I and it has the strongest affinity for IGF-II among the IGFBPs[101]. Human and rat IGFBP-6 lack two N-terminal cysteines and therefore the Gly-Cys-Gly-Cys-Cys motif, present in IGFBPs 1 through 5[102]. This is accompanied by preferential binding of IGF-II[101]. IGFBP-6 is *O*-glycosylated, and five serine/threonine

glycosylation sites in the non-conserved mid-region of human IGFBP-6 have been identified[103]. Glycosylation modulates the half-life in the circulation, cell association, and proteolysis of IGFBP-6[26-28]. IGFBP-6 is a relatively specific inhibitor of IGF-II actions and it has not been shown to potentiate IGF actions. It is often expressed in non-proliferative, quiescent state cells *in vitro* and differentiating agents increase its expression, invoking a role for IGFBP-6 as an autocrine inhibitor of cell proliferation. In this respect, it is interesting that IGFBP-6 expression is uniformly induced by retinoic acid, a potent differentiating agent[26,104].

IGFBP-6 and IGFBP-2 are the major IGFBPs in the cerebrospinal fluid[105]. IGFBP-6 is synthesized by the choroid plexus and astroglial cells[106]. It is also expressed in other human tissues such as placenta, lung, vascular smooth muscles, bone, skeletal muscle, prostate, and ovary[26]. Serum IGFBP-6 increases with age and it is higher in adult men than in women[107]. Elevated levels were observed in non-islet cell tumor induced hypoglycemia and in chronic renal failure[107].

2.7 Acid-labile subunit

Baxter has shown that ALS forms a ternary complex in the presence of IGF-I and IGFBP-3[108]. Leong *et al.* isolated full-length clones encoding the human ALS from human liver cDNA libraries[109]. ALS expression is developmentally and hormonally regulated and the majority of ALS is produced by hepatocytes under stimulatory control of GH, suggesting an important role in regulating the availability and action of the IGFs[40]. Association of IGFs into the ternary complex stabilizes levels of IGFs in the circulation and prevents rapid IGF efflux from the circulation into tissues[40,81,110]. In the presence of low GH levels before birth or during the early postnatal period in rodents, the ternary complex is not detectable in the circulation and most of the IGFs present are bound to other IGFBPs as binary complexes[111]. Under the influence of GH, hepatic expression of ALS and IGFBP-3 is enhanced postnatally, and the abundance of the ternary complex increases dramatically in the first few weeks of rodent life[111,112]. Unlike the binary complexes that can traverse the endothelial capillary barrier, the ternary complex is thought to be confined to the circulation[113].

The enhanced expression of ALS, with an eventual two- to three-fold molar excess over IGFBP-3[110], results in the gradual appearance of the ternary complex in the circulation in the first few weeks of rodent postnatal life. This change in the compartmentalization of IGFs in the circulation ensures formation of stable IGF reservoirs in the circulation, which appears to be important for normal postnatal growth[114]. In the rodent, IGF-II declines in importance during this period because of developmental down-regulation of expression[115], and IGF-I becomes the most abundant IGF in adult life. A

similar developmental pattern of ALS and ternary complex formation has been observed in humans. There are markedly lower levels of the ternary complex in second- and third-trimester fetal serum compared to adult serum[116]. In addition to low ALS levels, IGFBP-3 in present predominantly as a 29-kDa proteolytic fragment rather than an intact molecule during human fetal development, which further compromises ternary complex formation[116]. In postnatal life, serum ALS follows GH level increases during childhood, reaches a peak during puberty and declines in adult life[117]. Glycosylation and sialylation patterns of ALS carbohydrate moieties influence ALS binding to IGFBP-3 and association to ternary complex[118].

3. ALTERATIONS IN IGFBPS IN DEVELOPMENT

3.1 IGFBP-1

A physiologic role for IGFBP-1 during fetal development is suggested by the demonstrated inverse relationship between fetal serum or cord blood IGFBP-1 and fetal size in mid and late gestation[119-121]. IGFBP-1 is elevated in the fetal circulation of human and animal pregnancies complicated by intrauterine growth retardation (IUGR) caused by placental insufficiency and in utero hypoxia [122,123]. It is believed to restrict fetal growth by sequestering free IGFs.

Popovici *et al.* established highly pure primary cultures of human fetal hepatocytes *in vitro* and investigated the expression of IGFBP-1 and the effects of hypoxia on expression of IGFBP-1 mRNA and protein[124]. The study has shown that hypoxia up-regulates fetal hepatocyte IGFBP-1 protein and mRNA levels and IGFBP-1 is the major IGFBP derived from these fetal hepatocytes. These data also support a role for the fetal liver as a source of elevated circulating levels of IGFBP-1 in fetuses with *in utero* hypoxia and IUGR.

Barreca *et al.* reported a child with approximately 20-fold elevation of IGFBP-1 above levels normal for age, which manifested with prenatal growth retardation that further deteriorated postanatally[125]. The child had no detectable endocrine or metabolic disorders and normal IGF-I and ALS, lower IGFBP-3 and IGF-II and mildly elevated GH serum concentration, compared to age appropriate controls. Almost all IGF-I activity, basally as well as after the GH stimulation test, was in the 35- to 45-kDa complex, corresponding to the IGFBP-1 peak. GH therapy resulted in increased growth velocity in this child[125]. This report supports a growth inhibitory

effect of IGFBP-1 *in vivo* and is in agreement with phenotype of IGFBP-1 transgenic (Tg) mice discussed later.

The insulin receptor is a ligand-activated tyrosine kinase. Mutations in the corresponding gene cause rare inherited insulin-resistant disorders in humans, leprechaunism, and Rabson–Mendenhall syndrome (RMS). Patients with the most severe syndrome, leprechaunism, manifest intrauterine growth retardation, hypoglycemia, and early death (usually before 1 year of age). RMS is caused by mutations in the insulin receptor gene with less severe disruption of insulin signaling. RMS manifests clinically with severe insulin resistance with hyperglycemia, short stature, and survival up to 5–15 years of age. Neonates with RMS are small for gestational age and infants are characterized by absence of subcutaneous fat, premature dentition, acanthosis nigricans, fasting hypoglycemia in infancy, and postprandial hyperglycemia in the presence of extremely high insulinemia[126,127]. Levels of IGFBP-1 in early infancy are higher than in adult human subjects and the high IGFBP-1 plasma levels in newborns could be important in protecting them from hypoglycemia[128]. Interestingly, low IGF-I, low IGFBP-3, high GH, and high IGFBP-1 have been reported in children with leprechaunism and RMS[126,129,130]. High IGFBP-1 levels may further attenuate biologic activity of low IGF-I in this condition. The actual levels of free IGFs have not been reported in RMS or leprechaunism to our knowledge; however, high IGFBP-1 is likely to contribute to the observed growth and metabolic abnormalities.

Therapy with rhGH (up to 0.5 mg/kg/week) did not improve growth and failed to increase the levels of circulating IGF-I and IGF-binding protein-3 over a 14-month period in a child with RMS. Subsequently, rhIGF-I was given by daily subcutaneous injection and no increase in growth velocity was observed over a 14-month period[131]. The lack of efficacy of IGF-I treatment may be related to several factors, such as the poor metabolic state of the patient, the observed deficiency of IGFBP-3, an increased clearance of the IGFs, IGF-I resistance in target cells at a receptor or postreceptor level, or an inhibitory action of the mutant insulin receptors on IGF-I receptor signaling through IGF/insulin hybrid receptors[131,132]. Indeed, therapy of an infant with leprechaunism with combined subcutaneous and intravenous administration of rhIGF-I, which resulted in sustained high IGF-I in circulation, caused a substantial improvement of growth velocity and improved metabolic control[129].

In accordance with observations in leprechaunism and RMS are recent seminal observations of Accili and coworkers in mice with variable cellular mosaicism for null insulin receptor (*Insr*) allele[133]. A complete targeted ablation of *Insr* causes early postnatal lethality and precludes investigation of growth and metabolic phenotype of *Insr* deficiency in adult life[134]. *Insr*

ablation in approximately 80% of cells caused extreme growth retardation, lipoatrophy, and hypoglycemia, a phenotypic constellation that resembles the human leprechaunism. *Insr* ablation in 98% of cells also resulted in growth retardation and lipoatrophy. In addition, it caused diabetes without β-cell hyperplasia. In accordance with the data from children with RMS and leprechaunism, the growth retardation in the presence of disrupted insulin signaling in these mosaic mice was associated with a greater than 60-fold increase in the expression of hepatic IGFBP-1 and elevated IGFBP-1 in the circulation[134].

3.2 IGFBP-3

IGFBP-3 is an integral component of IGF ternary complex[37]. As the ternary complex formation is strongly GH dependent, IGFBP-3 levels in circulation are decreased in GH-deficient states and increased in the presence of GH excess[82]. GH deficiency or resistance to GH caused by an aberrant GH receptor signaling both lead to low abundance of ternary complex components and increased IGF disappearance from circulation[82]. Low levels of the ternary complex in circulation are associated with impaired postnatal growth in both humans and rodents[114, 135].

3.3 ALS

Recently, Domene *et al.* reported a patient with inherited absence of ALS[135]. Sequencing of the *ALS* gene in this patient revealed a 1-bp deletion, resulting in the substitution E35K and the appearance of a premature stop codon. They described a 17-year-old boy with delayed onset of puberty and slow pubertal progress, but with normal psychomotor and neurologic development. At 14 years of age, the boy was referred for evaluation of growth pubertal delay and at this time his height was –2.05 SD. Growth hormone responses to provocative tests were normal, but there was marked reduction of both IGF-1 and IGFBP-3, which remained unchanged after stimulation with growth hormone. The ALS was undetectable in the serum before and after GH stimulation. After 6 months of treatment with recombinant human growth hormone, reduction in the subscapular skinfold thickness was observed but there was no beneficial effect on either the velocity of growth or the serum levels of IGF-I, IGFBP-3, and the acid-labile subunit.

4. DISORDERS WITH COMPLEX DISTURBANCES IN IGFBP SYSTEM

4.1 Chronic renal failure

Chronic renal failure (CRF) is a state of GH resistance and children with CRF often fail to achieve adult height consistent with their genetic potential and develop progressive growth retardation. CRF is characterized by simultaneous increase in IGFBP-1, -2, -4, -6, and proteolytic fragments of IGFBP-3 and -5 in serum[70,136,137,138]. Increased levels of IGFBPs, in the presence of relatively normal total IGFs, lead to decreased free IGF-I may contribute to decreased skeletal growth, peripheral substrate utilization, and tissue metabolism in CRF[136]. Improvement of linear growth with growth hormone (GH) treatment of uremic children is thought to be due to increased IGF-I/IGFBP ratio, resulting in increased free IGF-I levels during treatment[139,140].

4.2 Duchenne muscular dystrophy

Duchenne muscular dystrophy (DMD) is an X-linked hereditary disease characterized by progressive loss of muscle ability and concomitantly increasing fibrosis. When supplemented or overexpressed in the Tg mice, IGFs have been shown to improve skeletal muscle functioning in a mouse model of DMD[141,142]. IGFBP-5, produced in excess by DMD fibroblast, is a potent inhibitor of proliferation in myoblast from DMD patients[143]. Bakay *et al.* used microarray studies to demonstrate an increase in mRNA levels of IGFs as well as IGFBPs in DMD skeletal muscle[144]. Therefore, the beneficial effects of IGF-I or IGF-II supplementation in dystrophic muscle could result from prevention of dose-dependent sequestration of the IGFs by inhibitory IGFBPs[144].

4.3 Hypodactyly

Hypodactyly (Hd) mice manifest an impaired limb development with abnormal digit formation[145]. The distinctive pointed limb buds have been shown to be the result of a massive increase in apoptosis. Allan *et al.* examined IGF-1, IGFBP-2, and IGFBP-5 expression patterns during limb development in Hd mice and found down-regulated IGF-I expression in Hd limb buds in regions undergoing high levels of cell death, while IGFBP-5 was increased in the regions of cell death, consistent with reports of its association with apoptosis in adult tissues. This study suggested that IGF and

its binding proteins may play an important role in the process of limb formation[145].

4.4 Cornelia de Lange syndrome

The Cornelia de Lange syndrome (CdLS) is a multisystem congenital disorder characterized by typical facial features, IUGR, short stature, microbrachycephaly, hirsutism, limb anomalies, cognitive retardation, and other organ system involvement[146]. Pregnancy associated plasma protein-A (PAPP-A) is an IGF-dependent IGFBP-4 degrading enzyme, which has been shown to enhance IGF activity by IGFBP-4 degradation[147]. Interestingly, PAPP-A levels are significantly reduced in second-trimester maternal serum from women with CdLS newborns[146]. Low maternal levels of PAPP-A at 8-14 wk gestation are associated with an increased risk of IUGR[148]. Alteration in IGF system, caused by low levels of PAPP-A, may contribute to pathogenesis of CdLS.

5. INSIGHTS FROM IGFBP AND ALS TRANSGENIC AND KO MICE

In vivo studies of IGFBP action have been limited until recently by the lack of sufficient quantities of purified IGFBPs. Some insights into the physiological role of the IGFBPs are now emerging with development of genetically modified mice with either absence or increased levels of IGFBPs achieved through gene inactivation (knockout, KO) or overexpression of additional gene copies (Tg mice), respectively. KO models exist for all six IGFBPs in mice[149]. Tg mouse models have been developed with either targeted or generalized overexpression of IGFBPs. Targeted and generalized IGFBP overexpression address different questions. Generalized IGFBP overexpression ensures that both high circulating and tissue levels are achieved and can determine the overall effect of increased binding protein on the whole animal. Targeted IGFBP overexpression, achieved by the use of tissue specific promoters, allows for the investigation of local effects of IGFBP expression in specific tissues without perturbing circulating levels of the IGFBPs, IGFs, GH, and other components of the IGF system. Phenotypic manifestation of IGFBP transgenic mice are summarized in Table 3. The predominant effect of generalized overexpression has been growth retardation as would be anticipated from inhibitory effect of IGFBP excess on the actions of IGFs[150-154]. In addition, impaired glucose homeostasis has been observed in both IGFBP-1- and IGFBP-3 Tg mice[150,155,156]. Similarly, reduced fecundity has been observed in IGFBP-1, -3, -5, and -6 Tg mice[152-

[154,157]. Localized tissue overexpression results in tissue inhibition of IGF action in most, but not all studies (Table 3).

5.1 IGFBP-1

IGFBP-1 KO mice demonstrated normal development[149]. The absence of a marked phenotype in this, and other IGFBP KO mice, may result from compensatory mechanisms, although the nature of these mechanisms have not been elucidated. After a two-thirds hepatectomy, one of the most rapidly and highly induced genes and proteins in regenerating liver is IGFBP-1[158]. Leu *et al.* studied the influence of IGFBP-1 nullification on hepatic necrosis and regeneration. They used IGFBP-1 KO mice, control mice and mice with immunoneutralized IGFBP-1, to compare effect of IGFBP-1 deficiency on hepatic necrosis after of a sublethal dose of Fas agonist [159]. IGFBP-1 deficiency was associated with more massive hepatocyte apoptosis and caspase activation, which was attenuated by pretreatment with IGFBP-1. IGFBP-1-deficient livers had enhanced signaling via the integrin receptor after Fas agonist treatment. Elevated expression of active TGF-α1, a hepatocyte apoptogen, was observed in IGFBP-1-deficient livers that correlated with the appearance of the apoptotic process. IGFBP-1 KO mice also displayed increased injury in a toxic hepatic injury model caused by carbon tetrachloride. IGFBP-1 KO mice had abnormal liver regeneration after partial hepatectomy, characterized by liver necrosis and reduced and delayed hepatocyte DNA synthesis[160]. These findings indicate that IGFBP-1 functions as an important hepatic survival factor in the regenerating liver by reducing the level of proapoptotic signals. This IGFBP-1 effect may be IGF-independent, possibly mediated most via inhibition of integrin signaling, or IGF-dependent. Although mature hepatocytes have a low abundance of type I IGF receptors[161], it is possible that hepatocyte progenitor cells have more abundant IGF receptors. Since IGFBP-1 appears to be important for hepatocyte regeneration it is possibley that under these circumstances IGFBP-1 may function to enhance IGF-I delivery to hepatocyte progenitor cells.

Phenotypic manifestations of IGFBP-1 overexpression in Tg mice suggest that excess IGFBP-1 consistently inhibits IGF action *in vivo*. Ubiquitous overexpression of IGFBP-1 at high levels is associated with pre- and post-natal retardation and reduced brain size[150]. Impaired brain development is consistently observed also in other IGFBP Tg mice and it is thought to be a particularly sensitive indicator of impaired IGF action caused by IGFBP excess[152-154,162,163].

IGFBP	Transgene construct[1]	Transgene expression	Phenotypic manifestation	Reference
IGFBP-1	MT-hIGFBP-1	Brain, heart, kidney, liver, lung, testes	Abnormal brain development, marked inhibition of hepatic preneoplasia	161,196
	mPGK-rIGFBP-1	Ubiquitous	Reduction in birth weight, postnatal growth retardation, reduction in litter size, impaired glucose tolerance	150,157, 164
	α1AT-hIGFBP-1	Liver	Reduced brain weight with structural alteration, reduced body weight gain, impaired glucose tolerance, reduced fecundity, proteinuria and glomerular lesions, impaired spermatogenesis, delay I bone mineralization	
	hIGFBP-1	Liver	Impaired glucose tolerance, abnormalities in insulin action, growth retardation in early postnatal life	155,165
IGFBP-2	CMV-mIGFBP-2	Ubiquitous	Reduced postnatal body weight gain, reduced bone size and mineral content, inhibition of hypertrophic and hyperplastic effects of GH/IGF-I excess on adrenocortical cells	151,168, 169
IGFBP-3	mPGK-hIGFBP-3	Ubiquitous	Reduction in birthweight, postnatal growth retardation, reduction in litter size, impaired glucose tolerance, significant negative effect on bone formation	152,156, 170
	CMV-hIGFBP-3	Ubiquitous	Reduction in birth weight, postnatal growth retardation, catch-up growth after puberty, reduction in litter size, increased adiposity, impaired glucose tolerance and insulin sensitivity, significant negative effect on bone formation	152,156, 170
	MT-hIGFBP-3	Kidney, intestine	Selective organomegaly, spleen, liver, heart	201
	WAP-hIGFBP-3	Mammary epithelium in late pregnancy and lactation	Reduced size of alveoli at peak lactation, inhibition of the gland from undergoing programmed remodeling and apoptosis after weaning	171
IGFBP-4	mSMP-rIGFBP-4	Smooth muscle cells (SMC)	Smooth muscle hypoplasia, significant reduction in wet weight of SMC-rich tissues, including bladder, intestine, aorta, uterus and stomach, with no change in total body or carcass weight	172,173
	hOCN-IGFBP-4	Osteoblasts	Markedly reduced cancellous bone formation and turnover and severely impaired overall postnatal skeletal and somatic growth	174
IGFBP-5	Cβ-actin-IGFBP-5	Ubiquitous	Pre-and postnatal growth retardation, neonatal mortality, reduced female fecundity, impaired myogenesis	153
	β-Ig-	Mammary	Impaired mammary development during	175

IGFBP	Transgene construct[1]	Transgene expression	Phenotypic manifestation	Reference
	rIGFBP-5	epithelium	pregnancy	
	rOCN-rIGFBP-5	Osteoblasts	Transient decrease in trabecular bone volume, impaired osteoblstic function and osteopenia	177
IGFBP-6	mGFAP-hIGFBP6	CNS, gonads	Pre-and postnatal growth retardation, reduced brain size, reduced female fecundity	154
ALS	CMV-hALS	Ubiquitous	Modest postnatal growth retardation, reduction in litter size	185

Table 3. Phenotypic manifestation of IGFBP overexpression in transgenic mice. [1]h = human, m = mouse, r = rat, c = chicken, MT = metallothionein promoter, PGK = phosphoglycerate kinase I promoter, α1AT = α1antitrypsin promoter, CMV = cytomegalovirus promoter, WAP = whey acidic protein promoter, SMP = smooth muscle actin promoter, OCN = osteocalcin promoter, β-lg = β-lactoglobin promoter

Initial observation of hyperglycemic effects of acute IGFBP-1 administration in rats[57] has not been reproduced recently with IGFBP-1 elevated to levels observed in various catabolic conditions in humans[58]. However, the latter study showed inhibition of protein synthesis in the fast-twitch skeletal muscle, thus supporting a role for IGFBP-1 in muscle catabolism under stress conditions[58]. In two different models of IGFBP-1 Tg mice with high level of expression, chronic exposure to elevated IGFBP-1 levels leads to development of impaired glucose tolerance, which supports the notion of IGFBP-1 inhibitory role of IGFBP-1 in regulation of IGF-I and insulin action *in vivo*[150,155,164].

In addition to impaired glucose tolerance, Crossey *et al.* studied effect of decidual IGFBP-1 excess on fetoplacental growth in Tg mice overexpressing human IGFBP-1 gene. In this model, human IGFBP-1 expression has the same pattern as in human tissues[155]. Endogenous fetal IGFBP-1 overexpression was associated with an impairment of fetal growth in midgestation. Maternal decidual IGFBP-1 excess was also associated with impaired fetal growth in independent of fetal genotype[165]. The authors further showed that decidual IGFBP-1 overexpression had a marked effect on placental development and demonstrated a predominantly maternal origin of IGFBP-1 in amniotic fluid. Placental morphology was abnormal in Tg females due to altered trophoblast invasion and differentiation[165]. These data are in agreement with prenatal growth retardation observed with ubiquitous overexpression of IGFBP-1[150] and also with human studies showing association between IUGR and elevated IGFBP-1[119-123].

5.2 IGFBP-2

IGFBP-2 KO mice express no IGFBP-2 mRNA and have no detectable IGFBP-2 in the adult circulation are viable and fertile[165]. Heterozygous and homozygous animals showed no significant differences in prenatal or postnatal body growth. Analyses of organ weights in adult males, however, revealed that spleen weight was reduced and liver weight was increased in the absence of IGFBP-2. In addition, ligand blot analyses of sera from adult IGFBP-2 null males showed that IGFBP-1, IGFBP-3, and IGFBP-4 levels were increased relative to wild-type mice. These results demonstrate that up-regulation of multiple IGFBPs accompanies the absence of IGFBP-2 and this may represent a partial compensation for IGFBP-2 deficiency. However, IGFBP-2 has a critical role, either directly or indirectly, in modulating spleen and liver size[166].

IGFBP-2 has higher affinity for IGF-II than IGF-I and IGFBP-2 levels are increased in IGF-II Tg mice suggesting regulation of IGFBP-2 levels by its primary ligand[167]. IGFBP-2 Tg mice with ubiquitous IGFBP-2 overexpression had ~3-fold elevation of serum IGFBP-2 levels whereas normal serum levels of IGF-I[151]. There were no apparent abnormalities of glucose metabolism. These mice demonstrated mild growth retardation (~10% by day 23) with mild impairment of linear growth and with reduced carcass weight and reduced brain size in later postnatal life. In an extension of their study, they overexpressed a mutated form of IGFBP-2 lacking RGD motif and showed that this domain is not essential for IGFBP-2 interaction with cell membrane *in vivo* and that the mutation of this sequence does not alter growth inhibitory effects of IGFBP-2[168]. Interestingly crossbreeding of giant PEPCK-bGH Tg mice overexpressing GH with IGFBP-2 Tg mice resulted in significantly lower body weight (by 17%), attenuated linear growth, and reduced organ size, compared to the giant mice[169]. These data indicate importance of tissue concentration of inhibitory IGFBPs for regulation of IGF and GH effects on tissue growth.

5.3 IGFBP-3

IGFBP-3 KO mice are viable and fertile. Ubiquitous overexpression of IGFBP-3 leads to an extensive phenotype with prenatal and postnatal growth retardation in the presence of elevated IGFBP-3 and IGF-I levels in circulation[152]. Significant reduction of organ size has been observed. The elevated IGFBP-3 levels perturbed glucose metabolism as evidenced by increased fasting glycemia and insulinemia and impaired glucose tolerance[156]. Glucose uptake into skeletal muscle *in vivo* was significantly decreased under both fasting and insulin-stimulated conditions. IGFBP-3

females had mildly reduced fecundity. IGFBP-3 overexpression had significant negative effect on bone formation *in vivo*, inhibited osteoblast proliferation derived from transgenic mice *in vitro*, and accelerated bone resorption in IGFBP-3 transgenic mice. Total and cortical bone mineral density has been significantly decreased in IGFBP-3 transgenic mice[170].

On the contrary, ubiquitous overexpression of mutant form of IGFBP-3, devoid of IGF binding affinity, had no measurable effect on pre- or postnatal linear growth or body weight acquisition. No alteration of glucose metabolism or organogenesis with exception of mildly reduced brain weight or glucose metabolism was observed in these mice (Silha & Murphy, unpublished data). These data support the notion that IGFBP-3 functions predominantly by attenuating IGF activity through IGF-dependent mechanisms *in vivo.*

In contrast, in Tg mice expressing human IGFBP-3 specifically in the mammary gland tissue, delayed involution of the gland with decreased number of apoptotic cells was observed. However, mammary gland development during puberty and pregnancy was normal[171]. This study demonstrated anti-apoptotic effects of IGFBP-3 *in vivo* and provides evidence that IGFBPs may act as a bifunctional, pro- and anti-apoptotic agents depending on tissue and developmental stage.

5.4 IGFBP-4

IGFBP-4 KO mice are viable, fertile and have no marked phenotype[149]. Fagin and coworkers used Tg mice with selective IGFBP-4 overexpression in smooth muscle to demonstrate importance of IGFBP-4 protease activity for regulation of IGF system *in vivo.*

Targeted expression of a protease-resistant IGFBP-4 mutant in smooth muscle resulted in greater growth inhibition of smooth muscle rich organs than equivalent levels of native IGFBP-4[172,173]. Targeted overexpression of IGFBP-4 to osteoblasts using a human osteocalcin promoter markedly reduced cancellous bone formation and turnover and severely impaired overall postnatal skeletal and somatic growth while intrauterine development was normal. These data are in accordance with inhibitory role of IGFBP-4 on IGF action *in vivo*[174].

5.5 IGFBP-5

IGFBP-5 KO mice are also viable and fertile and lack a significant phenotype[149]. Ubiquitous overexpression of IGFBP-5 resulted in IUGR, postnatal growth inhibition, reduced female fertility and retarded skeletal

muscle development in Tg mice which is consistent with ubiquitous overexpression of IGFBP-3, the most closely related IGFBP[152,153].

IGFBP-5 production by mammary epithelial cells increases dramatically during an involution of the mammary gland. In contrast to IGFBP-3, IGFBP-5 overexpression in mammary gland revealed decreased duct branching, cellularity, proliferation and increased apoptosis. These abnormalities could be corrected by treatment with IGF analogue with no affinity for IGFBP-5, suggesting that inhibition of IGF action in mammary gland is responsible for the phenotype of this transgenic model[175].

Targeted overexpression of transcription factor AP-2γ, which is involved in embryonic development but also in tumorigenesis, to mammary gland and seminal vesicles epithelium, identified IGFBP-5 as a target gene involved in coupling of increased proliferation and apoptosis in both tissues[176].

Transgenic mice overexpressing IGFBP-5 in the bone microenvironment have impaired osteoblastic function, osteopenia, and a transient decrease in trabecular bone volume secondary to reduced trabecular number and thickness and a transient decrease in bone mineral apposition rate[177].

5.6 IGFBP-6

IGFBP-6 KO mice are viable and fertile and appear normal[149]. IGFBP-6 Tg mice overexpressing IGFBP-6 under the control of GFAP have high levels of transgene in brain and gonads. These mice show marked reduction in brain size and reduced female fecundity, which is consistent with inhibitory function of IGFBP-6 *in vivo*[154]. Reduced fecundity of IGFBP-6 Tg females lends support to earlier studies suggesting antigonadotropin activity of IGFBP-6[154,178].

5.7 ALS

Inactivation of the ALS gene in mice results in mild retardation of postnatal growth despite profound disruptions in the circulating insulin-like growth factor system[114]. A modest growth deficit was observed in ALS KO mice after 3 weeks of life and reached 13% reduction in body weight by 10 weeks. This modest phenotype was observed despite reductions of 62 and 88% in the concentrations of plasma IGF-I and IGFBP-3, respectively. Increased IGF turnover accounted for these reductions because indices of IGF-I synthesis in liver and kidney were not decreased[114]. This study demonstrates importance of ALS for postnatal accumulation of IGF-I and IGFBP-3 in the circulation. However, ALS is not essential for growth. This phenotype of mild growth retardation in the presence of profoundly disrupted IGF system in circulation is in agreement with the manifestation of

ALS absence in humans and also with the phenotype of genetic nullification of hepatic IGF-I expression (LID mice)[135,179,180].

Genetic nullification of hepatic IGF-I expression using cre/lox system in LID mice leads to a 75% decrease in IGF-I, decreased IGFBP-3, and normal free IGF-I in circulation while IGF-I expression in peripheral tissues remains normal[179]. Although there are no significant differences reported on body weight acquisition in these mice, development of skeletal muscle insulin resistance[43,181], decreased adiposity[181], increased amyloid deposition in the brain[182], decreased myelopoiesis[183], and impaired cortical bone formation in these mice do suggest an important role for circulating IGF-I in these tissues[184]. Some of these effects, such as increased skeletal muscle insulin resistance and decreased adiposity, are also mediated by reciprocal increase in GH secretion in LID mice[43,181]. Therefore, these studies support a role of circulating IGF-I and its intact ternary complex in physiologic functioning of IGF system.

In ALS Tg mice, overexpressing ALS ubiquitously under the control of CMV promoter, mild growth retardation was observed[185]. The mild growth deficit in the absence of alterations in IGF system in circulation suggests a role for tissue-specific restriction of ALS expression in postnatal life. The growth deficit was probably caused by decreased IGF-I availability in tissues, possibly due to formation of the ternary complex in tissues. Indeed, double transgenic mice overexpressing ubiquitously in tissues both human ALS and IGFBP-3 manifested more pronounced growth retardation compared to IGFBP-3 Tg mice[185]. These data suggest an inhibitory role for tissue ternary complex in growth regulation and restriction of the ternary complex into circulation may therefore confer growth advantage.

6. SUMMARY AND CONCLUSION

IGFBPs regulate growth and development by regulating IGF transport to tissues and IGF bioavailability to IGF receptors at cell membrane level. IGFBP excess leads predominantly to inhibition of IGF action and growth retardation with impaired organogenesis. Absence of human and also mouse ALS leads to decreased IGF-I levels in circulation and causes mild growth retardation. Although IGFBP KO mice demonstrate relatively minor phenotypes, the possibility of compensatory mechanisms that mask the phenotypic manifestation of lack of individual binding proteins needs to be further investigated. Recent studies of hepatic regeneration in IGFBP-1 KO mice and also with mutant IGFBP-3 Tg mice provide some limited support for the existence of IGF-independent mechanism of action *in vivo*.

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Chapter 4

GROWTH HORMONE, INSULIN-LIKE GROWTH FACTORS AND THE FEMALE REPRODUCTIVE SYSTEM

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1. INTRODUCTION

Insulin-like growth factors (IGFs) promote coordinated somatic growth during fetal and postnatal development. These growth-promoting effects are mediated by the IGF-I receptor, which binds both IGF-I and IGF-II with high affinity. The IGF-I receptor is a membrane bound, ligand-activated tyrosine kinase that is structurally and functionally homologous to the insulin receptor and is very widely expressed in animal tissues. The two IGFs are abundant in the circulation and synthesized locally in many different tissues, including the reproductive system of most mammals. Given the widespread availability of the ligands and receptor, it is thought that interactions with diverse family of IGF binding proteins (IGFBPs 1-6) may modulate local IGF effects. These proteins bind IGF-I and II with high affinity, regulating their transport and presentation to the receptor and prolonging their half-life in the circulation and other fluid compartments[1].

Growth hormone (GH) stimulates the synthesis and release of IGF-I from the liver and some local tissues, and this circulating IGF1 mediates most of GH's effects on somatic growth. It appears that GH's major effects on reproduction are mediated by IGF-I, but evidence for some facilitory effects by GH on ovarian function, mediated directly by the GH receptor, will be

reviewed briefly. This chapter concentrates in main on the IGF system in mammalian ovary and uterus, comparing observations in rodent models, in large animals such as cow, pig and sheep and in the primate. Rodent models have been very informative because of the power of transgenics and targeted deletions, although their reproductive habits are very specialized and differ substantially from those of larger species, especially humans. Because of agricultural interest in promoting fertility and fecundity, there is abundant information on the IGF system in farm animals. Finally, we review somewhat more limited data available for humans and nonhuman primates. The rhesus monkey serves as a useful model for IGF actions in the human reproductive system[2]. While we are focused on mammals, it is interesting to note that a connection between insulin-like signaling and reproductive capacity is observed in evolutionarily distant species such as *C. elegans* and *D. melanogaster*, in which interruptions in insulin-like signaling pathways result in infertility[3].

The IGF system is apparently involved in all aspects of female reproduction, including ovarian, uterine, and placental growth and function and the neuroendocrinological integration of nutritional status and reproductive competency. This chapter focuses on IGFs in the ovary and uterus (the IGF system and placenta is the subject of another chapter in this book). The IGF system is also important in mammary gland development, but that subject is beyond the scope of the present effort. Finally, there is a huge body of literature on *in vitro* effects of IGFs on various types of reproductive tissues that have been reviewed[4], and this chapter concentrates primarily on *in vivo* studies.

2. THE OVARY

2.1 GH

Sexual maturation is delayed in states of GH deficiency or resistance, in both mice and men. This delay may be attributed to direct or indirect effects of GH or IGF-I on gonadotropin release as well as gonadal function. Ovarian development appears normal, however, in GH-deficient/resistant females, and they are generally fertile, although in mice, litter size is reduced due to reduced ovulatory rate[5,6]. Evaluation of ovaries of adult females at estrus revealed that follicles were normal in appearance and steroidogenic apparatus, but preovulatory follicles and corpora lutea were significantly reduced in number in GH receptor null mice. One of these studies reported decreased IGF1 mRNA levels in the GH receptor null ovaries[5] while the other found no decrease[6]. We found no alteration in ovarian IGF-I mRNA

concentration after hypophysectomy, using *in situ* hybridization to directly measure hybrids in defined granulosa cell populations[7]. Thus it remains somewhat unsettled as to whether the reduced follicular growth in GH deficient or resistant females is due to reduced circulating IGF-I, reduced local IGF-I or to a non-IGF-I mediated effect of GH. While most of the anabolic effects of GH on ovarian function appear secondary to IGF-I, the presence of GH receptor expression in the ovary[8,9] leaves open the possibility for direct effects by GH on ovarian function not mediated by IGF-I.

GH treatment has been found to reduce the dose of exogenous gonadotrophin required to enhance folliculogenesis in women with hypogonadotrophic hypogonadism, but there is little evidence that GH is of clinical benefit in the management of patients with other ovulatory disorders, including polycystic ovary syndrome (PCOS), or in superovulation protocols for *in vitro* fertilization[10].

2.2 IGFs and IGF-I receptor

IGFs appear to be important regulators of ovarian follicular growth and selection, and luteal steroidogenesis. The rat and mouse have served as important experimental models for elucidation of IGF-I's role in folliculogenesis. The IGF-I receptor is highly expressed in the oocyte, in granulosa cells of follicles at all stages, from small primary to mature preovulatory follicles, and corpora luteal cells in both rat and mouse[7,11]. IGF-I expression is more selective, however, being limited to the granulosa of healthy, growing and selected follicles[7,12]. Ovarian IGF-I expression is selectively localized in follicle-stimulating hormone (FSH) receptor expressing follicles but does not appear to be regulated by gonadotropin or GH, while IGF-I receptor expression is dramatically increased by gonadotropin[7,13]. Local IGF-I expression is significantly correlated with granulosa cell proliferation in the murine ovary[14] and IGF-I receptor expression is correlated with high-level metabolic activity, reflected by glucose utilization (Fig. 1). It seems likely that increased energy requirements, associated with rapid follicular growth, in the case of pre-ovulatory follicles, and steroidogenesis, in the case of corpora lutea, are met by enhanced "insulin-like" IGF-I effects promoting nutrient acquisition at the follicular level.

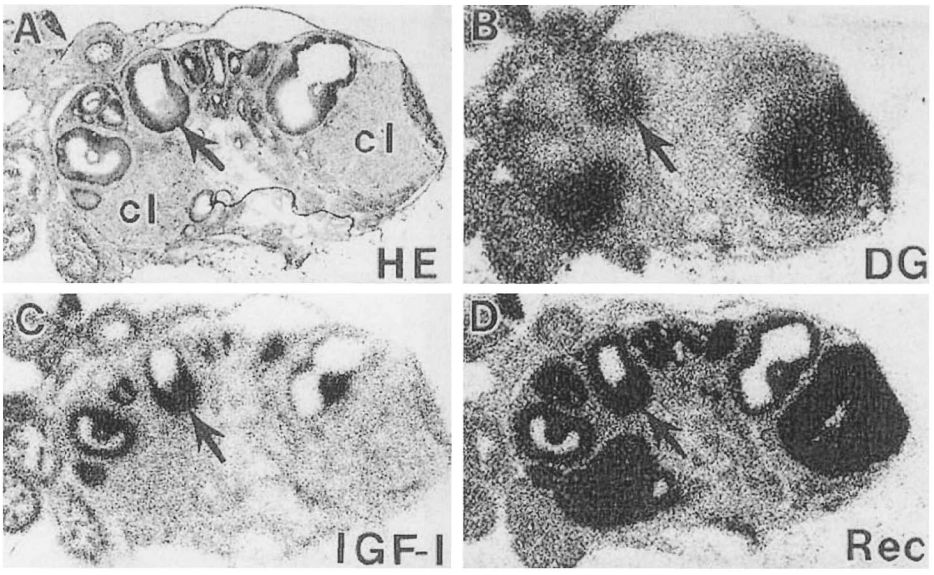

Figure 1. Ovarian IGF-I receptor expression reflects metabolic activity. Panel **A** shows an H&E stained section from a mature rat ovary and panels **B-D** are film autoradiographs from sequential ovary sections. **B** shows C-14-labeled 2-deoxyglucose uptake; **C** shows IGF-I and **D** IGF-I receptor mRNA by *in situ* hybridization. cl; Corpus luteum.

The cellular patterns of IGF-I and IGF-I receptor expression in the normal murine ovary correlate well with *in vitro* studies showing that IGF-I promotes proliferation in granulosa cells from the smallest follicles and steroid production in luteinized granulosa cells[4,15]. If, as we suggest, IGF-I's fundamental effects are to promote nutrient utilization, then IGF-I should effectively enhance whatever cellular processes are in progress. In the case of the murine ovary, local, granulosal IGF-I production promotes granulosa proliferation and hence follicular growth, while circulating IGF1 may act on the corpus luteum (CL) to promote steroidogenesis (Fig. 1). IGF-II is minimally expressed in the murine ovary, primarily in perifollicular blood vessels[2], and IGF-II deletion does not impair fertility in the mouse. It should be noted that IGF-II is much less abundant in the circulation and tissues postnatally in murine species than in other mammals, where IGF-II is significantly more abundant than IGF-I.

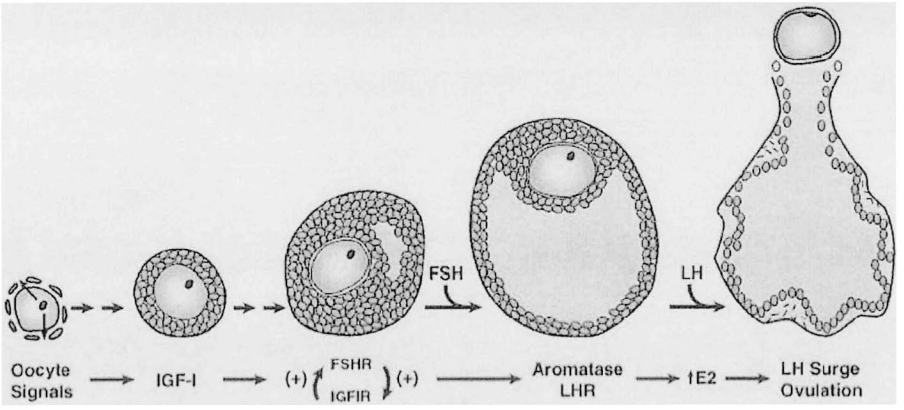

Figure 2. Schematic illustrating IGF-I's proposed role in follicular development. Oocyte autonomous signals are hypothesized to initiate granulosa cell proliferation and IGF-I production. IGF-I augments FSHR expression and FSH augments both IGFIR and FSHR expression. This mutual complementary positive feedback loop within the follicle is hypothesized to be critical for the amplification of FSH action to induce the formation of mature graafian follicles. In the absence of IGF-I or FSH, follicles arrest at a preantral/early antral stage of development. Maximal FSH action leads to mature antrum formation and granulosa cell aromatase and LHR expression. The resulting peak in follicular E2 synthesis stimulates an LH surge, which in turn stimulates ovulation. For the sake of simplicity, thecal layer development, which is also impaired in the IGF-I knockout mouse, has been omitted from the diagram. From ref. 13.

Targeted IGF-I gene deletion mice have small ovaries, consistent with their dwarf size; these ovaries appear anatomically normal with a full complement of primordial follicles, as determined for two independent deletions in different mouse lines[13,16]. Thus, IGF-I apparently is not essential for the normal development or differentiation of the ovary. IGF-I null mice are infertile, however, owing to impaired follicular growth, since although primordial follicle numbers are normal in *IGF-I*$^{-/-}$ mice, few antral follicles are evident, no pregnancies occur and no corpora lutea are formed[13]. Moreover, these IGF-I null mice do not ovulate in response to gonadotropin hyperstimulation[16]. We showed that glucose transporter, FSH receptor and aromatase expression were all significantly reduced in granulosa cells of the IGF-I null ovary[13,17], and that administration of IGF-I systemically restored their expression to wild-type levels, suggesting that the reduced gonadotropin responsiveness of these mice may be at least partially explained by reduced FSH receptor expression. Based on these observations, we proposed that local IGF-I production and reception promotes an amplification cycle as illustrated in Fig. 2. Local IGF-I produced by granulosa cells in small growing follicles enhances FSH receptor expression

and thus follicle response to FSH. FSH, in turn, stimulates more IGF-I receptor expression providing a positive feedback loop allowing rapid follicular growth. The specific molecular pathways engaged by IGF-I and its cognate receptor in the granulosa cell have been elucidated and reviewed by JoAnne Richards[18]. She has identified major signaling targets of IGF-I receptor action in the ovary, including the PI-3 kinase cascade, PKB/Akt and SGK, and members of the forkhead transcription factor family. She has also demonstrated that estradiol participates in the growth amplification cycle we have outlined earlier by augmenting IGF-I receptor expression.

The ovarian IGF system has been investigated in large farm animals such as pig, cow, and sheep as recently reviewed[19]. Interestingly, the sow is the only large animal that selectively expresses IGF-I in growing follicles similar to the murine example[20], and is also similar to murine species in being polytocous, in contrast to cattle and sheep. IGF-I and IGF-I receptor are selectively concentrated in healthy follicles, characterized by expression of aromatase and the gonadotropin receptors, in the sow ovary (Fig. 3). IGF-I expression does not characterize the emergence of ovulatory follicles in any of the other species that have been examined to date, although increasing IGF-I concentration, presumably derived from the circulation, is described for dominant follicle formation in cattle[21]. IGF-II is expressed in the theca-interstitial compartment of porcine, bovine, and ovine and primate ovaries and is abundant in the fluid obtained from large follicles of these species, as is IGF-I, apparently derived from the systemic circulation[19,22]. Evidence suggests that circulating IGF-I plays a role in follicular growth in domestic animals, since reduction in GH secretion and hence IGF-I levels leads to reduced follicular development, while increased IGF-I levels enhance follicular growth[23-25].This may be relevant to nutritional aspects of reproduction in farm animals, since nutritional deficiencies are associated with reduced circulating IGF-I levels and reduced follicular maturation/ovulation[24,26].

We have investigated IGF system expression in the nonhuman primate (rhesus macaque) and human ovary. As reported above for monotocous farm animals, IGF-I expression is not normally detected in rhesus or human growing follicles while IGF-II is expressed in the theca-interstitial compartment without apparent selectivity for follicle or cycle stage (Fig. 4B)[27-29]. IGF-II is, however, expressed in the membrana granulosa of large pre-ovulatory and ovulatory follicles in the monkey (Fig. 4J). We are not sure whether this IGF-II is expressed by granulosa cells or by invading stromal elements in the periovulatory follicle. As in all the other species, the IGF-I receptor is abundantly expressed in developing follicles and corpora lutea[28].

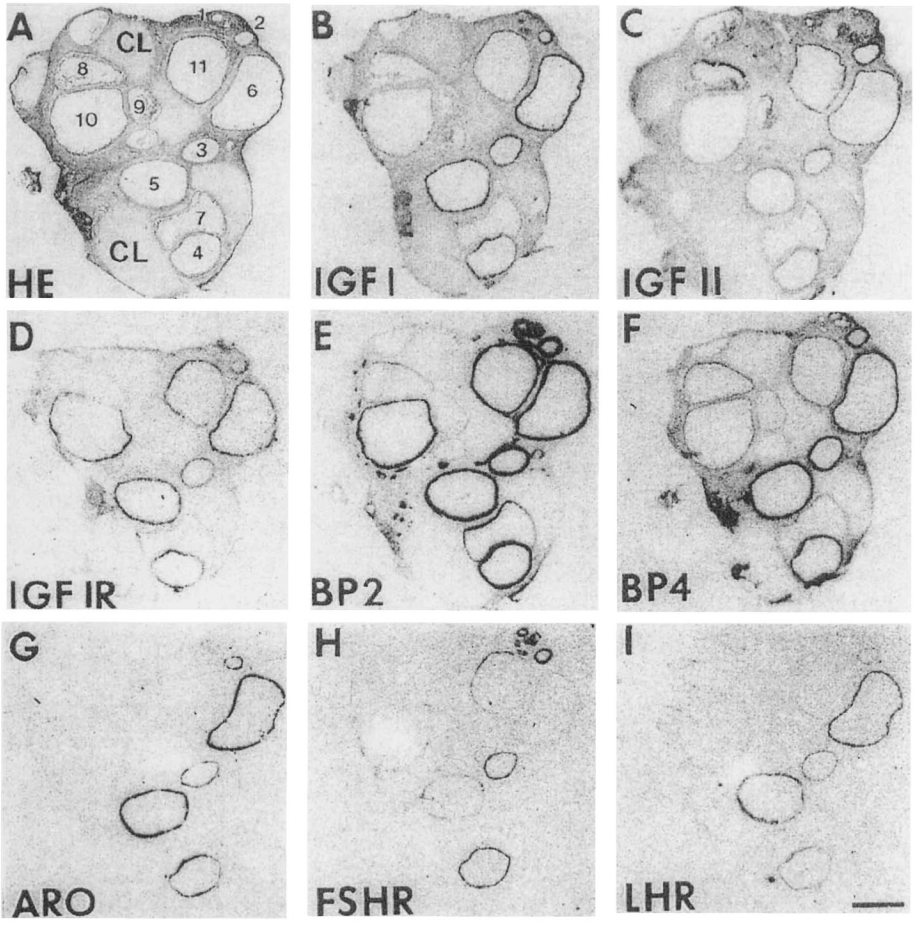

Figure 3. IGF system gene expression in the mid-follicular (day 16) gilt ovary. Panel **A** is an H & E-stained section and panels **B–I** are film autoradiographs of sequential sections hybridized to antisense RNA probes for the indicated mRNAs. Follicles 1-6 are healthy growing follicles of various sizes. Follicles 7-9 are clearly atretic as demonstrated by shredding of the membrana granulosa and by loss of the follicle's oval shape. Follicles 10 and 11 appear healthy by morphological criteria but are presumed to be destined for atresia based on absence of gonodotropin receptor or aromatase mRNA. ARO, aromatase; FSHR, FSH receptor; IGFIR,IGF-I receptor; LHR , LH receptor. Scale bar = 2.5 mm. From ref. 20.

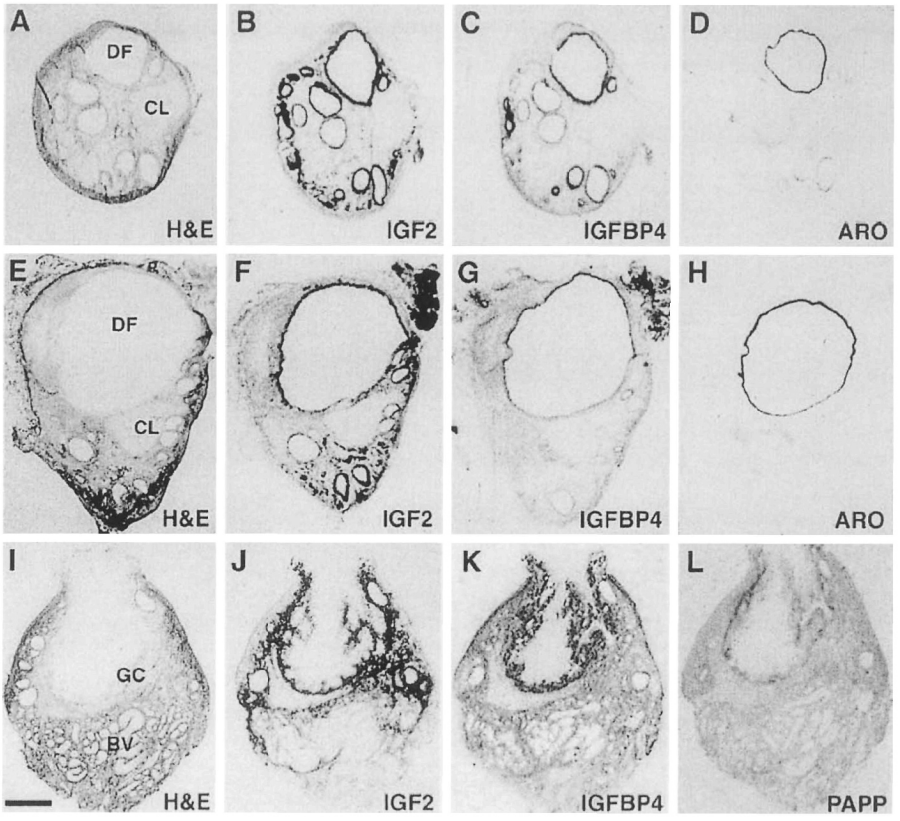

Figure 4. IGF-II and IGFBP4 gene expression in normal mid-cycle (day 14) rhesus monkey ovaries. On the extreme left of each row are H&E-stained ovary sections and the subsequent panels show film autoradiographs of sequential sections hybridized to cRNA probes for IGF-II, IGFBP4 and aromatase, or in panel **L**, the IGFBP4 protease, PAPP-A. A single dominant follicle (DF), defined by maximum cross-sectional diameter >2 mm and aromatase expression, is apparent in each ovary. The emerging DF in **A–D** has the highest aromatase expression, but other, smaller follicles still have discernible aromatase and IGFBP4 expression (**C and D**). In the more advanced DF seen in **E–H**, aromatase and IGFBP4 expression are restricted to the DF, while IGF-II mRNA is still evident in numerous smaller follicles. The DF in panel **I** is in the process of ovulating, and IGF-II, IGFBP4, and PAPP-A mRNAs are abundant in the exfoliating membrana granulose (J–L). This ovary is cut through the ovarian pedicle and the profusion of engorged blood vessels (BV) is quite impressive. DF, dominant follicle; CL, Corpus luteum. Scale bar = 0.8 mm. From ref. 29.

2.3 Androgen and the ovarian IGF system

While IGF-I is generally below detection limits in the normal primate ovary, increased IGF-I and IGF-I receptor expression are found in ovaries from androgen-treated monkeys[30]. We investigated the effects of androgen

on the monkey ovary to test the hypothesis that androgens may contribute to the development of the polycystic ovary syndrome[31]. Small preantral and antral follicles were significantly and progressively increased in number and thecal layer thickness in testosterone and dihydrotestoserone treated monkeys over 3–10 days of treatment. Granulosa and thecal cell proliferation, as determined by immunodetection of the Ki67 antigen, are significantly increased in these follicles. Androgens appear to boost the recruitment of resting, primordial follicles into the actively growing pool, with the number of primary follicles significantly increasing over time during androgen treatment[32]. Androgen treatment results in a three-fold increase in IGF-I and five-fold increase in IGF-I receptor mRNA in primordial follicle oocytes[32], suggesting that androgens may promote initiation of primordial follicle growth by augmenting IGF-I activity in the primordial follcile. In addition, androgen treatment results in significant, 3-4-fold increases in IGF-I and IGF-I receptor expression in granulosa, thecal and interstitial compartments (Fig. 5). Interestingly, FSH receptor expression was increased in parallel with IGF-I receptor in these androgen-treated animals[33] (Fig. 6), suggesting that in primates as in rodents, IGFs act to enhance FSH receptor expression and responsiveness (see Fig 2). Follicular atresia was not increased and there were actually significantly fewer apoptotic granulosa cells in the androgen-treated groups[31]. These findings show that, over the short term at least, androgens enhance IGF-I and IGF-I receptor expression along with follicular growth and survival in the primate ovary, suggesting that IGF-I and its cognate receptor may mediate androgen-induced ovarian follicular and thecal-interstitial growth.

The mechanism whereby androgens promote primordial follicle development appears to involve activation of oocyte IGF1 signaling. The IGF-I receptor is abundant in oocytes from all species investigated[7,11,20,28] and IGF-I stimulates oocyte metabolic activity and maturation *in vitro*[34,35]. Thus, enhanced IGF-I signaling through increased expression of both the peptide and its receptor in oocytes from androgen-treated monkeys may trigger oocyte activation and initiation of follicle growth. These observations provide a plausible explanation for the pathogenesis of "polycystic" ovaries in hyperandrogenism. Pathological studies show increased numbers of growing follicles, including primary follicles, in ovaries from hyperandrogenemic women with polycystic ovary syndrome[36]. Increased follicle numbers are also seen in testosterone-treated women[37,38] showing that androgens, whether derived from ovary, adrenal or exogenous sources, may stimulate excessive ovarian follicular growth. Further support for this notion is provided by the observation that treatment of women with PCOS with androgen receptor blockade results in diminution of follicle numbers[39].

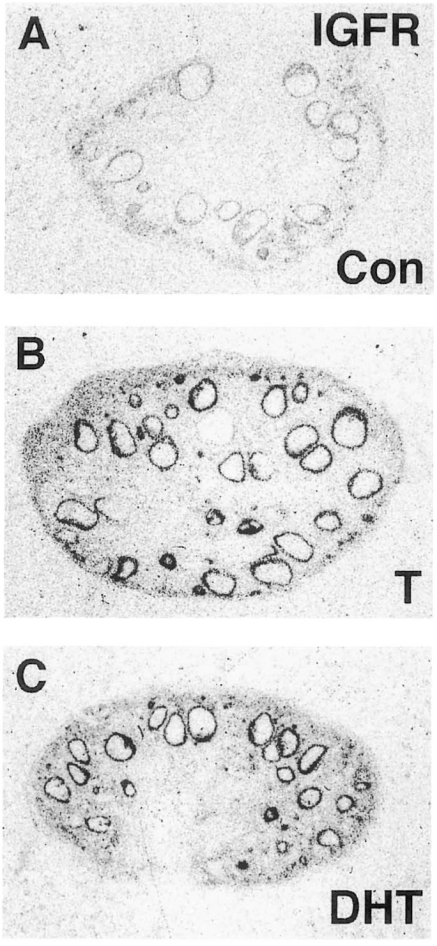

Figure 5. Effect of androgens on IGF-I receptor mRNA in the monkey ovary. Representative film autoradiographs illustrate in *situ* hybridization results from control, placebo-treated (**A**), testosterone-treated (**B**), and DHT-treated (**C**) monkeys. The IGF-I receptor films were exposed for 5 days. From ref. 30.

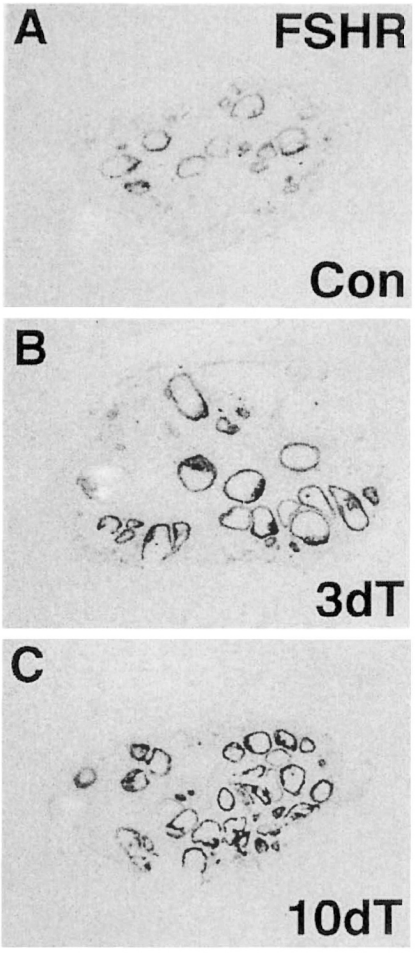

Figure 6. Increased FSHR gene expression in follicles from testosterone-treated monkeys. Representative film autoradiographs from follicular phase control (**A**), 3 day testosterone-treated (**B**), and 10 day testosterone-treated (**C**) monkeys are shown. Note the increased number of follicles in the androgen-treated ovaries. Bar = 2

In summary, although very little IGF-I is detected in the normal primate ovary, both IGF-I and IGF-I receptor expression are greatly increased by exposure to elevated androgen levels. In the model for generation of PCOS illustrated in Fig. 7, the polycystic ovarian phenotype may result from at least 2 different pathways. Excess systemic androgen levels, whether from adrenal hyperplasia, tumors or exogenous administration may stimulate increased follicle numbers and stromal growth, presumably acting through augmented IGF-I and IGF-I receptor activation. Alternatively, insulin resistance and hyper-insulinism may increase bioavailable IGF-I and testosterone by reducing the hepatic production of IGFBP1 and SHBG, so that increases in circulating free testosterone and free IGF-I may stimulate ovarian growth. Increased follicle numbers and stromal tissue result in increased production of inhibin and androgen, suppressing gonadotropins and impairing cyclic function. This scenario may explain why some women with PCOS respond to gonadotropins with hyperstimulation, since they have increased numbers of small follicles and enhanced FSH responsiveness. It also helps to understand why wedge resection of the ovary may be effective in normalizing cycles in women with this ovarian phenotype, presumably reduction in follicle number and stromal volume reduces excessive inhibin and androgen production, allowing resumption of normal gonadotropin entrainment of the ovary.

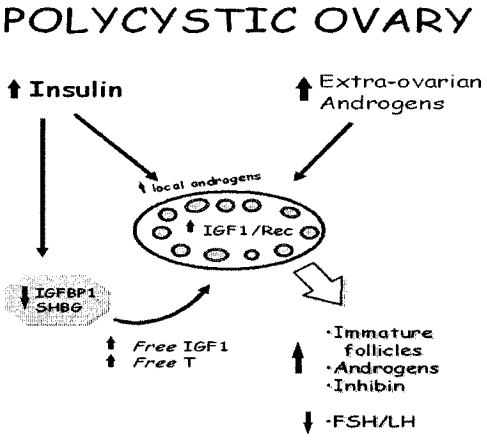

Figure 7. Potential routes to the polycystic ovary phenotype. Increased circulating insulin secondary to adiposity or "extreme" insulin resistance may have direct effects on the ovary to promote follicular growth and androgen production, and also suppresses hepatic production of SHBG and IGFBP1, resulting in increased free androgen and IGF-I levels in the circulation, which may then act on the ovary to promote follicular growth and androgen production. High circulating androgen derived from adrenal or exogenous sources stimulate the ovary directly, promoting early follicle growth and stromal hypertrophy through enhanced local IGF-I/IGF-I receptor expression. By either pathway, increased numbers of immature follicles produce androgen and inhibin, interfering with gonadotropin dynamics.

2.4 IGFBPs

IGFBPs 2–5 are expressed in diverse patterns in the rat and mouse ovary[11,40,41]. For example, IGFBP2 expression is confined to the ovarian stroma in the rat[11,40] but is expressed by granulosa cells in healthy follicles of mice[11,41]. IGFBP4 is found in atretic follicles in both rat and mouse[40,41], while IGFBP5 is reportedly localized in atretic follicles in rats[40,42] but not in mice[41]. Based on *in vitro* studies showing that IGFBPs inhibit IGF1's effects, it was thought that follicular IGFBP expression may lead to atresia. However, mice with targeted gene deletions for IGFBPs 2-5 display no obvious reproductive phenotype (43 and unpublished observations, J.E. Pintar). Given the variability in expression patterns and apparent insensitivity to deletion, it seems most likely that IGFBPs play dispensable, modulatory roles in murine ovarian function that are compensated by other IGFBPs or that are too subtle to be easily detected in the knockout models.

In larger animals and humans, there is also considerable variability is ovarian synthesis and circulating levels of IGFBPs. For example, equine granulosa cells produce only IGFBPs 2 and 5[44], while porcine granulosa produce IGFBPs 2, 3, 4 and 5[45]. Furthermore, physiological regulation of ovarian IGFBP expression also appears to be species specific[22]. IGFBP levels have been intensely investigated in follicular fluid, mainly by ligand binding studies. IGFBP levels generally are reported to decrease in dominant follicles and increase in atretic follicles, although IGFBP4 is found to increase markedly in ovulatory follicles in the sow[46]. In the sow, IGFBP2 mRNA is abundant in granulosa cells of small and preovulatory follicles and less abundant in atretic-appearing follicles (Fig. 3). IGFBP4 is concentrated in the theca interna of healthy, medium to large growing follicles, characterized by IGF-I, aromatase, and gonadotropin receptor expression, but is not detected in atretic follicles (Fig. 3). IGFBP4 appears in granulosa cells of peri-ovulatory follicles showing evidence of luteinization and luteinizing hormone (LH) receptor expression, consistent with the increase in IGFBP4 found in follicular IGFBP4 levels[46]. IGFBP4 is also abundant in the sow corpora lutea[20] and most other animals.

In the rhesus monkey, IGFBP4 expression is selective for LH receptor-expressing, steroidogenic thecal and granulosa cells of healthy follicles (Fig. 4) and is stimulated by the LH analog human chorionic gonadoropin (hCG) (Fig. 8). LH/hCG also increases IGFBP4 expression in the bovine and murine ovary[28,41,47]. The observation that IGFBP4 gene expression is selective for healthy follicles is supported by demonstration of thecal and granulosa cell proliferation, LH, and FSH receptor and aromatase expression in these follicles (Fig. 4). IGFBP4 is also expressed by healthy theca interna and corpora lutea in human ovaries[27,28]. Thus LH/hCG stimulates ovarian IGFBP4 expression in rodents, large animals, and primates, and IGFBP4 expression closely parallels LHR expression in healthy developing follicles

and corpora lutea. These observations suggest a role for IGFBP4 in LH-induced steroidogenesis or luteinization, rather than in inhibiting follicular development.

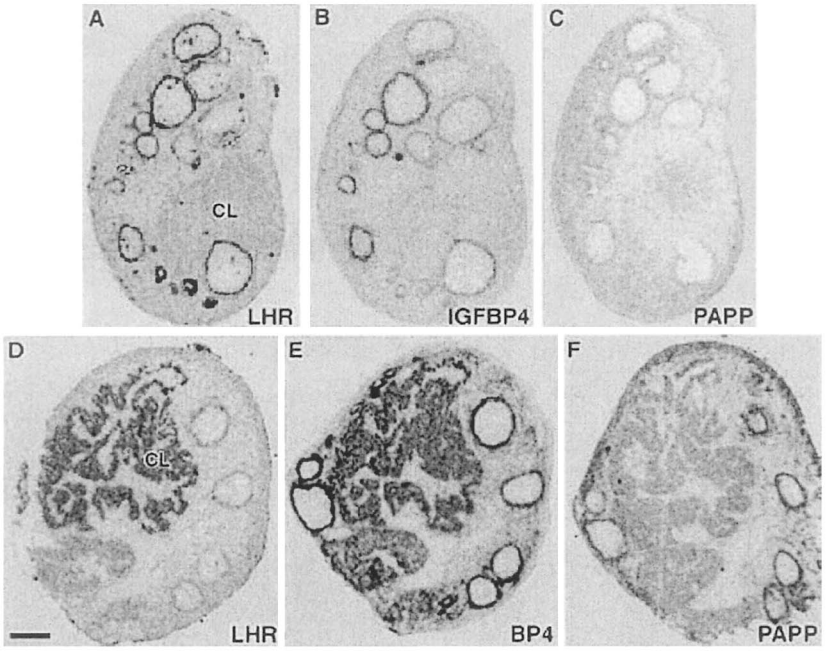

Figure 8. hCG increases IGFBP4 gene expression. Representative film autoradiographs comparing LHR, IGFBP4, and PAPP-A mRNA localization in sequential sections from an untreated mid-cycle monkey ovary (**A-C**) and from an hCG-treated mid-cycle monkey ovary (**D-F**). Both are nondominant ovaries with relatively "fresh" corpus lutea from the previous cycle. IGFBP4 and PAPP-A mRNAs are selectively localized in LHR-expressing follicles but little IGFBP4 or PAPP-A mRNA is detected in the corpus luteum (CL) of the untreated monkey ovary (as also seen in Fig. 1C). After hCG treatment, however, IGFBP4 and PAPP-A expression are abundant in the CL, and increased also in the antral follicles (**E and F**). There are a couple of small scars of old CL at the lower pole of the ovary which are also demonstrating heightened IGFBP4 and PAPP-A expression. Bar = 1 mm. From ref. 29.

The monkey ovary is replete with IGFBPs in addition to IGFBP4, although none of the others demonstrates an obvious connection with follicular development or sensitivity to gonadotropin regulation[48]. IGFBP1 is detected in theca-interstitial cells and at low levels in granulosa cells of atretic follicles. IGFBP2 is abundant in the ovarian surface epithelium and in granulosa cells of all antral follicles, including obviously atretic as well as dominant follicles. IGFBP3 is localized in oocytes, in the ovarian vascular endothelium, and the superficial cortical stroma where it is distinctly more

abundant in the nondominant ovary. IGFBP5 is selectively expressed by granulosa cells of mature ovulatory follicles in the monkey ovary[48]. IGFBP5 is also widely expressed in the ovarian stroma, where, in contrast to IGFBP3, it is distinctly more abundant in dominant compared with non-dominant ovary. IGFBP6 mRNA is detected at low levels in the ovary interstitium and theca externa, and in the ovary surface epithelium. These complex, differential patterns of IGFBP expression in the primate ovary certainly suggest that IGFBPs play some role in ovarian function, but this role(s) remain poorly understood.

A recent refinement of the notion that IGFBPs promote follicular atresia involves the idea that selective IGFBP proteolysis may promote formation of ovulatory follicles by increasing "free" or bioavailable IGFs[47,49,50]. This theory is based in large part upon findings of IGFBP fragments and increased free IGF-I in the follicular fluid of dominant follicles of cattle. IGFBP4 has been intensely studied in this context. One enzyme implicated in IGFBP4 proteolysis in follicular fluid is pregnancy-associated protein (PAPP)-A[47,51]. This enzyme displays proteolytic activity against IGFBP4 only in the presence of IGF-I/-II and is present in the circulation of pregnant women bound to a protein known as the proform of the major eosinophil basic protein (proMBP), which inhibits its proteolytic activity. The source and function of this protein complex in the circulation is unclear. The PAPP-A in ovarian follicular fluid is produced by granulosa cells and may be associated with proMBP. A complex series of inhibitory interactions has been proposed to explain the role of IGFBPs, in particular IGFBP4, in folliculogenesis, as illustrated in Fig. 9. IGF activation of its receptor is thought to be inhibited by IGFBP4 until such time as an IGFBP4 protease is unleashed from *its* inhibitor, allowing binding protein degradation. Although PAPP-A has received much attention, many other proteases and protease inhibitors are found in follicular fluid, particularly around the time of ovulation.

There are some unresolved issues related to this theory. If IGFs are in fact important effectors of follicular selection, then selective regulation of IGF expression would seem to be a more direct, economical mechanism than installing multiple layers of inhibitory factors. In fact, IGF-I expression appears to be selective for healthy follicles in murine species and swine, although not in ruminants or primates. It seems unlikely that IGFBP4's highly selective expression by steroidogenic cells in healthy follicles and corpora lutea and regulation by LH are consistent with a role in follicular demise. Why is IGFBP4 gene expression concentrated in steroidogenic cells of healthy developing follicles and corpora lutea (at least in the sow and rhesus monkey), if its role is to inhibit follicle function? Why would LH stimulate the production of a factor that inhibits steroidogenesis and follicular selection? Finally, IGFBP2 is abundantly expressed by follicles in all stages of development in the sow, mouse, and monkey ovary, not particularly suggestive of an inhibitory role.

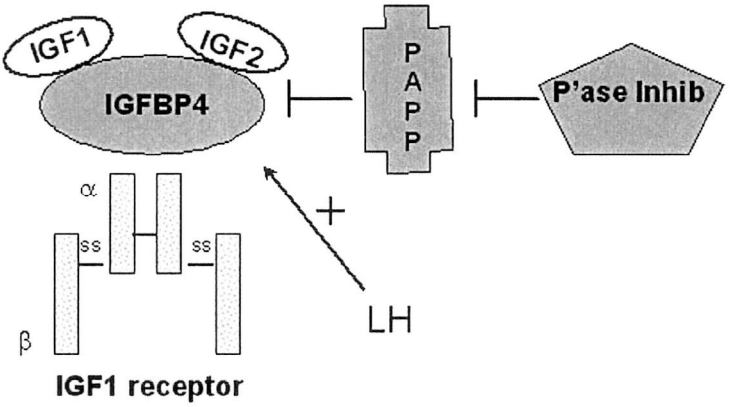

Figure 9. Illustration of the proposed IGFBP inhibitory network governing follicular IGF activity. The presence of intact IGFBPs, IGFBP4 in this example, is expected to inhibit IGF activation of the IGF-I receptor. IGFBP proteases such as PAPP-A are also present in the ovary and circulation, but may be inhibited by proteins such as proMBP. Follicular selection is proposed to occur when proteolysis of IGFBP liberates IGFs to interact with their receptor.

Most support for the view of IGFBPs as inhibitors of follicular selection is derived from studies of follicular fluid contents; however, interactions between IGFs and their receptor take place at the plasma membrane. In the developing follicle, IGF-I target cells exist in complex, interlocking local microenvironments comprised by the membrane granulosa and theca interna. IGFBPs produced by granulosa and thecal cells are expected to decorate cell membranes and the extracellular matrix within these highly specialized compartments. Thus the scene of action for IGFs produced by granulosa cells or entering the follicle from the blood stream will be in the membrane granulosa or theca interna, where binding proteins may reside to protect them from proteolysis, provide local storage sites, present the peptides to the receptor, or selectively inhibit their interaction with the receptor. It is not clear that the contents of the follicular fluid accurately reflect these interactions between IGF system components and cells of the functional follicular compartments. The increased proteolysis of IGFBPs noted in periovulatory follicles may simply reflect the general increase in protease activity as follicles prepare for rupture and transformation to corpora lutea. Nevertheless, given the convincing correlations between accumulation of free IGF-I and loss of intact IGFBPs in dominant follicle fluid from well-studied species such as cattle, it seems quite possible that there are important species differences in the mechanisms employed for emergence of ovulatory

follicles. Species that depend more on circulating than locally produced IGF for follicular growth may control emergence of a single dominant follicle through local regulation of IGFBP and protease production.

2.5 Summary on IGFs and ovarian function

Our understanding of the IGF role in ovarian function is most advanced in murine species, where IGF-I expression selectively characterizes growing, healthy follicles, and in which *IGF-I* gene deletion results in critically impaired follicular growth and infertility. While granulosa proliferation, glucose transport, and FSH receptor and aromatse expression are reduced in these mice, the fundamental molecular defects due to loss of IGF-I effect in follicular development remain unknown. Although IGFBPs are abundantly expressed in the murine ovary, targeted deletions of IGFBPs 2–5 have no apparent effect on female fertility, although situational roles, *e.g.*, reproductive adaption to stress, cannot be excluded. In domestic animals and primates, including humans, the story is less clear. The IGF-I receptor is highly expressed in developing follicles and corpora lutea of all species examined, suggesting that IGF-I or -II, whether produced locally in the ovary, or derived from abundant circulating reservoirs, effect ovarian follicular development and luteal function. However, in the absence of *IGF-I* or *IGF-II* gene deletions, we do not know if these are critical or simply facilitory effects. Further studies are required to elucidate the role of IGFBPs in ovarian function.

3. UTERUS

3.1 IGF-I as "estromedin"?

The uterus undergoes dramatic, sex-steroid regulated growth at puberty, during each menstrual cycle, and with pregnancy. Estrogen stimulates the production of IGF-I by uterine stromal cells[52-54], and the IGF-I receptor is highly expressed by uterine epithelium[54,55], making local IGF-I a likely candidate as mediator of estrogen's mitogenic effects in the uterus. This hypothesis has recently been examined in the IGF1 gene deletion mouse. The uterus is anatomically normal but extremely small in the IGF-I null mouse, as described in two independent deletions in different strains of mice[16,56]. This uterine hypoplasia could be due to diminished estrogen effect, since, as noted above, IGF1 null ovaries show poor follicle development and deficient estrogen production. Treatment of these mice with exogenous estrogen, however, failed to produce uterine growth[56,57]. Interestingly, DNA

synthesis was increased in the IGF-I null uterus comparable to that in similarly treated wildtype mice (Fig. 10), but cell size was smaller and mitotic figures were fewer in the IGF1 null mice[56]. These observations are especially interesting because IGF-I is usually regarded as a G_1 phase promoter, but uterine cells were arrested in G_2 in the estrogen-treated IGF1 null mice[56]. The retardation in mitoses was correlated with delayed cellular somatic growth in the absence of IGF-I, suggesting that a G_2 checkpoint based on cell size, similar to that seen in yeast, may explain the mitotic delay in the IGF-I null mice.

A recent study investigated whether transplantation of IGF-I null uteri into athymic female hosts could restore normal growth patterns[57]. In effect, the experiment tested whether exposure to circulating IGF-I in the normal host could normalize growth in an IGF1 null uterus. In fact it could, since the IGF-I null uteri reached normal size that matched the host uterine weight and epithelial cell size, 4 weeks after transplantation. Thus it appears that absence of locally produced IGF-I in the IGF-I null uteri did not prevent normal uterine growth in the context of normal circulating IGF-I1 and estrogen in the hosts. In addition, normal wild-type uteri were transplanted into estrogen-treated IGF-I null mice, where they demonstrated very little growth, suggesting that local, uterine IGF-I production is not sufficient to allow normal growth in the absence of circulating IGF-I[57]. These interesting observations suggest that circulating rather than locally produced IGF-I may be critical for mediation of estrogen's growth promoting effects upon the uterus in the mouse.

Another recent study approached the elucidation of estrogen–IGF-I interaction in uterine growth from another angle. These experiments treated ovariectomized, estrogen receptor-alpha null (ERKO) and wild-type (WT) mice with IGF-I to determine if IGF-I could stimulate uterine growth in the absence of estrogen effect[58]. IGF-I administration was reported to stimulate proliferation in WT, but not in ERKO uteri, suggesting that IGF-I's effects on uterine growth require ER-α. An issue with these observations, however, is that the degree of uterine proliferation induced by IGF-I treatment of WT mice appears to have been very low, and moreover was not quantified, so that it is unknown whether there were significant differences in IGF-I-induced proliferation between WT and ERKO uteri. We have not found any effect of IGF-I administration on uterine proliferation in WT or IGF-I null mice, and others have reported that IGF-I does not stimulate proliferation of uterine tissue in the absence of sex steroids[59]. Thus one possible explanation for these observations in different knockout models is that estrogen, acting through ER-α, triggers entry into the cell cycle and progression through S-phase in uterine cells – independent of IGF-I, but that local or systemic IGF-I is required to complete mitosis in a timely manner. The IGF-I requirement

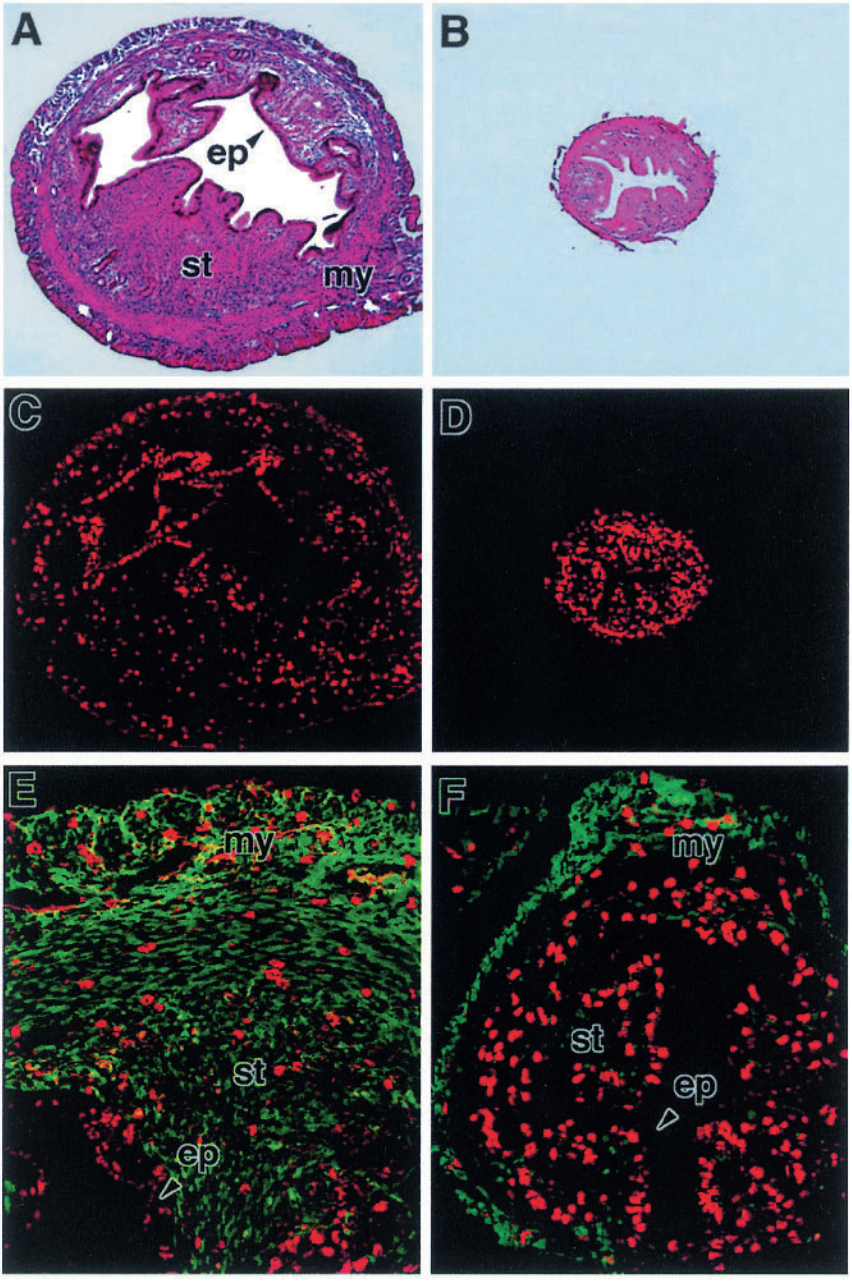

Figure 10. Autoradiographic visualization of E2-induced DNA synthesis in WT (**A, C, E**) and *Igf1* null (**B, D, F**) uteri. **A and C** and **B and D** are paired bright- and dark-field photomicrographs. **E and F** are higher magnification views showing localization of tritium-positive red nuclei in epithelium (ep), stroma (st) and myometrium (my). Mice were given a single intraperitoneal injection of 17-beta-estradiol (1 μg/g) at 0 h and injected with 3H-thymidine (2 μCi/g) one hour before sacrifice at 21 h. From ref. 56.

may, as a matter of speculation, be due to its anabolic effects on uterine cell growth required for cytokinesis. Previous *in vitro* work in cell lines has shown that IGF-I and other peptide growth factors may activate ER-α by phosphorylation, in the absence of ligand[60]. The recent observations in ERKO mice are taken as evidence that such cross-talk also takes place *in vivo*, *i.e.*, that IGF-I activation of ER-α is required for IGF-I's mitogenic effects[58]. However, as noted above, IGF-I does not have obvious mitogenic effects in the absence of estrogen in WT, ER-α expressing animals.

IGFBPs are also expressed in the murine uterus, but there has not been extensive study of this aspect of the IGF system, and as noted above, mice with targeted gene deletions for all the major IGFBPs have been fertile and without obvious abnormal reproductive phenotype, so will focus on IGFBPs in the primate uterus.

3.2 IGF system in the primate uterus

The IGF system (including IGF-I, IGF-II, the IGF-I receptor and IGFBPs) is highly expressed in the human[61-63] and rhesus monkey uterus[54,64,65]. Uterine IGF system expression is found early in fetal development[66,67], and during maturity is regulated by menstrual cycle stage[62] and exogenous steroids[54,56,65,68]. The IGF system is believed to be important in endometrial growth and remodeling during the menstrual cycle and during implantation and pregnancy[69]. As in the rat and mouse, IGF-I is expressed by the human endometrial stroma and increased in concert with increasing estrogen levels during the menstrual cycle[64]. IGF-II is also present in the human endometrial stroma, but is relatively more abundant in the secretory phase of the cycle. The IGF-I receptor is preferentially expressed in the uterine epithelium[64], although low level expression is detected in the stroma and myometrium. Several IGFBPs are detected in the human endometrium. IGFBP1 is abundant during the mid-late secretory phase and occurs in abundance in the decidua[68]. IGFBPs 2, 4, 5, and 6 are expressed throughout endometrial stroma[64]. IGFBP3, in contrast, is focally concentrated in endometrial capillaries and is increased in the secretory phase largely due to the intense vascularization of endometrial glands during this phase. IGFBP5 expression is more abundant in the proliferative phase, but all other IGFBP are relatively increased in the secretory phase of the menstrual cycle[64]. These findings support the view that the IGF system plays a fundamental role in endometrial biology, acting via autocrine and/or paracrine mechanisms, with IGF-I and IGFBP5 being dominant in the proliferative phase and IGF-II and the other IGFBPs predominant in the secretory phase of the menstrual cycle.

The IGF system is also highly expressed in the uterine muscle, where IGF overactivity has been implicated in the growth of uterine fibroids[61,68,70]. Studies in the ovariectomized rhesus monkey showed that IGF-I, IGF-II, and

the IGF-I receptor are coexpressed by uterine smooth muscle cells, supporting the possibility of autocrine/paracrine IGF action in stimulating myometrial growth[54]. IGF-I mRNA, which is barely detected in control myometrium, is significantly increased by E2 treatment and augmented even more by the combination of E2 and P4 treatment, with little change noted in IGF-II or IGF-I receptor expression. There is a significant correlation between local IGF-I concentration and local Ki-67-positive myometrial cells ($r = 0.891$; $P = 0.003$), implicating local IGF-I in both E2- and P4-induced myometrial growth[54]. IGFBPs 2-5 are all expressed by myometrial smooth muscle cells but each displays distinctive patterns of regulation by sex steroids. IGFBP2 is significantly increased by E2 and even more significantly by E2 and P4. IGFBP4 and 5 are readily detectable in control myometrium and significantly increased by E2 treatment, with no alteration by addition of P4. IGFBP3 is abundant in the control myometrium, but in contrast to other IGFBPs, it is significantly reduced by approximatley 75% by E2 and by E2 and P4 treatment. Interestingly, however, IGFBP3 is increased in the uterine vascular endothelium by both E2 and E2/P4 treatment. IGF-I and IGFBP2 demonstrate similar cellular and sex steroid-regulated patterns of myometrial expression. Both are dependent on E2 for significant expression and both are further augmented by the addition of P4 to E2 treatment. These observations indicate the potential for complex local interactions between IGF system components regulated by estrogen and progesterone

4. CONCLUSION

Reproduction in mammals is very energy expensive business, at least for females. The energy costs are so high that reproductive competency normally shuts down in times of nutrient scarcity or excessive energy output (e.g., extreme exercise, lactation). IGFs and IGFBPs are strongly regulated by nutritional status, with scarcity leading to suppression of free circulating IGF, as well as reduced IGF production in some tissues. While we usually think of nutritional cues acting at the neuroendocrine level, there is abundant evidence from domestic animals that the systemic IGF-I level is highly and positively correlated with the rate of ovarian follicle growth. Just as reduced energy stores and low insulin and IGF-I levels arrest follicular growth, nutrient excess elevates insulin and IGF levels, resulting in enhanced follicle recruitment and growth, sometimes to excess, as in the PCOS.

While local sources of IGF peptides and local distribution of IGFBPs in the ovary and uterus vary considerably between species, IGF-I receptor expression is quite constant. This receptor is most highly expressed in

growing follicles and in corpora lutea, in parallel with the level of metabolic activity in these structures, as reflected in glucose utilization. So whatever the source of the IGF ligand, and however the ligands are affected by the IGFBPs, the ultimate effect of IGF-I receptor activation is to promote anabolic processes required to support rapid cellular growth (follicle) and steroid biosynthesis (corpora lutea). IGF-I receptor expression is also highly abundant in the endometrial epithelium, which is mitotically and biosynthetically extremely active, undergoing rapid cycles of growth and differentiation and requiring abundant supplies of fuel. Somewhat beyond the scope of the present chapter, IGF-II serves a similar role in placental and fetal development, controlling the placental supply of nutrients available for fetal growth, irrespective of the maternal nutritional state. Thus, the IGF family appears to play an important role in nutrient partitioning, supporting the extraordinary, energy-expensive processes of follicular growth, steroidogenesis, and uterine hyperplasia and remodeling and fetal growth.

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Chapter 5

SKELETAL MUSCLE

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1. INTRODUCTION

Somatic growth is determined by several control mechanisms that integrate environmental signals with neuroendocrine cues. Growth factors are produced by many tissues and their effects are mainly local (autocrine/paracrine), whereas hormones exert their actions systemically, often far away from their tissue of origin. The growth hormone (GH)/insulin-like growth factor-I (IGF-I) system is essential for normal growth and development during embryonic and postnatal periods. Although GH is important for postnatal growth, IGF-I has been found to be critical for both stages of development. The original somatomedin hypothesis was initially postulated to demonstrate that GH stimulates growth through a factor produced by the liver (somatomedin C, called later IGF-I) [1]. Later, it was demonstrated that multiple tissues from the fetal mouse synthesized IGF-I[2,3] and that GH stimulated local production of IGF-I in bone [4,5]. This raised the importance of locally produced IGF-I in mediating the growth effects of GH. A revision of the original somatomedin hypothesis came with the results of two independent mouse models of liver IGF-I disruption [6,7]. Target deletion of liver IGF-I synthesis produced normal growth in mice leading to the suggestion that autocrine/paracrine IGF-I may be quite important in mediating postnatal somatic growth. More recent studies have, however, demonstrated that endocrine IGF-I is of major importance [8].

Taking together the extensive literature, it appears that whole body growth may be the sum of endocrine and autocrine/paracrine actions of the

GH/IGF-I axis. Thus, the endocrine function of somatic growth may correspond to the overlapping activity of GH and liver IGF-I, and possibly GH-dependent IGF-I production in a few extrahepatic tissues. Paracrine/autocrine effects may represent the GH-independent role of IGF-I in the extrahepatic tissues.

During the development of skeletal muscle, myoblasts cease to proliferate and differentiate into myotubes by activating a number of muscle-specific genes and are further remodeled into myofibers. In adult muscle a class of cells with embryonic characteristics named satellite cells remains. These cells are responsible for the regenerating capacity of the adult skeletal muscle after injury[9]. Myofibers are grouped into fiber types, type I (slow-twitch fibers) and type II, with subgroups IIA, IIX, and IIB (fast-twitch fibers). The different types of myofibers exhibit distinctly different contractile and metabolic properties. Type I and type IIA demonstrate oxidative metabolism whereas type IIB fibers are more glycolytic[10].

The IGF system plays an essential role in the formation and maintenance of skeletal muscle. IGF-I and IGF-II and their cognate tyrosine kinase receptor (IGF-I receptor) have been shown to elicit major growth-promoting effects[11]. While IGF-II is essential for normal embryonic growth [12], IGF-I operates throughout prenatal and postnatal development[13]. Unlike other growth factors, IGF-I stimulates both myoblast proliferation and differentiation[14,15]. In proliferating myoblasts, IGF-I increases the expression of cell cycle progression factors[14]. After withdrawal of myoblasts from the cell cycle, IGF-I promotes muscle differentiation by inducing the expression or activity of myogenic regulatory factors[16].

There are still controversial points of view on how GH regulates growth in skeletal muscle *in vivo*. The relatively scarce literature on this issue suggests that GH apparently has no direct growth-promoting effects in the skeletal muscle. In this chapter we summarize the overall literature of the GH/IGF-I system in the development of the skeletal muscle and its implications in aging, regeneration, diabetes, and myopathies.

2. THE GH SYSTEM

2.1 Growth hormone

GH and the GH receptor (GHR) belong to the superfamily of cytokine peptides[17] and receptors[18]. Somatotroph cells of the adenohypohysis

synthesize and store GH. Rodents and humans show a sexual dimorphism in the pulsatile secretion of GH[19]. GH secretion is tightly regulated by two hypothalamic factors, the stimulating GH releasing hormone (GHRH)[20,21] and the inhibitory hormone, somatostatin (SS)[22]. An inhibitory feedback loop on GH secretion is provided by circulating IGF-I that regulates GH actions in peripheral tissues. Other metabolic factors also regulate GH release; free fatty acids (FFAs)[23] inhibiting it, whereas leptin, neuropeptide Y (NPY), hexarelin and ghrelin have been shown to stimulate GH release[24-29].

2.2 The GHR and GHR signal transduction

The GHR was the first member of the type I cytokine receptor family to be cloned. GHR is a single-transmembrane receptor that mediates the actions of GH. It is now well established that binding of one molecule to two adjacent receptors is required for optimal activity[30,31]. GHR is present on the surface of most cells[18]. GH-binding protein (GHBP) is formed either by proteolytic cleavage in humans and rabbits[32-34] or alternative splicing in mice and rats[35,36] of the GHR extracellular ligand binding domain. GHBP stabilizes GH in plasma acting as a physiological buffer[37].

As with most cytokine receptors, GHR utilizes the JAK-STAT signal transduction pathway[38]. Upon GH binding, the GHR associates with the tyrosine kinase janus kinase 2 (JAK2). JAK2 phosphorylates STATs -1, -3, -5a, and -5b on tyrosine residues[38] allowing their translocation to the nucleus where they activate gene transcription upon binding to DNA sequences[39,40] (Fig. 1).

Gene expression of IGF-I and various IGF-binding proteins (IGFBPs) is regulated by GHR stimulation although a GH-responsive element has been found only in the promoter for the acid labile subunit (ALS) of the IGFBP-3 complex[41].

It has been proposed that there may be cross-talk between GH and IGF-I at the level of their signal transduction pathways. Upon GHR activation, JAK2 induces tyrosine phosphorylation of the insulin receptor substrate (IRS) proteins IRS-1 and IRS-2 and their association with phosphatidylinositol-3'-kinase (PI3K)[42,43] (Fig. 1). As mentioned later, IRS proteins are tyrosine phosphorylated by the activation of the IGF-I receptor (as well as by the insulin receptor). Effects of GH on insulin receptor-related metabolic signal transduction have been described[42,43]. However, there are no studies relating GH-induced IRS tyrosylphosphorylation to IGF-I-mediated mitogenic and metabolic effects.

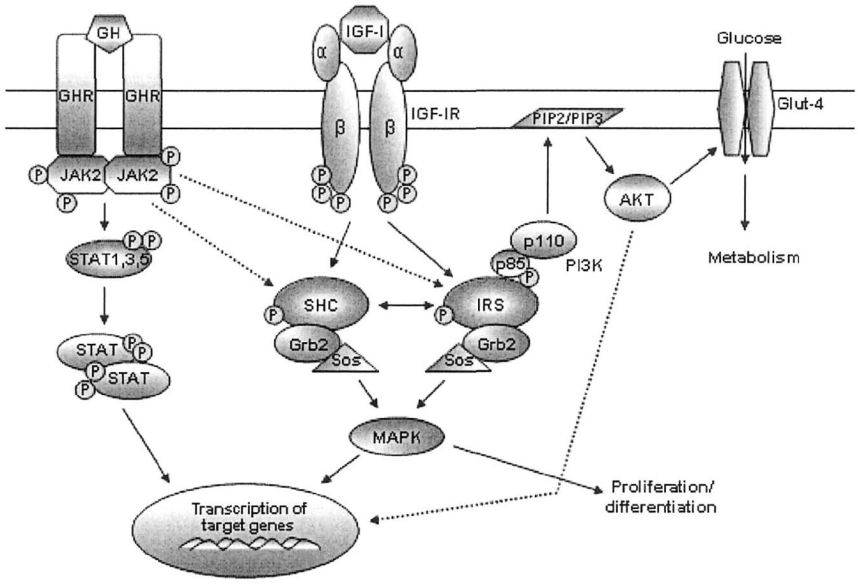

Figure 1. Signal transduction pathways used by GH and convergence between IGF-I and GH signaling. GH-induced dimerization of GHR drives activation of JAK2 and subsequent phosphorylation of STAT proteins which translocate to the nucleus to activate gene transcription. Crosstalk (dotted lines) between IGF-I and GH signaling is thought to be mediated by JAK2 tyrosyl-phosphorylation of SHC and IRS proteins leading, in turn, to activation of MAPK and PI3K pathways involved in growth and metabolism actions, respectively.

2.3 GHR in skeletal muscle

Although reports of direct effects of GH on muscle are rare, *in situ* hybridization studies in rats showed that both the GHR and GHBP genes are expressed in a number of tissues, including skeletal muscle[44]. At least five variant forms of the GHR mRNA were found in rats with the V3 variant being found in skeletal muscle. In human skeletal muscle, GHR transcripts are at low and variable levels among individuals[45]. Although there are very few reports of GH-GHR direct effects, expression of the GHR in many cell types has led to the suggestion that they might all be responsive to GH. However, there has been a great degree of difficulty in demonstrating GHR binding, particularly in skeletal muscle[46].

Expression of the GHR and GHBP are apparently under both tissue-specific and developmental controls. For example, there is a developmental switch from a nonbinding variant of the GHR in the embryo to a functioning

form shortly before birth in sheep and rats[47,48]. This was in agreement with the general view that GH has little or no effect on fetal growth (see later). During human development, GHR and GHBP mRNAs are present at low levels in fetal liver, increasing considerably after birth[44]. It was concluded that levels of liver GHR mRNA probably correlate with the influence of GH on growth of the animals. These levels were positively correlated with plasma IGF-I and growth rate. On the contrary, muscle GHR mRNA levels correlate with metabolic control[49]. Based on these studies, it appears that the GHR gene is expressed in skeletal muscle, but whether it might stimulate myogenesis either directly or by inducing autocrine expression of the IGFs remains to be elucidated.

2.4 The physiological role of GH on skeletal muscle

2.4.1 Effects of GH on muscle growth

GH is a pleitropic hormone with physiological actions that involve multiple organs and physiological systems[50]. Studies on the effects of GH in skeletal muscle have been performed in a variety of models, from cultured cells to intact and hypophysectomized animals and in humans. Hypophysectomy in young animals causes a substantial decrease in fast type 1 fibers in both soleus (primarily slow) and extensor digitorum longus (primarily fast) muscles, and treatment with GH reverses this effect[51]. Administration of GH to hypophysectomized animals causes an increase in muscle mass[52], although GH has no effect in intact young rats. Treatment of intact rats with a polyclonal antiserum to rat GH substantially reduced the weight, total protein and RNA content of hind limb muscles[53], but probably because there was a marked decrease in serum IGF-I levels.

It is well known that adults with GH deficiency have a generalized reduction in skeletal muscle mass[54,55]. Treatment of such patients with recombinant human GH (rhGH) increases lean tissue and skeletal muscle mass at all ages[56,57], although long-term exposure produces potential drawbacks[58]. However, other studies have reported beneficial effects of extended GH treatment on the musculature of GH-deficient humans[59,60]. Taken together these studies suggest that GH has the same effects in both human and animal muscles, but they do not prove that these effects do or do not involve elevation of circulating or muscle-expressed IGFs. Indeed, GH stimulates both circulating and local levels of IGF-I in various tissues[50]. Hence, whether the effects of GH on skeletal muscle growth are direct or IGF-I dependent remains to be elucidated.

Evidence for the synthesis of GH in a number of extrapituitary sites suggests that GH may have local paracrine/autocrine effects that might be distinct from the known classic circulating IGF-I-dependent effects. These local effects may be mediated either by local production of IGF-I or by other additional factors[61].

2.4.2 Effects of GH on muscle metabolism

GH is primarily an anabolic hormone and in skeletal muscle it induces positive nitrogen balance and protein synthesis[62]. Treatment with GH reduces fat mass and increases lean body weight by enhancing protein synthesis, with no effect on protein degradation[63,64]. In elderly patients, rhGH treatment had similar effects. Infusion of GH, IGF-I, and insulin into the forearm showed that GH and IGF-I increased amino acid uptake, whereas IGF-I and insulin, but not GH, inhibited amino acid release. Moreover, IGF-I is capable of inhibiting proteolysis[65]. Although there is no consensus as to whether IGF-I may or may not mediate the effects of GH on nitrogen balance and protein synthesis in muscle[66], these studies strongly support the suggestion that many of the anabolic effects of GH in muscle may be IGF-I dependent.

GH has a lipolytic action on fat and muscle. Acute GH administration causes a rise in the levels of circulating FFAs and glycerol by inhibition of lipoprotein lipase[67]. Long-term effects of GH include decreased deposition of fat and increased fat mobilization. The acute insulin-like activity of GH on carbohydrate metabolism has been seen both *in vitro* and *in vivo* and is independent of both IGF-I and insulin[68]. GH-induced tyrosine phosphorylation of IRS-1 and/or IRS-2 may be responsible for this effect[69]. Prolonged GH stimulation also enhances hepatic gluconeogenesis and glycogenolysis, resulting ultimately in hyperglycemia. This inhibitory effect on insulin activity may be indirectly caused by the GH-induced lipolysis and elevated plasma FFA levels that inhibit insulin activity at its target tissues[70].

2.4.3 GH actions on skeletal muscle not mediated by IGF-I

Fetal growth is not dependent on GH, as demonstrated by the fact that children with congenital absence of the pituitary and GH or GHR deletions are born near normal in size. However, postnatal growth and development are dependent on normal pulsatile secretion of GH[71], an effect that is especially prominent during puberty. There is extensive evidence for the effects of IGFs on isolated skeletal muscle cells, and a general lack of such

evidence for GH. Although there is evidence for a growth-promoting effect of GH that is independent of IGF-I in some tissues such as bone[72], as to date there is no evidence of such effect in skeletal muscle development.

Most studies of GH actions on isolated muscle cells have given negative results[46]. A report of a direct effect of GH on muscle cells involved BC3Hl cells. These cells exhibited metabolic responses to GH (*i.e.*, the insulin-like stimulation of glucose metabolism), but the authors did not show any effect on either growth or myogenesis in these cells[73]. Therefore, there are no reports establishing a direct effect of GH in stimulating the growth or differentiation of muscle cells. A clinical observation suggesting a direct GH effect on muscle is that local infusion of GH into the forearm of humans stimulated protein synthesis[74] and myosin heavy chain mRNA accumulation[75]. These studies do not exclude the possibility that GH may be acting by inducing expression of IGF-I in the musculature.

A more convincing observation for a direct effect of GH on skeletal muscle is a study showing that tyrosine phosphorylation of JAK2 and STAT5 occurs in skeletal muscle and liver of rats after intravenous administration of GH[76]. Although this is a more convincing report for some direct effects of GH on skeletal muscle, again it does not demonstrate that an effect on growth or myogenesis is or is not mediated by induction of IGF-I expression in skeletal muscle.

2.4.4 Effects of GH on expression of the IGF-I gene in muscle

The role of IGF gene expression in mediating the effects of GH on skeletal muscle growth is not clear. It has been known for more than a decade that cultured myoblasts release IGFs[77], but the relationship of this effect to GH treatment is ambiguous. There are a number of reports of GH-independent expression of IGF-I mRNA in skeletal muscle. Levels of IGF-I mRNA in extrahepatic tissues in the GH-deficient dwarf chicken are close to normal[78]. In most studies there was a similar lack of correlation between GH levels, growth, and muscle IGF-I mRNA levels[79-81]. However, in exercized rats, the anabolic response to training involved both GH-dependent increases in liver IGF-I mRNA and GH-independent increases in muscle IGF-I mRNA. This study also reported that the GH dependence/independence was related to different spliced variants of IGF-I mRNA[81] (see later). However, in one study, it was reported that GH does affect IGF-I mRNA gene expression in muscle. GH treatment of hypophysectomized rats caused an increase in IGF-I mRNA content in the gastrocnemius muscle[82,83].

It can be concluded that IGF-I gene expression can occur in muscle with or without GH stimulation. The possibility remains that at least some of the reported direct effects of GH might be mediated by autocrine/paracrine actions of IGF-I, although it has not been easy to demonstrate functioning GH receptors on skeletal muscle cells (see earlier).

2.4.5 Effects of GH on circulating levels of IGFs and IGFBPs

The stimulation by GH on liver secretion of IGF-I has been widely reported in a number of reviews and it is well established[84,85]. There is a general consensus that the liver is the primary source of circulating IGF-I (and IGFBP-3), but there is considerable uncertainty about the extent to which circulating IGF-I (in contrast to autocrine/paracrine secretions) affects specific tissues in specific situations.

3. THE IGF SYSTEM

The IGF family of growth factors is composed of three ligands: IGF-I, IGF-II, and insulin; their cognate receptors, IGF-I receptor, insulin receptor, and the IGF-II mannose-6-phosphate receptor (IGF-II/M-6-P receptor); and six high-affinity binding proteins (IGFBPs)[11,86]. Gene targeting studies revealed that the IGF-I receptor mediates the cellular responses of IGF-I and IGF-II. The insulin receptor mediates the biological (metabolic) functions of insulin (and some of the mitogenic actions of IGF-II in the embryo)[87]. A subset of alternately spliced insulin receptors with increased affinity for IGF-II has been described[88]. In contrast, the IGF-II/M-6-P receptor has no major role in IGF signal transduction, but is primarily responsible for clearing the extracellular IGF-II during fetal development[89]. The IGF-II/M-6-P receptor also plays a major role in transporting lysosomal enzymes between intracellular compartments.

The IGF-I receptor is a member of the tyrosine kinase growth factor receptors with high homology to the insulin receptor, especially in the tyrosine kinase domain[11]. Both receptors are formed by a heterotetrameric configuration of two α and two β subunits. The extracellular region is composed of the α subunits and forms the ligand binding domain. The β subunits form the transmembrane region of the receptor and contain the cytoplasmic tyrosine kinase domain. The tyrosine kinase activity of the

receptor is activated upon binding of the ligand, initiating various signaling cascades that result primarily in cellular proliferation or differentiation. In a similar way, insulin binds the insulin receptor to mediate primarily metabolic responses in insulin-responsive cells. Upon tyrosine phosphorylation of the IGF-I receptor, multiple endogenous substrates bind to its phosphotyrosine domain[11]. These substrates include the insulin related substrate (IRS) family of proteins (IRS-1 through -4) and the SHC family of adapter proteins, with SH2 domains[90,91]. Both docking proteins bind to the IGF-I receptor at the juxtamembrane region and then are able to recruit other substrates that lead to activation of a two principal signaling pathways, the mitogen-activated protein kinase (MAPK) pathway and the phosphoinositide 3′-kinase (PI3K) pathway[92,93] (Fig. 2).

The IGF-I receptor mediates most actions of IGFs in skeletal muscle. *In vitro* and *in vivo*, the IGF-I receptor mediates proliferation and differentiation, and several anabolic actions such as stimulation of protein synthesis and glucose uptake and inhibition of protein degradation[46] (Fig. 2). Owing to the close homology of the IGF-I and insulin receptors, hybrid receptors consisting of one IGF αβ-half receptor and one insulin αβ-half receptor have been described in many tissues, including skeletal muscle, that bind with high affinity to IGF-I, but not insulin[94].

Unlike insulin, regulation of IGF-I and IGF-II signaling is further complicated by their union with six high-affinity binding proteins (IGFBP-1 to -6)[86]. IGFBP-3 was the first IGFBP identified and forms part of the ternary complex. Recent data suggest that IGFBP-5 also forms a complex with IGF-I and ALS[95,96]. The IGFBPs function as carrier proteins for IGFs in the circulation, protecting them from proteolytic degradation, transporting them to specific tissues, and also modulate IGF actions[86]. In addition to their major roles in the circulation, most target tissues also express IGFBPs where they further regulate the local action of IGFs. Most IGFBPs can either inhibit or potentiate IGF-stimulated actions of IGF-I receptors, depending on the cell type. In addition, IGFBPs may have IGF-independent effects on cell function. Whereas IGFBP-3 functions primarily as the major transporter of the IGFs in the circulation, all six IGFBPs have been shown to be produced by individual tissues in a cell type specific combination.

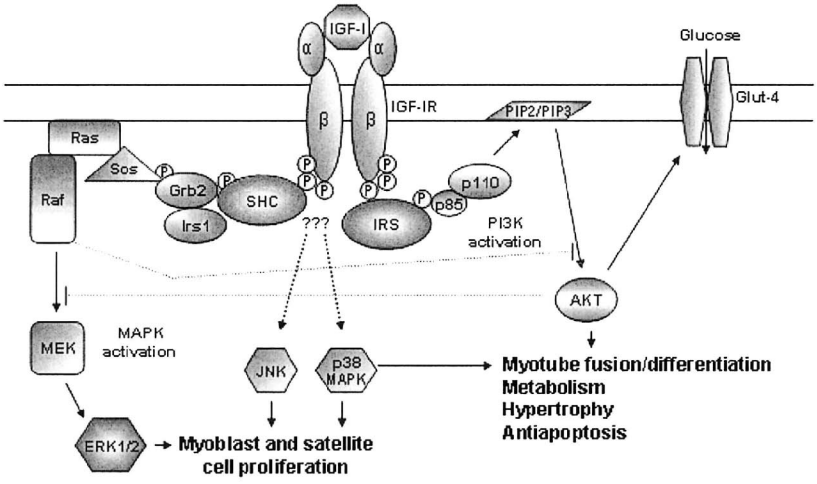

Figure 2. IGF-I receptor signaling pathways in skeletal muscle. Following IGF-I binding, IGF-I receptor autophosphorylates and recruits IRS and SHC proteins to activate two divergent downstream signaling cascades, MAPK and PI3K pathways. Grb2 linked to Sos activates Ras-Raf with subsequent MAPK activation of cell proliferation. p38 MAPK is also involved in both proliferation and differentiation of muscle cells (dashed lines). Similarly, PI3K activation is mediated upon tyrosylphosphorylation of its regulatory subunit p85 by IRS proteins. Then, inositol triphosphates phosphorylate AKT leading to cell differentiation, hypertrophy, resistance to apoptosis, and metabolism of muscle cells. A balance between MAPK and PI3K marks the proliferation and differentiation state of skeletal muscle growth (dotted lines). Myoblast proliferation is mediated by MAPK signaling. Moreover, active Raf also suppress IGF-I mediated PI3K signaling, favoring proliferation. In contrast, myotube differentiation is driven by PI3K and AKT can also bind and inactivate Raf, inhibiting MAPK signaling and thus suppressing proliferation.

Of the six IGFBPs, four have been shown to be secreted by various skeletal muscle cell lines. These include IGFBP-2, -4, -5, and -6[97-99]. IGFBP-1 and -3 were not secreted by any of the cultured muscle cell types examined. IGFBP-4 secretion appears to be associated with proliferation and IGFBP-5 is induced during differentiation[100,101]. IGFBP-2, -4, -5, and -6 can all act to inhibit muscle differentiation, but under proper conditions IGFBP-5 has the additional capability of stimulating myogenesis. IGFBP-6 also plays a role in quiescence[97]. *In vivo*, mRNAs of IGFBP-2, -4 and -5 have been shown to be highly expressed in skeletal muscles of rodents[102-104].

3.1 IGF-I in skeletal muscle

The mature IGF-I polypeptide is highly conserved across species and has many structural similarities to the proinsulin molecule. IGF-I is a single-chain polypeptide composed of 70 amino acid residues in four highly conserved domains (A,B,C,D) (Fig. 3). The IGF-I gene sequence is comprised of six exons. IGF-I premRNA can be alternatively spliced leading to five known mRNA precursors. IGF-I contains two promoters initiating at the 5´end of exons 1 and 2. IGF-I transcripts initiating at exon 1 are widely expressed in all tissues. Transcripts initiating at exon 2 predominate in the liver and are the major endocrine effectors of GH[105]. Three different E peptides are generated by alternative splicing at the 3´ end of IGF-I transcripts. These E peptides contain a common N-terminal 16-amino-acid sequence and alternative C-terminal sequences. Transcripts containing Eb sequence are more abundant in liver, whereas Ea-containing transcripts are widespread in extrahepatic tissues[105,106]. A third E peptide splice variant (Ec) has been described that results in the inclusion of a novel splicing site in the intron preceding exon 6. This novel transcript was found in muscles subjected to stretch and was called "mecano growth factor" (MGF)[107,108]. Exons 3 and 4 encode for the entire sequence of the mature polypeptide. Circulating IGF-I isoforms synthesized in the liver are very GH-responsive and therefore the principal endocrine effectors of GH.

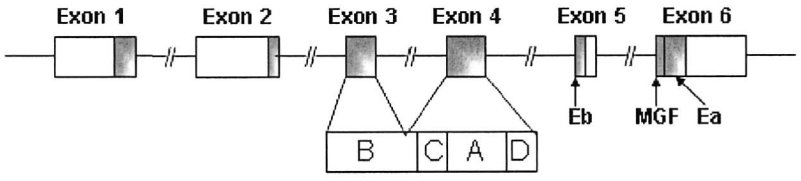

Figure 3. Schematic representation of *IGF-I* gene and peptide structure. BCAD represent the various domains of the mature peptide. The *IGF-I* gene contains two promoters initiating at exons 1 and 2 leading to two different tissue-specific transcripts. Moreover, tissue specificity is also determined by alternative splicing at 3' end of IGF-I transcripts (Ea or Eb). 1Ea IGF-I transcripts are predominately expressed in resting skeletal muscle whereas MGF is found in skeletal muscles subjected to stretch.

The class of 1/Ea IGF-I transcripts is expressed in all tissues during development[109-112]. At embryonic and early postnatal ages, the 1/Ea IGF-I mRNAs are highly expressed in the liver in a GH-independent manner[113-115]. In contrast, class 2Eb transcripts are detected in the liver postnatally and are tightly GH-regulated[113,114,116]. Other IGF-I isoforms predominately synthesized in skeletal muscle tissue presumably operate in an autocrine or paracrine manner and are less affected by circulating GH levels. The class of 1/Ea IGF-I transcripts have been found in the resting skeletal muscle, whereas the 1/Ec or MGF isoforms were prominent in stretched skeletal muscle[107,108].

3.2 Physiological effects of the IGFs in skeletal muscle

3.2.1 Biological significance of the IGFs demonstrated by knockout and transgenic tools

Naturally occurring mutations in the IGF system are extremely rare. A mutation of the *IGF-I* gene in a single patient has shown both intrauterine and postnatal growth retardation[117]. Congenital absence of IGF-II or IGF-IR has not been reported, suggesting that these mutations may be lethal *in utero* or in infancy. In humans, plasma IGF-I levels correlate with body size. Tall children have elevated plasma IGF-I levels[118] and mice selected for high IGF-I levels show increased body weight[119]. Moreover, infusions of rhIGF-I also enhance body weight and size in a number of models, further suggesting a role for circulating IGF-I in growth.

Homologous recombination techniques have allowed the biological significance of the IGF system to be elucidated[120] (Table 1). IGF-I and IGF-II and their cognate IGF-I receptor have been shown to elicit major growth promoting effects[11].While IGF-II is essential for normal embryonic growth[12], IGF-I operates throughout prenatal and postnatal development[13]. The results of these studies have demonstrated that the IGFs are very important in muscle growth and development. Mouse lines lacking IGF-I or IGF-II or IGF-I receptor show impaired embryonic development. Although the embryos are viable, they die immediately after birth because of breathing failure [12,13,121]. Mice with a targeted IGF-I deletion show generalized organ hypoplasia, including the muscles[13,121]. Mice lacking the IGF-I receptor exhibit marked skeletal muscle hypoplasia and die soon after birth because they fail to breathe[13]. In contrast, mice lacking functional IGF-II/M-6-P receptors also die around birth, but exhibit fetal overgrowth. These mutants

have elevated circulating levels of IGF-II. These findings support the theory that one function of the IGF-II/M-6-P receptor is to remove excess IGF-II from the circulation[89].

Mouse	IGF alteration	Skeletal muscle phenotype	Reference
IGF-I $^{-/-}$	Global KO of IGF-I	Hypoplasia	13,121
IGF-I receptor $^{-/-}$	Global KO of IGF-I receptor	Hypoplasia	13
IGF-II $^{-/-}$	Global KO of IGF-II	Hypoplasia	12
IGF-II/M-6-P receptor $^{-/-}$	Global KO of IGF-II/M-6-P receptor	Muscle overgrowth	89
IGF-I Tg	Global IGF-I overexpression	Muscle overgrowth	122
IGF-II Tg	Global IGF-II overexpression	Decreased muscle mass	123
IGF-I Tg targeted	Viral over-expression of IGF-I in skeletal muscle	Hypertrophy	124-126
Dom-neg IGF-I receptor Tg targeted	Overexpression of dom-neg IGF-I receptor in skeletal muscle	Hypoplasia at birth/ Hyperplasia in adulthood	127

Table 1. Genetically altered IGF mouse models and their skeletal muscle phenotypes. Dom-neg, dominant-negative; -/-, homozygous KO; KO, knockout; Tg, transgenic.

Using the opposite approach, transgenic mice overexpressing IGF-I have increased circulating IGF-I levels and increased weight and DNA synthesis in most organs, including skeletal muscle[122]. In contrast, mice expressing very high levels of IGF-II grow more slowly than their control animals[123] (Table 1).

Likewise, mice overexpressing IGF-I in muscle develop increased muscle mass and muscle hypertrophy[124-126]. We developed a transgenic mouse overexpressing a dominant-negative IGF-I receptor in skeletal muscle. The results showed marked hypoplasia in skeletal muscles just after birth, although a compensatory muscle hyperplasia was undergone by the satellite cells in adulthood[127] (Table 1). Thus, the IGF system plays an important role in both embryonic and postnatal growth and is essential for the formation and maintenance of skeletal muscle.

3.2.2 IGF-I effects on cell growth (proliferation and differentiation)

The development of skeletal muscle is a multistep process wherein pluripotent mesodermal cells give rise to myoblasts. Myoblasts permanently withdraw from the cell cycle and begin to express muscle-specific genes and proteins. These processes lead to the fusion of myoblasts into multinucleated differentiated myotubes. Prior to the onset of muscle differentiation,

proliferating myoblasts express two muscle-specific basic helix-loop-helix (bHLH) transcription factors, MyoD and Myf5. Upon activation, MyoD and Myf5 induce myoblast withdrawal from the cell cycle and the expression of other myogenic bHLH regulatory transcription factors, including myogenin and MRF4[128-132]. Unlike other growth factors, IGF-I stimulates both myoblast proliferation and differentiation[14,15]. These effects are temporally separated. In proliferating myoblasts, IGF-I increases the expression of cell cycle progression factors[14]. After withdrawal of myoblasts from the cell cycle, IGF-I promotes muscle differentiation by inducing the expression or activity of myogenic regulatory factors[16,133-136].

3.2.3 IGF-I effects on skeletal muscle metabolism

IGF-I and insulin have both shared and unique actions on muscle metabolism. In skeletal muscle and satellite cells, IGF-I increases protein synthesis and inhibits proteolysis. These actions are distinct from the metabolic effects of insulin, which acts primarily by inhibition of proteolysis. This evidence strongly supports the idea that IGF-I is acting via the IGF-I receptor and not the insulin receptor in muscle[46,65,137]. IGF-I also enhances glucose uptake into peripheral tissues, which is an insulin-like effect[138]. It has been proposed that this effect could be mediated by hybrid IGF-I/insulin receptors in both rodents and humans[94,139,140]. Indeed, we showed that in transgenic mice overexpressing a dominant-negative IGF-I receptor specifically in skeletal muscle the diabetic phenotype observed may be mediated by the formation of hybrids between IGF-I and insulin receptors[141]. In contrast, long-term administration of GH inhibits glucose uptake by inducing a state of insulin resistance at the liver and peripheral tissue level as a result of a postreceptor effect[142,143]. These findings further suggest that certain actions of GH occur independently from IGF-I.

3.2.4 Mechanism of IGF actions in skeletal muscle

Mice with targeted disruption of the IGF-I receptor exhibit marked skeletal muscle hypoplasia and die soon after birth[13]. In contrast, mice overexpressing IGF-I specifically in muscle develop increased muscle mass and hypertrophy[124-126]. Conversely, overexpression of human IGF-I receptors in rodent skeletal muscle cells enhances IGF-I–induced proliferation and differentiation[15]. Furthermore, functional specific inactivation of the IGF-I receptor in skeletal muscle of mice results in a delay in the proliferation and differentiation processes in skeletal muscle cells[127]. In accordance with these

in vivo results, transfection with a kinase-inactive IGF-I receptor abolished IGF-I–induced proliferation and delayed differentiation in mouse myoblasts in culture[144].

Unlike other growth factors, IGF-I stimulates proliferation, as well as differentiation of myoblasts acting on myogenic regulating factors and effectors of the cell cycle[14,15,127]. The proliferative versus the differentiating functions of IGF-I appear to be mediated by distinct intracellular signaling pathways[145]. Studies suggest that the MAPK pathway mediates cellular proliferation, whereas the PI3K pathway is activated during differentiation[145-148]. In addition to its function on fusion of myoblasts into myotubes, PI3K signaling also mediates IGF-I's anabolic effects on protein and glucose uptake, and cell survival or resistance to apoptosis in skeletal muscle[148-151] (Fig. 2).

However, recent studies have reported that ERK1/2 MAP kinases are differentially regulated in mitogenesis and myogenesis and that the stress-activated protein kinase p38 is critical for regulation of terminal differentiation[152-155]. All of these studies were carried out in cultured muscle cells. The discrepancy might be due to the distinct types of culture cells used in the various studies. The mechanism by which IGF-I–induced activation of these signaling pathways regulates muscle differentiation *in vivo* has not been clearly determined. Indeed, in mice with an inactivated IGF-I receptor in skeletal muscle (MKR mice) there is multistep regulation of the myogenic program by the p38 and ERK MAP kinase pathways; wherein ERKs have a biphasic profile during myogenesis and p38 is particularly activated for terminal differentiation[127]. These results indicate that the two MAP kinase signaling pathways (ERK and p38) must act in parallel and that both are required for the compensatory myogenic proliferation and differentiation in MKR mice. However, the PI3K pathway may also be important for muscle cell survival, since even though adult MKR mice experience compensatory muscle hyperplasia, they still have reduced muscle mass[127].

The exact mechanism by which myoblasts switch from a proliferative to a differentiation signal is yet unknown. A crosstalk mechanism between the two opposing pathways has been postulated, depending on the differentiation state of myocytes. Constitutively active Raf-1 promotes proliferation through Erk, and also inhibits IGF-I-induced myoblast fusion[156]. Thus, the MAPK pathway seems to predominate in proliferating myoblasts. In contrast, initiation of myotube fusion is subjected to an increase in Akt [157]. Postmitotic myotubes show increased metabolism, hypertrophy, and survival that is mediated by the PI3K–Akt signaling pathway[149-151,158-160]. Moreover, Akt is able to inhibit Erk activation by binding to and inactivating Raf in myotubes, but not myoblasts[159,160]. It has been suggested that IGF-I protects myoblasts from apoptosis during fusion into mature myotubes by activation of Akt

through PI3K[150] (Fig. 2). Taken together, these studies establish that the IGF-I receptor acts as an important regulator of muscle cell differentiation by regulating muscle-specific genes under tight control of its signaling pathways.

3.3 Autocrine function of the IGF system in skeletal muscle

Two mice models of liver IGF-I deficiency, in which body growth remained unaltered[6,7], challenged the somatomedin hypothesis that circulating IGF-I levels mediated by GH secretion are the principal effectors of body growth. These results pointed out the importance of autocrine/paracrine IGF-I secretion in extrahepatic tissues to mediate growth. These data may be also interpreted to suggest that IGF-I may function alone in certain tissues, whereas it may mediate the effects of GH in other tissues as in bone[161,162]. As we mentioned earlier, gene targeting studies of the IGF-I system in skeletal muscle have clearly shown the role of skeletal muscle IGF-I and its receptor in mediating proliferation and differentiation as well as anabolic actions. Since GH binding in skeletal muscle cells has been very difficult to assess[46], it may be possible that the autocrine/paracrine effects of IGF-I and its receptor are exerted in a GH-independent manner.

3.3.1 IGF-I and diabetes

Diabetes results from the inability of insulin secretion from pancreatic beta cells to control blood glucose levels. Under normal conditions, insulin regulates hepatic glucose production and stimulates glucose uptake into peripheral tissues. A primary defect in type 2 diabetes mellitus is insulin resistance, since the liver continues to produce glucose and the uptake of glucose into muscle is impaired.

In humans, rhIGF-I has been used successfully as a coadjuvant to insulin therapy in patients with type 1 and type 2 diabetes. Plasma glucose concentrations are reduced after acute or chronic administration of rhIGF-I [163-165]. By contrast, GH therapy leads to increased insulin resistance. In type 1 patients with poorly controlled diabetes, levels of circulating IGF-I are reduced and GH levels are elevated. Administration of rhIGF-I often restores the high levels of GH to normal, leading to improved insulin sensitivity and enhance glucose uptake in peripheral tissues[166]. In type 2 diabetic patients, who are commonly insulin-resistant, IGF-I therapy leads to enhance muscle glucose uptake[167-170].

In mice, genetic manipulations were aimed to elucidate insulin-stimulated glucose uptake into muscle as potential therapeutic targets for type 2 diabetes. Skeletal muscle expresses substantial levels of both the insulin receptor and the insulin-like growth factor I receptor (IGF-IR). The inhibition of insulin receptor function in skeletal muscle using a dominant negative insulin receptor transgene failed to exhibit severe insulin resistance or diabetes[171]. A similar conclusion was reached in mice with muscle-specific deletion of the insulin receptor which developed very mild insulin resistance and remained normoglycemic[172]. One possible explanation for the failure of these latter two mouse models to show insulin resistance and diabetes is that IGF-IRs remained functional in these animals and can compensate for the loss or inactivity of the insulin receptor[173]. Indeed, in our MKR model, both insulin receptor and IGF-IR were affected, because of the formation of hybrid receptors. These mice showed a clearly type 2 diabetic phenotype. Further support for this hypothesis came from the studies of muscle GLUT-4 knockout mice[174], in which both insulin- and IGF-I-induced glucose uptake in muscle were abrogated, and this resulted in severe insulin resistance and mild diabetes. In humans, a reduced responsiveness of muscle to insulin has also been related to the formation of IGF-I/insulin hybrid receptors in the skeletal muscle of obese and diabetic patients[139,140]. Thus, those findings showed that the IGF-I receptor may be essential to control glucose overall metabolism.

3.3.2 IGF-I in regeneration, aging and myopathies

IGF-I plays a critical role in muscle regeneration. It promotes the proliferation and differentiation of satellite cells in the muscle, enabling them to fuse to existing muscle fibers and repair damaged regions[14,46,105,175].

Normal muscle responds to exercise or stretch with hypertrophy accompanied by a rapid increase in IGF-I and markers of satellite cell differentiation[176]. In response to mechanical stretch, myocytes produce both IGF-I and a splice variant of IGF-I, the mechano-growth factor (MGF)[108]. MGF is proposed to exert a paracrine effect which is essential for myocytoskeletal repair[9]. IGF-I thus serves as a link between muscle mechanical stress and antiapoptotic and hypertrophic repair response[9].

Cytoskeletal remodeling and proliferation and fusion of satellite cells to existing myotubes occur in muscle degeneration by denervation or structural injury. Following denervation IGF-I, IGF-II, and IGF-IR expression is upregulated in satellite cells localized to the area of regeneration[177,178].

Numerous studies in which IGF-I has been introduced by recombinant virus or overexpressed in transgenic animals have demonstrated that IGF-I

causes significant muscle hypertrophy and strength in postnatal skeletal muscle[16,124-126,179,180]. The IGF-I hypertrophic response is mediated by the activation of the serine/threonine protein phosphatase calcineurin in muscle cells[181,182]. Moreover, recent data using transplanted bone marrow cells in damaged muscle of mice overexpressing local IGF-I have shown that IGF-I enhanced muscle regeneration by promoting the recruitment of these stem cells to sites of muscle damage[183].

It has clearly been described that in aging a loss of lean body mass and increased adipose tissue occurs. GH/IGF-I axis function declines with aging. Thus, GH secretion and plasma IGF-I levels decline steadily after age 30 in humans. In rat muscle, the cell surface IGF-IR expression decreases between infancy and young adulthood with a subsequent decrease during senescence[184,185]. Moreover, increased insulin resistance in aging is associated with dramatically decreased IGF-I-stimulated muscle protein synthesis and glucose uptake[184]. In patients with GH deficiency, treatment with GH or IGF-I clearly increases lean body weight, muscle mass, muscle strength, and muscle protein synthesis. Exercise partially restores muscle anabolic responsiveness to IGF-I[185,186] in aging muscle. In young men, exercise alone is sufficient to stimulate muscle anabolism, and GH treatment does not augment either strength or protein synthesis[187]. In GH-treated elderly men, resistance exercise did not improve strength or increase muscle IGF-I or IGF-IR mRNA expression and muscle mass[188]. In another study, there is no clear correlation between IGF-I serum concentrations and strength in older men[189].

During aging, declines in muscle mass and strength are due to impaired regenerative capacity. Studies have shown that postmitotic expression of a local isoform of IGF-I preserves regenerative capacity in senescence via the stimulation of satellite cell proliferation and differentiation[124,126]. Furthermore, IGF-I promotes protein synthetic pathways that can override muscle degeneration[179,190]. These data clearly show that IGF-I can prevent aging-related loss of muscle function[124,191].

Clinical experience with IGF-I was described for treatment of diabetic patients with severe insulin resistance. IGF-I improves glucose control in these patients, at least in part by decreasing secretion of GH, which has been shown to exacerbate insulin resistance[164,192]. IGF-I myotherapy in humans and animals has focused on IGF-I's anabolic effects on skeletal muscle. Administration of IGF-I, GH, or both increased muscle mass and/or strength in burn injury[193]. Myopathic injury is reported from direct inhibition of postreceptor IGF-I signaling through PI3K in skeletal muscle. IGF-I largely prevents muscle atrophy associated with acute glucocorticoid (acute quadriplegic myopathy) use in rodents and when injected intramuscularly

causes potent localized muscle hypertrophy[179,194]. However, IGF-I treatment of structural myopathy is poorly tested. IGF-I is of clear benefit in murine muscular dystrophy. In dystrophic mice IGF-I together with a high-protein diet significantly reduced protein degradation, enhanced protein synthesis, and increased hindlimb strength[195]. Moreover, postmitotic expression of muscle IGF-I preserves regenerative capacity in dystrophic mice[196]. However, analogous trials for human myopathies have not been reported. From these data, it is possible that IGF-I can prevent or diminish muscle loss associated with disease.

At present, systemic use of IGF-I is unconceivable due to elevated cost and poor absorption of IGF-I. Recently, administration of IGF-I in a stable binary complex with IGFBP-3 has reported to minimize side effects in diabetic patients[192]. Although, muscular injection of a recombinant adeno-associated virus directing overexpression of IGF-I into aged rats and in mice with Duchenne muscular dystrophy has shown to ameliorate muscle decline[124,196], muscle injection in humans remains impractical for structural myopathies.

4. CONCLUSIONS

Many of the effects of GH on various systems maybe affected by IGF-I produced by the liver and reaching that organ via the circulation. On the other hand GH may have direct effects on skeletal muscle for example since GHRs are expressed in those tissues. The source of IGF-I could also be local autocrine/paracrine in origin. While the studies presented in this chapter suggest that all these possibilities are at work when considering the role of the GH/IGF system on skeletal muscle growth and differentiation, more work remains to firmly establish these concepts. With regard to the effects of the GH/IGF system it is apparent that it forms the basis for an important element in skeletal muscle growth and differentiation, given the results of gene-deletion and transgenic mouse models as well as gene therapy with IGF-I. The metabolic effects of the GH/IGF-I system on muscle are also interesting and require further experimentation. GH generally causes insulin resistance in muscle and IGF-I enhances glucose uptake; the overall effect on insulin action and diabetes needs clarification.

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Chapter 6

CARDIOVASCULAR AND RESPIRATORY SYSTEMS

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1. STRUCTURAL AND FUNCTIONAL ASPECTS OF THE CARDIOVASCULAR SYSTEM

1.1 The myocardium and cardiac hypertrophy

The myocardium is the muscle layer of the wall of the heart. It consists of cardiac muscle fibres (cardiomyocytes), connective tissue (connective tissue cells and extracellular matrix), and blood vessels in the capillary microcirculation. Approximately 90% of left ventricular tissue volume in small rodents is occupied by cardiomyocytes, while 85% of the cells are interstitial cells.[1] The interstitium is comprised of more than 95% of type I and type III collagens[2].

The term hypertrophy defines an increase in cellular size based on a concordant increase in all of the cellular constituents[3]. Cardiac hypertrophy is an important compensatory mechanism in left ventricular pressure or volume overload states. The hypertrophy is then induced locally, triggered by the increased wall tension[3]. Besides increased load, extrinsic trophic hormonal or neurogenic factors may trigger cardiac hypertrophy. Load-dependent and load-independent hypertrophy share some common

intracellular as well as autocrine-paracrine mediators[3]. Moreover, there are common transcriptional regulatory pathways during cardiac hypertrophy, including re-expression of protein isoforms normally expressed in the developing heart (such as skeletal α-actin, atrial and brain natriuretic factor, and β-isoform of myosin heavy chain), as well as immediate-early proto-oncogenes. Besides control of gene transcription, increased translation efficiency represents a means of initiating the hypertrophic response[3].

1.2 Calcium in the contractile process and in hypertrophy-signaling

Calcium ions (Ca^{2+}) have multiple functions within the cardiomyocyte, although the most well-studied is their role in the contractile process[4]. Following a membrane potential, Ca^{2+} enter the cardiomyocyte through the L-type Ca^{2+} channels and this Ca^{2+} influx in turn triggers release of Ca^{2+} from the sarcoplasmic reticulum through the ryanodine receptor. The Ca^{2+} then bind to troponin C and the contractile elements are activated. The sarcoplasmic reticulum ATPase (SERCA) then rapidly pumps back Ca^{2+} into the sarcoplasmic reticulum. Ca^{2+} are also excreted from the cell, mainly through the Na^+/Ca^{2+}-Exchanger, which is driven by the electrochemical gradients for Na^+ and Ca^{2+}.

Na^+/K^+-ATPase has a key role in the active transport of Na^+ and K^+ across the cell membrane and establishing a negative electrical potential inside cells. Cardiac glycosides, *e.g.* digoxin, increase cardiac contractility by an inhibition of Na^+/K^+-ATPase, which results in an increased intracellular concentration of Na^+ ions[5]. As the Na^+/Ca^{2+}-Exchanger transports Na^+ and Ca^{2+} in both directions, increased Na^+ levels are followed by increased Ca^{2+} levels within the cell and thereby contractility is enhanced.

Besides being involved in the contractile process, Ca^{2+} acts as an activator of signal transduction pathways responsible for hypertrophic cardiac growth[4]. Proteins involved in Ca^{2+} handling, such as calmodulin and calsequestrin, seem to play important roles as overexpression of these proteins may cause cardiac hypertrophy[4]. Recent studies have reported increased expression and/or activity of the Na^+/Ca^{2+}-Exchanger in experimental models of hypertrophy[6], and inhibition of Na^+/K^+-ATPase has also been linked to cardiac hypertrophy[7].

1.3 Peripheral resistance and vascular endothelial function

Arterial blood pressure is determined by two prime factors: cardiac output and peripheral resistance. Cardiac output is almost exclusively regulated by changes in total peripheral resistance, and is adjusted in order to

maintain arterial pressure at a normal level[8]. In hypertension, increased peripheral resistance is a consistent finding, as it may be either a primary (vasoconstrictor hypertension) or secondary (volume-loading hypertension) event[8]. The small prearteriolar arteries are the major determinants of local blood flow as well as peripheral resistance. They have a thick smooth muscle layer and a high myogenic basal tone that is modulated by local regulatory mechanisms (mechanical and chemical) as well as higher control systems such as hormonal factors and sympathetic activity.

The endothelium, a continuous cellular monolayer lining the entire vascular tree, is highly involved in the regulation of vascular tone by transmitting signals to the vascular smooth muscle. The endothelium also has a wide range of other homeostatic functions, including blood coagulation, fibrinolysis, and platelet and leukocyte interactions. Endothelial dysfunction has been reported to exist in numerous pathological conditions, such as insulin resistance and congestive heart failure, and it is generally regarded as important in the pathogenesis of atherosclerosis[9]. Endothelial function is impaired in most studies of laboratory animals and humans with chronic hypertension, and it is plausible that endothelial dysfunction is both a cause and an effect of hypertension. According to one definition, endothelial dysfunction implies an imbalance between vasoconstricting/prothrombotic factors (*e.g.,* angiotensin II, endothelin-1) on the one hand, and vasodilating/fibrinolytic factors (*e.g.,* nitric oxide (NO), endothelium-derived hyperpolarizing factor, prostaglandin I_2) on the other[10].

NO is produced mainly in the endothelium, by endothelial NO-synthase from the precursor L-arginine[9]. The production of NO is triggered by various factors, including acetylcholine stimulation and shear stress. Among other functions, NO has a relaxant effect on vascular smooth muscle cells and thereby causes vasodilatation. Functional inhibition of NO-synthase *in vivo* (*e.g.,* with N[w]-Nitro-L-arginine methylester hydrochloride; L-NAME) increases blood pressure, illustrating the importance of NO in the regulation of vascular tone[11].

1.4 Factors determining cardiac output

Cardiac output, the volume of blood ejected from the heart in 1 minute, is the product of stroke volume and heart rate. Stroke volume is determined by both systolic (ejecting) function and diastolic (relaxing) function. Left ventricular systolic function, in turn, is the product of interaction of four variables[12]:

1. *Preload, i.e.,* the hemodynamic force exerted on the ventricular wall during filling. In practice, end-diastolic volumes/dimensions are

considered reliable indices of ventricular preload. By intrinsic regulation of cardiac pumping through the Frank–Starling mechanism (*i.e.*, stretched muscle contracts with greatly increased force), cardiac output increases with increased preload. Preload is largely regulated by venous return to the heart.

2. *Afterload*, *i.e.*, the load against which the muscle exerts its contractile force during systole.
3. *Left ventricular* myocardial mass, *i.e.*, more muscle generates more force. With hypertrophy of the left ventricular wall, wall stress also decreases, and systolic function is thereby enhanced.
4. *Myocardial contractility*, *i.e.*, the contractile state of the cardiac muscle.

1.5 Cardiac remodeling

Left ventricular remodeling is the process by which ventricular size, shape, and function are regulated by mechanical, neurohormonal, and genetic factors[13]. It may be physiological/adaptive or pathological, *e.g.,* due to myocardial infarction or hypertension. Post-infarction remodeling may be divided into an early phase (infarct expansion) and a late phase with global left ventricular dilatation and mural hypertrophy[13]. During infarct expansion and ventricular dilatation, cardiac pumping function is partly restored through the Frank–Starling mechanism and this process is associated with alterations in the structure and function of the extracellular matrix. Hypertrophy of the surviving myocardium occurs as a counterregulatory mechanism to decrease wall stress. In the case of a large myocardial injury, the remodeling fails to normalize wall stress, resulting in a progressive dilatation and deterioration of contractile function and subsequently heart failure.

2. GH/IGF-I AND THE CARDIOVASCULAR SYSTEM

Besides its growth-promoting and metabolic effects, the GH/IGF-I axis has an important role during cardiac development and in maintaining the structure and function of the heart[13,14]. This arrangement is well suited to its purpose, GH/IGF-I increases cardiac output to meet the peripheral metabolic demands elicited by its own actions. GH/IGF-I influences the vascular system and may have a role in the regulation of vascular tone and thereby peripheral resistance. It was recently shown that the *IGF-I* gene locus is linked to both systolic blood pressure and cardiac dimensions[15].

The myocardium and vessels express IGF-I[16-18] and functional receptors for both GH[19-21] and IGF-I[22,23] and IGF-I production is up-regulated in

response to GH[17]. Thus, there are possibilities of direct actions of GH as well as endocrine or autocrine/paracrine effects of IGF-I on the cardiovascular system. However, although interaction of the GH/IGF-I axis and the cardiovascular system has been extensively studied, the relative importance of direct effects of GH and local and endocrine IGF-I remains unclear.

2.1 GH/IGF-I and peripheral resistance

In normal rats, intravenous administration of IGF-I acutely decreases mean arterial blood pressure within a few minutes[24,25]. In humans, stroke volume and cardiac output were increased but blood pressure remained unchanged a few hours after a single injection of IGF-I[26]. In heart failure patients, IGF-I infusion increased cardiac output and decreased peripheral resistance within approximately 2 h[27], while a similar effect of GH was seen after 24 h when serum IGF-I was concomitantly increased[28]. These data suggest that IGF-I has potent, acute functional effects on the cardiovascular system. In a recent study, it was shown in healthy subjects that GH directly increased forearm blood flow in parallel with a decrease in peripheral resistance[29]. Moreover, these changes were suggested to be mediated by stimulation of endothelial function through the NO system. However, the effect was not seen until 4 h after GH infusion and it is possible that the GH effect was mediated by local production of IGF-I in the periphery.

Several studies have shown that the vascular actions of IGF-I may be exerted by stimulation of release of NO and/or other vasodilators from the endothelium. IGF-I stimulates NO release from cultured endothelial cells[30], vascular smooth muscle cells[31], as well as aortic preparations[25]. Pretreatment with the NO-synthase inhibitor L-NAME may abolish the vasodilating effect of IGF-I, as shown in large arteries[24,32-34]. There are also reports of eicosanoids as mediators of the vasodilating effects of IGF-I and that indomethacin may prevent vasodilatation by IGF-I[32,34,35]. Moreover, IGF-I may cause vasorelaxation by non-endothelium-dependent actions[36], possibly by increasing the activity of the Na^+,K^+-ATPase in vascular smooth muscle cells[37]. Despite the accumulating data on IGF-I as a vasodilator, there have only been a few studies addressing the role of IGF-I in physiological regulation of peripheral resistance. In 1996, Lembo *et al.* showed that mice with a mutant IGF-I allele and 30% of wild-type IGF-I levels present in all tissues and serum have elevated blood pressure[38]. In a recent study from our laboratory of transgenic mice with a liver-specific knockout of IGF-I (LI-*IGF-I*$^{-/-}$ mice), resulting in a 80% decrease of circulating IGF-I, we found a similar elevation of blood pressure as in the IGF-I mutant mice with a similar decrease in serum IGF-I, indicating that the decrease in endocrine-acting IGF-I is responsible for this blood pressure-elevating effect[39].

2.2 GH/IGF-I and cardiac hypertrophy

In vitro, the results of many studies have shown that IGF-I increases protein synthesis[40,41] and the size of cardiomyocytes[42]. Many studies have failed to show direct, IGF-I-independent hypertrophic effects of GH on cardiomyocytes[41,42] , but it has been suggested that GH may cause alterations in cardiomyocyte metabolism and stimulate hypertrophy independently of IGF-I[43,44].

Genes involved in cardiac hypertrophy. It has previously been shown that IGF-I stimulates the expression of muscle-specific genes[42]. In hypophysectomized (hx) rats, GH and IGF-I treatment stimulated similar cardiac gene expression (skeletal a-actin, atrial natriuretic factor), but neither IGF-I nor GH caused a shift of myosin heavy chain isoforms[45]. Moreover, altered Na^+,Ca^{2+}-exchanger expression[6] and inhibition of Na^+,K^+-ATPase[7] have been associated with development of hypertrophy. Interestingly, it has been shown that expression of activated calcineurin mimics the hypertrophic effects of IGF-I in skeletal muscle[46]. Taken together, these data suggest a role for calcium as a mediator of GH-induced hypertrophy.

Effects on extracellular matrix. Cardiac hypertrophy requires concomitant remodeling of the heart and attention has recently been focused on the role of the extracellular matrix in the remodeling process[47]. Matrix metalloproteinases, which are capable of degrading all the matrix components of the heart, are the driving force behind myocardial matrix remodeling[47]. It has been shown that IGF-I promotes collagen synthesis by fibroblasts[48] and that GH increases the collagen deposition rate in the heart[49], while in GH-induced hypertrophy the volume fraction of collagen is normal[50,51]. Thus, available data are in line with an increased synthesis, as well as breakdown of extracellular matrix with a finally unchanged cardiac collagen concentration by GH.

Increased number of cardiomyocytes? Beşides promoting cardiomyocyte growth, GH/IGF-I may influence the trophic status of the myocardium by preventing cardiomyocyte loss through apoptosis. It has been shown that apoptosis is ongoing even in the normal heart[52] and there is substantial evidence that IGF-I acts as an inhibitor of apoptosis and that GH/IGF-I may be "cardioprotective" [53-56]. However, although an increased number of nuclei in the heart of GH-treated adult rats has been reported[51], there is still no evidence that GH or IGF-I affects cardiac myocyte number in the normal heart unless overexpressed from the embryonic cell stage[52].

2.3 GH/IGF-I and cardiac contractility

In line with the findings on cardiac hypertrophy, there are several *in vitro* studies demonstrating direct effects of IGF-I on intrinsic cardiac contractility[53-55], while there is thus far no evidence of direct, IGF-I-independent effects of GH on cardiac contractility. However, if animals are treated with GH *in vivo*, allowing stimulation of IGF-I, subsequent *in vitro* assessment shows improved contractility[56,57]. Accordingly, decreased contractility has been demonstrated in dwarf rats with GH/IGF-I deficiency[58-60]. In contrast, a paradoxical enhancement of cardiac contractility was observed in the IGF-I mutant mouse[38].

At least three different mechanisms have been suggested for the GH/IGF-I-induced increase in cardiac contractility: (1) altered intracellular Ca^{2+} transients, (2) increased myofilament Ca^{2+} sensitivity, and (3) a myosin isoform shift.

Intracellular Ca-transients. IGF-I has been shown to acutely affect Ca^{2+} currents within the cardiomyocyte, with increased peak Ca^{2+} levels[54,61], and an altered time course of the current[54] in association with increased contractility. Specifically, the activity of L-type Ca^{2+}channels was acutely increased by IGF-I *in vitro*[62]. In cardiomyocytes from acromegalic rats, the action potential duration was increased as a result of a decrease in density of a transient outward current carried by K^+, which, in turn prolongs the Ca^{2+} influx through L-type Ca^{2+} channels[63]. In contrast to findings in other *in vitro* studies, the acute increase of inotropy by IGF-I was associated with decreased peak Ca^{2+} levels but increased Ca^{2+} sensitivity of the contractile elements in isolated whole heart preparations[55]. No influence of GH on Ca^{2+} currents has been seen in acute settings[55,61], while after more long-term treatment *in vivo*, GH has been suggested to increase peak intracellular Ca^{2+} levels measured *ex vivo*[56,57]. Accordingly, reduced peak intracellular Ca^{2+} levels as well as slowed intracellular Ca^{2+}clearing has been demonstrated in GH/IGF-I deficiency[60], while others report peak intracellular Ca^{2+} levels to be unchanged in GH/IGF-I deficiency[59].

To date, little is known about possible genes involved in the action of GH/IGF-I in altering Ca^{2+} handling. An up-regulation of SERCA levels has been suggested to contribute to the increased contractile function elicited by GH after myocardial infarction[57] and in rapid pacing heart failure[54], while another study[64], did not detect any change in SERCA expression. SERCA may increase contractility by enhancement of the so-called contractile reserve, *i.e.*, the Ca^{2+} storage within the sarcoplasmic reticulum, allowing higher peak Ca^{2+} levels on stimulation. Ueyama *et al.* also suggested that

GH treatment in cardiomyopathic, but not normal, hamsters preserved cardiac ryanodine receptor density[65].

Inhibition of Na^+,K^+-ATPase increases intracellular Na, which causes a net Ca^{2+} influx through the Na^+,Ca^{2+} exchange system and thereby enhances cardiac contractility[5], in addition to a possible role in the development of hypertrophy[7]. However, the activity of cardiac Na^+,K^+-ATPase has previously been reported to not be significantly changed by GH treatment in pigs[66] or GH-deficient dwarf rats[58].

Myofilament Ca-sensitivity and myosin isoform shift. GH/IGF-I has been suggested to increase myofilament Ca^{2+} sensitivity[55,67,68] and maximum Ca^{2+} activated force[55,56,67]. However, data are conflicting, and others report unchanged[61] or even decreased[56] myofilament Ca^{2+} sensitivity by GH/IGF-I. In dwarf rats, unchanged myofilament Ca^{2+} sensitivity has been reported[59,60], although maximum Ca^{2+} activated tension was less[59]. In animal models of GH excess, a shift toward a myosin isoform with lower ATPase activity has been demonstrated, which may decrease the energy demand of the contractile process[67,69].

Taken together, available data suggest that GH/IGF-I may increase cardiac contractility through alterations of intracellular Ca^{2+} transients, myofilament Ca^{2+} sensitivity and myosin isoform expression, although the findings vary between different settings and between studies. Besides regulation of ion channel activity, GH/IGF-I may regulate the expression of ion channels.

2.4 GH deficiency

In line with a trophic effect of GH/IGF-I on the heart, GH deficiency has been associated with a reduction of left ventricular mass and cardiac output in both experimental[58,70] and clinical[71] studies. GH substitution may improve cardiac output, as well as left ventricular mass and/or wall thickness[58,70-72]. Both experimental and clinical studies have shown that GH deficiency is associated with an increased total peripheral resistance that may be reduced by GH substitution[70,72]. Despite increased total peripheral resistance, blood pressure may be unchanged or even decreased in experimental models of GH deficiency[58,70,73], as well as in studies of young adults with GH deficiency[71,74,75].

Hypopituitary patients on routine pituitary hormone replacement therapy, excluding GH, have an increased mortality from cardiovascular disease[76] and several cardiovascular risk factors (abdominal obesity, insulin resistance, decreased fibrinolysis, disturbed lipoprotein pattern, atherosclerosis) have been identified in GH-deficient patients[77]. These factors may underlie the increased prevalence of treated hypertension reported in a cohort of patients with adult onset GH deficiency[77], a notion

that is supported by a recent study showing increased systolic blood pressure in familial isolated GH deficiency[78]. Taken together, the reports on blood pressure levels in GH deficiency are somewhat conflicting, and it is likely that the time of onset, as well as the duration of the GH deficiency, determines both its cardiac and vascular consequences.

2.5 GH/IGF-I excess

Some of the first experimental studies of the hemodynamic effects of chronic GH hypersecretion utilized a rat model with an implantable GH-secreting tumor[79]. These rats displayed cardiomegaly and increased cardiac contractility and output, while peripheral resistance was decreased[79]. In human acromegalics two stages of cardiovascular disease have been identified, an early "hyperkinetic" stage with increased cardiac output and decreased total peripheral resistance, and a late stage characterized by cardiomyopathy, hypertension, atherosclerosis, and ischemic heart disease[13]. This is in agreement with experimental studies suggesting beneficial effects initially, but later impaired cardiac performance in mice with overexpression of IGF-I in the heart[80] or overexpression of bovine GH (bGH) in mice[81,82]. The acromegalic cardiomyopathy is characterized by cardiomegaly, ventricular hypertrophy, fibrosis, and degeneration of cardiomyocytes[83]. Hypertension has been reported in the bGH mouse model[84] and different studies report a prevalence of hypertension of 20–50% of acromegalic patients[83]. Although some of these late complications can be ascribed to the metabolic derangements in acromegaly rather than the GH hypersecretion *per se*[85], the prevalent opinion today is that there is an independent detrimental cardiovascular effect of chronic exposure to excessive GH levels[83].

2.6 The GH/IGF-I axis in cardiovascular pathology

Considering the importance of the GH/IGF-I axis in cardiovascular physiology, one might expect that the GH/IGF-I axis plays a role, either mediary or compensatory, in cardiovascular pathophysiology. Indeed, local IGF-I, systemic IGF-I, and GH levels may all be affected in different cardiovascular pathological states. IGF-I expression is known to increase in cardiomyocytes[86], vascular smooth muscle cells[87], and whole heart preparations[86] in response to stretch *in vitro*. Moreover, cardiac IGF-I expression is up-regulated in both pressure and volume overload states[20,88].

Hypertension. In accordance with the aforementioned, hypertensive rats have increased vascular and cardiac IGF-I expression[21,89], while serum IGF-I has been reported to be unchanged[90]. Serum IGF-I has been reported to be

increased[91,92] and to be an independent determinant of cardiac mass[93] in human hypertension. Others have suggested that serum IGF-I correlates negatively to blood pressure in borderline hypertension[94]. Decreased IGF-I in serum, with a good negative correlation to mean arterial pressure, has also been reported in obese hypertensive patients[95]. Taken together, data on the relationship between serum IGF-I levels and blood pressure are conflicting, possibly reflecting alternatively causative or compensatory roles of IGF-I in different cohorts.

Myocardial infarction. As in all conditions with acute stress, serum levels of GH and IGF-I are elevated in acute myocardial infarction[96,97]. It has been shown that patients with poor outcomes have lower serum IGF-I in the acute infarction phase[97] and that low serum IGF-I is associated with increased the risk of ischemic heart disease[98]. Accordingly, a polymorphism in the IGF-I gene causing low serum IGF-I levels increases risk of myocardial infarction[99]. Following the increased load, auto/paracrine IGF-I is overexpressed within 6 h after experimental myocardial infarction, especially in the zone of viable myocardium immediately adjacent to the infarct[100]. After the acute infarction phase, local IGF-I remains elevated for at least 20 days[100].

2.7 GH/IGF-I in the treatment of heart failure

Several research groups have studied the effects of GH and/or IGF-I in states of impaired cardiac function. In an established rat model of congestive heart failure following ligation of the left coronary artery, GH and IGF-I has been found to increase stroke volume and cardiac output[101,102], also in the presence of angiotensin-converting enzyme (ACE) inhibition[103]. GH treatment of rats with experimental myocardial infarction has also been found to improve myocardial bioenergetics[104] and long-term survival[105].

In studies of patients with congestive heart failure due to ischemic or dilated cardiomyopathy, both acute administration of GH and more chronic treatment evoked beneficial effects[106,107]. Recently, however, randomised, placebo-controlled studies have failed to show any significant GH-mediated improvement of cardiac performance in patients with heart failure, despite significant increases in IGF-1[108,109]. Acquired GH resistance might be an important feature of severe heart failure and may explain the diverse responses to GH therapy observed in different patients[110]. Moreover, the duration of treatment has been relatively short (3 months) and the studies include a small number of patients, which may also contribute to the negative results. Hence, whether GH treatment will finally find a place in the treatment of heart failure remains to be established.

3. GH/IGF-I AND THE RESPIRATORY SYSTEM

Although traditionally the respiratory system is not considered to be under major endocrine regulation, it has become clear that hormones can influence respiration in several ways. In principle, hormones can influence breathing at the level of CNS, at peripheral chemoreceptors, or by altering basic metabolic rate. Long-term indirect effects of hormones on the respiratory system include alterations of acid/base balance, body temperature and body composition (for review, see ref. 111). Studies related to the GH/IGF-I axis and respiration are essentially limited to sleep-disorder conditions such as obstructive sleep apnea syndrome (OSAS), chronic obstructive pulmonary disease (COPD) and Prader–Willi syndrome (PWS).

Patients with OSAS have decreased GH secretion that can be normalized with continuous positive airway pressure (CPAP) treatment[112]. There are also a few reports suggesting a beneficial effect of GH treatment of patients with COPD on peak expiratory flow, respiratory muscle strength[113] and maximal inspiratory pressure[114].

PWS is a neurogenetic disorder in which clinical characteristics include short stature, mental retardation, behavior disorders, muscular hypotonia, hypogonadism and hyperphagia often resulting in severe obesity. In addition, many PWS patients frequently suffer from excessive daytime somnolens and apnea during sleep. These disturbancies could be in part related to obesity, but PWS patients also have decreased sensitivity of peripheral chemoreceptors to changes in blood oxygen and CO_2[115]. GH treatment of patients with PWS results in increased ventilation, central inspiratory drive, airway occlusion pressure and hypercapnic respiratory response[116].

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Chapter 7

HEMATOPOIESIS

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1. INTRODUCTION

The crosstalk between the neuroendocrine and hematopoietic systems is now well established. Similar ligands and receptors are used by these systems, providing physiological intra- and intersystem communication circuitry, which seems to play a relevant role in homeostasis. Increasing evidence has placed growth hormone (GH) and insulin-like growth factors (IGFs) among potent modulators of the hematopoietic system, in both health and disease (reviewed by Venters[1] and Zumkeller[2]). Herein, we focus on the effects of GH and IGF-I upon hematopoietic organs including bone marrow, thymus, and peripheral lymphoid organs. Nevertheless, before discussing such effects, it seems worthwhile to summarize background data concerning the general features of hematopoiesis, in terms of developing cells as well as the corresponding microenvironments in which differentiation takes place.

2. HEMATOPOIETIC MICROENVIRONMENTS AND THEIR ROLE IN HEMATOPOIESIS

Bone marrow and thymus are the primary hematopoietic organs responsible for the generation of myeloid and lymphoid precursors of the various cellular blood components. As briefly described in the following, these series of complex proliferation/differentiation and death processes occur in the context of specific microenvironments in each organ.

In the bone marrow, cell differentiation occurs from a common stem cell that progressively gives rise to further precursors, now committed with the distinct cell lineages, as illustrated in Fig. 1.

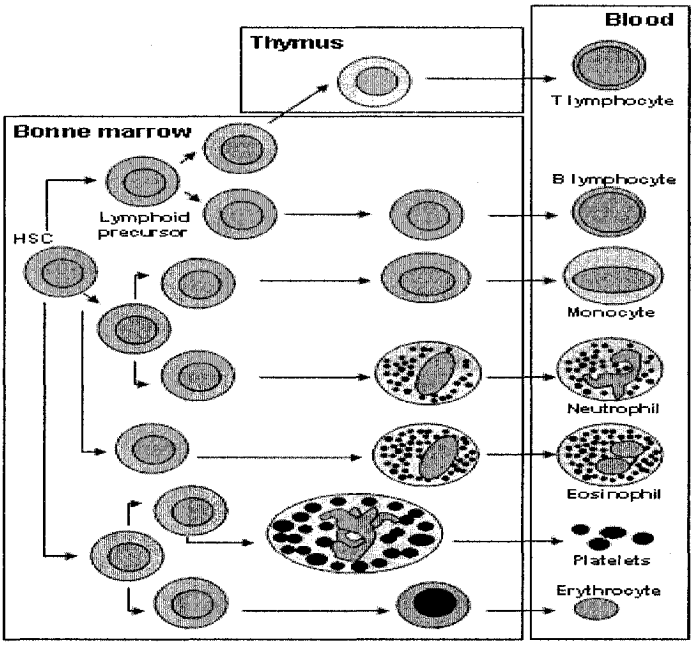

Figure 1. Hematopoiesis in the bone marrow. In this simplified scheme, we can see that starting from a common hemopoietic stem cell (HSC), both lymphoid and non-lymphoid blood components are generated in the bone marrow. As regards lymphoid cells, a common progenitor differentiates into a T-cell precursor (that leaves the organ toward the thymus) and a B-lymphoblast that will eventually differentiate within the bone marrow into a mature B lymphocyte. Intermediate precursors for the monocyte/granulocyte series will further differentiate and give rise to monocytes and neutrophils. Other precursors are committed to the generation of eosinophils or basophils (not shown). In addition, distinct HSC-derived precursors can differentiate toward the megakaryocytic series, eventually giving rise to platelets or the erythroid precursors that will differentiate into erythrocytes. Once each mature cell in the bone marrow is formed, it will leave the organ to gain the blood vessel.

Such progressive differentiation can be traced morphologically, phenotypically, and functionally. For example, CD34 is a membrane protein typically found in bone marrow precursors of the various lineages, including lymphoid, macrophage/granulocytic, megakaryocytic, and erythrocytic. By contrast, coexpression of CD34 plus CD2 denotes a pre-T commitment, whereas $CD34^+CD19^+$ precursors will progress toward B lymphocyte differentiation. Immature B cells will rearrange their immunoglobulin genes and the resulting products will form the core of the B-cell receptor. Ultimately, cells formed in the bone marrow leave the organ migrating through a sinusoid capillary wall, and gaining the blood flow.

Maturation of the distinct cell lineages in the bone marrow largely depends on the local interaction with microenvironmental components, which comprise reticular cells, endothelial cells, fibroblasts, and macrophages, as well as adipocytes and osteoblasts. Accordingly, hematopoiesis is influenced by a large number of soluble products secreted by the microenvironmental cells, including typical cytokines, growth factors and chemokines, comprising among others, interleukin (IL)-1, IL-3, stem cell factor, colony stimulating factors for granulocytes (G-CSF), and granulocyte/macrophages (GM-CSF), and the chemokine CXCL12 (reviewed by Metcalf[3]). In addition, the hormone erythropoietin, normally produced in the kidney, is crucial for the development of the erythroid lineage, whereas thrombopoietin is important for the production of megacaryocytes, and ultimately for platelet differentiation.

Important is the concept that distinct combinations of cytokines in the milieu generate the preferential expansion and differentiation of specific cell lineages. For example, the presence of IL-3, stem cell factor, plus GM-CSF directs differentiation toward the granulocyte/macrophage lineages, whereas IL-3 plus GM-CSF alone tends to induce proliferation/commitment of eosinophilic precursors. In addition, chemokines are involved in bone marrow physiology, as exemplified by the role of CXCL12 and its receptor CXCR4 in stem cell mobilization[4].

Cell–cell interactions are also crucial for normal hematopoiesis in the bone marrow. For example, membrane receptors such as the syalomucin CD34; the proteoglycan CD44 (that binds to hyaluronic acid and fibronectin); and the integrin $\alpha4\beta1$ (CD49d/CD29), which binds fibronectin and the vascular cell adhesion molecule, are relevant in the mobilization of $CD34^+$ hematopoietic precursors[4,5]. It is noteworthy that such an important event is also under control of matrix proteinases, including MMP2 and MMP9 (see review[4]).

In the thymus, bone marrow-derived T-cell precursors undergo differentiation (Fig. 2A), ultimately leading to migration of positively selected thymocytes to the T-cell–dependent areas of peripheral lymphoid

organs[6]. Such a process involves sequential expression of various proteins and rearrangements of the T-cell receptor (*TCR*) genes. The most immature thymocytes express neither the *TCR* complex nor the CD4 or CD8 accessory molecules, being called double-negative thymocytes. Maturation progresses with the initiation of rearrangements of *TCR* genes and then acquisition of CD4 and CD8 markers, yielding the $CD4^+CD8^+$ cells. Productive rearrangements result in the membrane expression of the *TCR* (complexed with the CD3) in low densities (TCR^{low}). Thymocytes that do not undergo a productive *TCR* gene rearrangement die by apoptosis, whereas those expressing productive *TCR* will interact with peptides presented by molecules of the major histocompatibility complex (MHC), expressed on microenvironmental cells. This interaction will determine the positive and negative selection events, crucial for normal thymocyte differentiation. Positively selected thymocytes progress to the mature $TCR^{high}CD4^+CD8^-$ or $TCR^{high}CD4^-CD8^+$ single positive stage, and ultimately leave the organ to form the large majority of the peripheral T cell repertoire[6].

Thymocyte differentiation occurs as cells migrate within the thymic lobules: $TCR^-CD4^-CD8^-$ and $TCR^+CD4^+CD8^+$ are cortically located, whereas mature $TCR^+CD4^+CD8^-$ and $TCR^+CD4^-CD8^+$ cells are found in the medulla.

Along their way, thymocytes interact with components of the thymic microenvironment, a tridimensional network formed of thymic epithelial cells (TEC), macrophages, dendritic cells, fibroblasts, and extracellular matrix[6,7]. The TEC network is the major component of the thymic microenvironment, being heterogeneous in morphology and phenotype. One lymphoepithelial complex, the thymic nurse cell, corresponds to a multicellular structure formed by one epithelial cell and variable numbers of thymocytes (mostly $CD4^+CD8^+$ cells), being located in the cortical region of thymic lobules. Once obtained TNCs can be used as an *in vitro* model of thymocyte migration in the TEC context. In addition to the interaction involving the TCR/peptide-MHC, in the context of CD8 or CD4 molecules, the thymic microenvironment influences thymocyte maturation via adhesion molecules (Fig. 2B). For example, TEC express intercellular adhesion molecule (ICAM)-1 and lymphocyte function-associated antigen (LFA)-3, which respectively bind to LFA-1 and CD2 present on thymocyte membranes[6]. Moreover, TEC–thymocyte interactions can be mediated by various extracellular matrix (ECM) moieties, and this is relevant for thymocyte migration[7,9].

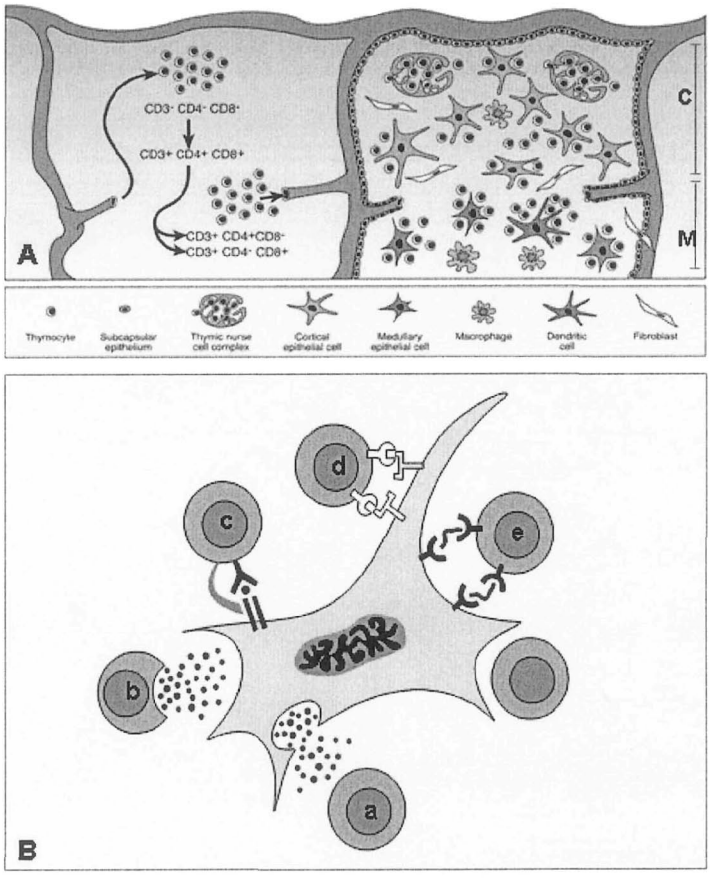

Figure 2. Thymopoiesis and the thymic microenvironment. Panel **A** shows, on the left side, one thymic lobule with a simplified view of thymocyte differentiation. Bone marrow–derived precursor cells enter the organ and localize in the outer cortex. At this stage they do not express the CD3-T-cell receptor complex, or the accessory molecules CD4 or CD8. As they differentiate, they acquire all these markers and become $CD^+CD4^+CD8^+$ cells, being located in the inner cortex. Those cells undergoing positive selection migrate from the cortex (C) to the medulla (M) and become either $CD4^+$ or $CD8^+$ single positive cells. These cells are able to leave the organ. The right part of panel **A** shows that thymocyte differentiation occurs in the context of the thymic microenvironment, composed by various epithelial cells, including the thymic nurse cells, as well as macrophages, dendritic cells, and fibroblasts (see the legend underneath the figure for each cell type). When migrating, differentiating thymocytes encounter microenvironmental cells with which they interact. Some of these cellular interactions are seen in panel **B**, where we can see that they interact with each other via soluble products, produced either by the microenvironmental cell (a) or the developing thymocyte (b). In addition, membrane interactions take place, such as those involving the TCR/CD3 complex with the major histocompatibility complex coupled with an endogenous peptide (c), crucial for defining positive versus negative selection. In addition, they can interact by means of classical adhesion molecules (d) and through extracellular matrix ligands and receptors (e).

Microenvironmental cells also modulate thymocyte differentiation by soluble polypeptides, comprising: (1) cytokines, such as interleukin-1 (IL-1), IL-3, IL-6, IL-7, IL-8, and stem cell factor; (2) chemokines, as for example CXCL12, CXCL10 and CCL25; and (3): thymic hormones, including thymulin, thymopoietin and thymosin-α1[6,10-12].

In addition to terminal differentiation of B cells (including hypermutation of immunoglobulin genes and further negative selection events), activation-induced proliferation and plasma cell differentiation occur in the lymphoid follicles of the various peripheral lymphoid organs, whereas antigen-induced T-cell proliferation takes place in the so-called T-cell zones of these organs, for instance, in the paracortical region of lymph nodes. Again, microenvironmental cells of these tissues are important in lymphocyte proliferation, differentiation, and migration.

3. GH AND IGF-I ENHANCE PRODUCTION OF SOLUBLE PEPTIDES BY HEMATOPOIETIC MICROENVIRONMENTS

Particularly in the thymus, accumulating data indicate that the GH/IGF-I axis influences the production of a variety of cytokines, including the chemokine family. Exogenous GH increases the *in vitro* production of IL-1α and IL-1β by bovine thymic microenvironmental cells[13]. In addition, IL-6 secretion is augmented by GH, an effect partially exerted through the IL-1 production pathway. Interestingly, IL-6 production by thymocytes is also up-regulated by *in vivo* injection of GH in aging animals[14]. More recently, we showed that CXCL12 production by TEC is enhanced, at both mRNA and protein levels, in GH-transgenic mice[15].

In vivo and *in vitro* fluctuations in GH or IGF-I levels modulate thymic hormone secretion. Thymulin levels are low in dwarf mice and in children bearing deficient GH production, whereas GH treatment consistently restored this thymic endocrine function, as early as 24 hours after injection[16-18]. On the other hand, elevated thymulin serum levels have been detected in acromegalic patients, and were correlated with the circulating IGF-1 levels[19]. It is interesting to note that both thymulin and GH serum levels decrease along with age. In this respect, *in vivo* injection of GH in ageing animals significantly increases thymulin serum levels.[14]

Peripheral blood cells can also be modulated by the GH/IGF-I axis, as regards secretory products. One example is the GH or IGF-I-induced enhancement of secretion of superoxide anion by neutrophils and

macrophages, which is important for their ability to kill bacteria (reviewed by Venters[1]).

4. GH/IGF-I EFFECTS ON PROLIFERATION AND DEATH OF HEMATOPOIETIC CELLS

Several data indicate that the GH/IGF-I axis stimulates erythropoiesis (see review Halvorsen and Bechesteen[20]), in particular when the levels of erythropoietin are low or absent[21]. Such effects are actually abrogated with anti-IGF-I receptor antibodies[22,23], which is in keeping with the demonstration of IGF-I receptors in cells of the erythroid lineage[24]. Interestingly, administration of IGF-I in patients suffering from Laron syndrome (primary GH insensitivity) results in a strong stimulatory effect on erythropoiesis[25]. In the same vein, erythropoiesis, which is impaired in adult GH-deficiency, is stimulated once the patients undergo GH treatment[26]. The monocytic lineage is also influenced by the GH/IGF-I axis, since *in vitro* proliferation of bone marrow-derived macrophages is enhanced by exogenous IGF-I[27].

The expression of proliferation-related genes such as cyclins A and E (respectively controlling the entry into and transition through the S phase of the cell cycle) is enhanced in GH receptor expressing Ba/F3 pro-B cell line after GH treatment[28]. Furthermore, it has been shown that in 32D myeloid precursor cell line, as well as in Ba/F3 cells, IGF-I synergizes with IL-4 to enhance cell proliferation, an effect that requires MAPK and STAT-6 signalling pathways[29]. Furthermore, it has been shown that IGF-I synergizes with IL-7 in inducing proliferation of pro-B cells[30]. These data are in keeping with those showing that IGF-I bone marrow stimulates B lymphopoiesis[31].

Studies performed in 32D cells also revealed that in some conditions, activation of the IGF-I receptor results in differentiation towards the granulocytic pathway similar to the granulocyte colony-stimulating factor[32].

Proliferation of thymocytes was enhanced when they were exposed *ex-vivo* to supernatants from GH-treated TEC cultures[33]. In addition, GH itself synergizes with anti-CD3 in its direct stimulatory effect on thymocyte proliferation[34]. In the same vein, an enhanced concanavalin-A mitogenic response and IL-6 production by thymocytes is observed in GH-treated aging rats[14], as well as in IGF-I-treated mice[35]. Finally, it is noteworthy that human thymocyte-derived GH-induced proliferation in thymocyte suspensions is apparently mediated by IGF-I[36]. As further discussed in the following sections, these findings indicate an autocrine/paracrine influence of the GH/IGF-I axis in the physiology of the thymus.

In addition to the data obtained in cell culture or *ex-vivo* conditions, a series of *in vivo* observations further supports the notion that GH and IGF-I exert enhancing effects upon hematopoiesis, and more particularly, thymopoiesis. Injections of GH3 cells (a pituitary adenoma cell line, able to produce GH) to old rats reversed the age-dependent thymic atrophy, with a consequent increase in thymocyte numbers[37]. Similar results were obtained with injection of IGF-I[38]. In addition, it was shown that cyclosporin A-induced thymic atrophy was restored by *in vivo* treatment with recombinant GH or IGF-I[39], and that IGF-I was able to induce repopulation of the atrophic thymus from diabetic rats[38]. These findings are supported by a clinical case of an acromegalic patient with high circulating levels of GH and IGF-I associated with thymic hyperplasia[40]. Moreover, as seen in Fig. 3, transgenic mice overexpressing GH or GH-releasing hormone exhibit overgrowth of the thymus and peripheral lymphoid organs.

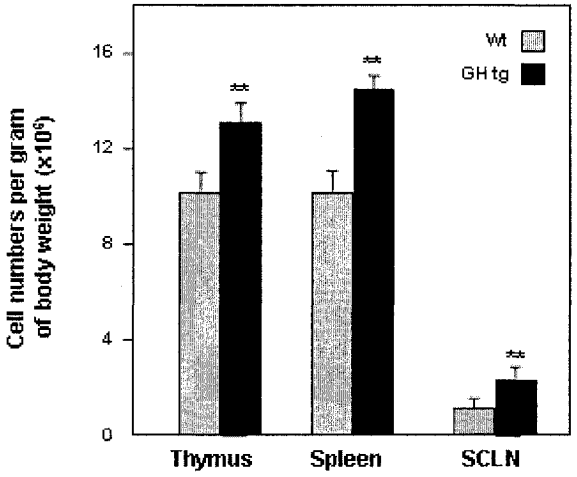

Figure 3. Increase of cell numbers in primary and secondary lymphoid organs in GH-transgenic mice. Considering that GH-transgenic animals are larger in size and weight, we calculated cell numbers per gram of body weight of each group. In thymus, spleen, and subcutaneous lymph nodes (SCLN) relative cells numbers of GH transgenic mice (GH tg) were higher than the corresponding wild-type controls (Wt). $^{**}p < 0.01$, as ascertained by unpaired Student's t-test.

Furthermore, short-term intrathymic injection of GH in mice, as well as long-term treatment with GH (in association with classical therapy) in HIV-positive patients, promoted an increase in the size of the thymus[41,15]. Also, GH or IGF-I administration together with bone marrow cells into syngeneic old recipients resulted in an increase of thymus cellularity, when compared

to the transfer of bone marrow cells alone[42]. In the same study, the authors showed that IGF-I potentiates the colonization of fetal thymus organ cultures with T-cell precursors, clearly indicating that IGF-I enhances the entrance of cell precursors into the thymus. This is in keeping with the replenishment of the thymus in cats infected with feline immunodeficiency virus. These animals exhibit a severe cortical thymocyte depletion, which is restored after long term treatment with IGF-I[43]. GH/IGF-I-induced cell proliferation is not restricted to primary lymphoid organs: exogenous GH and IGF-I increase cellularity in the spleen, an effect that can be observed not only in fetal age but also in aging animals[37,44]. This is in keeping with the specific increase in cell numbers in spleen and lymph nodes of GH-transgenic mice, as compared to their age-matched wild-type counterparts (Fig. 3).

In keeping with this, GH exhibits a synergistic proliferative effect on concanavalin A- or anti-CD3-stimulated spleen-derived T cells[34], thus telling us that the periphery of the hematopoietic system is also a target for the GH/IGF-I axis. IGF-I also stimulates peripheral $CD4^+$ T cells as well as B lymphocytes in the spleen (reviewed by Zumkeller[2]).

In addition to enhancing cell proliferation, a role of the GH/IGF-I in modulating apoptosis has been documented. For example, it has been shown that GH prevents apoptosis in the Ba/F3 cell line[45,46], and so has been shown for IGF-I, which actually enhanced the expression of the anti-apoptotic molecule Bcl-xL[47]. Moreover, in the rat Nb2 T cell lymphoma, GH inhibits the pro-apoptotic effect of dexamethasone, in a dose-dependent manner. Lastly, DNA breakdown in human erythroid colony-forming cells is largely reduced by IGF-I. Taken together, the data summarized in the foregoing strongly indicate that the ultimate enhancing impact of the GH/IGF-I axis in hematopoiesis is a combined effect of increase in cell proliferation plus decrease in programmed cell death.

5. THE GH/IGF-I AXIS MODULATE THE TRAFFIC OF HEMOPOIETIC CELLS?

The traffic of hematopoietic cells is also a target for the GH/IGF-I axis. Although the literature remains poor concerning normal bone marrow cells, it has been shown that exogenous recombinant GH in aging or irradiated mice not only induces hematopoiesis, but also mobilizat on of hematopoietic precursors and significant increase in blood cell counts[48].

Bone marrow stromal cell-derived IGF-I can play a role of chemoattractant for multiple myeloma cells[49]. In addition, IGF-I induces multiple myeloma cell adhesion to and migration through fibronectin via integrin-type fibronectin receptor stimulation[50], which fits with the data

showing that IGF-I enhanced transendothelial migration of these cells[51]. Recombinant GH increases human T-cell engraftment into the thymus of severe combined immunodeficiency (SCID) mice, an effect that seems to be mediated by adhesion molecules and extracellular matrix, since it can be abrogated with anti-β1 or anti-β2 integrin antibodies[52]. Moreover, as mentioned earlier, it has been demonstrated that IGF-I potentiates the colonization of fetal thymus organ cultures with T cell precursors[42], clearly indicating that IGF-I also enhances the entrance of cell precursors into the thymus. We showed that intrathymic lymphocyte traffic is enhanced in vitro by GH and IGF-I, via modulation of extracellular matrix ligands and receptors[53]. It is noteworthy that insulin does not promote significant effects upon the intrathymic nurse cell traffic[54], suggesting that IGF-I effects are triggered by the IGF-I receptor itself, and not via the insulin receptor. *In vivo*, we showed that, 16 h following a single intrathymic injection of GH in normal mice, there is an increase in numbers of $CD4^+$ recent thymic emigrants in the lymph nodes[15]. Moreover, increasing numbers of circulating $CD4^+$ recent thymic emigrants were seen in the blood of HIV patients receiving GH therapy[41].

Recent data from our laboratory indicate that GH also influences migration of lymphocytes from peripheral lymphoid organs. As depicted in Table 1, ECM-driven, as well as chemokine-driven lymphocyte migration is enhanced in GH transgenic animals, as compared to corresponding age-matched wild-type controls. Since the density of the respective receptors in peripheral lymphocyte surface does not change, these experiments also indicate that GH overexpression yields an activation status of ECM and chemokine receptors, above the levels seen in normal individuals.

	Wild type ($x10^4$)	**GH-Tg** ($x10^4$)
BSA	2.53 ± 0.18	2.38 ± 0.21^{NS}
LN	4.85 ± 0.17	6.45 ± 0.43*
CCL21	7.55 ± 0.16	11.30 ± 0.46*
LN + CCL21	23.90 ± 1.74	29.60 ± 2.26*

Table 1. Enhancement of migratory capacity of peripheral cells from mesenteric lymph nodes of GH transgenic mice, as compared to wild-type controls*

6. GH/IGF-I CIRCUITS IN HEMATOPOIETIC TISSUES

To further understand the various endocrine effects of GH and IGF-I in hematopoietic tissues, it is necessary to define the molecular mechanisms involved in triggering such biological responses. In this respect, expression of receptors for both hormones has been detected in both hematopoietic and microenvironmental cells of bone marrow, thymus (Fig. 4), as well as secondary lymphoid organs.

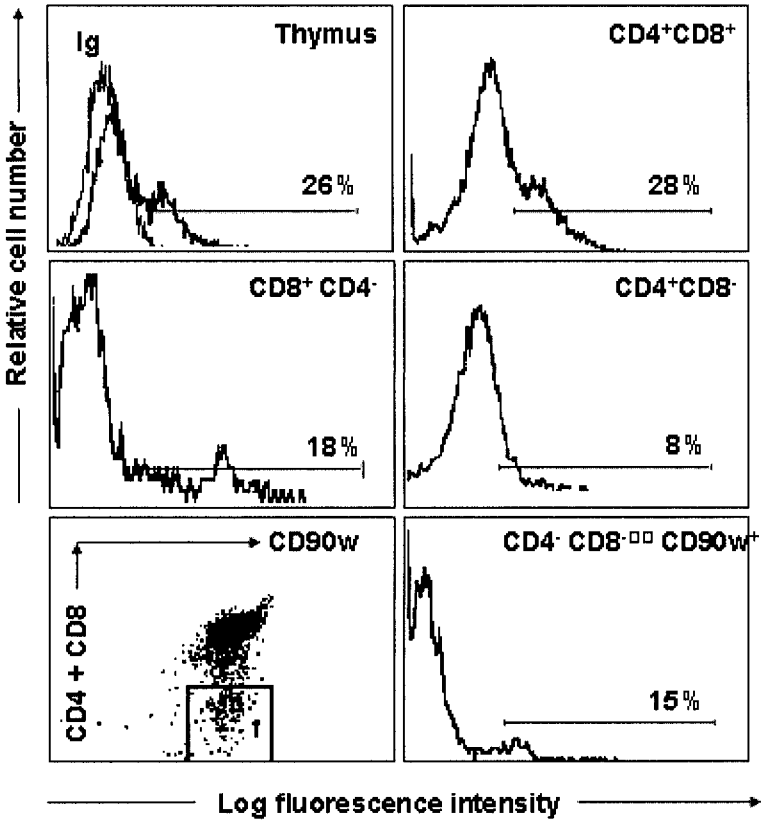

Figure 4. Flow cytometry analysis of GH receptor expression by thymic lymphocytes. The upper left panel shows the whole labeling of thymocytes, whereas the others depict GH receptor in CD4/CD8-defined subsets. Mean percentage levels are shown within each profile. Since the $CD4^-CD8^-$ double-negative compartment also contains non-T lymphocytes, we first gated double-negative cells in the positive region for CD90w (a pan T-cell marker) and negative region for CD4 plus CD8, as seen in the bottom left panel. The cells within the square, thus corresponding to $CD4^-CD8^-CD90^+$ thymocytes, were then evaluated for the presence of GH receptor, as seen in the bottom right panel.

Expression of GH receptor by human TEC has been demonstrated by means of ligand-specific binding assay, immunocytochemistry, *in situ* hybridization and reverse transcriptase-polymerase chain reaction (RT-PCR) (reviewed by Savino and Dardenne[6]) Additionally, GH receptor is present on thymocytes, particularly in the $CD4^-CD8^-$ immature subset[55,56]. Together, these findings point to the possibility of indirect as well as direct role of GH in thymocyte differentiation.

In peripheral lymphoid organs as well as in circulating blood cells, we also detected GH receptors in both T and B lymphocytes, as well as monocytes and granulocytes[57], thus indicating that hematopoietic cells may be responsive to GH, not only in early phases of their differentiation but also once they are mature, which fits, for example, with the data showing that GH improves resistance to bacterial infections, acting directly on phagocytic cells[58,59].

IGF-I receptor has been found in hematopoietic tissues. In the bone marrow, IGF-I receptor has been detected in erythroid, granulocytic/macrophage, as well as lymphoid lineages (reviewed by Venters[1] and Zumkeller[2]). IGF-I receptor is also expressed in thymocytes and TEC[60-63]. Interestingly, we found that IGF-I receptor density on TEC membranes increases following *in vitro* GH treatment[62], clearly suggesting that in hematopoietic tissues, IGF-I receptor expression is also partially under GH control. Lastly, functional IGF-I receptor is also present in peripheral lymphocytes, monocytes, and granulocytes[59,63].

In addition to the GH/IGF-I endocrine loops discussed earlier, these molecules likely act on an autocrine/paracrine way. In fact, it has been largely shown that cells of hematopoietic tissues produce GH, as well as IGF-I. Bone marrow stromal cells, as well as hematopoietic precursors in the organ can secrete both hormones[1]. Actually, IGF-I is secreted by human $CD34^+$ precursors, as well as *ex vivo* expanded granulocyte-macrophage, erythroid, and megakaryocyte precursors[63].

Moreover, both TEC and thymocytes can produce GH and IGF-I[35,53,56]. From a functional point of view, it was found that thymocyte proliferation induced by thymocyte-derived GH occurs via IGF-I production by these cells[64], indicating an autocrine/paracrine circuit involving the GH/IGF-I axis. In fact, thymocytes and epithelial cells constitutively express IGF-I, and such expression is enhanced by GH[62]. Accordingly, anti-IGF-I receptor blocking antibodies block thymocyte differentiation and expansion in fetal thymus organ cultures[65].

Overall, at least concerning lymphopoietic tissues, we can hypothesize that the GH/IGF-I axis exerts its modulatory effects simultaneously by means of endocrine and autocrine/paracrine loops.

7. HEMATOPOIESIS IN GH GENETICALLY ENGINEERED ANIMALS

The various aspects discussed in the preceeding quite strongly point to a role of GH and IGF-I in regulating various aspects of hematopoiesis, not only *in vitro* but, most importantly, in normal adult life. A putative mandatory role of GH and IGF-I upon the early development of hematopoietic organs has been investigated using genetically engineered mice, either for GH, GH receptor, or IGF-I.

In both GH receptor knockout and GH transgenic mice, we can see progression from immature $CD4^-CD8^-$ thymocytes through $CD4^+CD8^+$, ending up with CD4 and CD8 simple positive cells[66] (our unpublished results). Nevertheless, the thymic microenvironment of GH transgenic and GH receptor knockout animals is altered. Taking thymulin serum levels as one parameter for TEC activity, we found an enhancement in GH-transgenic and a decrease in GR receptor knockout mice, as compared to age-matched wild-type counterparts[54]. In addition, recent data revealed an increase in laminin production and CXCL12 expression by TEC from GH transgenic animals that parallels increased laminin- or CXCL12-driven thymocyte migration in these mice (our unpublished results).

Studies have also been conducted in IGF-I knockout mice[6,7], and the relative numbers of CD4/CD8 defined thymocyte subsets appear normal, which tells us that thymocyte development can occur in the absence of IGF-I. Yet, in this study several other parameters of both the lymphoid and microenvironmental compartments were not dissected. So, we cannot discard the possibility that some thymic functions are altered in these animals.

8. CONCLUDING REMARKS AND MAJOR ISSUES TO BE ADDRESSED IN THE FUTURE

The findings discussed herein clearly indicate that GH and IGF-I participate in the physiology of hematopoiesis. Although data from genetically engineered mice indicate that these molecules are not mandatory for initial development of hematopoietic tissues, it is apparent that circulating levels of these hormones in adult individuals regulate various biological functions related to both microenvironmental and lymphoid cells.

Also necessary for a better understanding of the role of the GH/IGF axis in hematopoiesis will be the dissection of the intracellular signaling pathways triggered by the GH/GH receptor ligation and activation of the IGF-I circuit in distinct cell types of hematopoietic organs. This issue,

together with an accurate gene profiling study for determining which genes are modulated by these molecules, will provide a more solid molecular background for the biological responses elicited by GH/IGF-I axis.

The possibility that GH and IGF-I improve some thymic functions, including thymocyte proliferation and migration, places this molecule as a potential adjuvant therapeutic in immunodeficiency conditions associated with thymocyte decrease and loss of peripheral T cells, as in HIV infection. Peripheral blood mononuclear cells from HIV-infected patients treated with GH for 3 months exhibited an upward trend in the IL-2 production after *in vitro* stimulation with HIV-HIV-1 envelope peptides[8]. More recently, 6-month treatment of AIDS patients with GH or IGF-I plus anti-virus therapy resulted in an increase of the thymus, with augmentation of $CD4^+$ recent thymic emigrants and total numbers of circulating $CD4^+$ T cells[41]. In addition, it has been recently shown that IGF-I was able to enhance lymphoid as well as myeloid cell reconstitution in mice, following allogeneic bone marrow transplantation, without side effects in terms of aggravating graft *versus* host disease[69]. Overall, the potential therapeutic use of GH and IGF-I fits with the hypothesis for a general anti-stress effect of these hormones upon the hematopoietic system[70,71].

Lastly, it should be pointed out that the effects of the GH/IGF-I axis upon hematopoiesis should be placed in a broader context. Actually, the neuroendocrine control of hematopoiesis appears to be far more complex, with other hormones, neuropeptides and neurotransmitters being involved[6,70,72].

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Chapter 8

BRAIN DEVELOPMENT

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1. INTRODUCTION

A relatively large body of evidence accumulated in recent years establishes an essential role for insulin-like growth factor-I (IGF-I) in central nervous system (CNS) development. Evidence that growth hormone (GH) has a role in brain development, both by stimulating IGF-I expression and through its direct actions, also is emerging. This chapter focuses on recent studies of the CNS that have contributed to our understanding of the roles played by IGFs and GH on neural proliferation, differentiation, survival, and maturation.

The IGFs, IGF-I and IGF-II, IGF receptors, and IGF binding proteins (IGFBPs), often are collectively termed the IGF system. Compared to GH, much more research has focused on the roles of IGFs in CNS development. Both IGF-I and IGF-II appear to signal their actions predominantly, if not exclusively, by interacting with the type I IGF receptor (IGFIR)[1,2]. Furthermore, each of their actions is modulated by IGFBPs that can either inhibit or augment their activity[3,4]. In addition, the cation-independent mannose-6-phosphate receptor (ciM6PR) binds IGF-II (but not IGF-I) with high affinity, and therefore, also is termed the type II IGF receptor (IGFIIR)[1,2]. It is, however, not clear whether the ciM6PR/IGFIIR transduces IGF-II growth-promoting signals. Because it traffics mannose-6-phosphate-

containing proteins and IGF-II to lysosomes for degradation, the ciM6PR/IGFIIR is capable of regulating IGF-II abundance and in this way likely alters IGF-II's influence. A wide array of studies, including those of genetically engineered mutant mouse lines[5], has demonstrated the pivotal role IGFs play in development and maintenance of the CNS. Components of the IGF system have been implicated in virtually every aspect of neural development including proliferation, survival, differentiation, and maturation (see later).

Although IGF-I and IGF-II are transported across the blood-brain barrier (BBB)[6,7] the relative contribution of peripherally produced IGFs to CNS development, as compared to that produced locally, remains to be entirely clarified. Nonetheless, several observations make it seem certain that IGF developmental influences derive from the actions of IGFs expressed within the CNS: (1) Each component of the IGF system is expressed in the CNS during development; and (2) Circulating IGFs do not appear to contribute significantly to somatic growth until a time in postnatal life when CNS development is nearly complete. Evidence from mutant mouse lines engineered to have low blood IGF-I levels, but normal tissue IGF-I expression, do not begin to exhibit growth retardation until the 3 to 4 weeks of age[8,9].

2. EXPRESSION

2.1 GH and GH receptor

GH is produced in the anterior pituitary gland and is believed to reach the CNS via receptor-mediated transport across the BBB[10,11]. Chronic administration of GH results in an increased GH concentration in CSF[10]. There is, however, evidence that GH can be synthesized within the CNS, specifically in the lateral hypothalamus, caudate putamen, striatum, ventral thalamus, formation reticularis, and the basal cortex [12,13].

The cognant GH receptor (GHR) is expressed in a number of brain regions during development. Studies assessing GHR expression during early development are limited. GHR immunoreactivity is detected as early as embryonic day 3 (E3) in all regions (diencephalon, telencephalon, mesencephalon) of chick brain[14]. By E14, however, GHR expression appears to be restricted to the molecular and pyramidal cell layers of the cerebral cortex, the grey matter of the cerebellum, the walls of the ventricles, and the choroid plexus[14]. In rats, GHR immunoreactivity can be detected between E12-E14 in derivatives of neural plate ectoderm, and in most brain regions by E18[15]. Lobie *et al.*[16] carried out an extensive postnatal ontological

analysis of GHR expression in the early postnatal rat and rabbit. Both neuronal and glial cells demonstrated GHR immunoreactivity. In rats, the strongest immunoreactivity was found in layers 2, 3, 5 and 6 of the cerebral cortex; neurons of the hypothalamus; retinal ganglion cells; and Purkinje cells of the cerebellum. GHR immunoreactivity then gradually decreased from postnatal day (P)2 to P90. GHR mRNA in rabbits exhibits a similar distribution pattern and developmental change[16].

In the adult rats, GHR expression is highest in the choroid plexus although it can be found in other areas, including the hippocampus, hypothalamus, and pituitary[17-19]. Zhai *et al.*[19] also report moderate expression in the cerebral cortex. These results are consistent with those observed in human studies[20,21].

2.2 IGF system proteins

CNS expresses most components of the IGF system, including IGF-I, IGF-II; their receptors; and IGFBP-2, -4, -5, and -6 during development. Each of these proteins is expressed during development in spatially specific patterns that are temporally coordinated. These patterns suggest important and dynamic roles during CNS development. IGFBP-1 and IGFBP-3 appear not to be expressed in the brain during normal development, although IGFBP-3 expression is induced during injury[22]. Each, however, appears to be transported to the CNS and exert biologic actions. For example, IGFBP-1 has been identified in the rat cerebellum[23], and IGFBP-1 when ectopically expressed in the brains of transgenic (Tg) mice markedly retards brain development[24-27].

2.2.1 IGF-I and IGFIR

IGF-I and IGFIR expression are widespread both during development and in the adult. Table 1 summarizes the temporal and region distribution of IGF-I in rodent CNS during development. Brain IGF-I is expressed in a region-specific pattern. While neurons are the major cells of IGF-I expression, IGF-I appears to be expressed by all other major neural cell types, including astrocytes, oligodendrocytes, and microglial[28-32], as well as neural stem cells (NSC) and their lineage-restricted progenitors[33,34]. During rat early postnatal development high IGF-I expression is found localized to somatosensory and brain stem nuclei including intralaminar nucleus, anterior pretectal nucleus, parafascicular nucleus and retroflexus nucleus[35] (Table 1). The timing, magnitude, and location of IGF-I expression closely correlate with dendritic growth and maturation, and with synapse formation in projecting neurons in these regions.

Region	Prenatal	Perinatal	Adult
Cerebrum	D	D	D
Meninges	D	D	D
Cerebral cortex	ND	ND	D
Piriform cortex	ND	ND	D
Corpus callosum	NA	NA	ND
Olfactory	D	D	D
Mitral cell layer	D	D	D
Glomerular layer	D	D	D
Hippocampus		D	D
Dentate gyrus	NA	D	D
Stratum oreins	NA	D	D
Stratum radiatum	NA	D	D
Stratum luc. mol.	NA	D	D
Stratum pyramidal	NA	ND	ND
Cerebellum		D	D
Cerebellar cortex	ND	D	D
Purkinje cell layer	NA	D	D
Ext. granular cell layer	NA	ND	ND
Int. granular cell layer	NA	ND	ND
White matter	ND	ND	ND
Deep nuclei	ND	D	D
Red nucleus	ND	D	D
Other			
Inferior olive	D	D	D
Inferior colliculus	ND	D	D
Medial habenula	D	D	D
Pretectal nucleus	D	D	D
Intralaminar nuc.	D	D	D
Parafascicular nuc.	D	D	ND
Retroflexus nuc.	D	D	ND

Table 1. Temporal and regional IGF-I mRNA expression during development. **D**, mRNA expression detected. **ND**, mRNA expression not detected. **NA**, area not assessed. Adapted from refs. 35-37.

The temporal expression of IGF-I also is associated with neural cell proliferation. IGF-I mRNA has been detected as early as E11 in most areas of murine brain, including olfactory bulb[28,38,39]. As judged by Northern blot analysis of rodent whole brain, IGF-I expression increases rapidly during late prenatal and early postnatal development and peaks in the first week of

postnatal life, times preceding and during rapid proliferation, differentiation and maturation of neural cells[40]. IGF-I expression then gradually decreases to adult levels by the second postnatal week[40]. In general, it appears that IGF-I expression in specific brain regions follows a similar temporal pattern[36,37,41].

Similar to IGF-I expression, brain IGFIR exhibits widespread but distinct temporal expression pattern. IGFIR expression in the CNS begins during embryonic development, peaks perinatally, and then gradually decreases to adult levels in both rodents[42,43] and rabbits[44]. While the IGFIR appears to be expressed in all neural cell types and at each stage of development, its expression is concentrated in distinct areas. In rodents the areas that exhibit relatively high levels of expression include the cerebral cortex, hippocampus, olfactory bulb, cerebellum and choroid plexus[28,45,46]. In addition to the cell body, the IGFIR also is expressed on the growth cone[47] and synapses[48], consistent with a role for IGF-I signaling in dendritic growth and maturation, and synaptogenesis. In the cerebellum the IGFIR is localized to the postsynaptic membrane in the dendrites and soma of Purkinje cells and the presynaptic membrane of axon terminals associated with Purkinje cell soma, as well as mossy fiber rosettes in the cerebellar glomeruli[48].

2.2.2 IGF-II and ciM6PR/IGFIIR

IGF-II likely has a role in early CNS development. In contrast to IGF-I, which exhibits greater magnitude and widespread expression during postnatal life, IGF-II is expressed less broadly in the prenatal CNS. In rats, IGF-II mRNA has been found in the neuroectoderm as early as E10[49]. In addition to choroid plexus and meninges, IGF-II mRNA also is detected in the hippocampus, hypothalamus, brain stem, and floor of the 3rd ventricle from E13 to E18[38,50]. Unlike IGF-I, which is predominantly expressed by neurons during development, IGF-II appears to be primarily expressed in glia[40,50]. IGF-II expression in most brain regions gradually declines with maturation, and by the time of birth its expression appears to be restricted to the choroid plexus and meninges, where it continues to be expressed at relatively high levels throughout life[51-56]. Whether this pattern of IGF-II expression is replicated in humans is unclear, as reports are somewhat conflicting. One study of human fetal tissues reported that IGF-II mRNA and protein is restricted to the choroid plexus and meninges[57]. However, several studies using Northern blot hybridization and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of human fetal tissue report widespread IGF-II mRNA expression within both fetal and adult brain[58-60]. The relatively high magnitude of IGF-II expression in CNS is likely due to loss of parental IGF-II gene imprinting, *i.e.*, expression of both parental

alleles[59-61]. McKelvic *et al.*[62] report detection of IGF-II mRNA in cerebellum and hypothalamus and note that this expression diminishes or disappears at later stages. In the chicken, IGF-II expression is widespread and can be found in the brain stem nuclei, optic tectum, and cerebellum[63]. IGF-II Purkinje cell expression noted in embryonic stages is later replaced by IGF-I.

In contrast to the restricted expression of IGF-II, the ciM6PR/IGFIIR can be detected in a wide range of CNS regions during development and in the adult. Analysis of mRNA concentrations in whole brain from rats revealed that peak expression occurs prenatally and gradually tapers off during development to reach adult levels by P7[64]. A more detailed analysis of the distribution pattern of ciM6PR/IGFIIR protein done by Valentino et al.[65] confirmed this temporal pattern. In addition, transiently high levels of the ciM6PR/IGFIIR are found prenatally in the cortex, hypothalamus, hippocampus, subventricular zone, and the olfactory bulb[50,65,66]. Hawkes and Kar[67], using immunoblotting and immunohistochemistry methods, reported a distinct and widespread expression in adult rats, which includes expression in the mitral layer of the olfactory bulb, neuronal cell bodies of the striatum, layers IV and V of the cortex, Purkinje cells of the cerebellum, and granular and pyramidal layers of the hippocampus. In addition, substantial labeling in the neurophil suggests localization on neuron fibers. Couce *et al.*[68] report that they found no glial immunoreactivity, although they confirmed pyramidal cell expression in the hippocampus, along with reactivity in the polymorphic layer of the hippocampus and granular cell layer of the dentate gyrus.

In conclusion, the overall pattern of expression of IGF-II suggests that in early prenatal development IGF-II, although produced mainly by the meninges, has widespread influence over the CNS. The extensive expression of the IGF-II receptor in prenatal and early postnatal stages and subsequent reduction indicates that this protein plays an important, but transient, role in the development of the CNS. Whether this role is simply supplanted by IGF-I at later postnatal stages or whether each IGF serves a unique function remains unclear.

2.2.3 IGFBPs

The majority of the data on IGFBP expression come from studies during early postnatal development and in adult animals; but prenatal expression patterns also have been documented. The distinct spatial pattern of IGFBP expression suggests that each may play unique and regionally specific roles in development. Because IGFBPs have functions independent of their interactions with IGFs, such as stimulation of cell migration[3,4], they may

have distinct roles during CNS development. Expression patterns of various IGFBPs are shown in Table 2. At E15, IGFBP-4 mRNA is found in the hippocampus, choroid plexus, and the meningeal layer surrounding the cerebellum[69]. In the adult, expression of IGFBP-4 is more widespread. In addition to the meninges, it can be found in layers II and IV of the cerebral cortex, the olfactory peduncle, amygdala, thalamus, basal ganglia[69], and the pyramidal cell layer of the hippocampus[70]. The early expression pattern of IGFBP-6 contrasts with embryonic expression of IGFBP-4 in that expression appears much more restricted[71]. IGFBP-6 mRNA has been localized to the trigeminal ganglia at E16. During later stages of development (P21), expression is associated mainly with gamma-aminobutyric acid (GABA)ergic interneurons in the forebrain and cerebellum.

Region	Prenatal	Perinatal	Adult
Cerebrum	2	2	2,4,6
Cerebral cortex	2	2	2,4,6
Piriform cortex			2
Choroid plexus	2,4		
Olfactory bulb	2,5	5	2,4,5
Mitral cell layer		5	2,5
Glomerular layer			
Hippocampus	4	4,5	2,4,5
Dentate gyrus			5
Ammon's horn		5	5
Cerebellum	5	5	2,5,6
Cerebellar cortex			2
Meninges	4		
Deep nuclei		5	
Other			
Superior olive		5	2,5
Inferior olive		5	5
Inferior colliculus		5	2
Pretectal nucleus			2
Trigeminal ganglia	6		
Basal ganglia	4	4	4
Amygdala			4
Thalamus		5	4,5

Table 2. Temporal and regional distribution of IGFBPs 2, 4, 5, 6. BP numbers in bold refer to entire structure, those not in bold depict substructural localization where available. Information from refs. 28,37,50,54,69-77.

Studies addressing expression patterns of IGFBP-2 and IGFBP-5 have focused on adult animals. Both of these binding proteins demonstrate widespread expression. IGFBP-2 was found in the mitral layer of olfactory bulb[76] and in the choroids plexus[54,75]. Sullivan and Feldman[50] also report expression in these regions prenatally. A more detailed study of mRNA expression in early postnatal rats by Lee *et al.*[37] noted IGFBP-2 expression in astroglia in the cerebellum, hippocampus, and neocortex.

IGFBP-5 mRNA can be detected as early as E10 in neuroepithelium and its derived structures, such as notochord and the floor plate[73], and the epithelium that gives rise to the pituitary gland[74]. Later in prenatal development IGFBP-5 mRNA is found in the proliferative zone of the external granule layer of the cerebellum and the mitral neurons of the olfactory bulb[74]. IGFBP-5 expression is widespread in the postnatal brain, although cerebellum exhibits the highest levels of expression[70,72,77].

3. GH ACTIONS IN THE CNS

GH has been shown to influence brain growth and function (see reviews[78,79]). Whether GH actions are direct or mediated by IGF-I remains to be clarified; however, as with other organs, both mechanisms of action are likely. GH synthesis in the CNS appears to be restricted (see earlier), whilst GH immunoreactivity can be readily identified in most regions of the brain. GH is transported to the CNS either by crossing the BBB or by entry through the circumventricular organs[79]. Chronic administration of GH has been shown to result in increased GH in the cerebral spinal fluid (CSF)[10,11,80]. The presence of GH in the brain and the widespread expression of the GHR in the brain strongly indicates a role for GH in the CNS[78].

GH deficient mice, such as Snell dwarfs and *little* mice, exhibit reduced cerebral size with altered neuron size and number, and decreased myelin[81]. While Snell dwarfs mice carry a mutation in the pituitary transcription factor Pit-1, the *little* mice do not express the GH-releasing hormone receptor. Whether *little* mice have a reduction in brain myelin concentration, or reduced total myelin content because of their smaller brain size, however, has been debated[82]. In contrast, GH overexpressing transgenic mice exhibit brain weights that are not significantly heavier, although they have somewhat greater weights (7% increased)[83]. Because the magnitude of IGF-I expression has a major influence on brain size, neuron number and myelination (see later), the latter findings are consistent with GH having a regulatory influence on CNS IGF-I expression.

Several reports provide evidence that IGF-I mediates GH effects on the brain: (1) In mice carrying a fusion gene linking the 5'flank of the IGF-I

gene to a luciferase reporter gene, systemic GH administration stimulates luciferase expression in the CNS, but only modestly[41]. Nonetheless, this experiment suggests that GH can exert a degree of control over IGF-I transcription in the brain *in vivo*; (2) Peripheral administration of GH to rats increases IGF-I mRNA expression in the CNS and activates signaling pathways thought to be involved in mediating IGF-I anti-apoptotic effects[84] and (3) In a study of cultured embryonic rat cerebral cortical cells, Ajo *et. al.*[85] demonstrated that GH stimulates neuron progenitor proliferation and differentiation, and that these effects are blocked by antibodies to IGF-I. Finally, there is a significant body of evidence that GH is involved in a wide range of cognitive and behavioral CNS functions including memory, appetite, mood and sleep (see reviews in refs.17,80,86-88). Whether IGF-I mediates these GH actions, however, is not known.

4. IGF-I ACTIONS IN CNS

Much evidence indicates that IGFs play an important role in CNS development. While it appears that IGFs promote growth and development of all neural cell types, their specific actions likely depend on the developmental stage of each cell lineage and the local cellular milieu. IGF-I actions on neuron and oligodendrocyte genesis are relatively well studied, but there are few studies, especially *in vivo* studies, investigating other types of CNS neural cells, *i.e.*, astrocytes, microglia, radial glial, and ependymal cells. The paucity of data about IGF actions on the development of these cells is primarily attributable to the lack of a precise understanding of their development and developmental stage-specific markers.

4.1 IGF-I actions on neural stem cells

IGFs and IGF receptors are expressed in neural stem cells during early development, and appear to be essential for their proliferation, survival, and differentiation. In culture, IGF-I and/or IGF-II increase neural stem cell number of embryonic rat hypothalamic cells[89,90], chick immature sympathetic neurons[91], and neural stem cells derived from E14 mouse striatum[92,93]. It is not clear, however, whether this increase in cell number is the result of IGF's proliferative and/or anti-apoptotic action. Exposure to IGF-I also appears to be important for other growth factors. When IGF-I is removed from culture medium, epidermal growth factor (EGF) and fibroblast growth factor (FGF) are neither able to stimulate proliferation of neural stem cells nor to promote their differentiation into mature neural cells[92,93].

Recently, neural stem cells have been found to exist in the CNS of the adult human and rodent (see review in ref. 94). These cells are termed "adult neural stem cells", and their embryonic counterparts called "young or embryonic neural stem cells". Although there is no convincing evidence showing that adult neural stem cells act the same as their embryonic counterparts, adult neural stem cells are capable of proliferating *in vivo* and differentiating into each major neural cell type in culture. This raises the possibility of a therapeutic means to treat brain injuries and disorders by promoting adult neural stem cell growth.

IGF-I appears to promote both proliferation[95] and survival[33] of cultured adult neural stem cells in a fashion similar to its effects on young neural stem cells. Reports on IGF-I actions in adult neural cell differentiation, however, have not proved consistent. In 10-day cultures of FGF2-responsive adult stem cells derived from rat forebrain[96] or hippocampus[95], IGF-I stimulates adult neural stem cells to become neurons, as evidenced by an increase in the number of neurons without apparent changes in the number of $GFAP^+$ astrocytes and GC^+ oligodendrocytes[95]. Using a similar culture system, Gage and coworkers[97], however, reported that when hippocampal FGF2-responsive adult stem cells were cultured with IGF-I or IGF-II for 4 days, the number of cells in oligodendrocyte lineage was markedly increased, while neuron number was only minimally increased. The reasons that account for these discrepancies are not clear. One possibility is that the cells used before IGF-I treatment in these experiments were at different developmental stages and/or that IGF-I has differential effects on cells at different developmental stages. Other factors, such as animal species and the brain regions from which adult stem cells were isolated, may also contribute.

IGF *in vivo* actions on neural stem cells and fate-committed early neural precursors during development and in adults have not been studied in detail. While all neural cell types apparently can develop from neural stem cells in mice with blunted expression of *Igf-1, Igf-2,* or *Igfr* genes, the CNS size of these mutant mice is markedly reduced both prenatally and postnatally[98-103]. Fewer oligodendrocytes also develop when cells from forebrains of E18 *Igfr* KO mice are grown in culture[101]. These data suggest that proliferation and maturation of neural stem cells and/or oligodendrocyte precursors in *Igfr* KO mice is reduced. Consistent with this notion, Ni *et al.*[104] showed that in P2 Tg mice that overexpress IGFBP-1, an IGF inhibitor, the number of BrdU-labeled cells is decreased in the hippocampus and in the cerebral cortical ventricular (VZ) and subventricular zones (SVZ), the locales of dividing neural stem cells and precursors. These data, therefore, implicate IGFs in maintaining the number of neural cells with proliferative potential.

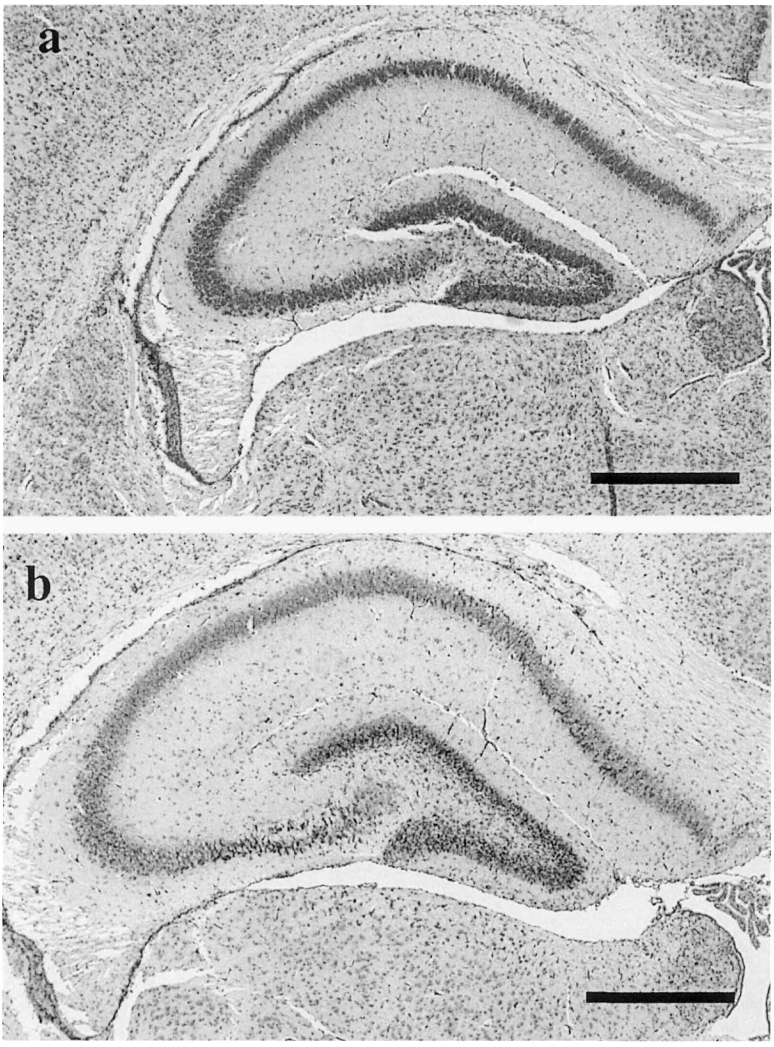

Figure 1. Cresyl violet staining of postnatal hippocampus from (**a**) control and (**b**) nestin-IGF-I overexpressing transgenic mouse. Note the dramatic increase in overall size of the hippocampus as well as the increase in white matter. Scale bar = 500 μm. See Popken *et al.*[39] for more information on nestin-IGF-I mice.

To directly examine IGF-I actions on neural stem cells and fate-committed precursors, Popken et al.[39] recently generated a line of transgenic (Tg) mice that overexpress a nestin promoter-driven IGF-I transgene in brain (nestin/IGF-I Tg mice) as early as E11. They showed that IGF-I significantly increases brain size, and the number of proliferating cells (by BrdU labeling)

in cortical VZ and SVZ at both E14 and E16. The IGF-I overexpression resulted in an increased neuron number (27-69%, depending on brain region) and glial cell number (by ~37%) in cerebral cortex of postnatal mice, indicating that IGF-1 promotes development of cells in both the neuronal and glial lineage. Fig. 1 depicts postnatal changes in hippocampal size. It remains to be elucidated, however, whether IGF-I increases survival of young neural stem cells and precursors.

4.2 IGF actions on neurons

4.2.1 Neurogenesis

Among the major CNS cell types, neurons are the first to develop. In mammals, neurogenesis is completed before birth in nearly all CNS regions, with the exception of the VZ, olfactory bulb, cerebellum, and hippocampus. Multipotent neural stem cells in the VZ, as well as those in the cerebellum and hippocampus, continue to proliferate and generate new neurons throughout postnatal life. It appears that IGFs play an important role in these processes.

A large amount of *in vitro* evidence demonstrates that IGFs enhance neuronal proliferation[91,105], survival[106-109], and maturation[92,96,110]. Postnatal overexpression of IGF-I in brain results in large brains with increased neuron number[111-114]. This increase in neuron number appears to be predominantly due to the capacity of IGF-I to promote neuron survival[114-116], but increased neuronal proliferation also occurs[114]. IGF-I also may influence neuron number by promoting neuronal differentiation[117]. It appears, however, that IGFs act *in vivo* in a regionally and developmentally dependent manner. Ye *et al.*[114] found that overexpression of IGF-I in Tg mice during postnatal life significantly increases the number of cerebellar neurons. At P50 the numbers of granule cells and Purkinje cells are increased by 82% and 20%, respectively. Using the same Tg mice, Dentremont *et al.*[111] reported the number of neurons is significantly increased in the nucleus of the solitary tract and dorsal motor nucleus of the vagus, but not in the hypoglossal and facial nuclei. In addition, peripheral injection of IGF-I for 20 days in wild type adult rats increases hippocampal granular neurons by 78%, but does not alter the number of astrocytes[118]. Conversely, ablation of IGF-I expression during development results in reduced brain size and a selective loss of neurons, such as striatal parvalbumin-containing neurons, but dopaminergic neurons and spinal cord motorneurons appear to be unaffected in IGF-I KO mice[99].

IGF-I may also promote recruitment of neuronal cells during development. Recently IGF-I was reported to be associated with radial cells

and to promote neuronal recruitment from the ependyma/subependyma in the adult songbird[119]. Whether IGF-I also serves a similar function in mammals needs to be confirmed.

4.2.2 Neurite growth and synaptogenesis

Both *in vitro* and *in vivo* data show that IGF-I promotes neurite growth. In culture, IGF-I not only increases the length of neurites[120-125], but also enhances the initiation of neurite outgrowth and neurite branching[122]. Unlike brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), which only increase the basal dendrites of pyramidal cells in rat primary somatosensory cortex, IGF-I increased the branching and total extent of both apical and basal dendrites[124]. Similarly, overexpression of IGF-I in the CNS greatly increases neuropil, which is occupied primarily by neuronal processes[27,112]. Reducing IGF-I availability by blunting its expression[126] or overexpressing IGFBP-1[27] decreases dendrite and axon growth. These IGF-I actions are associated with the stabilization of tubulin mRNAs during neurite formation[127]and an increase in neurofilament mRNA expression (our unpublished data).

IGF-I also promotes synaptogenesis during postnatal development[113,128]. In the hippocampal dentate gyrus and hypoglossal nucleus, the total number of synapses is greatly increased in IGF-I Tg mice from P14 to P130[113]. The synapse-to-neuron ratio, however, is increased only at P28 and P35, and returns to normal values by P130[113]. The latter indicates that synapse elimination, a normal event during the course of brain development, remains intact. Because increasing myelination appears to play a role in synapse elimination and IGF-I is a potent stimulator of myelination (see later), IGF-I may be indirectly involved in controlling the normal number of synapses. Regardless of the latter speculation, these data indicate that IGF-I promotes formation and maturation of synapses during development. This finding is supported by the data of Camarero *et al.*[117], which show that synapse distribution in the cochlear ganglion persists at an immature stage in the absence of IGF-I

4.3 IGF actions on oligodendrocytes and myelination

4.3.1 Oligodendrocyte lineage

Oligodendrocytes, arguably the most abundant CNS cell type, produce the myelin sheaths that surround axons and facilitate neuronal electrical transduction. As with the neuronal lineage, IGFs appear to affect cells of oligodendrocyte lineage during each stage of their development, and in turn

myelination. IGF-I and IGF-II significantly increase oligodendrocyte number in cultures derived both from rodents[31] and human[129] by promoting proliferation of oligodendrocyte precursors[130,131], survival of both mature oligodendrocytes and their precursors[132-134], and maturation of precursors[31,129,134-136]. Addition of IGFBP-1 or -2, two IGF inhibitory binding proteins, decreases oligodendrocyte differentiation in culture[137]. IGF-I actions on oligodendrocyte lineage seem to be developmental-stage specific. Mason and Goldman[138] reported that while in the presence of insulin at concentrations sufficiently high to interact with the IGFIR, IGF-I promotes differentiation of $O4^+$ oligodendrocyte precursors, but not $A2B5^+$ precursors, which are at an earlier development stage than $O4^+$ oligodendrocyte precursors.

Although the total number of oligodendrocytes is increased in IGF-I Tg mice[27,139,140] and decreased in mutant mice with reduced IGF-I availability[27,99,100], there are little *in vivo* data that directly address whether IGF-I acts to promote proliferation and/or survival of oligodendrocyte lineage cells during development. The lack of data stems from: (1) the relative paucity of stage-specific markers for cells in oligodendrocyte lineage and (2) the difficulty in ascertaining the identity of apoptotic cells during development. For example, the cell membrane lipid A2B5 is a well-recognized oligodendrocyte precursor marker in culture, but its rapid disintegration and removal of apoptotic cells abrogates its use as a marker of apoptotic oligodendrocytes *in vivo*. Nonetheless, several recent studies clearly demonstrate that IGF-I promotes survival of mature oligodendrocytes in IGF-I Tg mice during demyelinating injury induced by cuprizone[141] and axonectomy[142]. Importantly, in the presence of IGF-I, surviving oligodendrocytes have the capacity to induce remyelination[141].

4.3.2 Myelination

One of the most dramatic effects of IGF-I is its stimulation of increased myelin content. Myelin content is increased by four-fold in the brain of IGF-I Tg mice[143], and dramatically reduced in mice with reduced IGF-I availability (IGF-I KO and IGFBP-1 Tg mice)[25,27,104,139,144]. This increase in content results from IGF-I-induced increases in myelin sheath thickness (more sheaths enwrapped around each axon) and in more myelinated axons[27,139] (Fig. 2). As would be expected from these findings, the expression of myelin basic protein (MBP) and proteolipid protein (PLP), two proteins expressed only in mature oligodendrocytes and myelin, is increased in IGF-I Tg mice and reduced in mutant mice with reduced IGF-I availability[27,140,143]. Because the magnitude of changes in expression of

myelin-related protein genes cannot be fully accounted for by the changes in oligodendrocyte number, it appears that IGF-I specifically stimulates oligodendrocyte myelin synthesis.

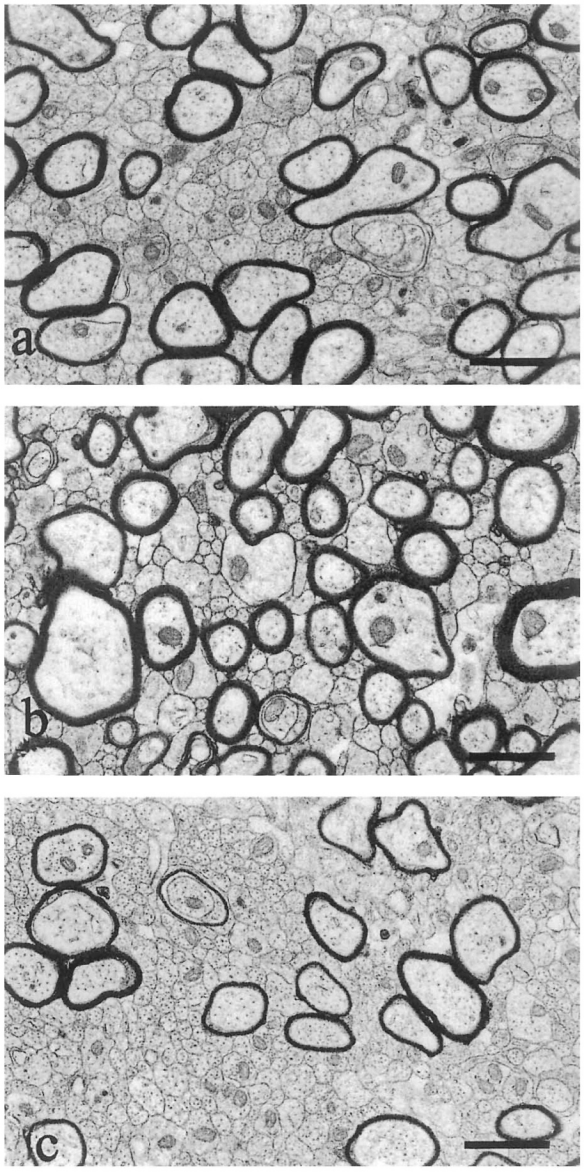

Figure 2. Electron microphotographs of posterior anterior commissure of a wild-type control mouse (**a**), a IGF-I Tg mouse (**b**), and a IGFBP-1 Tg mouse (**c**). Tg and wild-type control mice were sacrificed and perfusion-fixed at postnatal day 35. Scale bars = 1 μm. See Ye *et al.* [139] for more information on this study.

In the absence of IGF-I expression, IGF-II seems to be able to compensate, at least in part, for IGF-I actions on oligodendrocyte development and myelination in murine. Ye *et al.*[140] reported that the number of oligodendrocytes and the concentration of myelin-specific proteins are reduced during early postnatal development, but approach normal in adult IGF-I KO mice. The latter observation correlates with an increase in IGF-II abundance. A similar increase in serum IGF-II also was observed in a young man with an IGF-I null gene mutation[145].

4.4 IGF actions on astrocytes and microglia

Little is known about IGF actions in the development of astrocytes and microglia, although many studies have demonstrated that IGFs increase the number of astrocytes and microglia. In culture, addition of IGF-I significantly increases proliferation of astrocytes[146,147] and microglia[148]. Although astrocytes comprise their normal percentage of neural cells in adult IGF-I Tg mice, their total number is increased as is the total number of brain cells (our unpublished data).

During injury and in a variety of CNS disorders, both astrocytes and microglia become reactive and produce IGFs. While active astrocytes appear to produce only IGF-I[149-152], microglia secrete both IGF-I and IGF-II[148-150,152,153]. Because increased IGF expression by astrocytes and microglia occurs at or near sites of injury, the IGFs produced by these cells appear to act locally in an autocrine or paracrine fashion. Ectopic expression of IGFBP-1 retards brain development and reduces astrocyte response to injury[104]. In contrast, when IGF-I Tg mice are subjected to treatment with cuprizone, a copper chelator that induces oligodendrocyte damage, there is a dramatic increase in the number of microglia[141].

4.5 Mechanisms of IGF-I actions in the CNS

It is likely IGF-I acts in CNS primarily in a paracrine and autocrine fashion. As indicated earlier, neural stem cell precursors in the VZ and SVZ cells express IGF-I[154]. Addition of IGF-I antibody or inhibitory IGFBPs, or an IGFIR antagonist peptide (JB1) blunts IGF-I actions on proliferation[93,155], survival[107], and differentiation [96].

The intracellular signaling pathways that mediate IGF-I actions in CNS development remain to be precisely elucidated. Two major signaling pathways, the mitogen activated protein (MAP) kinase and the phosphatidylinositol-3 (PI-3) kinase pathways clearly play an important role in mediating IGF-I actions in neural cell development. IGFs bind and

activate the IGFIR, which in turn activates multiple signaling pathways including MAP and PI-3 kinase pathways. MAP kinase pathway appears to be involved in progenitor proliferation in both young and adult mice. Inhibition of MAP kinase activity ablates IGF-I-stimulated stem cell proliferation[95]. In mature neurons the IGF-I receptor may be coupled to an inhibitory G protein, which mediates G-dependent MAP kinase activation[156].

The PI3 kinase-Akt pathway plays a key role in the promotion of survival of neural cells, including neural stem cells, precursors, and mature neurons, and oligodendrocytes. In cultured dentate gyrus[107] and cerebellar granule cells[107,157] and immature oligodendrocytes[158,159], IGF-I activates PI-3 kinase and its downstream serine/threonine kinases, Akt and p70 S6 kinase. Activated Akt in turn acts by inhibiting forkhead transcription factor activity[160]. Another Akt-mediated pro-survival pathway leads to decreased caspase-3[115,161] and caspase-9[162] activity by its capacity to phosphorylate these caspases[162], which reduces their activity, and by reducing their expression[115].

Akt kinase activity also appears to be involved in IGF-I-mediated proliferation and growth of neural cells. For example, IGF-I-stimulated Akt activation enhances dendritic growth in cultured Purkinje cells[116] and proliferation of rat hippocampal adult stem cells[95]. These Akt effects are likely associated with its ability to regulate cell glucose transportation and energy production. IGF-I-stimulated Akt activation also significantly increases glucose transporter (GLUT4) expression and glucose uptake in mouse brain neurons[163,164]. In addition, this pathway appears to enhance Na^+K^+ATPase activity cultured rat astrocytes, which in turn may promote mitogenesis[146].

The upstream and further downstream events in these IGF-I signaling pathways are less clear. Blunting the expression of either IRS-1 or IRS-2 expression reduces brain growth[103,165], myelination[166], and neuronal proliferation (by 50%)[165], suggesting that both IRSs play an important role in mediating IGF actions on brain development. Further studies, however, show that blunting IRS-1 only minimally blocks IGF-I-stimulated brain growth and has no effects on myelin-associated protein expression[103], suggesting that IRS-1 is not essential for IGF-I signaling in oligodendrocytes, and that other IRSs, such as IRS-2 and IRS-4, likely play an important role in oligodendrocyte and brain development. Recent studies indicate that IRS-2 is essential for IGF-I stimulated neuron progenitor proliferation, but not for IGF-I anti-apoptotic action[165].

Activation of protein kinase C and stabilization of intracellular calcium levels also plays a role in mediating IGF effects on rat astrocyte proliferation[167], c-*fos* expression in postmitotic neurons[168], and neuron survival[169].

5. IGF SYSTEM AND INJURY

Much experimental evidence indicates that the IGFs, primarily IGF-I, have a central role in repair, recovery and/or amelioration, following a wide variety of brain injuries. This data comes both from experiments in cultured neural cells and in animals. Table 3 lists a number of studies performed in animals that support an *in vivo* role for IGF-I. As during development, IGF-I appears to ameliorate CNS injury by at least two mechanisms: (1) inhibition of apoptosis and (2) stimulating the proliferation either of neural stem cells and/or committed neural progenitors.

IGF-I's capacity to inhibit apoptosis both in cultured cells and during development is well established (see earlier). While most *in vivo* studies of IGF-I and injury have evaluated neuroprotection, relatively few have specifically addressed apoptosis. Nonetheless, there is clear evidence that IGF-I inhibits apoptosis in neurons[170-172] and in oligodendrocytes[141,161]. Presumably much, if not most, of IGF-I neuroprotection, such as reduction in infarct size following hypoxic-ischemic (H/I) injury, is due to its anti-apoptotic actions[173]. More recently, IGF-I has been shown to stimulate neuron progenitor proliferation in the dentate gyrus following middle cerebral artery ligation[174]. It is not clear, however, whether IGF-I is primarily stimulating proliferation and development in neural stem cells (NSC) or in neuron progenitors. Given that IGF-I appears to stimulate proliferation of cultured uncommitted NSC[33,93], both seem possible[173].

IGF neuroprotective actions appear to apply to virtually all brain injuries (Table 3), including ischemia, H/I, chemically induced injuries, cryo-injury, electrolytic injury, and so forth. The relationship of IGF system expression and action to H/I injury has been extensively studied, and may be applicable to many CNS injuries (see review in ref.173). Immediately after H/I injury, IGF-I expression falls, and this correlates inversely with the magnitude of apoptosis[170]. Beginning about 2 days after injury, IGF-I expression increases, peaking 5-7 days following injury (more slowly in adult animals). Astrocytes and reactive microglia appear to be the major sites of this expression[152,173-175], although expression by neurons, oligodendrocytes, and NSCs also seems possible and has not been excluded. Following other injuries, astrocytes appear to be the major site of IGF-I expression. Examples include stereotaxic injury[176], electrolytic lesion to the hippocampus[177], cuprizone[151], and experimental autoimmune encephalomyelitis[178]. Increased IGF-II expression appears to occurs much later following H/I (7-10 days), and appears to be derived from microglia and macrophages[152,173], but could also be derived from its usual sites of synthesis, such as the choroids plexus, etc.[175]. Changes in the expression of the IGFIR following H/I have not been clearly demonstrated.

Injury	IGF-I Treatment	Animal	Observation
Amyloid-β injury	Systemic	Rat	Reduces amyloid-β burden[179]
Cerebral artery occlusion	Intranasal	Rat	Reduced infarct volume[180]
	I.C.V,	Rat	Reduced infarct volume[181]
	I.C.V.	Sheep (fetal)	Reduced oligodendrocyte loss[161]
	I.C.V.	Rat	Increased neuron progenitor cell proliferation [174]
Cerebellar deafferentation	Systemic	Rat	Restored motor function; decrease apoptosis[171]
Colchicine injection in dentate gyrus	None	Rat	Decreased IGF-II binding; increased IGF-II gene expression; increased IGF-I binding and IGFBP-3 gene expression[175]
Contusion	None	Rat	Up-regulation IGFBP-2, -4 and IGF-I mRNA[182]
Cuprizone	None	Rat	Increased IGF-I expression in astocytes[151]
	Transgene expression	IGF-I over-expression, mice	Inhibition of oligodendrocyte apoptosis[141]
Cryogenic	None	Rat	Increase in IGF-I mRNA[183]
Electrolytic lesion to hippocampus	None	Rat	Increase IGF-I peptide[177]
Experimental Autoimmune encephalomyelitis	None	Rat	Increased IGF-I and IGFBP-2 expression surrounding lesions[178]
	Systemic	Rat	IGF-I ameliorates injury[184,185]
Hypoxia/ ischemia	None	Rat	Increased serum IGF-I, increase IGF-I in astrocytes and reactive microglia; increases in IGFBPs; IGF-IR unchanged[173]
		Rat	Increased IGF-I and IGFBP-5 expression[186]
	None Systemic GH	Rat	Increase IGF-I and GH receptor mRNA Moderate neuroprotection[187]
	None	Mouse	Induction IGF, IGFBP-2, -3, and -5 4 days post-injury[148]
Ischemia	None	Rat	Increased IGF-I expression[152]
			Immediate decreased IGF-I mRNA that correlates with increased apoptosis[170]
Penetrating	None	Rat	Induction of IGF-I expression in astrocytes[176]
		Rat	Increased serum IGF-II protein; chronic increase in IGF-II mRNA[56]
	Injection into injured area post-injury	Rat	Reduced apoptosis; reduced post-trauma weight loss; increased post-trauma motor activity[172]
Quinolinic acid	Intrastriatal injection of IGF N-terminal tripeptide	Rat	Rescued projection, cholinergic NADPH interneurons[188]

Table 3. Evidence from experimental animals that IGF system proteins are involved in recovery and/or amelioration of CNS injury.

Expression of IGFBPs is coordinated with that of IGF-I. The precise sites of expression appear to depend upon the area of the lesion[173]. In general, the

normally abundant expression of IGFBP-2 in the brain is increased further in the area surrounding the injury 3-5 days following H/I[152]. IGFBP-3, which does not appear to be widely expressed in the brain under homeostatic conditions, is induced in a similar temporal fashion and in association with the vasculature[22]. IGFBP-4 expression also is increased in the first 3-5 days following H/I, but it is located predominantly within the injured area. Later, IGFBP-5 expression increases in the injured area. IGFBPs modulate IGF actions, both by inhibiting and augmenting IGF activity, but they also have independent actions[3]. IGFBPs, therefore, may have role following injury that do not involve modulating IGF actions. Such IGFBP action remains to be identified.

IGF-I treatment clearly is protective against H/I injury[173]. Intracerebroventricular (ICV) IGF-I has dose- and time- dependent effects on H/I injury. ICV IGF-I administered within hours of injury can dramatically ameliorate neuron loss and infarct size. These effects, however, differ according to region, with the greatest IGF-I effect being in the lateral parietal cortex, followed by the striatum, the dentate gyrus, and thalamus, with the least amelioration being in the hippocampus. Specific neuron types appear to be differentially protected[189]. Other studies have demonstrated that systemic or intranasal IGF-I administration also can be effective in treatment of neural injury[184,185,190,191], including ischemia[191]. As these results strongly indicate, IGF-I can cross the blood-brain barrier[7].

IGF-I also has actions that may protect against chronic and inherited neurologic diseases. For example, IGF-I has been shown to decrease β-amyloid burden in Tg mice that overexpress β-amyloid[179]. This is supported by data showing that IGF-I protects cultured cells from β-amyloid peptide induced neuron death[192]. Taking together these results, it appears that IGF-I may ameliorate Alzheimer's disease. Studies in cultured cells also indicate that IGF-I can protect against the neurotoxicity caused by dopamine[193], glutaric acid[194] and the mutant Huntington protein[195], findings that suggest that IGF-I might be helpful in the treatment of early Parkinson's disease, glutaric acidemia type I, and Huntington's disease. In addition, systemic IGF-I infusion delays Purkinje cell degeneration in "shaker" mutant rats[196]. Finally, there is strong evidence that IGF-I promotes oligodendrocyte survival and myelination, both in culture[131] and *in vivo*[27,103,139,143], and may protect against a variety of demyelinating injuries[131,134,140,141]. These findings suggest that IGF-I may be useful in the treatment of a wide variety of demyelinating diseases and injuries.

6. HUMAN CLINICAL CORRELATIONS

A few mutations in IGF-I and IGFIR genes have now been identified in humans. Each of these mutations is characterized by severe intrauterine growth retardation (IUGR) and poor postnatal growth. Although highly variable, each also has been associated with findings consistent with CNS abnormalities seen in mutant animals. Woods *et al.*[145] reported a teenager with a homozygous deletion in the *IGF-I* gene. His head circumference at birth was 4.9 SD below the mean, commensurate with the magnitude of length (-5.4 SD) and weight retardation (-3.9 SD). He also exhibited delays in his developmental milestones and subsequent mild mental retardation, as well as sensorineural deafness. More recently, another child has been found to have very low IGF-I expression resulting from a point mutation in the polyadenylation sequence of the *IGF-I* gene[197]. This child also had severe IUGR, a small head circumference, and sensorineural deafness. Brain magnetic resonance imaging (MRI) studies, however, were reported as normal in both.

Two IGFIR gene mutations have been reported by Abuzzahab *et al.*[198]. One was identified as a compound heterozygous missense mutation in exon 2. This exon encodes the portion of the IGFIR alpha subunit, which binds IGF-I. When studied in the patient's cultured skin fibroblasts, this mutation was found to result in decreased IGF-I receptor affinity and decreased IGF-I-stimulated IGFIR phosphorylation (but with an apparent normal receptor number). At age 12 years her verbal IQ was 134, but performance IQ was 89 and she exhibited multiple behavioral abnormalities, such as anxious affect, psychomotor agitation, obsessive tendencies, and social phobias. Neither head circumference nor head imaging studies were reported. The second child reported manifested a heterozygous point mutation resulting in a stop codon in exon 2. Further evaluation of the patient's fibroblasts by flow cytometry indicated that the number of IGFIRs expressed was decreased. At birth this child had microcephaly (head circumference = -4.6 SD), again similar in magnitude to the IUGR (-3.5 SD). He is reported to have mild retardation of motor and speech development. Okubo *et al.* identified another child with a heterozygous IGFIR deletion on the basis of a 15q26.2 chromosomal deletion, an IGFIR specific fluorescence in situ hybridization (FISH) analysis indicating a single *IGF-I* gene, and reduced IGFIR expression and IGF-I-stimulated receptor phosphorylation of the patients cultured fibroblasts[199] . This child had moderately delayed motor and speech development, and at 10 years of age had learning difficulties.

The neurological and psychological manifestations exhibited by these children indicate that IGF signaling has a role in human brain development and function. In other words, it seems clear that either a decrease in IGF-I

expression or decreased IGF signaling, whether it be secondary to decreased IGFIR number or binding affinity, leads to brain abnormalities. Nonetheless, the nature of IGF actions in the human CNS is far from being defined. More precise brain imagining and functional studies performed during the course of development may help to better define brain abnormalities in such children. Regardless, these clinical situations raise numerous questions. As appears to be the case in mice[103] , can increased brain IGF-II expression compensate in part for the absence of IGF-I in the children with *IGF-I* gene mutations, and thus, mask some IGF-I actions? Does IGF-I or IGF-II expression increase in the CNS in response to decreased IGFIR signaling, as is the case in disorders resulting from resistance to other hormones?

7. FUTURE DIRECTIONS

While major insights into the actions of GH and the IGF system on CNS growth and development continue to accumulate, it is clear our understanding remains primitive. The fundamental questions and issues that remain are many, but some that seem particularly important follow. Distinguishing direct GH actions on the brain from those that result from GH stimulation of IGF expression is problematic. IGFs stimulate an extraordinarily wide variety of influences on neural cells, including neural progenitor proliferation, inhibition of neuron and oligodendrocyte apoptosis, neuritic outgrowth, oligodendrocyte myelin synthesis, and so forth. It is unlikely that IGF is sending instructive signals for each of these events. Rather, it seems certain that IGFs are acting in concert with other growth factors and signaling molecules that give instructive signals. The nature of these interactions, the signaling pathways they utilize, and their developmental context are crucial to understanding the roles of IGFs in CNS development. Some other mechanistic questions relevant to CNS development are: (1) Do IGF-I and IGF-II have distinct actions/roles? (2) What signaling pathways are activated by IGFs to determine their apparent myriad of distinctly different effects? (3) Does the ciM6PR/IGFIIR serve a role in IGF-II signaling? and (4) What are precise actions of IGFBPs, both those in modulating IGF actions and those that are independent of IGFs? Cognitive consequences of GH and IGF actions, which seem likely to occur, are poorly defined, and for the most part, not well documented. Finally and perhaps most importantly, will IGFs prove useful in the treatment of neurological injury and disease?

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Chapter 9

REGULATION OF VERTEBRATE SENSORY ORGAN DEVELOPMENT: A SCENARIO FOR GROWTH HORMONE AND INSULIN-LIKE GROWTH FACTORS ACTION

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1. INTRODUCTION

Sensorineural organs act as an interface between the nervous system and the world. Diverse sensorineural organs detect different kinds of environmental events using specialized sensorial receptor cells classified as chemoreceptor, thermoreceptor, mechanoreceptor, and photoreceptor cells. Chemoreceptor cells are associated with the senses of taste and smell and are sensitive to chemicals in the environment. The taste buds of mammals detect dissolved food molecules and are located primarily on papillae of the tongue[1]. The olfactory epithelium is designed for recognition of volatile molecules and is located in the nose in terrestrial vertebrates. The olfactory cells present olfactory cilia that specifically respond to a large number of different chemicals in the air, including as many as 1000 types of different

smells in humans[1,2]. Thermoreceptor cells are associated with the sense of touch. In mammals, changes in temperature are detected by cold, warmth and pain thermoreceptors[1]. Mechanoreceptor cells respond to many different stimuli, such as sound, touch, pressure, gravity, stretch, or movement. Touch receptors are located in the skin and perceive touch, pressure, and pain[1]. Other mechanoreceptors include those located at the base of hairs and bristles that respond to motion and inner ear receptor cells that sense sound and balance[1]. Finally, photoreceptor cells located in the retina use pigments to absorb and detect light. The sensitive rods are specialized in low-intensity light detection, while the cones work better with daylight and are able to analyze the different light wavelengths responsible for the colors[1]. Upon specific activation, all types of receptor cells generate changes in membrane potentials that initiate the transmission of information to the brain that then reads this information and generates the adequate body responses.

In this chapter we summarize the available studies concerning the development of sense organs under physiological and pathological conditions, with respect to GH/IGF action.

2. DEVELOPMENT OF SENSORINEURAL ORGANS

The vertebrate sense organs arise at early stages of embryonic development and have distinct developmental origins. A variety of extrinsic signals control sensorineural development, including members of different families of cytokines, and growth and neurotrophic factors. The insulin-like growth factor (IGF)/growth hormone (GH) axis plays a central role in the development of the sensory organs, where these proteins and their receptors are widely expressed (Table 1)[3-26]. A detailed description of the elements of the IGF system and signaling pathways is provided in other chapters of this book[27, 28].

GH availability, the expression of its receptor (GHR) and some actions on the sensorineural organs have also been reported, however, there is less information available than for the IGF system.

	IGFI	*IGFIR*	*IR*	*GHR*	*IGFII*	*BP-1*	*BP-2*	*BP-3*	*BP-4*	*BP-5*	*BP-6*
Early Embr. Dev.	OV CVG Ret Lens	OlfP Ret	OB	OpV	Ret	n.d.	OtP NP	n.d.	n.d.	n.d.	n.d.
Late Embr. Dev.	OB Ret	OE Ret	OB	Ret Lens	Ret	n.d.	Ret	n.d.	n.d.	OB	n.d.
Early Post. age	OB Ret Cp Corn	OE Ret Lens Conj Corn	n.d.	n.d.	n.d.	n.d.	Ret OB Corn Iris Conj Scl	Cp Chr Corn	OB Cp Chr Corn	OB Ret Cp Chr Corn	Ret Cp Chr Corn
Adult	OB Ret Cp Corn	OE Ret Lens Conj Corn	n.d.	n.d.	n.d.	n.d.	Corn Iris Conj Scl	Cp Chr Corn	BG Cp Chr Corn	Ret Cp Chr Corn	OB Ret Cp Chr Corn

Table 1. Expression of factors, their receptors and BPs during sensory organ development and postnatal growth. This table summarizes available data on mRNA expression of IGF-I, IGF-1R, IR, GHR, IGF-II and IGFBPs 1-6 (refs. 3-26). **Vision**: Chr, chromatophores; Conj, conjunctiva; Corn, cornea; Cp, choroid plexus; OpV, optic vesicle; Ret, retina; Scl, sclera; **Inner ear**: CVG, cochleovestibular ganglion; OtP, otic placode; OV, otic vesicle; **Olfaction**: NP, nasal placode; OB, olfactory bulb; OE, olfactory epithelium; OlfP, olfactory placode. Emb, embryonic; Dev, development; Post, postnatal; n.d., not determined.

Many sense organs have a placodial developmental origin[29,30]. The inner ear and the olfactory system arise from the otic and olfactory placodes, respectively. Placodal development starts as discrete ectodermal thickenings that, through complex morphogenetic and neurogenetic processes, generate the adult structures. Furthermore, placodes can modulate the development of related structures. The olfactory placode is essential for normal forebrain development[30] and the olfactory and otic epithelia induce chondrogenesis in the surrounding mesenchyme, providing a protective, rigid structure for these sensorineural organs, which also contributes to the structural scaffold of the head. The optic lens also has a placodial origin and it is essential for normal development of the retina and other adjacent structures, such as the iris, the ciliary body and the overlying cornea[30-34].

Effect	Neural Target	Factor
Increase cell size	Neurons (CG, OB)[36, 53]	IGF-I
Enhance proliferation	Neuronal precursors (Ret)[37-39]	IGF-I, IGF-II, Insulin
	Stem cells (OB)[64]	IGF-I, Insulin
	Neuronal precursors (CVG, ONE)[19, 40-43]	IGF-I, IGF-II, Insulin
	Schwann cells (OEG)[44,45]	IGF-I, IGF-II, Insulin
Decrease	Neurons (OMN, Ret)[46-52]	IGF-I, IGF-II, Insulin
apoptosis/enhance	Neurons (CVG, ONE, CG)[42,54,63,68,69]	IGF-I
survival	Schwann cells (CG)[36,55-59]	IGF-I
Differentiation	Neurons (Ret)[38,60-62]	IGF-I, IGF-II, Insulin
	Stem cells (OB)[64]	IGF-I, Insulin
	Neurons (CG, ONE, OB)[36,42,64]	IGF-I
	Schwann cells (CG)[45,65,66]	IGF-I, IGF-II, Insulin
Neuritogenesis/	Neurons (ION, OMN, Ret)[15,24,48,67]	IGF-I, Insulin
Axogenesis	Neurons (OB)[19,53,70,75]	IGF-I, IGF-II
	Olygodendrocytes (OB)[53,70,75]	IGF-I
Myelination	Neurons (CG)[36,71]	IGF-I, IGF-II
	Schwann cells[45]	IGF-I, IGF-II, Insulin
	Olygodendrocytes (OB)[53]	IGF-I
Synaptogenesis	Neurons (OC)[36]	IGF-I
Neuromodulation	Taurine (Ret)[73]	IGF-I, Insulin
	Calcium channels (Ret)[72,74]	IGF-I, Insulin

Table 2. IGFs actions on the sense organs. **Vision:** ION, Isthmo-optic nucleus; OMN, ocularmotoneurons; Ret, retina; **Inner ear:** CG, cochlear ganglion; CVG, cochleovestibular ganglion; OC, organ of Corti; **Olfaction:** OB, olfactory bulb; OEG, olfactory ensheating glia; ONE, olfactory neuroepithelium.

During the last decade, progress has been made in the understanding of the molecular basis of sensorineural organ development[5,16,29,30,35], as well as in the role of IGFs in neural development. IGFs are fundamental for the development, maturation, and functionality of the nervous system, being involved in the regulation of processes such as cell proliferation, survival, and differentiation[3]. Studies on sensorineural development (Table 2)[12,16,19,21,36-75], although less abundant, point in the same direction.

Diseases and Syndromes	Sensory System Dysfunction	GH/IGF axis Dysfunction
Idiopathic late onset cerebellar ataxia[75]	Reduction of olfactory function	Reduced IGF-I levels
Amyotrophic lateral sclerosis[75]	Reduction of olfactory function	Reduced IGF-I levels
Turner's syndrome[78]	Otitis media Sensorineural hearing loss	Reduced IGF-I levels
Laron's syndrome[80,81]	Deafness Retinitis pigmentosa	GHR defects Abnormalities of GH signal transduction Primary defects on IGF-I synthesis or secretion
Usher's syndrome[83]	Deafness Retinitis pigmentosa	n.d.
Igf-1 mutations[76,77,82]	Deafness	Elevated GH secretion Undetectable serum IGF-I Normal serum IGFBP-3
IGF1R mutations[84]	n.d.	Decreased IGF1R levels Reduced affinity for IGF-I Increased concentrations of IGF-I and IGFBP-3 Decreased concentrations of IGFBP-2
Leber congenital amaurosis syndrome[85]	Abnormal eye movement Vision defects	GH insufficiency
Septo-optic dysplasia[86]	Optic nerve hypoplasia and pituitary dysfunction	GH insufficiency

Table 3. Clinical alterations associated with defects in the GH/IGF axis.This table summarizes human diseases and syndromes that present a dysfunction of the sense system associated with alterations in the GH/IGF axis[75-86]. n.d., not determined.

Alterations in the GH/IGF axis are causal to defects in the development of human sense organs (Table 3)[75-86]. The study of animal models that present altered levels of expression of one or more elements of the GH/IGF axis has, indeed, provided further insight into some of these human diseases and syndromes[28,71]. An understanding of the regulation of sensorineural development may contribute to the elucidation of the origin or progression of human diseases such us blindness or deafness, and to the development of novel therapeutic strategies.

3. THE VISUAL SYSTEM

Although the retina is a part of the central nervous system (CNS), because of its sensorial function and peripheral location it is considered as a sensorineural organ. The sensorial attributes reside in the innermost layer of the eye, the neuroretina. The neuroretina and the adjacent pigmented epithelium, together with part of the iris and ciliary body, are derived from the neuroepithelial wall via optic vesicle and optic cup. The lens, and to a lesser extent the cornea, is generated from the placoda formed in the overlying surface ectoderm (Fig. 1A). The vertebrate neuroretina is a complex neuronal network comprising six neuronal types: the rod and cone photoreceptors, responsible for the absorption of various wavelengths of light; the bipolar, horizontal, and amacrine interneurons, which integrate photoreceptor information; and the retinal ganglion cells, which transmit this information to the brain for cognitive processing. In addition to these neuronal types, Müller glial cells also originate within the retina. The outer most layer of the eye is the sclera, which at the front of the eye is transformed into the transparent cornea that permits light rays to enter the eye. The middle layer includes the iris, the ciliary body, and the choroid.

GH gene expression occurs in extrapituitary tissues prior to, and even after, the organogenesis of the pituitary gland[87-90]. GH immunoreactivity is detected early in development in, among other nervous system locations, the chick otic and optic vesicles. Later in development, but still prior to the differentiation of pituitary somatotrophs, the chick neuroretina, the pigmented epithelium, and the epithelial lens fiber cells show intense GH immunoreactivity[88,91]. The distribution of the GH receptor mirrors that of GH. Since GH is absent from the circulation of early chick embryos [87,88], these observations suggest that extrapituitary GH expression has an autocrine/paracrine role during early embryogenesis, in particular in the development of the ear and the eye[87-90,92]. The presence of GH and its receptor correlates with a suggested involvement of GH in the regulation of ocular development by acting on the intraocular melanocortin system in the chick[91] or inducing retinal angiogenesis. Indeed, GH deficiency in humans is associated with reduced retinal vascularization[93], whereas exogenous GH promotes retinal angiogenesis[94].

The actions of GH during embryonic development could be mediated by other growth factors, particularly IGF-I[95,96]. The widespread expression of mRNAs for IGF-I, IGF-1R, and IGFBP-2 to IGFBP-6 in specific histological layers of the retina, choroids, ciliary body and cornea in the rat suggests specific roles of the IGF axis in the eye[3,9,10] (Table 1). The developmental expression of most IGF family members has been described in the eye of birds[3,5], mammals[3] and fish[6,7] when proliferation and

differentiation of neuroretinal cells occur. IGF is also involved in lens differentiation. The presence of IGF-I mRNA in ocular embryonic tissues suggests an autocrine/paracrine function of the IGFs.

In addition to GH/IGF-I axis involvement in normal development, its deregulation has pronounced physiological effects such as dwarfism (associated with low levels of GH), gigantism, and acromegaly (associated with high levels of GH)[96,97]. More specifically in the visual system, Leber congenital amaurosis syndrome is associated with short stature, growth hormone insufficiency and vision defects[85]. Septo-optic dysplasia (SOD), a disorder of multifactorial etiology that includes gestational diabetes, is characterized by optic nerve hypoplasia with pituitary dysfunction[86]. Children with SOD may manifest a variety of visual and/or physical symptoms that range from mild to severe[86, 98-100]. A case of a 14-year-old boy with optic hypoplasia and pituitary dwarfism due to a complete deficiency in GH has also been reported[101]. A rare case of GH and gonadotropin deficiency associated with dysmorphic features has been reported in a 16-year-old boy with clinical characteristics that included left anophthalmia (absence of the left eye), microphallus, bilateral cryptorchidism, and mental retardation[102]. All of these features are attributed to the hypothalamic dysfunction and are very similar to the features of septo-optic dysplasia, but mutation analyses revealed no mutations or polymorphisms in the SOD associated gene *HESX1*[102].

Vascularization of the retina normally occurs during fetal development, with little or no vascularization after birth[103]. A role for GH in normal retinal vascular development has been suggested because children with congenital GH deficiency have reduced retinal vascularization[93]. GH may exert its effects through circulating or locally produced IGF-I since the lack of IGF-I during the early neonatal period is associated with lack of vascular growth and retinopathy of prematurity, a blinding disease initiated by lack of retinal vascular growth after premature birth[104,105]. IGF-I probably influences angiogenesis and the development of retinal neovascularization through interaction with locally produced factors such as vascular endothelial growth factor (VEGF), by acting as a permissive factor for maximum VEGF stimulation of angiogenesis[104]. IGF-I above a specific threshold level is necessary for maximum VEGF activation of the MAPK and Akt pathways, pathways important for endothelial cell proliferation and survival[104]. However, ischemia-induced retinal neovascularization is only partially suppressed in transgenic mice expressing a GH antagonist gene[106].

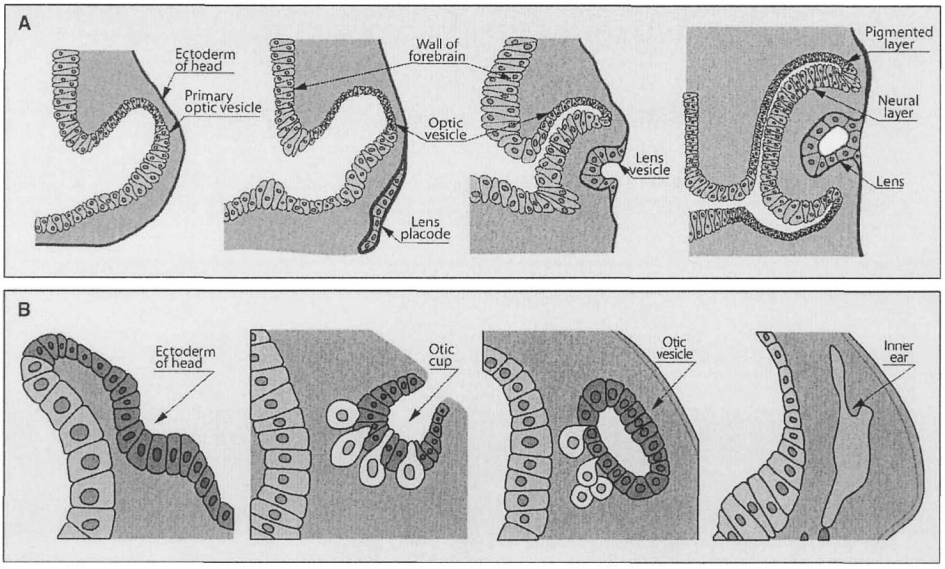

Figure 1. (**A**). Neuroretina development. (**B**). Inner ear early development.

4. THE OLFACTORY SYSTEM

Most elements of the olfactory system are generated from the most rostrally placed placodes, the olfactory placodes. In mammals, these placodes give rise to four types of neurons: olfactory, sense or vomeronasal, nervus terminalis, and migratory gonadotrophin-releasing hormone (GnRH) neurons[107], plus other cell types such as receptor cells or glial and Schwann cells. The olfactory neurons follow distinct patterns of cell differentiation and migration and, *pari pasu* the nasal epithelium involutes and induces the chondrogenic capsule in the surrounding mesenchyme. A characteristic of GnRH neurons is that they are generated outside the CNS and migrate toward it[30,108,109]. Also remarkable is the fact that olfactory sense neurons are renewed from progenitor cells present in the olfactory neuroepithelium in adult mammals[110,111]. Therefore, the olfactory system is a source of adult stem cells[112]. The axons of the olfactory sensory neurons are surrounded by a special type of glial cell, called olfactory ensheathing glia, which are also derived from the olfactory placode[113-116]. The olfactory epithelium has intrinsic growth factors that regulate development and can support the genesis and survival of new neurons[18]. The members of the IGF system, in particular IGF-I, are expressed in the olfactory system cells and can influence neuronal generation, survival, and/or differentiation (Tables 1 and

2). Expression of both IGF-I and its high-affinity receptor IGF-1R decreases and/or becomes more restricted to specific cell types in the olfactory structures with age in different vertebrates[16,17,24]. However, it is important to note that the olfactory bulb is one of the areas in the CNS that retains relatively high expression of IGF-I and the IGF-1R, with this higher expression generally being associated with areas of adult neurogenesis[3]. Regarding the function of IGFs during olfactory system development, expression patterns in the olfactory bulb suggest a role for IGF-I from early neurogenesis onwards and, accordingly, *Igf-1* knockout mice present severe alterations in the formation of the mitral cell layer of the olfactory bulb, as well as altered morphology of radial glia[64].

5. THE GUSTATORY SYSTEM

The development of the vertebrate taste system has been relatively understudied compared with other sensory systems. Tongue development starts when the first pharyngeal arch forms a swelling called the median tongue bud, shortly afterwards another pair of lateral swellings, the distal tongue buds or lateral lingual swellings, is formed, which rapidly overgrow the median tongue bud. These structures continue to grow throughout development and form the anterior two-thirds of the tongue. From the second pharyngeal arch develops a midline swelling called the copula that is later overgrown by the hypopharyngeal eminence, which gives rise to the posterior one-third of the tongue. The hypopharyngeal eminence expands mainly by the growth of the endoderm of the third pharyngeal arch, with a small contribution from the fourth pharyngeal arch to the most posterior aspect of the tongue[117]. The capacity of the pharyngeal endoderm to generate taste buds is probably determined by the end of gastrulation, but the molecular bases of this process are yet to be elucidated. The sensory afferent axons from the VIIth, IXth, and Xth cranial nerves invade the lingual epithelium before taste bud differentiation and it has been proposed that innervation may play a role in taste buds differentiation[118]. The neurotrophic factors brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) participate in the innervation of the developing tongue in mouse and humans. It has been reported that mice with deficits in BDNF or NT-3 present gustatory or somatosensory alterations, respectively[119-122]. In addition, alterations in size, number and morphology of gustatory papillae and taste buds present in BDNF null mutant mice demonstrate the neural dependence of developing taste organs[123].

To our knowledge, there are no studies on the actions of the GH-IGF axis on the development of the gustatory system.

6. THE TACTILE SYSTEM

The sense of touch allows the perception of stimuli such us contact, pressure, temperature or pain. Its sensory organ is the skin. The perception of stimuli is carried out by skin specific sensory mechanoreceptors, thermoreceptors and nociceptors located throughout the layers of the skin (epidermis, dermis and hypodermis). For example, Meissner's corpuscles sense the onset and end of continuous light pressure, Pacinian's corpuscles sense firm pressure, Ruffini's corpuscles and Krause's corpuscles respond to changes in temperature and pressure on the skin. There are four types of mechanoreceptive afferent neurons that innervate the skin and respond to cutaneous motion and deformation in different ways[124]. The association of specialized receptor cells with peripheral terminals of sensory axons forms the sensory receptors in the skin. The specificity of the receptor is determined by the subtype of transducer that is stimulated and by the structure of the receptor that surrounds each of these nerve terminals[124, 125]. The development of the innervating sensory nerve cell occurs *pari pasu* with that of the receptor cell. The neurons promote receptor cell differentiation, which in turn provide neurotrophic support to the sensory neurons. The signals that mediate these actions are not known in detail, but the influence of neurotrophic factors on the development of the tactile system has been confirmed by the study of transgenic mice lacking neurotrophins or their receptors[126-128].

Actions of the IGFs in the regulation of tactile corpuscles development or in the perception of the stimuli have not yet been reported.

7. THE AUDITORY AND VESTIBULAR SYSTEMS

The vertebrate inner ear is derived from a thickening of the head surface ectoderm, adjacent to rhombomeres 5 and 6, named the otic placode that invaginates and pinches off the ectoderm to form the otic vesicle or otocyst. Figure 1B shows the early stages of vertebrate inner ear development. The otic vesicle is a transient structure that undergoes multiple morphogenetic movements and developmental changes associated with cell proliferation, differentiation and cell-death. This results in the ear labyrinth: the cochlea,

the utricle and saccule, and the semicircular canals, each containing their corresponding sensory organs[35,129,130] with the mechano-transducing hair cells and the neurons that connect them with the central nervous system. Local mechanical perturbations are transduced by hair cells into synaptic potentials, which elicit the activation of auditory (cochlear) and vestibular neurons, that project towards the homonymous central nuclei, the first input station of the auditory pathway. Auditory neurons inform the brain of the intensity and spectral properties of sound and vestibular neurons carry information about position, velocity and acceleration. Generation of otic neurons is a sequential process. First, otic neurons are specified in the otic epithelium, neuronal precursors then delaminate to form the cochleo-vestibular ganglion (CVG), where they proliferate and differentiate. The CVG neurons project extensions back to innervate the vestibular and cochlear (auditory) sensory epithelium.

Diffusible factors like fibroblast growth factors, IGF-I and the nerve growth factor family of neurotrophins are locally synthesized during development and elicit a network of interconnected signaling pathways that finally instructs the cell to proliferate, die or differentiate. The elements of the IGF system are expressed in the early developing chicken inner ear[3,14] (Table 1). GH immunoreactivity has also been detected during early development of the chicken otic vesicle[88,90-92]. In the developing vertebrate inner ear, IGF-I acts as a survival and growth factor [135]. IGF-I is also expressed during maturation of the rodent auditory system and in adult hair cells[131,132]. During the early postnatal period of mouse inner ear development, from postnatal day (P)5 to P20, IGF-I is expressed in the Organ of Corti and cochlear ganglia[36]. The cochlear and vestibular ganglia also express insulin, IGF-II and their receptors (Fig. 2A). Recently, the analysis of a human fetal cochlear cDNA library indicated the presence of IGF-I and IGFBP-1, -3 and –5. Analysis of gene expression profiles of the rat cochlea demonstrated the presence of IGF-II and IGFBP-2 and -6[133,13]. Studies on primary cell cultures and genetically modified animal models have shown that IGF-I is essential for the normal development and function of the vertebrate inner ear[135] (Table 2). Endogenous IGF-I is essential for generation of the CVG in chicken embryos, with the blockade of IGF-I actions being associated with an increase in cell death, a reduction in cell proliferation and a reduction in the levels of expression of neuroblasts and neuronal markers[140].

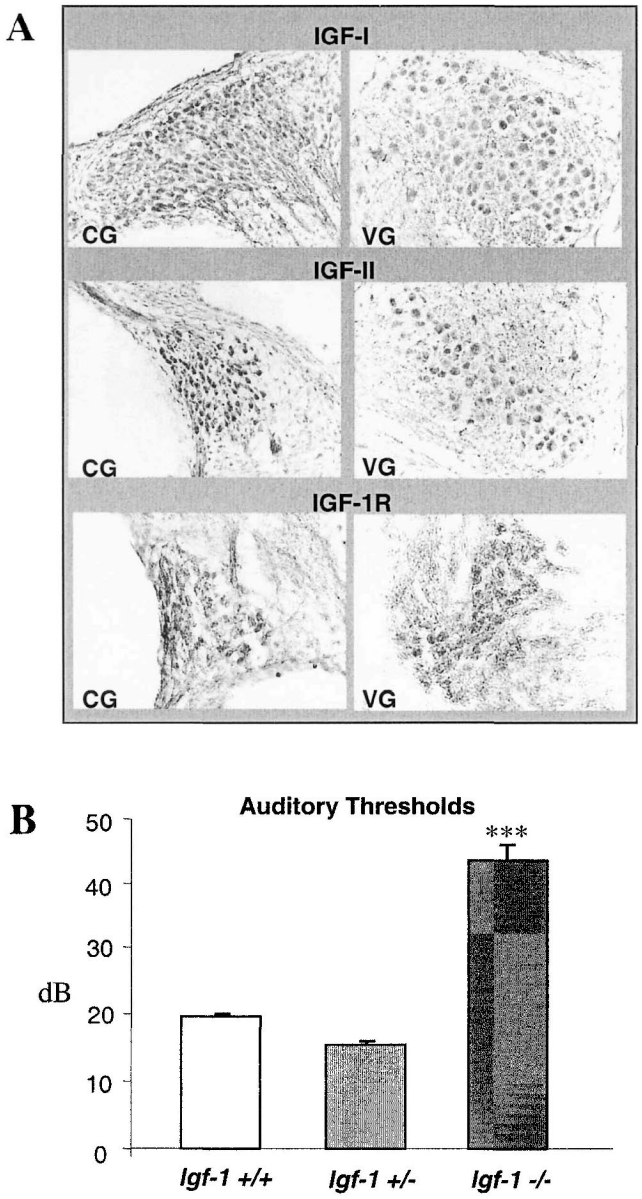

Figure 2. **A.** Expression of insulin-related factors and receptors in the cochlear and vestibular ganglia in P5 mice. Immunolocalization of IGF-I, IGF-II, and IGF1R was positive in the cochlear (CG) and vestibular ganglia (VG) of P5 mouse. Microphotographs show representative 25 μm paraffin sections from at least four mice studied in three different assays. Scale bars, 50 μm. **B.** Auditory thresholds of wild type (n=25), heterozygous (n=30) and knockout *Igf-1* mice (n=7) at P30. Statistical analysis reveal that the *Igf*-1 deficient (-/-) mouse has a two-fold increase in auditory thresholds (*** P<0,001) compared to control (+/+) and heterozygous (+/-) mice.

We have explored the signaling mechanisms that mediate IGF-I proliferative responses in otic cells. IGF-I binding to IGF1R increases the levels of inositol lipid mediators, activates the Raf/MAPK cascade, and induces the expression of the transcription factor AP-1 and proliferating cell nuclear antigen (PCNA)[40,43,136-138]. Anti-apoptotic effects of IGF-I are mediated by the activation of the Akt/protein kinase B pathway and modulation of the levels of the pro-apoptotic lipid mediator ceramide[139].

Interestingly, the early actions of IGF-I on cell proliferation are mimicked by other factors of the family, insulin and IGF-II, but later actions on cell differentiation are specific to IGF-I[43,140]. Accordingly, during the mouse inner ear postnatal period of maturation, from birth to P20, the neurons of the cochlear ganglia become strictly IGF-I dependent and its deficit causes retarded maturation and decreased cell survival[36,71]. Moreover, IGF-I deficiency causes decreased neuronal differentiation in the mouse cochlear ganglion, a sustained deficit in the cochlear nerve and ganglia myelination, and aberrant synaptogenesis at the Organ of Corti, suggesting that IGF-I is required to reach full auditory function[36,71]. We do not yet know whether the alterations observed in cochlear neurons of *Igf*-1 null mice could compromise mid-term survival of hair cells of the organ of Corti. We have confirmed by using auditory brainstem response tests that *Igf-1* knockout mice at day P30 have an increase in auditory thresholds compared to control (*Igf-1*$^{+/+}$) and heterozygous (*Igf-1*$^{+/-}$) mice[135] (Fig. 2B). These results confirm the key role of IGF-I in the development and maintenance of auditory function. Indeed, a deficiency in IGF-I results in sensory-neural deafness in humans[76,77,82] (Table 3), but a partial deficiency in IGFR1 is not associated with deafness in humans, although no detailed study of the auditory function of these patients has been reported[84].

Hearing and balance impairment caused by hair cell loss or dysfunction is a high-prevalence multifactorial disease that currently has no restorative treatment available. IGF-I and insulin, alone or in combination with other growth and neurotrophic factors, protect otic cells from ototoxic damage and promote *in vitro* hair cell regeneration[141-149]. The potential of IGF-I in the treatment of neurodegenerative diseases, together with the reported actions of IGF-I in inner ear development and function, support the hypothesis that this factor is a good candidate for inner ear regeneration therapy.

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Chapter 10

ROLE OF INSULIN-LIKE GROWTH FACTORS IN NEURONAL PLASTICITY AND NEUROPROTECTION

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Key words: Brain function; nerve cell plasticity; neurodegenerative diseases; neuro-repair; synaptic contacts.

1. INTRODUCTION

The notion that insulin-like growth factor (IGF-I) is an important modulator of brain function is now reasonably well established. The classical role assigned to IGF-I in the brain is to promote neuronal survival during development. Indeed, the canonical pro-survival pathway in developing neurons; which includes the ser-kinase Akt, was first described downstream of the IGF-I receptor[31]. But the biological role of IGF-I is not circumscribed to the developmental period; accumulating evidence indicates that this growth factor exerts a wide-spectrum protective action in the adult brain[19]. The last decade has witnessed a spur of studies on the neurobiological actions of IGF-I. The result is that we now may consider IGF-I as a prototypical neuroprotective factor. Much less is known however about the potential role of the other known member of the family, IGF-II. This is mostly attributable to the fact that the reported actions of IGF-II in the brain are mediated by the IGF-I receptor, and since IGF-II has a lower affinity for this receptor, it is widely accepted that the major player in brain physiology is IGF-I. This situation has led to a reduced interest in determining the role

of IGF-II in the brain, and for that matter, elsewhere. However, high levels of IGF-II in particular brain locations[11] and widespread distribution of its receptor[42] support the idea that this growth factor has a role in brain physiology other than to mimic IGF-I actions.

IGFs are synthesized by the developing brain[10,53,54], with mRNA levels becoming much lower in the adult tissue, except for very specific locations such as the choroid plexus and brain vessels for IGF-II [45,63,80] or a few scattered brain regions for IGF-I[3,6,36]. However, IGF protein levels, in particular IGF-I, do not decrease in many brain regions as much as mRNA levels, which suggested the existence of an extracerebral source of IGFs[84]. Recent studies have confirmed that a substantial amount of brain IGF-I is taken up from the circulation and that this uptake is modulated by physiological changes[16,17]. The case of IGF-II is easier to explain because cerebrospinal fluid (CSF)-derived IGF-II produced by the choroid plexus can reach the brain parenchyma. This may suggest that there is a switch from a paracrine/autocrine pathway during development to an endocrine pathway in the adult in the action of IGFs in the brain. Nevertheless this idea is tempered by the fact that extracerebral IGFs can similarly access the brain during development because they are abundant in the periphery, and locally synthesized IGFs, albeit at lower levels than during embryogenesis, may also be important in local modulation of adult brain function.

At any rate, IGFs are present in the developing and adult brain and modulate a wide variety of brain functions in a developmental and anatomically regulated fashion. In the present chapter we discuss the contribution of IGF-I to two major brain properties that are intimately interconnected, neural plasticity and neuroprotection. The two processes underscore major functional traits in brain organization.

Neural plasticity is a broad, widely used concept that refers to an intrinsic property of the brain: the ability to adapt to a constantly changing environment. Although it is quite likely that all cell constituents within brain tissue participate in its plastic properties, the current bias favoring neurons as the cells that confer the nervous system its functional properties has led to analysis of mechanisms underlying neuronal plasticity in much greater detail than plastic processes in other cell types such as glia or endothelia. Fortunately, this trend is changing, and glial cells are becoming accepted partners in neural plasticity processes[8]. Since neuronal function means cell communication, neuronal plasticity translates into regulated variability in neuronal transmission. Because neuron-to-neuron communication takes place at the synapse, in the majority of cases changes in synaptic plasticity have been the actual focus of studies addressing neuronal plasticity. But changes in the size of neuronal populations, in neuronal excitability, or in the

metabolic status of neurons, to name a few, are undoubtedly affecting the ability of neurons to cope to changing demands.

Neuroprotection is also a comprehensive concept that includes a wide variety of processes. Usually we use the term neuroprotection when referring to therapeutic maneuvers to counteract brain disease; but we can also employ this term when analyzing endogenous mechanism that allow the brain to cope with potentially pathological disturbances. In the latter sense, IGF-I is indeed a neuroprotective signal[83]. A functional link between neuroprotection and neuronal plasticity is easily inferred; neuroprotective processes must aim plastic processes if they are meant to preserve neuronal function.

2. NEURAL PLASTICITY

The developing brain is considered to exhibit a high level of cell plasticity. Both genetic and epigenetic cues allow a substantial degree of flexibility in the final number of cells formed and in the establishment of connections among cell populations. Abundant literature documents in detail the role of growth factors in controlling these aspects of cell plasticity during brain formation[81]. Although in this regard, IGF-I is not usually included as a neurotrophic factor in brain development, a field dominated by the neurotrophin family of growth factors, genetic analysis has firmly established an essential role for IGF-I in controlling brain size by modulating number of cells produced, degree of fiber myelination, and connectivity[7,21].

Once brain development is completed, plasticity will rely on the resources acquired during development, *i.e.,* cell numbers and connectivity, as well as on emerging properties inherent to functional brain cells. As commented earlier, these plastic properties have been studied in neurons with a prominent focus on synaptic plasticity mechanisms because the level of activity of neural circuitries relies to a great extent in the strength of the synapses involved. In turn, synaptic strength will be determined by the amount of neurotransmitter released by the presynaptic terminal, the abundance of functionally active postsynaptic neurotransmitter receptors, and by the intrinsic excitability of the postsynaptic neuron. The number of synapses recruited, which in turn is related to the size of the neuronal population involved, will also determine overall activity of the circuitry. All these parameters are modulated by IGF-I.

Release of several types of neurotransmitters has been reported to be regulated by IGF-I under experimental conditions. These include major neurotransmitter systems such as acetylcholine[57,76], glutamate[34], and GABA[22,76]. Although modulation of GABA release by IGF-I has been documented to take place also *in vivo*, we still do not know the physiological

importance of these observations. In the case of acetylcholine, both stimulatory and inhibitory effects have been reported, while glutamate levels at the synaptic cleft are regulated indirectly by IGF-I through regulation of the expression of glutamate transporters.

The number of postsynaptic neurotransmitter receptors is also controlled by IGF-I by modulating their membrane trafficking and insertion. These effects are independent of the classical trophic actions of IGF-I in controlling the abundance of neurotransmitter receptors through modulation of their synthesis[50,78,92]. Insertion or internalization of neurotransmitter receptors by IGF-I allows for a rapid modulation of synaptic strength. Specifically, IGF-I modulates the cell surface distribution of AMPA and kainate subtype of glutamate receptors, albeit in opposite ways[38,60], and possibly also of GABA receptors[88]. Although *in vivo* evidence supports a physiological role for IGF-I modulation of glutamate receptor availability on synaptic activity[20], it is still premature to conclude what are the functional consequences *in vivo*, mostly because different functional outcomes can be envisaged.

By far, the best documented action of IGF-I as a modulator of neuronal plasticity is through modulation of neuronal excitability by interacting with different types of neuronal membrane ion channels. These include Cl^-, Ca^{++}, and K^+ channels[9,47,70,73,74]. In all instances, IGF-I potentiates the activity of these currents through mechanisms involving allosteric modification of ion channel function or translocation to the membrane. Although the net effect on neuronal excitability is difficult to anticipate because stimulation of these broad set of ion channels will have a disparate effect on membrane potential, *in vivo* recordings after systemic IGF-I administration reveals increased neuronal excitability in very specific brain nuclei[16]. Intriguingly, parallel expansion in the receptive fields of stimulated neurons is observed[70].

Although still speculative, coupling of increased neuronal excitability to enlargement of the receptive field after IGF-I exposure may contribute to learning-related plastic mechanisms operating in neurons. Modifications in the size of neuronal receptive fields are postulated to underlie learning processes[13]. Indeed, training in a particular task results in enlargement of the neuronal population involved in task performance[69]. This enlargement is possible through recruitment of new synapses within the circuitry and recruitment of new synapses may be brought about through increased excitability of the neurons in the circuit. This increased in neuronal excitability may develop in the following sequence (Fig. 1): 1) engagement of a neuronal population in a given task will increase their activity; 2) through coupling of increased neuronal activity to increased blood flow, a phenomenon that forms the basis of fMRI analysis, blood-borne IGF-I will accumulate into active neurons (this activity-dependent uptake of systemic IGF-I is theoretically possible because entrance of circulating IGF-I into the

brain is stimulated by physiological stimuli such as physical exercise that stimulates brain blood flow); 3) in turn, neurons accumulating IGF-I will become more excitable. Therefore, circulating IGF-I may constitute a physiological mediator of activity-dependent changes in the size of the receptive field of neurons. This introduces a fascinating new role of blood-borne IGF-I as an important modulator of cognition. Notably, serum IGF-I levels are correlated with cognitive status in humans[56,86].

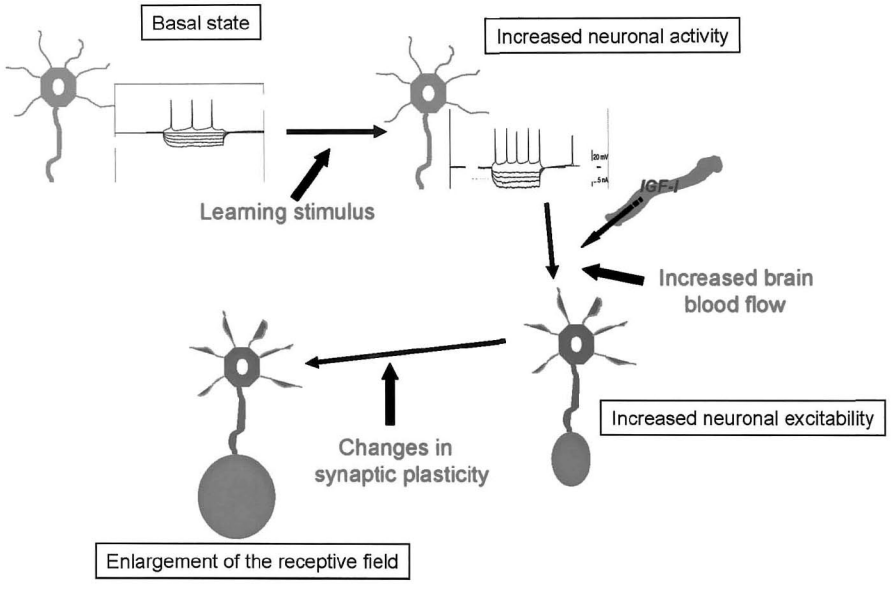

Figure 1. A role for circulating IGF-I in learning processes. Expansion of the receptive field of neurons brought about by accumulation of serum IGF-I by neurons may underlie changes in circuitry properties associated to the learning process. Stimulation of neuronal activity after behavioral engagement (as during learning) leads to increased blood flow to the stimulated area. In turn, serum IGF-I will accumulate in activated neurons which will increase their excitability[16] associated with an increase in the size of their receptive field[70].

Synaptic remodeling refers to the dynamic status of synapses. Recent work indicates that synaptic turnover at dendritic spines is more frequent and faster than previously thought. It is easy to infer that regulation of this event will contribute to neuronal plasticity processes as the number of synapses engaged will heavily determine the activity of the circuitry. Ultrastructural studies indicate that the density and size of dendritic spines depends on IGF-I input to target neurons such as Purkinje cells in the cerebellum[67]. As commented earlier, the expansion of the receptive field on exposure to IGF-I

may theoretically involve not only strengthening of existing synapses, but also the formation of new ones. We hypothesize that IGF-I acts as a synaptic "stabilizer," favoring the stable formation of synapses through mechanisms involving synaptic-specific expression of proteins involved in pre- and postsynaptic interactions such as membrane adhesion molecules. This is based on the observation that IGF-I modulates the polarity and motility of many different types of cells by controlling the expression of proteins involved in cell-to-cell interactions[61]. However, this possibility remains entirely speculative at present.

3. NEUROPROTECTION

Physiological neuroprotective mechanisms aim to maintain neurons alive and functional. Owing to the restricted neuronal turnover capacity of the adult brain, maintenance of neuronal populations appears to be crucial for proper function. A widely used strategy in the mammalian nervous system is to contain functionally overlapping cell populations. In this way, function is compromised only after extensive neuronal loss occurs, such as observed in Parkinson patients, in whom motor deficits appear only after more than 80% of the dopaminergic neurons in the substantia nigra are dead or atrophied[25]. Genetic manipulation has shown that IGF-I governs the size of neuronal populations. While excess IGF-I leads to bigger brains, its absence produces microcephaly[27]. Therefore, a first protective action of IGF-I already takes place during brain development by controlling the final size of neuronal populations.

Albeit limited in the adult period in mammals, another neuroprotective strategy is to produce new neurons, as seen in the hippocampus and olfactory bulb. Because the functional significance of adult neurogenesis is not yet established[51], we can only speculate about its role as a neuroprotective strategy. However, recent work indicates that the ability of IGF-I to control adult neurogenesis is markedly neuroprotective. In the absence of appropriate IGF-I input, IGF-I-deficient mutant mice show substantial decreases in new neuron formation together with enhanced susceptibility to injury[85]. Hence, a reduced capacity to produce new neurons affects the ability of the brain to cope with insults[4].

Apart from these preventive measures oriented to produce excess neurons and replace lost ones, the brain has developed self-reparative mechanisms against insults. Therefore, loss of function will eventually develop only if preventive and reparative processes are overcome. IGF-I participates also in this aspect of neuroprotection as a potent reparative agent. The best

characterized action of IGF-I is as an antiapoptotic signal[29]. However, IGF-I participates also in other reparative processes, acting as an anti-inflammatory agent by counteracting the actions of pro-inflammatory cytokines, for instance[28,71]. IGF-I also participates in reactive angiogenesis occuring after injury and in this way ensures proper nutrient supply to damaged areas (unpublished observations). Re-establishment of lost connections is also an important aspect of IGF-I-dependent repair[33,41]. The cellular and molecular pathways recruited by IGF-I in these processes are still poorly characterized.

IGF-I protects against almost any kind of *in vitro* or *in vivo* injury to brain cells, from those of ample occurrence such as oxidative damage to disease-specific derangements such as ß-amyloidosis (Table 1). This remarkable ability to protect against so widely different types of insults suggests that neurorepair by IGF-I proceeds through a fundamental protective pathway, such as activation of Akt, the canonical pro-survival kinase downstream of the IGF-I receptor[31].

In vitro	Reference	*In vivo*	Reference
Oxidative stress	43	Ischemic insult	40
Neurotoxic drugs	12,26	Neurotoxins	33,72
Hypoxia	90	Trauma	49
β Amyloid	68	Alzheimer amyloidosis	18
Huntingtin	44	Motor neuron diseases	48,6
Hypoglycemia	23	Cerebellar ataxia	17,32
		Brain senescence	62
		Nerve transaction	46
		Diabetic neuropathy	91

Table 1. In vitro and *in vivo* neuroprotection by IGF-I

Alternatively, we recently suggested that the reason underlying the ability of IGF-I to protect against so many different types of insults is that pathological changes develop only after a tonic protective action of IGF-I upon neurons is compromised[35]. Specifically, we speculate that common pathological processes such as inflammation, excitotoxicity, or accumulation of deleterious molecules such as ß-amyloid or glycated products lead to neuronal death, at least in part through their ability to counteract the protective actions of IGF-I[85] (Fig. 2).

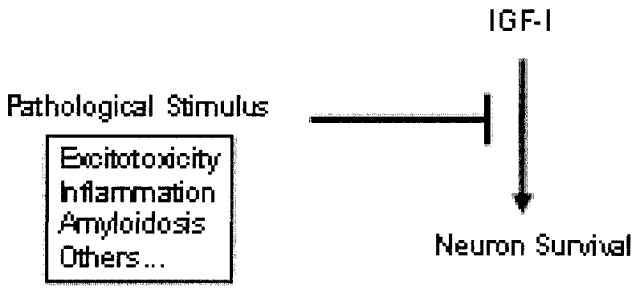

Figure 2. Pathological derangements lead to neuronal demise at least in part by inhibiting pro-survival actions of IGF-I.

What are these tonic protective actions of IGF-I on the adult brain? Indeed, fundamental homeostatic processes are modulated by this multi-faceted growth factor. IGF-I ensures adequate nutrient sources to neurons by increasing neuronal glucose uptake and lactate production by astrocytes[24,77]. Albeit still controversial, lactate from astrocytes has been proposed to contribute to metabolic demands in active neurons[87]. In this way IGF-I would stimulate neuronal excitability and at the same time ensure proper nutrient supply to stimulated neurons. In this vein, IGF-I also increases brain blood flow and the formation of new brain vessels[37] (and unpublished observations), allowing nutrient supply on demand together with proper oxygen levels. Conceivably, the latter is achieved through an activity-dependent process similar to that already described for IGF-I-mediated changes in the receptive field size of activated neurons (see Fig.1). Since a low rate of basal angiogenesis in the adult brain is thought to be maintained to match functional demands, activity-dependent uptake of serum IGF-I, a potent angiogenic promoter, would result in local increases in angiogenesis on demand.

More specialized processes regulated by IGF-I include brain clearance of potentially hazardous substances such as ß-amyloid peptides through a complex regulatory mechanism involving a network of β-amyloid transport proteins interacting at the blood-brain interface[18]. This novel mechanism of IGF-I neuroprotection has been discussed in detail recently[15] and points to an intriguing possibility: Alzheimer's neuropathology may develop as a consequence of a gradual loss of sensitivity to IGF-I during aging. However, this remains to be shown.

As suggested earlier, IGF-I may also be necessary to maintain appropriate synaptic contacts. This is also a fundamental way to maintain healthy neuronal populations since changes in the number of synaptic contacts lead to changes in neuronal excitation which in turn modulates neuronal

performance through changes in synaptic gain[55]. Therefore, the ability of systemic IGF-I to increase neuronal excitability may contribute to strengthening synaptic contacts between stimulated partners.

4. PATHOLOGICAL SIGNIFICANCE AND THERAPEUTIC POTENTIAL OF IGF-I IN BRAIN DISEASE

Genetic ablation of IGF-I has shown that this growth factor is important for appropriate organ growth, including the brain[7]. Although brain-specific null IGF-I mutants have not been yet developed, genetic models interfering with IGF-I actions on the brain have shown that insufficient IGF-I input to the brain affects its size. This reduction may be due to decreased generation of neurons, reduced neuron survival or both. Probably because of its critical role, null IGF-I mutants have not been found among the human population (only a small proportion of null IGF-I mice survive for several weeks). However, children with very low levels of IGF-I has been reported in whom brain deficits accompanied mental retardation[14]. Although a few recent examples of IGF-I receptor defects have been documented in children with growth retardation[1], these have not yet been analyzed for possible brain anomalies, which can be expected. At any rate, lack of IGF-I during brain development will compromise cell resources and adequate connectivity in the adult, leading to functional derangements.

As for the adult brain, we speculate that if a constant trophic input to the brain through IGF-I is required for adequate function, abnormal IGF-I signaling will have pathological consequences. Restoration of adequate IGF-I signaling through novel therapeutic strategies can be anticipated to be beneficial. However, these two notions are far from being proved yet. Albeit compelling evidence indicates that low IGF-I input probably contributes and even leads in specific cases to brain disease[85], this possibility needs to be further substantiated. While IGF-I, as well as complementary strategies such as potentiation of endogenous IGF-I signaling[59], have been tested as therapeutic tools in various brain diseases, a combination of shortcomings and distorting factors precludes any firm conclusion about the true therapeutic potential of IGF-I for neurodegenerative diseases[2,32,39,40,48,52,58,64,65,72,75,82]. The fact that controversial epidemiological data suggests that IGF-I may have tumorigenic effects[5,30,79,89] has considerably hindered further progress with additional clinical assays. Because new evidence supports a therapeutic use of IGF-I in diseases such as Alzheimer's dementia[18] or Huntington's chorea[44], we can anticipate a deeper and more detailed insight into this largely neglected area of research

with IGF-I. To circumvate potential carcinogenic actions of IGF-I, the pharmaceutical industry is actively looking into synthetic compounds mimicking neuroprotective pathways used by IGF-I. This is a very logical and promising strategy that will hopefully yield new useful drugs for neurodegenerative diseases.

5. FUTURE PERSPECTIVES

We anticipate that the therapeutic use of IGF-I in neurodegenerative diseases is going to be one "hot-spot" in research in this field. Relying on ongoing clinical trials with IGF-I in diabetes and other endocrine diseases, soon-to-come assays in amyotrophic lateral sclerosis and hopefully in cerebellar ataxia, analysis of the therapeutic potential of IGF-I in Alzheimer's disease and in Huntington's chorea will be very likely implemented. Insight into the cellular and molecular pathways used by IGF-I for its extraordinarily ample protective action on the brain will also be developed. This aspect is particularly relevant because knowledge of physiological neuroprotective mechanisms will allow targeting of new molecules for drug research.

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Chapter 11

AGING AND LIFE SPAN

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Key Words: Body size; dwarf; glucose regulation; longevity; stress resistance; therapy; transgenic

1. INTRODUCTION

The factors that regulate aging processes are poorly understood. However, the closely related molecules, growth hormone (GH) and insulin-like growth factor-I (IGF-I), and corresponding downstream signaling factors have been shown to play a major role in the regulation of aging and life span. This mechanism appears to be well conserved evolutionarily, as evidence for altered signaling of these pathways extends the life span of yeast to mammals. Genetic models of aging have provided extensive data implicating a role for these hormones in both premature and delayed aging. These models are under active investigation to relate the GH/IGF-I/insulin pathway to the physiological changes associated with aging.

2. GH DECLINES WITH AGE

It is well documented that growth hormone levels begin to decline soon after the peripubertal period of rapid growth[1]. The progressive decline of GH

secretion with age has been termed the "somatopause" in humans. Reduced GH secretion results from a loss of the high-amplitude release that occurs nocturnally. Further studies indicate that hypothalamic growth hormone releasing hormone (GHRH) and somatostatin may become dysregulated with age[2,3]. Decreased plasma IGF-I levels parallel the decline in GH pulses[4]. The lower levels of plasma GH and IGF-I in the elderly are thought to cause many aspects of physical change in aging including decreased muscle mass, strength, skin thickness and bone mineral density and increased fat mass and overall loss of energy. These changes parallel those observed in adult GH deficiency. GH replacement successfully alleviates the symptoms of adult GH deficiency. The beneficial effects of GH therapy in aged individuals have also been reported along with both observed and potential adverse side effects including arthralgia, carpal tunnel syndrome, edema, weight gain, myalgia, hyperglycemia, cancer and colonic polyps[5,6]. Therefore, GH therapy in the aged is controversial, at best.

3. GH DEFICIENCY AND LIFE SPAN: MAMMALS

Endocrine mutants have provided tremendous insight into the role of hormones in aging (Table 1). Several types of GH/IGF spontaneous mutants and genetically engineered mice have been studied and include the Ames dwarf, Snell dwarf, GH receptor/binding protein knockout, Little, GH receptor antagonist, IGF receptor knockdown, and GH transgenic mice. Each of these mutants is described in the context of life span followed by specific information regarding possible mechanisms of delayed and premature aging.

Ames dwarf mice have a primary pituitary deficiency resulting in the absence of GH, prolactin (PRL), and thyrotropin (TSH). These deficiencies result from a point mutation in the *prop-1* gene[7] that promotes appropriate differentiation of the pituitary cells into somatotrophs, lactotrophs, and thyrotrophs. Consequent to GH deficiency, these mice have undetectable levels of plasma IGF-I[8]. Ames dwarf mice live 49-64% longer than their wild-type siblings (males and females, respectively)[9]. Ames mice are one of the longest living endocrine mutants and serve as an example of delayed aging. They are characterized by their diminutive body size (one third of normal) and reduced fertility.

Phenotype	Yeast (*Sch9, RAS*)[32,154]	Worms (*daf2, age-1*)[33-35]	Flies (*Chico, INR*)[38,39]	Mice (*prop-1, pit-1*, GHR/BP KO)[9,10,12]	Mice GH over-expressing[44,45,179]
GH/IGF1/ Insulin signaling	↓	↓	↓	↓	↑
Body size	↓	↓	↓	↓	↑
Reproduction	↓	↓	↓	↓	↓
Glucose metabolism	↓	↓	↓	↓	↑
Stress resistance	↑	↑	↑	↑	↓
Longevity	↑	↑	↑	↑	↓

Table 1. Phenotypic characteristics of mutant yeast, worms, flies and mice.

Snell dwarf mice are phenotypically identical to Ames mice lacking GH, PRL, and thyrotropin owing to lack of appropriate pituitary differentiation because of a mutation in the transcription factor *pit-1* (downstream of *prop-1*). These mice also have significantly reduced plasma IGF-I levels and live significantly longer (40–45%) than normal littermates[10].

More evidence that reduced GH/IGF-I leads to life extension is derived from GH receptor/binding protein knockout (GHR/BP KO) mice[11]. These mice have high plasma GH but significantly reduced IGF-I levels because of the lack of receptor binding in target tissues. The GHR/BP KO mice are smaller than wild-type mice, exhibit delayed puberty, and the average increase in life span is 31–46%[12,13].

Mice with a spontaneous mutation in the GH releasing hormone (GHRH) receptor were first described by Eicher and Beemer[14] and termed "Little" mice[15,16]. These mice are deficient in GH and smaller in size, but exhibit normal body proportions, reduced fertility, and a 25% increase in life span compared to wild type controls when fed a low-fat diet[10].

Mice engineered to overexpress a GH antagonist (mutated bovine GH) phenotypically resemble "Little" mice but do not exhibit extended life spans compared to their normal counterparts[13,17]. In addition, these mice have greatly reduced plasma IGF-I levels.

Attempts at creating IGF-I knockout mice have usually resulted in embryonic lethality[18,19], although more recently Liu and LeRoith[20] reported a

42% postnatal survival rate in mice created to lack IGF-I gene expression. These mice are severely growth retarded both *in utero* and postnatally (35% at birth and 65% as adults). No life span data have been reported in these mice. However, an IGF-I receptor knockdown mouse was recently shown to live 26% longer than normal mice in this line[21]. These mice exhibit a 50% reduction in IGF-I receptor expression, slightly smaller body weights, and normal fertility. In addition, a liver-specific IGF-I gene deficient mouse (LID) was created by Yakar and coworkers[22] but no data regarding life span of these mice are available at this time. These mice develop and grow normally but exhibit altered carbohydrate metabolism[22,23].

The evidence is overwhelming and clearly demonstrates that reduced GH/IGF-I signaling via reductions in plasma GH/IGF-I hormone levels or altered hormone receptor interactions extends life span in rodents. Table 2 summarizes some of the general clinical features of GH deficiencies and corresponding mutations. A human correlate of the mutation in Ames dwarf mouse was described by Krzisnik and coworkers[24] and showed that some members of this human "dwarf" population (*prop-1* gene mutation) also outlived normal individuals from the same population. These individuals were not extremely long lived (>100) thus it is postulated that the potential detrimental effects of GH/IGF-I deprivation may not be pronounced or even realized in a 2 to 3 year-old-mouse living in a protected laboratory environment versus a 100-year-old human[25]. Other populations of *prop-1* mutant people have been reported and showed no significant increases in life span over that of the normal population[26-28]. In addition, GHR/BP gene knockout mice are the mouse correlate of Laron dwarfism in humans. This syndrome is caused by a mutation in the GH receptor and resembles clinical GH deficiency. Individuals with Laron syndrome exhibit high plasma GH levels, very low circulating IGF-I concentrations, short stature, obesity, and impaired physical development[29]. Regarding mental status, the older literature suggests that impairment is common; however, Kranzler and coworkers found normal intelligence in a homogeneous population exhibiting GHR deficiency[29,30]. No differences in mortality are reported between those with Laron dwarfism and the normal population. Robust data on these populations are unlikely because so few individuals are available for study (~250 individual cases have been identified worldwide). GH deficiency in humans has been associated with increased fat mass, decreased muscle and bone mass, insulin resistance, and reduced life span[31]. However, some GH-deficient hypopituitary patients are also deficient in adrenocorticotropin (ACTH), likely accounting for the observed increase in mortality of this population. Clinical presentation is often non-specific and may include fatigue, lack of energy, poor concentration, memory loss in adults, and short stature in children. Human defects in IGF-I production are

extremely rare, most probably as a result of intrauterine lethality[20]. In addition, multiple examples in invertebrates support and extend that found in mammalian systems.

Phenotype/ Mutant Gene	PROP1[a]	POU1F1 (pit-1)[a]	GHRHR[b]	GH1[c]	GHR[d]
GH/IGF1/Insulin signaling	↓	↓	↓	↓	↓
Growth failure/ Short stature	+	+	+	+	+
Body fat	increased	increased	increased	increased	increased
Reproduction					
Neonate	Microphallus	Microphallus	N/A	N/A	Small genitalia/gonads
Adolescent	Puberty delayed	Puberty delayed	Puberty delayed	Puberty delayed	Puberty delayed
Adult	Infertility	Infertility	Normal fertility		Normal fertility
Glucose metabolism					
Neonate	Hypoglycemia	Hypoglycemia	Normal	Hypoglycemia	Hypoglycemia/
Adult	Fasting symptoms	Fasting symptoms	-	-	hyperinsulinema Fasting symptoms
Longevity	Possibly increased[24] Decreased[183]	?	Normal	?	Normal[29] Decreased[31]

Table 2. Phenotypic characteristics of GH/IGF-I deficiency/insensitivity in humans.[183]
[a]GH deficiency due to hypothalamic/pituitary dysfunction. [b]GH releasing hormone receptor – isolated GH deficiency.[c]Isolated GH deficiency. [d]GH insensitivity.

3.1 GH deficiency and life span: Invertebrates

Inactivation of the pathways that promote growth extend life span in yeast, worms, and flies. In prokaryotes such as yeast, down-regulation of glucose signaling by mutations in *Sch9*, *RAS2,* or *CyR1/PKA* genes extend life span up to 300%[32]. The pathway becomes more complex with the evolutionary progression to nematodes (*C. elegans*) and flies (*D. melanogaster*) and includes insulin for glucose regulation and IGF-I-like pathways (Table 3). The *daf-2* gene encodes an insulin/IGF receptor, and when disrupted, significantly extends life span in nematodes[33]. Disruption of downstream components of the *daf-2* signaling pathway including *age-1* [a homolog of the mammalian phosphoinositide-3-kinase (PI3K)] and *Akt/PKB* also leads to life span extension. Elegant genetic experiments in the nematode have yielded great insight regarding the life history of this organism and mechanisms such as this that evolved to protect the worm from periods of low food availability. Some *daf-2* and *age-1* mutants can

live 150% longer than normal worms and have normal fertility and development[34-36]. Interestingly, restoration of daf-2 signaling in neurons alone returned the life span of *daf-2* mutants to wild-type values while genetic rescue of muscle and intestinal daf-2 signaling had no effect[37]. These data suggest that the nervous system is involved in regulation of longevity in the nematode. In fruit flies, mutation of the gene encoding the insulin-like receptor (*INR*) or of the gene encoding the insulin receptor substrate (*chico*) increases life span[38,39]. This extension in life span is likely related to juvenile hormone (JH) deficiency (*INR* mutants lack JH). Juvenile hormone is a growth factor known to regulate larval development and reproduction, suppress responses to external stress, and influence insect adult longevity[40,41]. Functionally, JH exhibits some homology to thyroid hormones in mammals. Treatment of long-lived mutant female flies with an analog of JH shortens life span[38,42]. Each of these studies involving mutants in organisms ranging from yeast to mammals strongly implicates the GH/IGF-I/insulin signaling pathway with aging and life span.

	Yeast	**Worms**	**Flies**	**Mammals**
Ligands				Growth hormone ↓
	Glucose ↓	Insulin/IGF-I-like ↓	Insulin/IGF-I-like Chico ↓	IGF-I ↓
Receptors	Gpr1 ↓	DAF-2 ↓	INR ↓	IGF-I receptor ↓
Downstream signaling molecules	Cyr1 (cAMP) Sch9 PKA ↓	AGE-1 Akt/PKB ↓	PI3K Akt/PKB ↓	PI3K Akt/PKB ↓
Physiological outcome	Stress resistance Growth	Stress resistance Growth	Stress resistance Growth	Stress resistance Growth
Reduced pathway signaling	Extended longevity	Extended longevity	Extended longevity	Extended longevity

Table 3. Common longevity signaling molecules in different species.

4. GH EXCESS AND LIFE SPAN

In contrast to these deficiencies in GH and IGF-I that result in delayed aging, GH excess can hasten the process of aging. Several GH transgenic rodent models have been developed over the last two decades. The mice were created to overexpress a GH transgene (rat, bovine, ovine, human) resulting in high to supraphysiological levels of GH in the plasma. Life

expectancy in these mice is reduced to less than 50% of their normal counterparts[43-45]. In addition to a shortened life, these animals exhibit insulin resistance and hyperinsulinemia[47-48] (but are not diabetic) and show several signs of premature aging. Relatively young mice (6–8 months of age) begin to show physical signs of aging including weight loss, scoliosis, and coat deterioration. Further examination reveals significant evidence of premature aging in mice overexpressing GH including reduced replicative potential[43], reduced antioxidative defense capacity[49-51], increased free radical processes[52], reduced reproductive function and life span[53-55], increased astrogliosis[56], deficits in learning and memory[57], and increased plasma corticosterone[56,58]. The primary cause of death in these models of GH overexpression is related to significant kidney pathologies (glomerular nephritis and sclerosis)[59,60]. It is most likely the excess GH, not the detrimental effects of heterologous GH expression, that is causative in the premature aging and significant reduction in life span of GH transgenics animals. Supporting evidence for this assertion is observed in mice expressing a transgene for GHRH (MT-GHRH) [61]. The MT-GHRH mice exhibit enlarged pituitary glands, significant elevations in homologous (mouse) plasma GH levels and considerably shortened life spans[62]. A reduction of life span is also observed following antisense GH transgene expression in rats[63]. Moreover, overexpression of IGF-II in smooth muscle cells of mice shortens life span significantly, especially in males[64]. High-dose GH treatment in rats has also proved to be toxic.[65] Although the majority of reports indicate that GH treatment decreases life span, there are data in mice administered low or moderate doses of GH that resulted in either slightly increased life span or no effect on longevity, respectively[66,67]. In the study by Khansari and Gustad[66], human GH was administered to mice, leading to the question of specificity as human GH has both lactotropic and somatotropic activity in rodents.

Pathological GH excess occurs in humans with acromegaly. Pituitary adenomas can release excessive GH, resulting in significantly increased incidence of diabetes, cardiovascular disease, and tumors[68-72]. In the normal population, these same diseases increase in incidence with age. Patients with acromegaly are treated with somatostatin analogs (octreotide) that act to decrease plasma GH concentrations and subsequently reduce IGF-I levels, resulting in clinical improvement[73]. High levels of IGF-I have been associated with increased risk of several diseases in humans including breast, lung, colorectal, and prostate cancer[74-78]. Similarly, IGF promotes tumors in mice[79-81]. The antiapoptotic effects of IGF-I, as well as its mitogenic activity are likely related to these observations. High normal plasma GH levels have been identified as an early mortality risk factor[82]. In one study, treatment of critically ill elderly patients with GH significantly increased mortality[83].

Moreover, a recent study demonstrated that children treated with GH experience statistically significant increases in incidence of cancer as adults[84]. Therefore, excessive GH/IGF-I is detrimental and can result in significant disease and reduced life span in mammals.

5. MECHANISMS OF LIFE EXTENSION: GROWTH AND BODY SIZE

It has been proposed that during evolution, the common IGF/insulin pathway diverged into two, one to regulate cell division and growth and the other to control metabolism and partitioning of energy resources[35,85]. In nematodes, flies and mammals, these metabolic pathways regulate energy, reproductive activity, and stress responses such that when food is abundant, growth, sexual maturation, and reproduction dominate. When food is scarce, resources are used to favor survival and directed away from growth and reproduction to increase stress resistance and repair processes leading to delayed aging and longevity.

Potential mechanisms of longevity causally related to GH and IGF-I include body size, metabolism, reproduction, and resistance to stress. Physiological factors affected by deficiencies or excesses in GH/IGF/insulin signaling are significant. Somatic growth is driven by components of this pathway; therefore, body size differences are obvious in many of these mutants. Those mutants with reduced signaling of this pathway are significantly smaller than wild-type controls. In worms, flies, and rodents, the range of differences is related to the degree of suppression of the IGF-I pathway, in particular. IGF-I receptor knockdown mice are slightly smaller (8% in males) than wild-type mice while Ames and Snell dwarf mice are 66% smaller (one third the size of wild-type). The GHR/BP knockout mice are 40% the size of wild type siblings as adults. Flies with disrupted insulin receptors or insulin receptor substrate genes are also considered dwarfs. Furthermore, the fact that small body size is strongly associated with longevity also holds true for domestic dogs[86] and likely for humans[87]. The relationship between small body size and longevity is further supported by reports showing that mice selected for reduced body sizes live longer[88] and calorie restriction also reduces growth and adult body size[89]. Therefore, growth negatively influences life span in mammals[90] and likely other species. Growth and oxidative metabolism are causally linked by signal transduction pathways[90]. Low levels of reactive oxygen species stimulate cell proliferation and signal transduction[91-93].

In addition to reductions in body size and weight, Ames dwarf mice exhibit the expected declines in lean muscle mass. However, these mice also

have lower bone mineral densities and lower percent body fat compared to age-matched wild-type mice[94]. Less body fat may appear paradoxical in view of the deficiency of GH as GH is a lipolytic factor. The absence of GH should result in increased fat mass. However, the low insulin levels may counter this effect by decreasing storage of this fuel. Finally, the brain accounts for a greater proportion of body weight in Ames dwarf than in corresponding wild-type mice.

5.1 Mechanisms of life span extension: glucose regulation

Hormonal regulation of metabolic pathways is a key factor in the determinants of longevity in several organisms. The actions of GH on glucose regulation are well known. This hormone is a diabetogenic factor in that it opposes the actions of insulin. GH elevates plasma glucose concentrations by stimulating gluconeogenesis and glycogenolysis and inhibiting glucose uptake at the tissue level. Therefore, elevated GH levels such as those observed in acromegalic patients and genetically engineered mice with elevated plasma GH levels exhibit hyperinsulinemia, hyperglycemia, and/or insulin resistance[48,95]. More than 50% of humans with supraphysiological GH levels become diabetic and develop micro- and macrovascular complications associated with hyperglycemia. In sharp contrast, GH-deficient animals have low blood insulin, low glucose, and increased insulin sensitivity[96-99]. Administration of a single dose of insulin induced a larger decrease in blood glucose levels in Ames and GHR/BP KO mice than in corresponding wild-type mice[100]. However, disposing of glucose was reduced in both types of mice, indicating a decreased ability to increase insulin secretion in response to an acute glucose challenge. Two different mechanisms may be involved. GHR/BP KO mice have increased numbers of insulin receptors while Ames mice have higher levels of IRS-1 and IRS-2, two downstream effectors of the insulin receptor. This evidence is supported by decreased numbers of pancreatic islets in the Ames mouse[101]. Caloric restriction, long known to increase life span in rodents[102-105], flies[106], and likely primates[107], also reduces insulin levels in the blood. Despite normal blood glucose concentrations and clearance, liver specific IGF-I deficient mice[22] are hyperinsulinemic and exhibit increased insulin resistance due to GH hypersecretion and β-cell hyperplasia. Therefore, these mice are not likely to exhibit life span extension, as signaling of this pathway is not truly reduced[23,108]. Mechanistically, reducing circulating glucose delays aging by decreasing the accumulation and detrimental processes associated with glycation end products[109,110] and reducing metabolism (less fuel) and associated metabolic ROS generation.

Insulin sensitivity declines with age[111]. In addition, insulin sensitivity is specifically related to visceral fat stores; sensitivity increases with reduction in visceral fat[112,113]. A case in point is the FIRKO mouse that lacks expression of the insulin receptor specifically in fat tissue. These insulin receptor knockout animals have significantly reduced fat mass, are protected against age-related obesity and decreased insulin sensitivity. The decrease in fat mass occurs in the absence of reduced food intake. In addition, these mice do not develop diabetes or glucose intolerance and, importantly, live 18% longer than wild-type control mice[114,115]. Although studies of centenarians have not yet shown statistical differences in IGF-I levels, greatly enhanced insulin sensitivity is strongly correlated with longevity in this unique population[116,117]. Moreover, polymorphic variants of the IGF-I receptor and PI3K genes were shown to affect plasma IGF-I levels and human longevity, suggesting that life span control is evolutionarily conserved[118]. Low serum IGF binding protein 2 concentrations have also been found to be a strong indicator of overall physical function in elderly men[119].

5.2 Mechanisms of life span extension - reproduction

GH and IGF-I are known to effect development of numerous organ symptoms. Therefore, lifelong reduction of this hormone produces some secondary endocrine alterations. For example, GHR/BP KO mice have decreased thyroid hormones, increased glucocorticoids, and decreased insulin and glucose as previously mentioned. Their phenotype is similar to Ames and Snell dwarf mice that lack thyroid hormone and exhibit increased corticosteroids and decreased blood insulin and glucose concentrations[95].

Reproductive competence is significantly affected by the GH/IGF signaling pathway. The GHR/BP KO mice exhibit delayed puberty and most animals are fertile. However, the Ames and Snell dwarf mice have significant delays in sexual maturation; the females are infertile while males are considered subfertile[120]. The IGF-I receptor knockdown mice exhibit normal puberty and fertility although the males have not been examined thoroughly. Concomitant with lower plasma IGF-I concentrations, calorie-restricted rodents exhibit delayed puberty, reduced litter size, and lower fecundity[121-123]. The effects of caloric restriction (CR) on reproductive function result from alterations in neuroendocrine factors including IGF-I. When CR is confined to adulthood, life span is also extended although not to the same extent as that in young mice[104]. Although CR has been documented to extend life span in yeast, worms, rotifers, flies, spiders, fish, rodents, and likely nonhuman primates, there are some species that are not affected[105,124]. Flies with mutations in the insulin-like receptor or in the insulin receptor

substrate (IRS) exhibit infertility or reduced fecundity[38,39]. However, IRS heterozygotes show evidence of increased longevity without reductions in fecundity or body size[39]. Juvenile hormone in insects is likely a secondary longevity factor downstream of insulin/IGF-I that regulates reproduction and growth as mentioned earlier[125]. In *C. elegans*, reproductive ability is coupled to metabolism such that when conditions are favorable for reproduction, metabolic demand is high. Nutritional signals act through insulin/IGF-I and stimulate production of nuclear hormone receptors that promote reproduction (such as daf-9) [126,127]. Ablation of nematode germ line derived cells results in life span extension of 60% suggesting a major hormonal influence on life span[128]. Germ line cells and the somatic gonad may exert antagonistic actions on aging as the removal of both did not affect life span.

Reproductive function of GH transgenic mice is altered by the high plasma GH concentrations. Female GH transgenic mice exhibit early puberty and increased ovulation rates; however, fertility is reduced and the reproductive life span is severely shortened when compared to wild-type mice (degree of suppression appears to depend on level of circulating GH) [58,129,130]. Male mice with elevated GH levels are fertile, but exhibit alterations in sexual behavior, reproductive pituitary hormone secretion, and a reduced reproductive life span[57,130,131].

5.3 Mechanisms of life span extension: Stress resistance

Resistance to oxidative stress is another factor significantly affected by altered signaling of the GH/IGF pathway and closely regarded in the aging field as a major player in life span determination. The free radical theory of aging proposes that endogenously generated reactive oxygen species (ROS) cause aging via damage to DNA, proteins, and lipids[132,133]. The effects of GH and IGF-I on oxidative metabolism and oxidative damage have been documented in numerous reports. GH is an anabolic factor that increases cellular metabolism. Increased metabolic activity (glucose oxidation and oxygen consumption) leads to increased oxidative phosphorylation and increased production of ROS as byproducts of metabolism. Rollo and coworkers[52] showed that GH overexpression increased superoxide radicals and oxidative damage to membrane lipids (lipid peroxidation). Perhaps adaptively, tissues from mice with elevated plasma GH exhibit significantly reduced levels of antioxidative enzymes including manganese superoxide dismutase (MnSOD; dismutates superoxide anion into H_2O_2 and O_2), copper-zinc SOD (similar to MnSOD), catalase, and glutathione peroxidase [both of which reduce hydrogen peroxide (H_2O_2) to H_2O and O_2 in cytosol and mitochondria, respectively] [49-51]. In addition, direct effects of GH and IGF-I *in vitro* strongly support the *in vivo* data showing that these two hormones

directly down-regulate catalase activity, glutathione peroxidase activity, and protein and MnSOD protein in hepatocytes[134]. Other proteins hormones tested (luteinizing hormone and prolactin) had little if any effect on antioxidant enzymes, indicating specificity of GH and IGF-I[134].

In stark contrast to suppressed antioxidative defense induced by GH, there are several reports demonstrating enhanced defense capacity of this system in GH deficiency. Ames dwarf mice exhibit elevated catalase levels (activity, protein, and/or mRNA) in liver, kidney, heart, and hypothalamic tissues[49,50,135]. Glutathione peroxidase (GPX) and superoxide dismutase (SOD) activities are also elevated in GH-deficient dwarf mice (unpublished data) [49]. Muscle tissue GPX activity is preserved in dwarf mice at several ages following both acute and chronic exercise while that from wild-type mice declines with age[136]. Interestingly, GH replacement in dwarf mice down-regulates catalase, glutathione peroxidase, and MnSOD proteins and activities in both young and adult animals[137]. Nonenzymatic antioxidant molecules are also elevated in dwarf mice. Metallothionein and glutathione both exhibit ROS scavenging abilities, and levels of these are significantly increased in multiple tissues from the dwarf mouse[138,139].

In general, circulating GH/IGF-I status is correlated with antioxidative capacity; high levels of plasma GH suppress this defense mechanism while the absence or low levels of GH/IGF-I enhance the ability of an organism to counter oxidants. The signal transduction pathways used by GH, IGF-I, and insulin overlap and influence redox-regulated transcription factors[140]. The PI3K pathway is defective in GH transgenic animals while animals with reduced GH/IGF-I signaling (dwarf and calorie-restricted mice) exhibit increased PI3K sensitivity[120,141,142]. Signaling via the PI3K pathway is significantly affected by free radicals. Insulin resistance in diabetes and obesity is improved by antioxidants[143-145]. This point is further illustrated in $p66^{shc}$ mice with disrupted IGF-I/insulin signaling. The $p66^{shc}$ protein is an adaptor protein that is crucial to IGF-I/insulin receptor signaling. Disruption of this gene results in mice with enhanced resistance to oxidants and extended life span when compared to wild-type control mice[146].

Oxidative damage to DNA, proteins, and lipids can occur when reactive oxidants are not appropriately countered by compounds of the antioxidant system. Mitochondrial oxidant production (liver H_2O_2) is significantly lower in dwarf mice, possibly indicating decreased metabolic activity in the absence of GH[147]. Consequent to reduced ROS and elevated antioxidants, dwarf mice exhibit lower DNA and protein oxidative damage[147]. Importantly, Sanz and coworkers[148] have also shown that mitochondrial DNA damage in brain and heart tissues is dramatically lower in Ames mice when compared to wild-type control mice. Functionally, these GH-deficient mice out-survive their GH sufficient counterparts following administration

of the systemic oxidative stressor paraquat[120]. The IGF-I receptor knockdown mice challenged with paraquat also lived significantly longer than mice with normal levels of IGF-I receptors (wild-type)[21].

Long-living invertebrate IGF/insulin mutants also exhibit enhanced stress resistance. Catalase and SOD proteins and activity levels are up-regulated in the *daf-2* and *age-1* mutant nematodes[149-151]. In addition, these investigators showed that these mutants consistently express resistance to oxygen, H_2O_2 and paraquat[150-152]. Moreover, one of four *C. elegans* SOD genes (*SOD3*) is directly regulated by the IGF/insulin signaling pathway[153]. Reduced signaling of this growth and metabolic pathway in *Drosophila* enhances oxidative stress resistance by increasing the expression of SOD[38,39]. Genes that extend life span in yeast confer resistance to oxidative stress and heat shock[32,154]. Reduced GH/IGF/insulin signaling is also associated with increased resistance to other stressors including heat shock, UV, and gamma irradiation. Overall, the reported studies strongly support the notion that GH/IGF-I/insulin signaling pathways are intimately involved in the modulation of oxidative stress. The suppressive effect of GH on multiple components of the antioxidant system and consequent oxidative damage may be one mechanistic reason that levels of this hormone decline with aging.

GH-deficient dwarf mice and rats resist cancer development following administration of chemical carcinogens[155-157] and exhibit reduced growth of transplanted tumors[158]. Spontaneous tumor incidence appears to be delayed and the severity reduced in the hypopituitary dwarf mice[10,159]. Moreover, tumor growth in IGF-I deficient mice is reduced relative to control mice[160]. Cancer incidence is also lessened in calorie-restricted rodents.[104] In addition, GH/IGF-I deficient mice develop significantly less osteoarthritis than wild-type mice[161]. It has been postulated that stress resistance is coordinately increased (heat shock proteins, antioxidants, detoxification systems, metal chelators, and repair systems) and this up regulation results in multi-stress resistance to different stressors[90,162,163]. Recent work by Murakami and coworkers[164] exemplifies this idea well in showing that fibroblasts from the skin of long-living Snell dwarf mice are resistant to multiple forms of cellular stress including heat, paraquat, H_2O_2, UV light, and the toxic metal cadmium.

6. GH THERAPY IN THE ELDERLY

GH therapy to reduce or reverse aging is becoming quite popular. A highly cited study in favor of this treatment was conducted by Rudman and coworkers[5]. In this study, elderly men with very low levels of IGF-I were treated for several months with recombinant human GH, resulting in

increased muscle mass, decreased fat mass, and increased bone density at one site (1.6%; lumbar vertebral). Since this time, other studies have confirmed these findings but also suggest that the benefits are neither clear nor consistent[6] as no parallel functional improvements (strength, endurance, mood, and mental status) were observed in this study as well as in others[165,166]. GH administration for anti-aging purposes in humans thus far has been confined to short-term studies using relatively small numbers of individuals. Despite the lack of consensus regarding the beneficial or detrimental effects of this somatotropic hormone, GH is being marketed as the "fountain of youth". GH therapy in healthy elderly individuals is controversial, and extreme caution should be exercised owing to unproven and potentially dangerous side effects[6,166,167] and the enhanced responsiveness of this population to this form of hormone supplementation[168].

GH therapy has been reported to enhance cognitive function in elderly patients[169,170]. Animal studies provide support for these observations showing that GH improves brain vascularity, blood flow, and cognitive function[171,172]. IGF-I has also been observed to protect and stimulate the central nervous system in animal experiments[173-175]. However, an improvement in memory following GH treatment in young but not old rats possibly reflects a down-regulation of GH receptors in the old animals[176]. In contrast, there is also compelling evidence in animals suggesting that GH deficiency is associated with maintenance of cognitive function. Ames dwarf mice do not exhibit the age-related decline in cognitive function (including memory) and behavior that is observed in wild type control mice[177]. Similarly, GHR/BP knockout mice show no decline in cognitive function when age-matched wild-type littermates are significantly impaired[178], suggesting that the absence of GH action enhances memory retention. Other than hypophysectomy, which decreases brain IGF-I mRNA, direct evidence of brain dysfunction in GH/IGF-I-deficient animal models is lacking[180]. Correlative data are available and show that deficits in the IGF-I axis occur in aged brain and that IGF-I levels are associated with cognitive function[181,182]. Experiments teasing out the effects of age versus the effects of low GH or IGF-I levels have yet to be done.

7. CONCLUSIONS

The natural age-related decline in plasma GH levels and the concomitant decrease in IGF-I that occurs in mammals is likely a protective mechanism to decrease metabolic activity and cellular division. High levels of either of these hormones throughout life could play a contributory role in pathological

changes associated with aging such as increased oxidative damage and cancer. Decreasing IGF-I and insulin signaling without a concomitant decline (or increase) in GH concentrations may provide an optimal balance of hormones that would maintain muscle and bone mass yet enhance stress resistance and prevent many of the physical signs of aging. In addition, this type of treatment may result in decreased incidence of age-related diseases such as cancer, diabetes and cardiovascular disease. Reduced activity of the somatotropic axis is key in the quest to slow or delay aging and promote life extension.

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Chapter 12

INTERACTIONS OF INSULIN-LIKE GROWTH FACTOR-I AND ESTROGEN IN THE BRAIN

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1. INTRODUCTION

Insulin-like growth factor exerts different effects on the development and plasticity of the nervous system and is a potent neuroprotectant (see Chapters 8 and 10). As in other organs, the actions of IGF-I in the nervous system may be affected by the interaction with other molecules. In the brain, these include neurotransmitters, neuromodulators, hormones and other growth factors. For instance, growth hormone regulates IGF-I expression in the brain in an anatomically specific manner[1] and leukemia inhibitory factor has been shown to regulate IGF-I levels in peripheral nerves[2]. The cross-talk between IGF-I intracellular signaling with the signaling pathways of other factors may also play an important role in the actions of IGF-I in the nervous system. This is the case of the interaction of IGF-I and nerve growth factor (NGF) in peripheral nerves, where the phosphatidylinositol-3 kinase (PI3K)-Akt-glycogen synthase kinase 3 (GSK3) pathway seems to underlie the synergism of these two factors on axonal growth[3]. Neurotransmitters, such as glutamate, may affect the actions of IGF-I in the brain by interfering with IGF-I receptor signaling. Recently it has been shown that glutamate at high

concentrations induces loss of sensitivity to IGF-I by phosphorylating the IGF-I receptor docking protein insulin-receptor-substrate (IRS)-1 in Ser(307) through a pathway involving activation of protein kinase (PK) A and PKC[4].

One of the best characterized factors that affect IGF-I's actions in the nervous system is the ovarian hormone estradiol. Evidence has accumulated over the past years indicating a close interdependence between the actions of IGF-I and estradiol in the brain. Both factors have in common the duality of being hormones, as well as locally produced neuromodulators, and they exert similar pleiotropic actions in the developing and adult brain. During the development of the nervous system, IGF-I and estradiol promote the differentiation and survival of specific neuronal populations. In the adult brain, IGF-I and estradiol act as neuromodulators, regulate synaptic plasticity, are involved in the response of neural tissue to injury, regulate neurogenesis, and protect neurons against different neurodegenerative stimuli[5].

IGF-I and its receptors are expressed at high levels by neurons and glia in the developing rodent brain. However, the expression of IGF-I is dramatically reduced in many brain areas, excluding the hypothalamus, by the time of puberty. In contrast, expression of the IGF-I receptor is maintained in neurons and glia in the adult brain[6], suggesting that under physiological conditions the actions of peripheral IGF-I are predominant in adult life[7,8].

Estradiol is mainly produced in the ovary and reaches the brain from the circulation. In addition, estradiol is formed in the brain from androgen precursors present in plasma and probably also from androgen precursors locally synthesized from cholesterol. Local production of estradiol is important in the developing brain, as inferred from the high level of expression of aromatase, the enzyme that converts testosterone into estradiol[9]. In the adult rodent brain, the level of aromatase expression is substantially reduced, suggesting a decrease in the local formation of estradiol. Interestingly, both IGF-I and estradiol synthesis are induced in damaged brain regions following brain injury, suggesting a possible role for the local production of these factors in brain recovery[10,11].

In this chapter we focus on the functional implications and the molecular mechanisms involved in the interaction of IGF-I and estradiol in the brain.

2. INTERACTION OF IGF-I AND ESTROGEN IN NEUROENDOCRINE AND REPRODUCTIVE EVENTS IN THE BRAIN

The nervous system is a target for the ovarian hormone estradiol. This hormone regulates brain development and function, acting on neurons, synapses, and glial cells[12]. For many years, the actions of estradiol in the brain were thought to be restricted to areas involved in neuroendocrine regulation and the control of sexual behavior. Today it is evident that estradiol exerts a broad spectrum of actions in many brain areas that are not directly related to reproduction[12-15].

One of the main mechanisms of action of estradiol in the brain, as in other organs, is the activation of nuclear estrogen receptors. These receptors belong to the steroid/thyroid nuclear receptor superfamily, and once activated they homo- or heterodimerize, interact with DNA, and recruit a cohort of transcriptional cofactors to the regulatory regions of target genes. The two mammalian estrogen receptors cloned to date, denominated α and β, are widely distributed throughout the central nervous system (Table 1). In addition, estradiol exerts rapid membrane effects on neural cells, modulating ion channels, neurotransmitter transporters, intracellular calcium levels, and phosphorylation of different kinases[5,12-15].

As an endocrine signal, IGF-I represents a link between the growth and reproductive axes, and the interaction between IGF-I and estradiol in the brain is of particular physiological relevance for the regulation of growth, sexual maturation, and adult neuroendocrine function. IGF-I regulates gonadotrophins by acting at the level of both the hypothalamus and pituitary[16-18]. IGF-I from peripheral origin reaches the rat hypothalamus, where it appears to be a signal related with the initiation of puberty[17]. Coincident with the onset of puberty, IGF-I mRNA and protein levels increase dramatically in the rat mediobasal hypothalamus and median eminence[19,20]. Decreased IGF-I plasma levels in growth hormone deficient mice, as well as in growth hormone receptor deficient mice, are associated with reproductive deficits, a delay in female puberty, and alterations in the functioning of the hypothalamic-pituitary-gonadal axis. In addition, deletion of insulin receptor substrate-2 (IRS-2), a component of the insulin/IGF-I receptor signaling cascade, causes infertility in females[19].

Interactions between estrogen and IGF-I have been reported in brain areas involved in neuroendocrine control. One such area is the hypothalamic arcuate nucleus, a key center for neuroendocrine regulation of the growth and reproductive axes. This nucleus shows estrogen-induced and estrous cycle-related plastic changes in synaptic connectivity and glial-neuronal interactions in female rodents[20]. During the preovulatory and ovulatory phases of the estrous cycle, there is a transient disconnection of axo-somatic

γ-aminobutyric acid-(GABA)ergic synapses on the arcuate somas of adult female rats. This synaptic remodeling is induced by estradiol and is linked to plastic modifications in astroglial processes[20].

During the estrous cycle, estradiol increases IGF-I accumulation in the arcuate nucleus[21]. IGF-I levels increase in the afternoon of proestrus and remain high in the morning of estrus. This effect may be in mediated in part by the increased expression of insulin-like growth factor binding protein-2 by hypothalamic tanycytes, specialized glial cells that express IGF-I receptors and accumulate IGF-I from the cerebrospinal fluid[22]. Furthermore, results from *in vivo* experiments using intracerebroventricular infusion of specific receptor antagonists have shown that both estrogen receptors and IGF-I receptors are involved in the induction of synaptic and glial plastic modifications in the arcuate nucleus that take place during the estrous cycle and are linked to luteinizing hormone release[23]. Under normal conditions, the number of synaptic inputs on arcuate neuronal somas of cycling female rats decreases between the morning of proestrus and the morning of estrus. This phasic synaptic and glial remodeling is blocked when the selective and specific IGF-I receptor antagonist JB1 is administered in the lateral cerebral ventricle to neutralize the local actions of IGF-I. In contrast, JB1 did not affect the number of synapses in proestrus rats, suggesting that IGF-I receptor activation is necessary for the estrogen-dependent synaptic plastic changes, but not for the normal maintenance of synaptic inputs[24].

Arcuate neurons express IGF-I receptors[6] and may, therefore, be a direct target for the actions of JB1. This antagonist may affect pre- and/or postsynaptic mechanisms, since ultrastructural studies have shown that IGF-I receptors are present both in axo-somatic presynaptic terminals, as well as in neuronal somas of the rat arcuate nucleus[6]. Arcuate astrocytes are another possible cellular target for the IGF-I receptor antagonist, since they also express IGF-I receptors[6]. Arcuate astrocytes appear to be directly involved in the regulation of synaptic inputs to arcuate neurons[20]. Synaptic disconnection of arcuate neurons in estrous females is accompanied by increased expression of glial fibrillary acidic protein (GFAP) and by an increase in the ensheathment of neuronal surfaces by astrocytic processes[20]. Interestingly, studies on hypothalamic tissue fragments from ovariectomized rats have shown that IGF-I receptor activation is needed for the induction of GFAP changes by estrogen in the arcuate nucleus[25].

Another alternative mechanism involved in the blocking effect of JB1 on arcuate nucleus synaptic plasticity may be the regulation of local IGF-I levels as a consequence of the antagonistic effect of JB1 on IGF-I receptors in tanycytes. As mentioned previously, tanycytes, a specialized form of glial cells present in the walls of the third ventricle and in the median eminence, may play an important role in the regulation of local IGF-I levels in the

arcuate nucleus. Tanycytes express IGF-I receptors, are able to accumulate IGF-I from extrahypothalamic sources, and show changes during the estrous cycle in IGF-I immunoreactivity and IGF-I accumulation. Therefore, tanycytes could represent a possible site of action for JB1 in the inhibition of synaptic plasticity. Indeed, administration of JB1 in the rat lateral cerebral ventricle is able to block the accumulation of IGF-I by arcuate nucleus tanycytes[26].

Quesada and Etgen[27,28] have reported several functional interactions between estrogen and IGF-I receptor in neuroendocrine brain areas. These authors have shown that estradiol treatment induces alpha(1B)-adrenoceptor expression in the hypothalamus and preoptic area and that this hormonal effect is blocked by intracerebroventricular infusion of the IGF-I receptor antagonist JB1. Furthermore, estradiol potentiates the effects of IGF-I on alpha(1)-adrenergic receptor activation in the preoptic area and hypothalamus. Blockade of the IGF-I receptor during estrogen priming blocks estrogen-induced luteinizing hormone release and partially inhibits hormone-dependent reproductive behavior. Quesada and Etgen have proposed that the interaction of estrogen with IGF-I receptor may help coordinate the timing of ovulation with the expression of sexual receptivity[28].

3. INTERACTION OF IGF-I AND ESTRADIOL ON NEURONAL DEVELOPMENT

The interactions of estrogen and IGF-I are also relevant for brain development. The interaction between these two factors contributes to the development of structural sex differences in the brain through regulation of the survival and differentiation of developing neurons in brain areas involved in the regulation of neuroendocrine events and reproduction[29]. Several studies have demonstrated an interdependence of estrogen receptors and the IGF-I receptor in promoting the survival and differentiation of developing hypothalamic neurons[30,31]. Both estradiol and IGF-I promote neuronal survival and differentiation in primary neuronal cultures grown in a defined medium deprived of serum and hormones. In these cultures, the induction of neuronal survival and differentiation by IGF-I is prevented by inhibiting the synthesis of estrogen receptor α by the addition of a specific antisense oligonucleotide[31]. The effect of IGF-I can also be prevented by the estrogen receptor antagonist ICI 182,780[31]. In turn, the promotion of neuronal survival and differentiation by estradiol is prevented by blocking the synthesis of IGF-I in the cultures by using a specific IGF-I antisense

oligonucleotide[31], as well as by the pharmacological blockade of the MAPK and the PI3K signaling pathways, both used by the IGF-I receptor[32].

4. INTERACTION OF IGF-I AND ESTRADIOL ON THE RESPONSE TO BRAIN INJURY AND ADULT NEUROGENESIS

Estradiol and IGF-I may also interact in areas of the brain that are not directly involved in the control of neuroendocrine events and reproduction, as has been shown in studies assessing the neuroprotective effects of these two factors. IGF-I and estradiol have neuroprotective properties and prevent neuronal cell death in different experimental models of neurodegenerative diseases[33,34]. The interaction of IGF-I and estradiol in neuroprotection has been assessed in ovariectomized rats, using systemic administration of kainic acid to induce degeneration of hippocampal hilar neurons[35], an experimental model of excitotoxic cell death. Both systemic administration of estradiol and intracerebroventricular infusion of IGF-I prevent hilar neuronal loss induced by kainic acid. The neuroprotective effect of estradiol is blocked by intracerebroventricular infusion of the IGF-I receptor antagonist JB1, while the neuroprotective effect of IGF-I is blocked by the intracerebroventricular infusion of the estrogen receptor antagonist ICI 182,780[35]. Similar results have been obtained after the unilateral infusion of 6-hydroxdopamine into the medial forebrain bundle to lesion the nigrostriatal dopaminergic pathway[36], a model of Parkinson's disease. Pretreatment with estrogen or IGF-I significantly prevents the loss of substantia nigra compacta neurons and the related motor disturbances. Blockage of IGF-I receptors by intracerebroventricular JB-1 attenuates the neuroprotective effects of both estrogen and IGF-I. These findings suggest that the neuroprotective actions of estradiol and IGF-I after brain injury depend on the coactivation of both estrogen and IGF-I receptors.

Another functional outcome of the interaction of IGF-I with estrogen in the brain is the regulation of adult neurogenesis. Neural precursors located in the subgranular zone of the dentate gyrus of the hippocampus proliferate in adult rodents. The newly generated neurons are functional, integrate in hippocampal circuits and may be involved in certain forms of hippocampus-dependent learning. IGF-I and estradiol are among the molecules that have been identified as modulators of adult neurogenesis[8,37]. A recent study has shown that intracerebroventricular administration of the estrogen receptor antagonist ICI 182,780 blocks IGF-I-induced neurogenesis in adult ovariectomized rats[38], suggesting that estrogen receptors are involved in the

action of IGF-I on adult hippocampal neurogenesis (Fig. 1). This interaction of IGF-I with estrogen receptors may occur directly in the proliferating cells, since they express receptors for both IGF-I and estrogen[38].

5. MECHANISMS OF INTERACTION BETWEEN IGF-I AND ESTRADIOL IN THE BRAIN

Analysis of the distribution of the IGF-I receptor and of the two known forms of estrogen receptors (α and β) in the rat brain reveals that most neural cells expressing IGF-I receptor also express estrogen receptors[39]. Colocalization of estrogen receptor α and IGF-I receptor immunoreactivity is observed, by confocal microscopy, in neurons in the preoptic area and hypothalamus. IGF-I receptor immunoreactivity is detected in the cytoplasm and estrogen receptor α immunoreactivity is located in the cell nucleus. Some neurons in the ventromedial nucleus and the arcuate nucleus are immunoreactive for both receptors. Many neurons in the hypothalamus and preoptic area show estrogen receptor α immunoreactivity only; however, most IGF-I receptor immunoreactive neurons are also estrogen receptor α immunoreactive[39].

In the hippocampal formation, colocalization of estrogen receptor α and the IGF-I receptor is seen in many pyramidal neurons in the lateral portion of the CA1 field. The number of double labeled cells is lower in the CA2 layer and no double labeling is observed in the CA3 or the dentate gyrus[39].

The cerebral cortex presents abundant immunohistochemical colocalization of IGF-I receptor and estrogen receptor α. There is abundant colocalization in layers III and IV from the frontoparietal cortex motor area and to a lesser extent in layer VI. Similarly, many neurons show colocalization of estrogen receptor α and IGF-I receptor in the primary olfactory cortex, whereas colocalization is less abundant in the posterior cingulate cortex and scarce in the frontoparietal cortex somatosensory area[39].

Estrogen receptor β is expressed in almost all IGF-I receptor immunoreactive neurons in the preoptic area and the hypothalamus. Double labeled cells are abundant in the preoptic area and in the supraoptic, paraventricular and arcuate nuclei. In most neurons the labeling for estrogen receptor β is observed in the cell nucleus; however, cytoplasm labeling is faint, but consistent, in cells with estrogen receptor β in nuclei. Colocalization of estrogen receptor β and IGF-I receptor is observed in the cytoplasm. In magnocellular neurons of the supraoptic and paraventricular nuclei, estrogen receptor β immunoreactivity is predominantly located in the cell nucleus and there is little colocalization of estrogen receptor β and IGF-I receptor immunoreactivity in the cytoplasm[39].

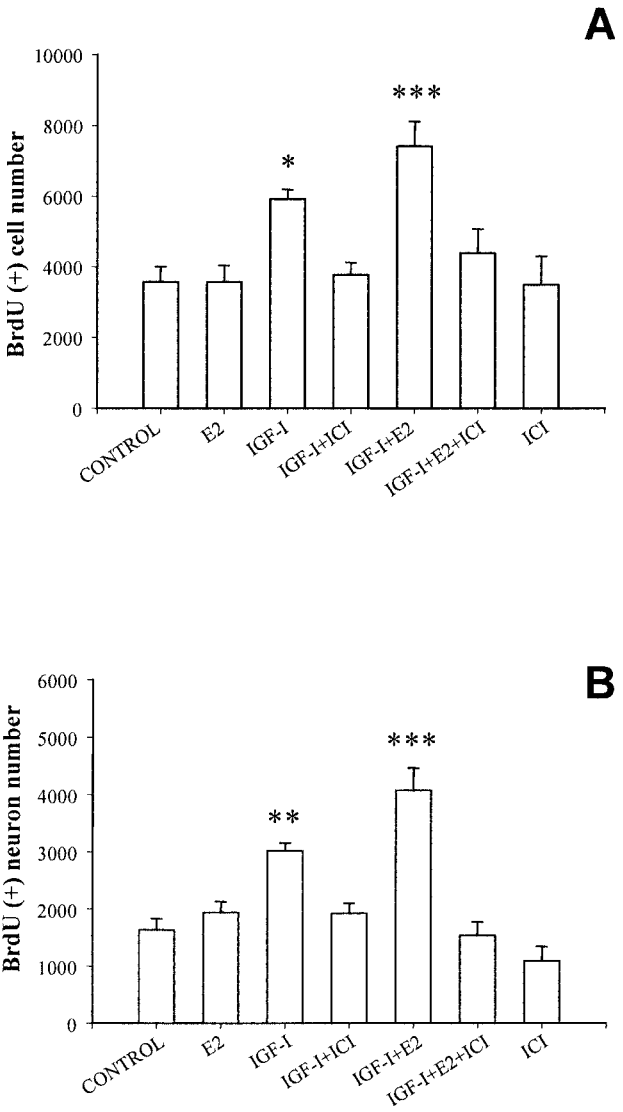

Figure 1. Total number of BrdU-labeled cells (A) and total number of BrdU-labeled neurons (B) in the granule cell layer and subgranular zone of the dentate gyrus of ovariectomized rats. Animals received a daily intraperitoneal injection of 10 mg/ml BrdU (5-bromo-2-deoxyuridine; Sigma, St. Louis, MO.) in 0.9% NaCl solution during six consecutive days (daily dose: 50 mg/kg b.w.). Animals were killed 24 h after the last injection of BrdU. Data are expressed as means ± S.E.M. E2, Animals treated with 17-β estradiol; IGF-I, animals treated with insulin-like growth factor-I; ICI, animals treated with the estrogen receptor antagonist ICI 182,780. The number of animals in each experimental group was: control (n = 5); estradiol (n = 6); IGF-I (n = 5); IGF-I + ICI (n = 6); IGF-I + E2 (n = 8); IGF-I + E2 + ICI (n = 6); ICI (n = 7). Asterisks = significant differences (*$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) versus control values.

Many pyramidal neurons of the CA3 layer from the hippocampus are immunoreactive for both estrogen receptor β and IGF-I receptor, with overlapping colocalization in the cytoplasm. In the dentate gyrus there is cytoplasmic colocalization of both receptors in many interneurons. The colocalization of estrogen receptor β and IGF-I receptor is low in the rest of the hippocampal fields[39].

Area studied	Colocalization of IGF-I receptors with ER alpha	ER beta
PREOPTIC AREA	N	N,A
Medial preoptic nucleus	-	N,A
HYPOTHALAMIC NUCLEI		
Supraoptic & paraventricular	-	N,A
Ventromedial & Arcuate	N	N,A
HIPPOCAMPUS		
CA1	N	A
CA2	N	N,A
CA3	-	N,A
Dentate gyrus	-	N,A
CEREBRAL CORTEX		
Primary olfactory cortex	N	N,A
Posterior cingulate cortex	N	N,A
Frontoparietal cortex		
Motor area		
Layer I	-	N,A
Layers II-V	N	N,A
Layer VI	N	N
Somatosensory area		
Layers I-VI	N	N,A
CEREBELLAR CORTEX		
Molecular layer	-	A
Purkinje cell layer	-	N
Granule cell layer	-	A
White matter	-	A

Table 1. Coexpression of IGF-I receptor and estrogen receptors in selected brain areas. A, Astrocytes; N, neurons. Based on ref. 39.

Estrogen receptor β and IGF-I receptor are colocalized in the frontoparietal cortex motor area from layer II to layer VI. The co-expression is more abundant in layers II and III. There is scarce colocalization of estrogen receptor β and IGF-I receptor in the posterior cingulate region and the frontoparietal cortex somatosensory area. A high proportion of neurons show coexpression of estrogen receptor β and IGF-I receptor in the primary

olfactory cortex. Cytoplasmic coexpression of the estrogen receptor β and IGF-I receptor is observed in the Purkinje cells of the cerebellum[39].

Colocalization of immunoreactivity for estrogen receptor β and IGF-I receptor is observed in glial cells with the morphological appearance of astrocytes in all brain areas. These glial cells are particularly abundant in the hilus of the dentate gyrus and in the white matter of the cerebellum[39].

Table 1 is a summary of the distribution of immunoreactive cells in the preotic area, the hypothalamus, the cerebral cortex, the hippocampus and the cerebellar cortex, in which colocalization of IGF-I and estrogen receptors has been observed.

The abundant coexpression of estrogen receptors with IGF-I receptors in neurons and glia in the brain indicates that interactions of the intracellular signaling pathways of IGF-I receptors and estrogen receptors are possible in many brain cells. In different cell lines, including neuroblastoma cells, IGF-I may activate estrogen receptors in the absence of estradiol[40]. Whether or not this is also valid for the brain *in vivo* is unknown. However, both *in vitro* and *in vivo* studies have shown that in the brain estradiol may activate the two main signal transduction cascades coupled to the IGF-I receptor: the PI3K and the MAPK signaling pathways.

In primary cultures of astroglia and in explants of cerebral cortex, estradiol induces the rapid activation of ERK[41,42]. The estradiol-induced activation of ERK may be detected *in vivo* after systemic administration of the hormone[43]. Estradiol also induces the phosphorylation of Akt in dissociated neurons from the rat cerebral cortex and the hippocampus, in cerebral cortical explants and in the adult rat brain *in vivo*[43,44]. Furthermore, IGF-I and estradiol act synergistically to increase Akt activity in the rat brain[44]. Figure 2 shows the result of an experiment in which IGF-I was infused with an osmotic minipump into the lateral cerebral ventricle of ovariectomized rats to determine if a sustained increase in intracerebral IGF-I levels affects the activation of Akt/PKB by estrogen in the hippocampus. Animals were infused for 6 days with IGF-I and then injected with estradiol and killed 24 h later. A significant increase in the phosphorylation of Akt/PKB was observed in rats injected with estradiol alone compared to animals treated with vehicle. The infusion of IGF-I in the lateral cerebral ventricle was, by itself, unable to increase the phosphorylation of Akt/PKB. However, the hippocampus of animals that received the intracerebroventricular infusion of IGF-I showed a striking increase in Akt/PKB phosphorylation in response to estradiol compared to animals infused with vehicle. This synergistic effect of IGF-I and estradiol was not observed for ERK1 and ERK2[44].

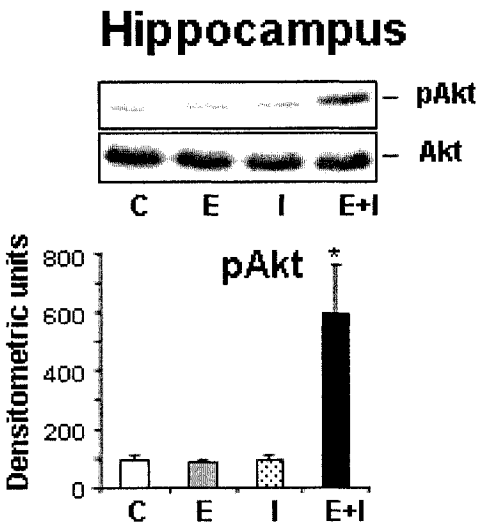

Figure 2. Levels of phosphorylated Akt (pAkt) in the hippocampus of ovariectomized rats killed 24 h after systemic administration of estradiol (E), after 7 days of intracerebroventricular infusion of IGF-I (I) or 24 h after systemic administration of estradiol and 7 days after intracerebroventricular infusion of IGF-I (EI). Levels of basal Akt were used as a control for protein loading. C, Control rats injected with vehicles. Data are normalized to control values and represent the mean ± SEM of arbitrary densitometric units. Samples from 6 rats were analyzed for each experimental group. Asterisks represent significant differences ($p < 0.05$) versus control values.

Glycogen synthase kinase 3 (GSK3), downstream of Akt, may also be a point of interaction of estrogen and IGF-I signaling. Physiological phosphorylation of microtubule-associated proteins by GSK3β may be involved in the regulation of microtubule dynamics, neuritic growth, synaptogenesis and synaptic plasticity[45]. However, under pathological conditions, GSK3β may be responsible for the hyperphosphorylation of tau in Alzheimer's disease[46] and its inhibition is associated with the activation of survival pathways in neurons[47]. Interestingly, estradiol regulates the activity of GSK3 and decreases the phosphorylation of tau in the rat hippocampus in vivo[48]. Furthermore, estradiol increases the association of tau with phosphorylated GSK3β, with the p85 subunit of the phosphatidylinositol 3-kinase and with β-catenin, another substrate of GSK3[48]. These observations are coherent with results showing that systemic administration of estradiol to adult ovariectomized rats results in a transient increase in tyrosine phosphorylation of the IGF-I receptor, in a transient interaction of the IGF-I receptor with the estrogen receptor α, but not β, and in an enhanced interaction of estrogen receptor α with the p85 subunit of PI3K in the brain (Fig. 3)[49].

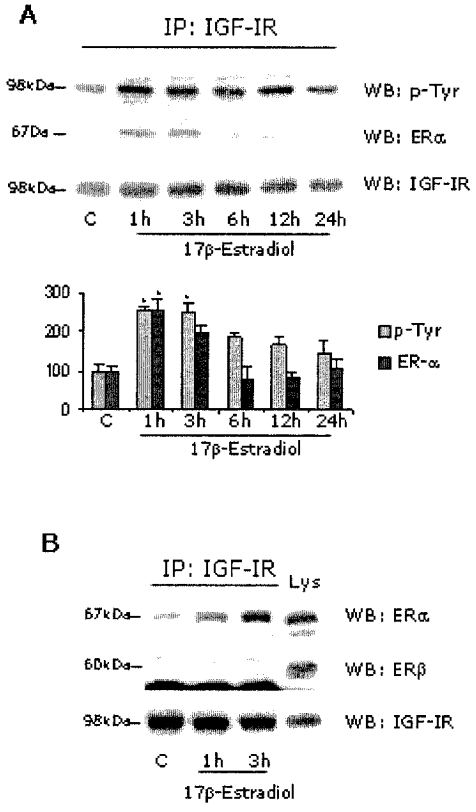

Figure 3. **(A)** Estrogen treatment in vivo activates IGF-IR and induces the association between IGF-I receptor (IGF-IR) and estrogen receptor a (ERα) in the hypothalamus of ovariectomized rats. Animals were killed at the indicated periods of time after acute estrogen treatment. IGF-I receptor was immunoprecipitated from total lysates. The immunocomplexes were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and probed with an antiphosphotyrosine antibody (p-Tyr, upper gel), an antibody against estrogen receptor a (middle gel). To verify equal loading, membranes were probed with an antibody against IGF-I receptor (lower gel). The histogram shows the densitometric analysis of the Western blots. Values are the percentages of control levels and are represented as means ± S.E.M. from three different animals. Data were analyzed by ANOVA followed by the Bonferroni's test. Asterisks indicate significant differences ($p < 0.05$) versus the values of control animals injected with vehicle **(C)**. **(B)** Estrogen treatment induces the association between IGF-I receptor and estrogen receptor α, but not estrogen receptor β (ER β) in the hypothalamus of ovariectomized rats. Animals were treated with estrogen for 1 h, 3 h or with vehicle. Hypothalamic lysates were immunoprecipitated with IGF-I receptor antibody and probed with estrogen receptor α, estrogen receptor β and IGF-I receptor antibodies. The ability of these antibodies to recognize the antigens in total hypothalamic lysates (Lys) is also shown. **(C)** Parallel immunoprecipitations were performed using a nonimmune (n.i.) serum to verify the specificity of the bands detected by Western blotting.

Together these findings suggest that estrogen receptor α may affect IGF-I actions in the brain by a direct interaction with some of the components of IGF-I signaling, such as the IGF-I receptor, PI3K and GSK3β (Fig. 4).

5.1 Functional Implications of the Interactions in the Signaling of IGF-I Receptor and Estrogen Receptor-α in the Brain

The interaction of estrogen receptor-α with the signaling pathways of IGF-I receptor in the brain may explain the interaction of estradiol and IGF-I in the regulation of different neural events. ERK and Akt may be involved in the interaction of IGF-I and estradiol in the regulation of neuronal differentiation, synaptic function, synaptic remodeling, neuroprotection, and sexual behavior[1,43,50]. The synergistic interaction of IGF-I and estradiol in the phosphorylation of Akt may be critical for the regulation of glucose transport and metabolism[51,52] and for neuroprotective actions. Akt regulates several transcription factors that may be involved in the control of neuronal survival, such as CREB,[53] NF-kappaB[54], and several members of the forkhead family[55-57]. In addition, activation of Akt results in the phosphorylation of the Bcl-2 family member Bad and this may suppress Bad-induced cell death[58,59]. Furthermore, Akt activation enhances Bcl-2 promoter activity[53] and both IGF-I and estrogen induce Bcl-2 expression in neurons. Interestingly, IGF-I receptor activation is necessary for the induction of Bcl-2 by estradiol in the adult brain[43]. Also downstream of Akt, IGF-I and estradiol may interact on the regulation of microtubule dynamics, neuritic growth, synaptogenesis, synaptic plasticity, and neuronal survival, acting on GSK3β and its substrates, β-catenin, and tau[48].

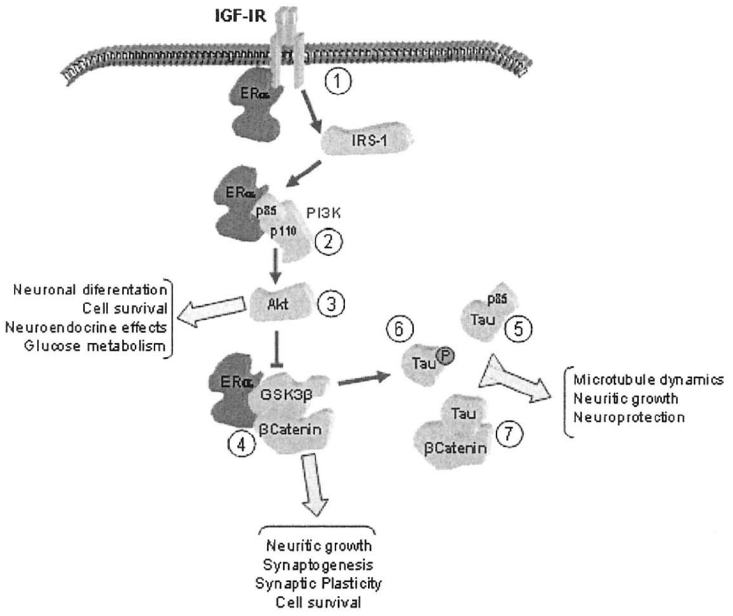

Figure 4. Proposed model for the interaction of estradiol and IGF-I in the brain. Estradiol interacts with IGF-I at different levels, regulating the activity and association of different proteins involved in the cellular response to IGF-I. Estradiol regulates the activity of protein kinases, increasing IGF-IR (1) and Akt (3) phosphorylation. E2 decreases GSK3 β activity (4), which in turn decreases tau phosphorilation (6). In addition, ERα interacts with GSK3 β (4) and estradiol treatment modulates the interaction between Erα, IGF-IR (1), and p85 (2). The association of p85 (5) and β catenin (7) with tau is also increased on estradiol treatment in the brain.

6. CONCLUDING REMARKS

The studies reviewed in this chapter indicate that the mechanisms of intracellular signaling of IGF-I and estradiol are intimately associated in the nervous system. Estrogen receptor α appears to be part of the signaling mechanism of IGF-I in the brain. In turn, estradiol activates IGF-I receptor signaling in neurons and glia. The interaction between IGF-I and estradiol appears to be relevant during brain development and in adulthood and participate in the regulation of neuronal differentiation, neuronal survival, synaptic connectivity, neural plasticity, neuroendocrine regulation, and behavior. The coupling of the signaling pathways of IGF-I and estradiol in neural cells may act as a coincidence signal detector to timely coordinate the endocrine and paracrine effects of both factors.

Finally, it should be noted that the crosstalk between the signaling of estradiol and IGF-I are not a peculiarity of neural tissue, since both factors interact in other cellular systems as well.[60-62] Among the most studied examples are breast cancer cells. Both factors play a role in the development and proliferation of breast cancer cells and show synergistic interactions in the activation of cell cycle components, resulting in increased proliferation rates.[63,64] The cross-talk between the receptors for estradiol and IGF-I in breast cancer cells show many similarities to that observed in neural cells.[63,65-67] Therefore, it is possible that the interactions, reviewed in this chapter, between IGF-I and estradiol may represent a general mechanism operating in different tissues.

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Chapter 13

CANCER

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Key words: Apoptosis; autocrine; cancer; endocrine; epidemiologic studies; malignant transformation; mitogenesis; paracrine; risk factors.

1. INTRODUCTION

The growth hormone (GH)/insulin-like growth factor (IGF) axis is the principal endocrine system controlling somatic growth. Hormones are defined as molecules that are released by endocrine glands into the circulation for delivery to target tissues, where they exert their effects via specific receptor binding and post-receptor signaling. Hence, endocrinologists have traditionally measured circulating hormone levels as a major marker of endocrine system action. Epidemiologic data have associated cancer risk with changes in circulating levels of several GH/IGF axis components. The reproducibility of these findings has focused a lot of attention recently on the possible role the GH/IGF axis may contribute to cancer development and progression.

There are two important shortcomings with this approach. First, associations can never prove causation, because the direction of causation remains unknown and there may be confounders. Second, as unexpectedly demonstrated by liver-specific *igf1* gene-deleted (LID) mice, endocrine IGF is but part of the story. Despite a 75% reduction in circulating IGF-I concentrations, LID mice had normal postnatal growth[1]. Just as the relative contributions of endocrine versus local (autocrine or paracrine) GH/IGF axis

components are being questioned in normal growth, so too is their significance in the neoplastic process.

Thus, there are two levels of possible GH/IGF contributions, each supported by different types of data. Endocrine action is investigated in epidemiologic studies and some *in vivo* animal tumor models, whereas autocrine/paracrine actions are studied in *in vitro* experiments of cellular signaling and some *in vivo* animal models. The relative significance of and the relationships between these two levels in carcinogenesis remain unclear, but data are accumulating for both. Currently, there is no strong evidence that GH or IGF-I causes cancer, but the data suggest a possible promoting role for preexisting lesions.

2. ENDOCRINE GH/IGF AXIS

2.1 GH

Epidemiologic data associating circulating GH levels with cancer risk have come from various patient populations, which serve as human models of high or low GH levels, and more recently, variations within the normal population. Animal models have also contributed to this field.

2.1.1 GH Deficiency

Concern for a possible carcinogenic effect of GH was raised in 1988 by a letter to Lancet, in which the authors reported a higher incidence of leukemia in their GH recipients than children of the general population[2]. Subsequent reanalyses identified the greater presence of leukemic risk factors, such as prior neoplasms and radiation therapy, among GH-deficient children as a confounder[3,4]. Many patients on recombinant human GH (rhGH) therapy are enrolled in post-marketing surveillance studies, including the National Cooperative Growth Study, KABI International Growth Study, and others, which serve as powerful research tools that complement the safety monitoring by the Food and Drug Administration's MEDWATCH system. A review of 12,209 patients in one such study, representing more than 51,000 patient-years at risk, revealed 10 new cases of extracranial neoplasms, which did not differ from the expected incidence derived from the National Cancer Institute's SEER (Surveillance, Epidemiology, and End Results) Program[5]. Additional data are continuously being accumulated, and the current tally exceeds 200,000 patient-years at risk.

A recent retrospective cohort study renewed the scare following years of reassuring experience. In the United Kingdom, 1848 patients treated with cadaveric pituitary-derived GH from 1959 to 1985, were followed up for cancer incidence through 1995 and mortality through 2000. The authors found a higher incidence of colorectal cancer (n = 2) and higher rates of mortality from colorectal cancer (n = 2) and Hodgkin's disease (n = 2) compared to the general population[6]. The small numbers make inference difficult, as does the switch from cadaveric pituitary-derived GH to rhGH in 1985. Implications for clinical rhGH therapy will be addressed in Section 16.5.1, but this study certainly underscores the need for long-term surveillance of GH recipients into adulthood, when the incidence of cancer normally rises.

Regarding untreated GH deficiency, a retrospective review of 333 patients consecutively diagnosed between 1956 and 1987 revealed 3 male deaths from malignancy (expected 10.1) and 4 female deaths (expected 4.1)[7]. But the major finding of this study was a shortened life expectancy, associated with an increased mortality from cardiovascular disease.

2.1.2 Acromegaly

Acromegaly, a condition of pathologic GH excess most commonly caused by a GH-secreting pituitary adenoma, has been studied as a natural model of GH effects on carcinogenesis. Because the underlying pituitary tumor is benign (adenoma), and stereotactic radiation therapy is reserved for surgery- and medication-resistant cases, changes in cancer development in these patients are generally related to the GH excess. The conclusions are controversial (for an excellent point/counterpoint, see ref. 8 and 9), for two main reasons. First, acromegaly is rare (prevalence of 4 to 6 per million), so many studies are uncontrolled, retrospective, small series reports. It is difficult to accurately determine the true cancer incidence among acromegalics from such data, and ascertainment bias may be affecting the results. Second, prior to therapeutic advances, premature mortality (primarily from cardiovascular disease) prevented acromegalic individuals from reaching older ages, when cancer risk is greatest. Thus, meta-analyses comparing early versus more recent reports may show different results, and the appropriateness of the selected control populations may be questionable. By virtue of dying younger than the control population, the acromegalic cancer risk may be under-appreciated. To resolve the debate, multicenter acromegaly registries are being compiled in the United States and United Kingdom.

Data thus far suggest, if anything, an effect of acromegaly on colorectal neoplasia. Still, findings of an increased incidence of colon polyps and

adenocarcinoma have been inconsistent[8,9]. Again, experimental design issues, including control reference data and examination techniques (flexible sigmoidoscopy versus full-length colonoscopy), may be confounding the analyses. It is generally agreed that colorectal neoplasia behaves differently in this patient group. Acromegalics have increased length of total colon and sigmoid loop, mucosal hypertrophy, prolonged colon transit times, and higher levels of deoxycholic acid reflecting increased transit times and intraluminal bacterial activity[10,11]. In acromegalics, colonic lesions tend to be more right-sided, adenomas tend to be larger and more dysplastic, and polyps more often occur multiply[12].

Colon cancer in acromegaly is an excellent example of how epidemiologic associations are insufficient to prove causation. Acromegaly is the clinical model of GH excess. Yet IGF-I is also pathologically increased in acromegaly. So is it the GH or the IGF-I? Intestinal length and mucosal mass were increased in transgenic mice that overexpressed either GH and IGF-I or IGF-I alone (with GH suppression); increased crypt cell proliferation was observed only in the IGF-I transgenics, whereas GH excess stimulated more intestinal epithelial cell differentiation[13-16]. Furthermore, does the IGF-I excess promote colon cancer directly, through the mechanisms described later in this chapter, or is there a confounder? For instance, excessive IGF-I may lead to increased intestinal length, which prolongs transit time and thereby leads to greater deoxycholic acid levels[17]. Deoxycholic acid has been implicated in colonic mucosal proliferation[18] and colon cancer cell migration[19], and has been shown to stimulate proteasomal degradation of the tumor suppressor p53[20] and at high concentrations, inhibit expression of the tumor suppressor *BRCA1*[21].

The possibility of confounding has been similarly raised for prostate cancer in acromegaly. Accentuated and early benign prostatic hyperplasia has been reported in acromegaly[22]. This may lead to an ascertainment bias, as these men may be more likely to seek medical attention for prostate-related symptomatology and be more likely to have detection of subclinical prostate cancer[23]. The overall association of acromegaly with prostate cancer risk is still unclear, owing to the aforementioned effects of early mortality on study results. Thus, we await the outcomes of the multicenter acromegaly registries and additional mechanistic studies.

2.1.3 Normal GH variability

Recently, the consequences of subtle variations in circulating hormone levels within the normal population, rather than larger pathologic changes, have become the interest of epidemiologic studies. A T-to-A polymorphism

at position 1663 of the human *GH1* gene has been identified as a cause of such variability in GH and IGF-I levels. Data from two case-control studies in Hawaii are summarized in Table 1. The first was a population-based study of 535 case patients with colorectal adenocarcinoma and 650 control subjects; the second, a sigmoidoscopy screening-based study of 139 case patients with adenoma and 202 control subjects[24]. They showed that the *A* allele was associated with a lower ratio of plasma IGF-I/IGFBP-3 and a lower risk of colorectal cancer in the total population; the reduction in colorectal cancer risk was evident in the Caucasian and Native Hawaiian subgroups of the population, but not in the Japanese. Thus, additional ethnic genetic and/or environmental factors, such as diet or exercise, can modify the effects of GH variability on cancer risk.

	T/T	T/A	A/A	P (trend)
IGF-I (ng/ml)	172.3	175.0	152.3	0.05
IGFBP-3 (ng/ml)	2859	3070	2792	0.05
Height (cm)	170.0	169.5	170.0	0.78
Overall				
OR colorectal cancer	1.00	0.75 [0.58–0.99]	0.62 [0.43–0.90]	0.006
OR adenoma	1.00	0.76 [0.46–1.24]	0.62 [0.31–1.22]	0.17
OR colorectal cancer				
Caucasian	1.0	0.85 [0.50–1.43]	0.44 [0.21–0.93]	0.05
Native Hawaiian	1.0	0.39 [0.16–0.94]	0.20 [0.07–0.59]	0.003
Japanese	1.0	0.79 [0.56–1.12]	0.85 [0.53–1.36]	0.34

Table 1. Summary of data examining the association between T-to-A polymorphism and risk of colorectal neopalasia in Hawaiian populations. Based on data from ref. 24.
OR = adjusted odds ratio; [ ] = 95% confidence intervals.

2.1.4 Animal models

Animal models allow independent manipulation of both hormonal levels and environmental factors such as carcinogen exposure to test relationships between the two. Mammary carcinogenesis was studied in Spontaneous Dwarf rats (SDR), which are Sprague Dawley rats rendered GH deficient by a *GH* gene mutation[25]. When exposed to the direct-acting carcinogen *N*-methyl-*N*-nitrosourea, SDR exhibited lower mammary tumor incidence (3 of 15 rats) and lower tumor number (average 0.2 tumors/rat) compared to similarly exposed wild-type rats (10 of 10 rats and 5.3 tumors/rat,

respectively). The indirect-acting carcinogen 7,12-dimethylbenz[*a*] anthracine (DMBA) produced an average 0.21 tumors per SDR/SDR rat versus 4 tumors per wild type or heterozygous rat. Yet these rats also demonstrated an important role for GH in normal mammary gland development. SDRs had less alveolar development, but normal ductal branching; GH infusion induced epithelial cell proliferation and alveolar development similar to that of the wild-type rats.

Mammary carcinogenesis was also studied in dwarf rats (dw) of the Lewis strain, who harbor a recessive mutation specifically affecting GH synthesis[26]. Heterozygous *dw/+* rats are normal size; homozygous dw/dw rats have 10% circulating GH concentrations and 50% IGF-I concentrations of their heterozygous littermates. *dw/dw* and their *dw/+* littermates were treated with DMBA on day of life 50 and then 6 weeks of daily injections: dw/+ received normal saline, whereas the *dw/dw* rats were randomized among saline and low- and high-dose porcine GH. None of the *dw/dw* saline rats developed mammary tumors by 27 weeks after DMBA; 70% of *dw/+* rats had tumors. GH treatment dose-dependently increased mammary tumorigenesis in the dw/dw rats (83% of low-dose and 100% of high-dose). These studies indicate the GH/IGF-I deficiency confers resistance to carcinogen-induced tumor formation.

2.2 IGF-I

2.2.1 Epidemiologic studies

Multiple large case-control studies have found positive associations between high circulating IGF-I concentrations and increased risk for different types of cancer. The striking features of these studies are the reproducibility across cancer types and the significant effects of variations in IGF-I concentrations that fall within the normal range, *i.e.* the "high" IGF-I levels in these studies are not pathologically high from disease states, but rather the highest quartile or quintile of the general population compared to the lowest quartile or quintile. These studies are extensively discussed elsewhere[23,27], and summarized in Table 2[28-50].

Cancer	IGF axis component	Study design	Results	Ref.
Prostate				
	IGF-I IGFBP-3	Case (152)-control (152)	RR for highest quartile: IGF-I = 4.3 [1.8-10.6] IGFBP-3 = 0.41 [0.17-1.0]	28
	IGF-I IGFBP-3	Case (210)-control (224)	OR per 100 ng/ml increment of IGF-I: 1.51 [1.0-2.26] overall and 2.93 [1.43-5.97] for age less than 70 yrs No association with serum [IGFBP-3]	29
	IGF-I IGFBP-3	Case (149)-control (298)	Increased risk with increasing [IGF-I] (p = 0.02) and increasing [IGFBP-3] (p = 0.03) overall; for men age <59 yrs, association stronger for high [IGF-I] (p = 0.01), but [IGFBP-3] lost (p = 0.44)	30
	IGF-I	Meta-analysis	OR for highest quartile IGF-I similar to that for highest quartile of testosterone	31
	IGF-I IGFBP-3	Meta-analysis	OR for high IGF-I: 1.47 [1.23-1.77] OR for high IGFBP-3: 1.26 [1.03-1.54]	32
	IGF-I IGFBP-3	Case (530)-control (534)	For advanced-stage prostate cancer: RR for highest quartile IGF-I = 5.1 [2.0-13.2], high [BP-3] = 0.2 [0.1-0.6]. RR for combination high IGF-I/low BP-3 = 9.5 [1.9-48.4] Neither was predictive of early-stage cancer, nor associated with Gleason score.	33
	IGFBP-3 IGFBP-2	Pre-op in 120 men with localized disease (post-op in 51 of them) vs. 44 healthy men vs. 19 nodal mets vs.10 bone mets	[IGFBP-3] lowest in men with bone mets. [IGFBP-2] higher in cancer patients than healthy men and declined with prostatectomy. Among cancer patients, [IGFBP-2] was lower with advanced disease and larger tumor volume.	34
Gastro-intestinal				
Colo-rectal cancer	IGF-I IGFBP-3 IGF-II	case (193) – control (318) men only	RR for highest quintile IGF-I = 2.51 [1.15-5.46] IGFBP-3 = 0.28 [0.012-0.66] IGF-II not associated with risk.	35
	IGFBP-1,2,3	case (102) – control (200) women only	OR for highest quintile IGFBP-1 = 0.48[0.23-1.0] IGFBP-2 = 0.38[0.015-0.094] IGFBP-3 = 2.46[1.09-5.57]	36
	IGF-II	case (92)-	IGF-II elevated in Dukes A and B, but not	37

Cancer	IGF axis component	Study design	Results	Ref.
	IGFBP-2	control (57)	advanced disease. IGFBP-2 related to tumor burden and fell with curative resection	
Colo-rectal adenoma	IGF-II	case (52) – control (293)	OR for adenoma per SD change: IGF-II = 3.05[2.04-4.57] IGFBP-2 =2.26[1.53-3.44]	38
Gastric adenocar - cinoma	IGF-I IGFBP-3	26 cases pre-op and post-op days 14 and 50.	All 26 had IGF-I over the normal limits pre-op that significantly decreased post-op but not to normal. IGFBP-3 also high pre-op.	39
Lung				
	IGF-I IGFBP-3 IGF-II	case (204) – control (218)	OR for highest quartile IGF-I = 2.06[1.19-3.56] IGFBP-3 = 0.48[0.25-0.92] IGF-II not associated with risk.	40
	IGF-I IGFBP-1,2,3	case (93) – control (186) Women only	No associations found.	41
	IGF-I IGFBP-3	case (230) – control (659) Men only	IGF-I not associated. OR highest quartile of IGFBP-3: All cases: 0.50[0.25-1.02] Ever smokers (n = 184): 0.41[0.018-0.92]	42
GYN				
Breast cancer	IGF-I	case (397) – control (620)	Post-menopausal: no association. Premenopausal: RR for top tertile All = 2.33[1.06-5.16] Age < 50 yrs = 4.58[1.75-12.0] Adjusting for IGFBP-3 raised the RR's All = 2.88[1.21-6.85] Age < 50 yrs = 7.28[2.40-22.0]	43
	IGF-I IGFBP-1,3	case (63) – control (27)	IGF-I levels similar. OR for high: IGFBP-3 = 0.18[0.05-0.55] IGFBP-1 = 0.21[0.07-0.68]	44
	IGF-I IGFBP-3	case (40) – control (40)	OR for high free IGF-I = 6.31[1.03-38.72]. High total IGF-I/intact IGFBP-3 ratio, OR = 3.35[1.08-10.36]	45
	IGF-I IGFBP-1,2,3	case (149) – control (333) only post-menopausal.	No associations found.	46
	Number of CA repeats	Case (53) – control (53)	19 repeats: OR = 2.87[1.16-7.06] Both 19 repeats and high plasma IGF-I: OR	47

Cancer	IGF axis component	Study design	Results	Ref.
	in IGF-I gene		= 5.12[1.42-18.5]	
Ovarian cancer	IGF-I	Case (132) – control (263)	All: no association. Age < 55yrs: top tertile OR = 4.97[1.22-20.2]	48
Endo-metrial cancer	IGF-I IGF-II	Case (84) – control (84)	IGF-I: inverse association IGF-II: positive association	49
Bladder cancer	IGF-I IGFBP-3	Case (154) – control (154)	OR for highest quartile IGF-I = 3.10[1.43-6.70] IGFBP-3 = 0.38[0.19-0.78] IGF-I/IGFBP-3 molar ratio = 4.30[1.99-9.28]	50

Table 2. Summary of studies associating cancer risk with circulating levels of IGF and IGFBPs. * numbers in parentheses refer to sample sizes for the case-control studies. [ ]= 95% confidence intervals for odds ratios and relative risks.

2.2.2 Animal models

As described in the introduction, LID mice serve as the classic model to distinguish endocrine from autocrine/paracrine IGF-I, and have circulating IGF-I concentrations 25% of normal[1]. Six weeks of treatment with recombinant human IGF-I or saline further manipulated the circulating IGF-I concentrations such that control + IGF-I had the highest IGF-I levels, LID + saline had the lowest, and control + saline and LID + IGF-I fell inbetween. When mouse colon adenocarcinoma cells were transplanted onto the surface of the cecum of these animals, the aggressiveness of tumor behavior paralleled the IGF-I levels. Control + IGF-I mice had the greatest frequency of tumor growth, greatest mean tumor weight, greatest tumor vessel count, greatest frequency of hepatic metastases, and greatest numbers of metastases per liver; LID + saline mice had the lowest by all parameters, and the other mice were intermediary[51].

Athymic nude mice were injected with fibroblasts that contained either normal (16,000/cell) or experimentally increased (190,000/cell) density of the type 1 IGF receptor (IGF-1R). Systemic IGF-I treatment did not change tumor development in the mice injected with normal fibroblasts. However, for the mice with high IGF-1R fibroblasts, systemic IGF-I treatment decreased tumor latency, increased fibrosarcoma growth, and increased mitogenesis[52].

Circulating IGF-I had an effect on cancer behavior in both models, but it is important to note that both also involved local changes in the cell's growth regulatory mechanisms (preexisting adenocarcinoma or almost 12-fold over-

expression of IGF-1R). Thus, the current evidence may support a permissive effect of circulating IGF-I on existing cancers, but not a causal role in the creation of cancer.

2.3 IGFBP-3

Many of the same case-control studies showing positive associations of cancer risk with high IGF-I concentrations showed inverse associations of cancer risk with high IGFBP-3 concentrations. This suggests that theoretically the greatest risk of developing cancer comes from having high IGF-I coupled with low IGFBP-3 levels. GH raises both. The IGFBP-3 data are also summarized in Table 2[28-50].

3. AUTOCRINE/PARACRINE GH/IGF AXIS

Many studies have demonstrated that local perturbations in the GH/IGF axis can enhance cell survival and proliferation, and thereby promote cancer. These changes can occur in the cancerous cell itself or in the supporting stroma. Several caveats must be kept in mind. Foremost, multiple genetic changes are required for the creation and progression of each and every cancer, so the GH/IGF axis must be considered within that greater context. Second, none of the changes are universal; they are frequently cancer type-specific and even within a cancer type, stage-specific. Finally, an exhaustive review of all the published changes would exceed the scope of this chapter. What follows, then, is a selective review of illustrative examples demonstrating pathophysiologic principles by which GH/IGF axis changes may contribute to cancer.

3.1 GH

Although the pituitary gland is the main source of GH in the body, abnormal augmentation of local GH production can increase proliferation of the local cells. For example, expression of GH mRNA identical to pituitary GH mRNA has been shown in the ductal luminal epithelial and myoepithelial cells and scattered stromal fibroblasts of normal human mammary glands. This expression was increased in both the epithelial and supporting stromal compartments of three progressive proliferative disorders: benign fibroadenoma, preinvasive intraductal carcinoma, and invasive ductal carcinoma with lymph node metastases[53]. Normal, proliferative, and neoplastic lesions of the breast were also found to express

the GH receptor (GHR), as did the stroma to a lesser degree. GHR expression levels varied greatly among individuals, but did not correlate with lesion histology[54]. When MCF-7 human breast cancer cells were stably transfected with either the gene for human GH or a translation-deficient mutated *GH* gene, GH expression increased cellular proliferation and was synergistic with trophic factors such as IGF-I[55]. Mechanistically, GH caused transcriptional repression of the p53-regulated placental transforming growth factor-β (PTGF-β); reduced PTGF-β in turn led to decreased Smad-mediated transcription which resulted in decreased cell cycle arrest and apoptosis[55].

3.2 IGFs

Like GH, overexpression of the IGFs may enhance cell survival and proliferation. For example, IGF-I overexpression was found in 31 of 50 thyroid adenomas and 38 of 53 thyroid carcinomas examined, and correlated with carcinoma tumor diameter but not patient age, gender, or tumor stage[56]. IGF-I was not increased in sporadic adrenocortical carcinomas, but the amount of IGF-II protein in the malignant tumors far exceeded that in benign tumors or normal adrenal tissue[57].

The single-copy six-exon *IGF-I* gene is transcribed from two promoters (P1 and P2), located 5' to exons 1 and 2, respectively, resulting in RNAs with different 5' leader sequences; alternative RNA splicing and differential polyadenylation yield multiple mature transcripts[58]. Normally, local IGF-I expression can be increased by GH[59], estrogen[60], cAMP[61], and transforming growth factor-β (TGF-β)[62], and decreased by glucocorticoids[63]. In contrast, the most common mechanism of IGF-II overexpression involves loss of imprinting; IGF-II is normally expressed from the paternal allele only and is under reciprocal regulation with the maternally expressed tumor suppressor, H19[64,65].

As proof of principle, two sets of IGF-I transgenic mice created by DiGiovanni *et al.* supported the hypothesis that local IGF-I overexpression can enhance cellular proliferation and contribute to neoplasia. Transgenic mice overexpressing IGF-I in the basal epithelial cells of the prostate developed prostatic hyperplasia by 2 to 3 months of age, and atypical hyperplasia and prostatic intraepithelial neoplasia by 6 to 7 months. Well-differentiated adenocarcinomas were found in mice starting at age 6 months, and two of the older mice developed less differentiated (small cell) carcinomas. Of all the mice aged 6 months or greater, 50% had prostate tumors[66]. Transgenic mice overexpressing IGF-I in the basal cells of the epidermis had morphologic changes of their skin and ears, epidermal hyperplasia, hyperkeratosis, and about half the older mice developed squamous papillomas, some of which converted into carcinomas. Increased

skin proliferation was indicated by increased labeling index. IGF-I overexpression also increased susceptibility to carcinogens, as carcinogen-induced papilloma development in the IGF over-expressing mice was seven fold greater than in their nontransgenic littermates[67].

3.3 IGF receptors

Overexpression of the IGF receptor can also augment cellular proliferation, and when concomitant with IGF overexpression, forms an effective autocrine loop for self-stimulated growth. The growth-promoting actions of both IGF-I and IGF-II are mediated by the type 1 IGF receptor (IGF-1R), an $\alpha_2\beta_2$ heterotetrameric tyrosine kinase receptor closely related to the insulin receptor (IR). Not only are IGF-1R and IR structurally homologous, but they also share common signaling pathways, ligand cross-reactivity, and can form hybrid receptors. (Table 3)[68-73].

Receptor	Structure	Ligands*	Characteristics	Function
IGF-1R	Transmembrane $\alpha_2\beta_2$ tyrosine kinase	IGF-I IGF-II	Ubiquitous; important for normal growth	cell survival, mitogenesis, transformation
IGF-2R	Identical to mannose-6-phosphate receptor	IGF-II	Soluble receptor	clear IGF-II from the circulation
IR-A	Transmembrane $\alpha_2\beta_2$ tyrosine kinase	IGF-II insulin	Predominant IR isoform in fetal tissues; shorter (12 amino acids omitted from α-subunit by skipping exon 11)	fetal growth, metabolism
IR-B	Transmembrane $\alpha_2\beta_2$ tyrosine kinase	insulin	Longer IR isoform; found in metabolically responsive adult tissues (fat, liver, muscle)	glucose storage and oxidation, lipid storage, protein synthesis, regulation of gene expression
Hybrids	Transmembrane $\alpha_2\beta_2$ tyrosine kinase	IGF-I	Random assembly of an IR hemireceptor combined with an IGF-1R hemireceptor	cell survival, mitogenesis, transformation

Table 3. Summary of IGF and insulin receptors. *Ligands listed refer to high-affinity binding only.

Overexpression of IGF-1R, IR, and hybrid receptors have all been found in cancers, such as cancers of the breast[74] and thyroid[75]. In fact, IGF-I-

stimulated growth of thyroid papillary carcinoma cells in culture was attenuated by antibodies specifically targeting either IGF-1R or hybrid receptors[75]. Thus, the hybrid receptors are not merely structural errors from receptor over-expression, but serve growth-promoting functions in the malignant cells.

3.4 IGF/Receptor signaling

Ligand binding to the IGF-1R (and IR) causes autocatalytic phosphorylation of the receptor's tyrosine kinase domain, which also phosphorylates additional IGF-1R (and IR) tyrosine residues important for the recruitment of adapter molecules like IRS and Shc. These in turn activate kinase cascades, primarily the PI3 kinase/Akt (PKB) pathway and the MAP kinase pathway, respectively, and lead to signal transduction to the nucleus and mitochondrion[76].

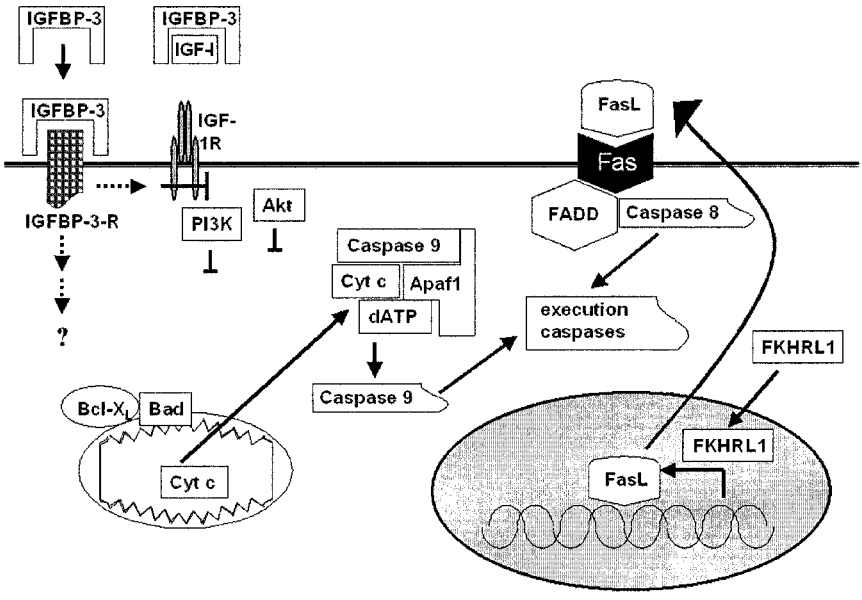

Figure 1. Activation of Akt by IGF/IGF-1R signaling stimulates cell survival. (Modified from ref. 76).

Enhanced IGF/IGF-1R signaling can contribute to each of the four stages of cancer progression[76]. In the first stage, malignant transformation, a cell must acquire the ability to both advantageously proliferate and escape the

body's protective mechanisms, *i.e.* cell cycle arrests that regulate cell growth and apoptosis, or cell suicide, that aborts any aberrant cells. Stimulation by growth factors, like IGF-I, is required for cell cycle entry and progression to the late G_1 phase restriction point, beyond which the cell is committed to completing a round of cell division. IGF-I stimulates the G_1 phase progression by inducing cyclin D1 for assembly with cyclin-dependent kinase (CDK)4[77,78]. IGF-I can further stimulate survival and proliferation through multiple branch points of its signaling pathways, one of which is summarized here and in Figures 1 and 2. As shown in Figure 1, IGF-1R binding by IGF leads to phosphorylation and activation of Akt via PI3 kinase[79]. Activated Akt, itself a kinase, phosphorylates multiple substrates including the Bcl-family member, Bad, and the forkhead transcription factor, FKHRL1. When phosphorylated, Bad and FKHRL1 are sequestered in the cytoplasm by binding to 14-3-3 proteins. Akt also phosphorylates caspase 9, directly inhibiting its function, and IKKα, thereby activating NFκB for nuclear localization and transcriptional activation of survival genes such as *c-myc*. Conversely, the consequences of Akt inactivation, when IGF/IGF-1R signaling is absent, are shown in Figure 2. Unphosphorylated Bad localizes to the mitochondrion, where it binds Bcl-X_L and releases cytochrome *c* to the apoptosome, thereby activating caspase 9. Similarly, unphosphorylated FKHRL1 localizes to the nucleus, where it transcriptionally activates numerous genes including IGFBP-1 and FasL. FasL is the ligand (hence, the name) of Fas, the membrane-bound death receptor that activates caspase 8 via the adapter molecule, FADD. Thus, both the mitochondrial and cytoplasmic caspase cascades are activated and converge on activating the execution caspases to complete apoptosis. In short, activation of Akt by IGF/IGF-1R accomplishes both, stimulating cell survival while avoiding apoptosis[76,80,81].

The second stage of cancer progression involves additional adaptations that enable continued growth of the clonally expanded, transformed cell as a bulky tumor, wherein nutrient delivery may become restrictive. As shown in colon[82,83], lung[84], and thyroid cancers[85], IGF-I induces the angiogenesis agent, vascular endothelial growth factor (VEGF), via increased synthesis of the HIF1α transcription factor. Colon cancer cells harboring a dominant-negative truncated IGF-1R, when injected into nude mice, developed smaller tumors, reduced VEGF expression, lower tumor vessel count, and decreased pericyte coverage of endothelial cells[86].

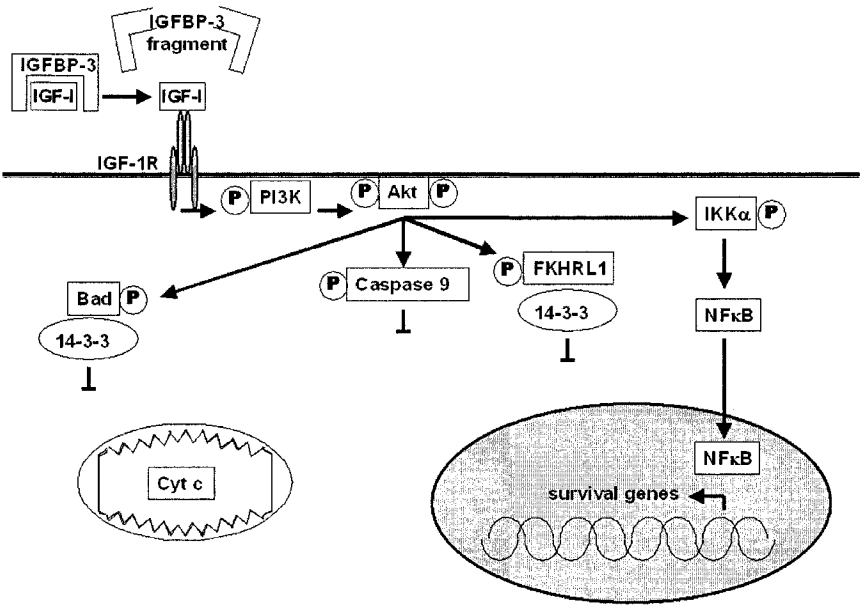

Figure 2. IGF-1R and Akt inactivation lead to apoptosis. (Modified from ref. 76.)

IGF-1R signaling further contributes to cell motility and cell environment interactions that are important for local invasion and the metastatic process. Adherence junctions connect epithelial cells into a normally growing sheet and consist of a core (transmembrane E-cadherin plus cytoplasmic α-, β-, and γ-catenins) that is coupled to microfilaments via α-catenin, either directly or indirectly through α-actinin and vinculin[87]. In MCF-7 breast cancer cells, IGF-1R signaling was shown to promote cell separation, via disassembly of the adherence junctions and redistribution of α-actinin, actin, and fascin into motile apicolateral spikes, as well as to cell migration, via reassembly of stress fibers, development of long membrane protrusions, and stimulation of myosin light chain kinase[88]. IGF-1R signaling also relocalizes integrins to the leading edge of migrating cells. Conversely, integrins, heterodimers that bind extracellular matrix molecules and transduce signals to the intracellular environment, can modulate IGF-1R signaling when they are activated by their own ligand binding[89]. While inducing cellular changes necessary for motility, IGF-1R signaling can help create a suitable microenvironment for the migrating cell by inducing expression of proteases like cathepsin D[90], matrix metalloproteinases[91,92], and urokinase plasminogen activator[93]. These proteases not only dissolve basement membranes in the

path of the migrating cell, but they can also cleave IGFBP-3, thereby releasing any bound IGF in the microenvironment for further cell stimulation[94]. Transfection of IGF-1R increased cell spreading on fibronectin, colony formation in soft agar, and metastatic behavior of M-27 Lewis lung carcinoma cells, which are usually poorly invasive and express low numbers of IGF-1R; use of site-directed mutants enabled further delineation of the various IGF-1R functions to different subsets of the normal tyrosine phosphorylation sites[95].

During the last stage, many cancers acquire resistance to therapeutic agents designed to kill rapidly proliferating cells. Conditions of increased IGF signaling led to increased resistance of a variety of cancer cell lines to such agents in *in vitro* experiments. These include resistance of breast[96] and colorectal cancer[97] cells to irradiation; breast cancer cells to herceptin[98], doxorubicin, and taxol[99]; colorectal cancer cells to 5-fluorouracil[97]; small cell lung cancer cells to etoposide[100]; pancreatic cancer cells to COX-2 inhibitors[101]; rhabdomyosarcoma cells to rapamycin[102]; metastatic sarcoma cells to doxorubicin[103]; leukemic cells to all-trans-retinoic acid (ATRA)[104]; and multiple myeloma[105] and thyroid cancer cells[106] to Apo2L/tumor necrosis factor-related apoptosis-inducing ligand (TRAIL).

3.5 IGFBPs

Another major mechanism for enhanced cellular survival and proliferation is the reduction of IGFBPs, either through decreased expression or increased proteolysis. Because the binding affinity of IGF for the IGFBPs exceeds that for IGF-1R, IGFBPs tend to reduce IGF/IGF-1R signaling through competitive binding. This has been demonstrated by numerous experiments in different cell types using Des-(1-3)-IGF-I, an IGF-I analog that binds IGF-1R and stimulates DNA synthesis but cannot bind IGFBP-3[107-109]. As the principal endocrine IGFBP, IGFBP-3 is the best studied and will be the focus of this discussion.

In addition to inhibiting growth by preventing IGF from binding IGF-1R, IGFBP-3 can also inhibit growth and induce apoptosis in an IGF-independent manner[68]. This has been demonstrated by experiments using IGF-free systems, *IGF-1R*$^{-/-}$ cells and rhIGFBP-3 fragments with negligible binding affinity for IGF-I[68,110,111]. IGF-independent actions of IGFBP-3 are presumed to occur via IGFBP-3-specific receptors, which were first detected on the surface of breast cancer cells[112,113] and involve the midregion of the IGFBP-3 molecule[114]. The exact signal transduction pathway of IGFBP-3-mediated apoptosis is still unclear, but proposed mechanisms include: direct inhibition of IGF-1R[115], increased intracellular calcium[116], nuclear

translocation and RXR-binding[117-120], changes in BcL family members[121], and mitochondrial actions[122].

Thus, a cell can increase its IGF bioavailability, enhance its growth and reduce apoptosis by decreasing the amount of IGFBP-3[110]. Changes in levels of the other IGFBPs, both up and down, have also been found in a variety of cancers, including prostate[94], lung[123], and adrenocortical tumors[57]. Although generally thought to be growth inhibitory, IGFBPs have been shown to be growth stimulatory under certain experimental conditions. By sequestering IGF from the IGF-1R, the IGFBPs may allow local accumulation of IGF without down-regulation of the IGF-1R; when this local pool is simultaneously released through IGFBP proteolysis into low-affinity IGF-binding fragments, a greater amount of IGF can bind the IGF-1R for a larger growth stimulus than had the IGF accumulated in a bioavailable form all along[68]. Hence, the literature contains conflicting reports of increased IGFBP levels as growth inhibitory or growth stimulatory[124], and further studies are needed. The growth stimulatory effect of IGF presentation is illustrated in Figure 1, and the growth inhibition, via inhibition of IGF-1R signaling or via IGFBP-3-receptor-mediated pathways, is illustrated in Figure 2.

3.6 IGFBP proteases

As mentioned, IGFBP proteases play a critical role in modulating the local bioavailability of IGF for binding IGF-1R; IGF binding affinity is greatest for the intact IGFBP, less for IGF-1R, and less still for IGFBP fragments. Prostate-specific antigen (PSA), whose serum level correlates with prostate cancer volume, was the first IGFBP protease biochemically identified.[125,126] The current list falls into three major categories: kallikrein-like serine proteases, matrix metalloproteinases, and cathepsins[68]. IGFBP proteolysis can be enhanced through increased protease expression by cancers or through protease activation by the tumor's acidic microenvironment[68,127].

Interestingly, IGFBP proteolysis also occurs at both the local and endocrine levels. Although the PSA elevation in prostate cancer patients' serum negatively correlates with the circulating level of intact IGFBP-3 (and positively with circulating IGFBP-2 levels) [128], the pattern of IGFBP-3 cleavage fragments in serum differs from that created by seminal PSA, so additional protease(s) must be involved[68]. The frequency of supranormal plasma IGFBP-3 proteolysis was also found to increase with increasing stage of primary breast cancers[129].

4. INDIRECT CONTRIBUTIONS

Cancer is a multigenetic phenomenon, and changes in the GH/IGF axis must be considered within the larger context of cellular signaling. Primary changes in the GH/IGF axis may impact the activities of other signaling pathways, and conversely, primary changes in other pathways may modify GH/IGF axis function.

4.1 GH

Although the somatomedin hypothesis identified IGF-I as the principal signaling target of GH, GH has been found to induce other downstream signaling molecules as well. These include: hepatocyte growth factor in the liver[130], epidermal growth factor (EGF) in the kidney[131], basic fibroblast growth factor in chondrocytes[132], interleukin-6 in osteoblasts[133], bone morphogenetic proteins 2 and 4 in fibroblasts[134], interleukin-1α and 1β in the thymus[135], and preadipocyte factor-1 in beta cells[136]. Whether GH contributes to the neoplastic process through downstream signaling molecules outside the IGF axis is yet unknown. In addition, GH was shown to induce phosphorylation of the EGF receptor (EGFR)[137]; because phosphorylation slowed the rate of EGF-induced EGFR intracellular redistribution and degradation, GH cotreatment potentiated EGF-induced EGFR signaling[138]. EGFR over-expression is one of the most common genetic lesions found in esophageal cancer[139], and IGFBP-3 induction was recently identified as a consequence of this over-expression[140]. Cross-talk between the GH/IGF axis and EGF/EGFR pathways in the pathogenesis of esophageal cancer remains to be elucidated.

4.2 IGF-IGF-1R signaling

IGF/IGF-1R signaling may contribute to cancer through interactions with oncogenes and other mitogens. For example, although IGF-1R was insufficient for growth in soft agar, it was required for malignant transformation by the SV40 large T antigen; stable transfection with SV40 large T antigen led to colony formation by *IGF-1R*$^{+/+}$ but not *IGF-1R*$^{-/-}$ cells[141]. Signal cooperation has also been shown between IGF-I and hepatocyte growth factor-scatter factor (HGF-SF) in hepatocellular carcinoma[142] and granulocyte-monocyte-colony-stimulating factor (GM-CSF) in acute myeloid leukemic cells[143]. Likewise, the IGF axis can interact with sex steroid systems to affect cancer development and behavior. This has

been studied most for estrogen in breast cancer and androgens in prostate cancer. The interactions are bi-directional and frequently stage specific. Estrogen and IGF-I were synergistic in stimulating MCF-7 breast cancer cell proliferation; the combination enhanced G_1 phase progression through complementary regulation of p21, cyclin D1-CDK4 and cyclin E-CDK2 complexes[144,145]. IGF1R overexpression has contributed to continued growth of estrogen-deprived breast cancer cells[146] and androgen-independent prostate cancer cells[147]. However, IGF-1R expression is lost as prostate cancers progress to metastases, an effect likely mediated by WT-1(Wilm's tumor gene product) [148]. One of the proposed mechanisms by which tamoxifen is beneficial for breast cancer treatment, beyond its estrogen inhibitory activity, is its reduction of serum IGF-I concentrations[149,150].

Many tumor suppressors, on the other hand, function at least in part by inhibiting IGF action. This may occur at the transcriptional or functional levels. Transcription of IGF-1R is repressed by WT-1[151] and the tumor suppressor p53[152]. p53 also represses transcription of IGF-II[153], but stimulates transcription of IGFBP-3[154] (see Section 4.3). Other tumor suppressors can inhibit IGF action at the signaling level, without affecting transcription levels. PTEN (phosphatase and tensin homolog) is a phosphatase that dephosphorylates Akt, thereby inhibiting one of IGF's major signaling pathways[155,156]. Germline *PTEN* mutations have been detected in Cowden syndrome, characterized by multiorgan hamartomas and increased cancer risk, and allelic losses and somatic mutations at the *PTEN* locus are frequently found in cancers, especially in later stages as they become more aggressive[157,158]. The von Hippel Lindau gene product (VHL) leads to reduced VEGF production via ubiquitin-mediated degradation of HIF-1α[159]. VHL was shown to also directly interact with protein kinase C-δ, causing its dissociation from IGF-1R and inhibition of IGF-mediated invasiveness[160]. VHL is important in the pathogenesis of renal cell carcinoma, and its germline mutation causes a dominantly inherited familial cancer syndrome[161].

4.3 IGFBP-3

As mentioned in the previous section, IGFBP-3 is transcriptionally induced by the tumor suppressor p53[154]. In fact, IGFBP-3 was shown to mediate p53-induced apoptosis during serum starvation[162], and multiple *p53* mutants that lost the ability to induce IGFBP-3 and Bax, but not p21, were unable to induce apoptosis[163,164]. IGFBP-3 is also induced by cytokines, retinoic acid, DNA damage (both irradiation and drug-induced), and hypoxia[110,165]. By inducing IGFBP-3 and repressing both IGF-1R/IGF-II,

p53 switches the IGF axis balance from growth stimulation to growth inhibition and apoptosis. p53 is the most frequently mutated gene among all human cancers, and its germline mutation is associated with Li-Fraumeni Syndrome, another dominant familial cancer syndrome[110].

Conversely, oncogenes can affect the IGF axis by inhibiting IGFBP-3 function. For example, oncogenic H-*ras* caused resistance to the growth–inhibitory effects of IGFBP-3 in breast cancer cells[166]. E7, a product of human papillomaviruses associated with cervical cancer, was shown to bind IGFBP-3, leading to IGFBP-3 proteolysis and inhibition of IGFBP-3-induced apoptosis[167].

5. CLINICAL IMPLICATIONS

5.1 rhGH treatment

Clinical practice with growth-promoting therapies has already been affected by the concern of possible cancer-promoting effects of GH and IGF-I. GH deficiency frequently develops as a consequence of irradiation or chemotherapy, and for intracranial neoplasms, from the brain tumor itself or from tumor resection[168]. Because the greatest risk of cancer recurrence is in the first year after treatment, current practice in many centers is to defer rhGH therapy until the patient is at least one year tumor-free. Yet when carefully studied, the evidence indicated that rhGH did not increase recurrence rates[169].

rhGH is currently accepted as a safe therapy, but there are two important considerations as we look to the future. First, rhGH therapy is evolving from limited physiologic replacement to increasingly pharmacologic use, in terms of escalating doses and additional indications[170]. Its safety profile must be reevaluated in this new context. To guide continued safety, IGF-I levels in all rhGH recipients should be closely monitored and rhGH dose titrated to avoid supraphysiologic IGF-I levels.

Second, the follow-up study of the United Kingdom cadaveric pituitary GH recipients bears mentioning[6]. The significance of the results for current rhGH recipients is unclear. It is difficult to generalize from a sample size of two cases, and change in product makes comparisons dubious. Dosing schedule for rhGH is different than that for pituitary GH, and infectious risks have been eliminated by the switch to recombinant technology[171]. Just as Creutzfeldt-Jakob disease painfully exposed the incomplete purity of the pituitary GH, perhaps viral oncogenes were also transmitted? Yet the study pointed out a very important concept: because cancer incidence is greatest in

older ages, ongoing surveillance of rhGH recipients into late adulthood is needed to fully assess the long-term safety.

5.2 Cancer prevention

The ultimate goal of understanding how the GH/IGF axis may contribute to cancer is to devise ways of manipulating the system to prevent or treat cancer. Calorie restriction has long been recognized as an effective means of reducing circulating IGF-I levels. In cancer-susceptible mouse models, calorie restriction has been shown to not only lower IGF-I levels, but also to delay spontaneous tumor development and suppress carcinogen-induced tumor progression[172,173]. For example, calorie restriction of p53-haploinsufficient mice suppressed progression of p-cresidine induced bladder tumors, but not when the circulating IGF-I levels were restored by pump infusion[174]. In human epidemiologic studies, overweight has been the most reproducible cancer risk factor, so now the focus has shifted onto more prevention-oriented studies; physical exercise has been shown protective for breast and colon cancers[175], and several studies are trying to dissect out specific dietary components as particularly risk-affecting[176-178]. The burgeoning obesity epidemic throws urgency and keen interest upon this area of investigation, and opens up a new line of query as well: since the insulin-IR system is so closely related to the IGF axis and hyperinsulinemia is a frequent complication of obesity, the possible contributions of insulin to cancer remain to be explored.

5.3 Cancer treatment

Forays into GH/IGF axis modulation as a means of treating cancer have fallen into two general categories: dampening the whole GH axis and more specifically targeting IGF/IGF-1R signaling. Table 4 summarizes the data from experiments using upstream targets[179-184]. To date, approaches for specifically inhibiting IGF-1R signaling include adenoviral dominant negative IGF-1R[185], IGF-1R antibodies[186], IGF-1R antisense[187], IGF-1R siRNAs[188], and IGF-I antisense[189]. The clinical efficacy of IGF-1R inhibition is yet unknown, as are the potential toxicities from inhibiting normal IGF-1R or cross-reactivity with IR. Thus this construes a hot area for on-going research.

GH antagonist	Model system	Effect	Ref.
GH-R antag (pegvisomant)	Meningiomas xenografted into athymic mice; animals treated X 8wks	Decr tumor volume and tumor weight. Serum [IGF-I] and [BP-3] decr; [BP-1] and [BP-4] incr. No difference in tumor [IGF-I]	179
-+Transgenic mice with GH-antag	DMBA-induced mammary tumors	Transgenic mice smaller, decr [IGF-I]. Decr tumor incidence.	180
GH-RH antag (MZ-4-71)	Caki-I (renal adenoCA) Tissue cx and xenografted into nude mice	Mice: decr tumor volume; decr tumor weight. Decr serum [GH] and [IGF-I]. Also decr [IGF-I] in liver and tumor; decr [IGF-II] in tumor. Cx: inhibit growth, but only at high concentrations.	181
GH-RH antags (MZ-4-71, MZ5-156 and JV-1-36)	HT-29 (colon CA): Tissue cx and xenografted into nude mice	Mice: decr tumor volume and weight by all 3 agents. Decr cell prolif, incr apoptosis; decr tumor IGF-II. No change in serum [IGF-I] or [IGF-II]. Cx: MZ-5-156 dose-dependently decr IGF-II and cell prolif.	182
GH-RH antag (JV-1-38); somatostatin analog (RC-160)	PC-3 (prostate CA): Tissue cx and xenografted into nude mice	Mice: tumor volume decr 49% by JV-1-38, 30% (NS) by RC-160, 63% by combination. Decr serum [IGF-I] by RC-160 only; decr tumor IGF-II mRNA by both; decr VEGF by both. Cx: cell prolif inhibited by JV-1-38, not RC-160 alone but moreso in combination.	183
GH-RH antag (JV-1-38)	MNNG/HOS (osteosarcoma) and SK-ES-1 (Ewing's sarcoma): Tissue cx and xenografted into nude mice	Mice: tumor volume and weight decr for both, incr tumor doubling time. Decr [IGF-I] in serum and liver mRNA; Decr [IGF-II] and IGF-II mRNA in both tumors. Cx: inhibited prolif of both cell lines.	184

Table 4. GH antagonism to reduce IGF-I and inhibit cancer.Antag, antagonist; cx, tissue culture; decr, decrease; incr, increase; prolif, proliferation.

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Chapter 14

GENETIC BASIS OF PROPORTIONAL SHORT STATURE

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Key words: Gene; GH; IGF-I; short stature.

1. INTRODUCTION

The considerable progression in molecular biology and genetic techniques during the last two decades, together with the unravelling of the human genome in recent years, has notably contributed to expand the knowledge of the genetic bases of pathological growth in humans. Furthermore, these dramatic advances have also provided us with a host of new molecular techniques that allow a better and quicker diagnosis of molecular anomalies related with the pathophysiology of the human growth hormone/insulin-like growth factor-I (GH/IGF-I) axis. Hence, the number of genes whose alterations appear related to, and/or associated with the different manifestations and classifications of human pathological growth has increased considerably. In the present chapter we review the genetic and molecular basis of proportional undergrowth due to GH or IGF-I deficiency or resistance. Thus, we will examine the different genes related with the GH/IGF-I axis and their various alterations that are currently known to be implicated in the etiopathogenesis of proportional undergrowth.

2. MOLECULAR BASES OF PROPORTIONAL UNDERGROWTH

Intensive investigations during the last two decades have demonstrated the critical role of the GH/IGFs system in all phases of mammalian growth, from intrauterine growth through to childhood and puberty.

A deficiency in the insulin-like growth factors (IGFs), in particular IGF-I, may be the consequence of a primary or secondary IGF-I deficit, manifested by the absolute or relative absence of detectable IGF-I in the serum or plasma (Table 1). GH insufficiency due to defects in the synthesis and/or secretion of GH is also manifested by low or undetectable circulating levels of GH and IGF-I (Table 1).

Aetiology	Serum GH levels	Serum IGF-I levels	PHENOTYPE
GH deficiency	↓↓	↓↓	Short stature,
GH resistance	→ or ↑	↓↓	prominent forehead and
IGF-I deficiency	→ or ↑	↓↓	underdeveloped nasal bridge
IGF-I resistance	→ or ↑	→ or ↑	

Table 1. Molecular determinants of proportional undergrowth.

Resistance to IGF-I due to defects in the IGF-I receptor or downstream the receptor would also result in a similar phenotype, but with normal or elevated IGF-I levels. Likewise, resistance to the actions of GH, due to defects in the GH receptor or downstream the post-receptor signaling pathway, result in a similar phenotype in the face of normal or elevated GH serum levels and depleted IGF-I concentrations.

A deficiency in IGF-I, regardless of its origin, results in a phenotype of short stature, prominent forehead and an underdeveloped nasal bridge (Fig. 1A). Birth length is normal or slightly reduced in the majority of children with IGF-I deficiency. However, postnatal growth is highly abnormal owing to progressive slowing of the growth velocity, especially after 6 months of age (Fig. 1B).

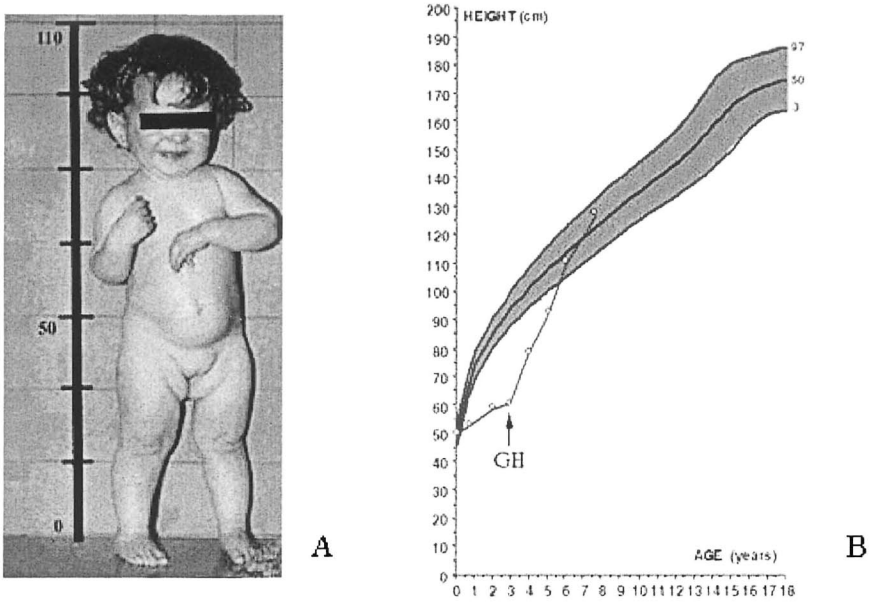

Figure 1. (**A**) The typical phenotype associated with a deficiency of IGF-I A (Adapted from Pérez-Jurado[1]). (**B**) Pathological and normal growth curves. The 3rd to 95th height percentiles are represented at each year of age. The pathological growth curve is indicated by the curve outside the percentile range. GH treatment was administered, as indicated by the arrow, and growth was restored to the normal range.

The complexity of the human GH axis regulation at the hypothalamus and pituitary gland, as well as in target cells and tissues, predicts the existence of a relatively large number of genes that could be potentially involved as the molecular basis of proportional undergrowth (Fig 2.).

3. GENETIC DETERMINANTS OF GH DEFICIENCY

The frequency and prevalence of GH/IGF-I deficiency is difficult to estimate, and may vary depending upon the different diagnostic criteria applied and the ethnical origin of the studied population. Previous studies estimated a prevalence of idiopathic GH deficiency of 1/3480[2]. Approximately 5% to 30% of patients with GH deficiency have an affected first degree relative, thus suggesting the possibility of an underlying genetic defect. Furthermore, the fact that only 20% of the sporadic cases of GH deficiency is the result of environmental factors or

hypothalamic/hypophyseal anatomical defects as revealed by magnetic resonance imaging, suggests also the possibility that a high proportion of the sporadic cases may be also caused by genetic defects[3].

Proportional undergrowth with a genetic basis may be caused by many different genetic determinants among the genes involved in the regulation of the GH/IGF-I axis. The associated phenotypes and their molecular basis will constitute the main body of this chapter's content.

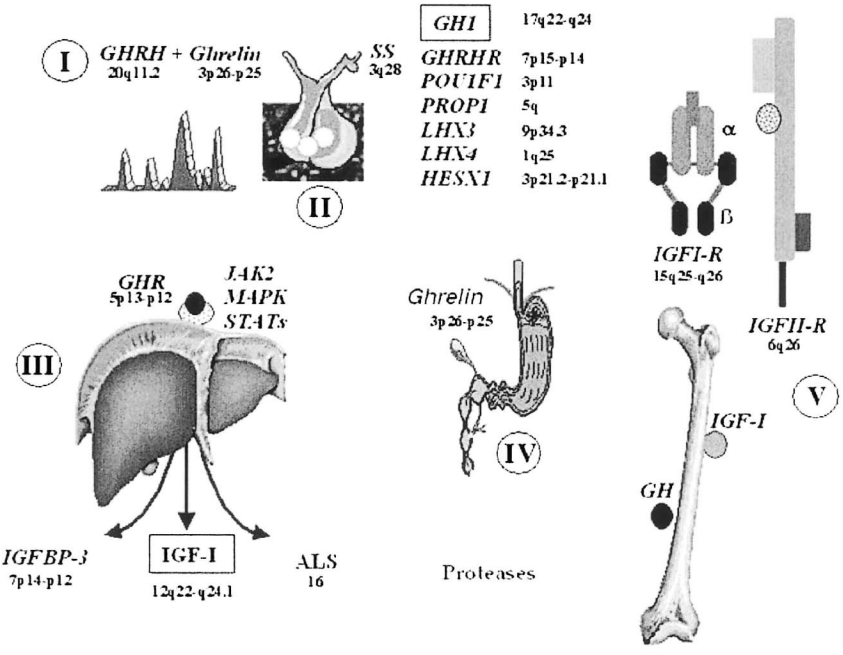

Figure 2. The complexity of the multilevel GH/IGF-I axis regulation. The chromosomal location of all involved genes is indicated. Level I: the hypothalamus with the releasing factor GHRH, ghrelin, and somatostatin (SS), that regulate pituitary GH secretion; level II: the anterior pituitary gland with the transcription factors involved in its embryonic development and cell lineage specification; level III: the liver, as the main organ for the synthesis of IGF-I, stimulated by GH and mediated by the GHR at the cell membrane and by the intracellular second messenger signaling cascade; level IV: the stomach, as the main producer of ghrelin; level V: the GHR and IGF-I receptors and downstream secondary elements in target tissues, *eg.*, the bone.

3.1 Familiar isolated GH deficiency (IGHD)

Four different forms of IGHD differing in the degree of GH deficiency, molecular basis, mode of inheritance, and response to recombinant-derived GH treatment have been described (Table 2).

IGHD type	Mode of inheritance	Endogenous GH	Clinical characteristics	Gene(s) involved (chromosome)	Most frequent mutations
IA	AR	Absent	Neonatal hypoglucemia; consanguiniety; presence of different antibodies against GH at the start of the GH treatment	*GH1* (17q22-24)	Homozygous deletions, missense mutations that generate null alleles
IB	AR	Reduced	Normal response to pharmacological GH stimulation tests; good response to GH therapy	*GHRH-R* (7p14)	Nonsense mutations and splice site mutations in homozygosity
				GH1 (17q22-24)	Compound heterozygosity of point mutations
II	AD	Reduced	Normal response to pharmacological GH stimulation tests; good response to GH therapy	*GH1* (17q22-24)	Heterozygous mutations that affect regulatory sequences such as splice sites or conserved residues
III	XL	Reduced	Hypogammaglobulinemia; increase in β-lymphocytes and immunoglobulins after the start of treatment	*BTK* (Xq21.3-q22)	Splice site mutations

Table 2. Familiar Isolated GH Deficiency (IGHD).

3.1.1 IGHD type IA (IGFD I; OMIM 262400)

This is the most severe form and its incidence is unknown. Patients affected with IGHD IA usually have a normal or slightly reduced birth length and may present with hypoglycaemia during the neonatal period. However, in all of these subjects there is a very reduced growth rate after 6 months of age. The levels of circulating GH are undetectable, both in basal conditions and after all classes of provocative stimuli.

IGHD IA has an autosomal recessive pattern of inheritance and in the majority of the cases is due to homozygous deletions within the GH gene cluster encompassing the pituitary *GH* gene (*GH1,* OMIM +139250). The most frequent genetic defect (approximately 70% of the cases) detected in IGHD IA consists of a deletion of 6.7 kb, but other deletions of 7.6 kb, 7 kb, 45 kb and a double deletion in the *GH* gene cluster have been also described[4]*.

As expected for an autosomal recessively transmitted disorder, a high incidence of consanguinity is observed in the families of patients with IGHD IA, which suggests that these patients inherited two identical mutated alleles. Interestingly, the heterozygous carriers of these deletions are not GH

deficient, indicating that the presence of one single *GH1* allele is sufficient for maintaining GH production.

The human *GH* gene cluster is located on the long arm of chromosome 17 (17q23) and spans 66.5 kilobases (kb) (Fig. 3). It is comprised of five genes aligned in the same transcriptional orientation. From centromere to telomere the pituitary *GH* gene (*GH1*), chorionic somatomammotrophin pseudogene 1 (*CSHP1*), chorionic somatomammotrophin gene 1 (*CSH1*), placental *GH* gene (*GH2*), and gene 2 for chorionic somatomammotrophin (*CSH2*) are located. Each of these five genes consists of five exons and four short introns with a high degree of homology throughout their entire sequence (92% to 98%), suggesting that all of these genes arose from a single ancestral gene as a result of homologous, but unequal, recombinations that resulted in gene duplications. The presence of numerous *Alu* sequences in the cluster suggests that they could have been implicated in the recombination processes that gave rise to the actual *GH* gene cluster[5]. *GH1* is expressed in the somatotrophs of the anterior pituitary and its major protein product is a pro-hormone of 217 amino acids that includes in the N-terminus a signal peptide necessary for its translocation into the endoplasmic reticulum (ER). Within the ER the cleavage of the 26-amino-acid signaling peptide occurs, giving origin to the biologically active form of GH, a 191-amino-acid and 22-kDa protein. Alternative splicing of the primary RNA results in at least four different mRNA transcripts, the majority of which contain the whole coding sequence whereas 10% lack the first 45 bp of exon 3 and less than 5% lack the whole exon 3, or both exons 3 and 4[6].

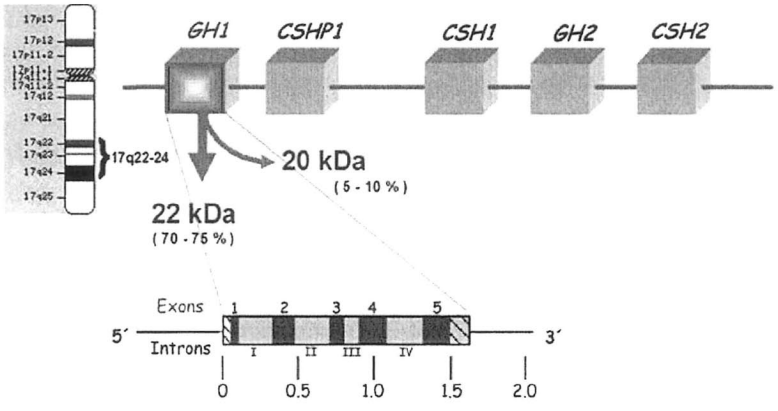

Figure 3. GH cluster on chromosome 17. The five genes, *i.e.*, the pituitary *GH* gene (*GH1*), the chorionic somatomammotrophin pseudogene 1 (*CSHP1*), the chorionic somatomammotrophin gene 1 (*CSH1*), the placental *GH* gene (*GH2*), and gene 2 for chorionic somatomammotrophin (*CSH2*) are oriented in the same direction along chromosome 17q22-24, and share a high degree (~90%) of homology.

In addition to deletions, other types of *GH1* mutations such as point mutations and microdeletions associated with the severe phenotype of IGHD IA and undetectable plasma GH levels have been described[7,8]. All of these mutations generate null alleles of *GH1*, producing truncated proteins that are probably degraded intracellularly.

3.1.2 IGFD type IB (IGHD IB; OMIM #173100)

IGHD IB is transmitted according to a Mendelian autosomal recessive pattern and is associated with low but detectable plasma GH levels after standard pharmacological stimuli. Other endocrine functions are not affected, and therefore the phenotype is less severe than in type IA.

The identification of a homozygous mutation in the mouse growth hormone releasing hormone receptor gene (*ghrhr*) as the molecular basis of the defect in the *Little* mouse, an animal model for autosomal recessive IGHD[9], suggested that the human homologue, *GHRHR,* could be a good candidate to explain at least some cases of familiar IGHD. Indeed, since then, several mutations in *GHRHR* have been demonstrated as the main cause of IGHD IB[10-17], whereas mutations in *GHRH* have been excluded[18].

GH secretion is regulated by the interaction of GH releasing hormone (GHRH) and somatostatin, being released in 10 to 20 pulses in each 24-h cycle. The timing of GH pulses is primarily supervised by intermittent somatostatin withdrawal[19], and the amplitude of GH pulses is driven by GHRH. Thus, GHRH acting through the GHRH receptor (GHRHR), plays a pivotal role in the regulation of GH synthesis and secretion in the pituitary. In humans the gene that codes for the GHRHR, *GHRHR,* has been located on chromosome 7p14[20]. Analysis of its genomic structure revealed the existence of 13 exons. The corresponding cDNA encodes a 423-amino-acid protein that has seven putative transmembrane domains, characteristic of G protein-coupled receptors. It is a member of the secretin family of G protein-coupled receptors and has 47%, 42%, 35%, and 28% identity with receptors for vasoactive intestinal peptide, secretin, calcitonin, and parathyroid hormone, respectively.

Splicing mutations in *GH1* have been also associated with IGHD IB. Cogan *et al.*, detected a G>C tranversion in the donor splice site of intron 4 of *GH1*[21]. The mutation activated a cryptic splice site 73 nucleotides upstream of the exon 4 splice donor resulting in the loss of amino acids 103 to 126 and in a reading frameshift that caused the incorporation of 94 novel amino acids. The authors suggested that the aberrant protein would not be secreted normally since the affected region, encoded by exons 4 and 5, is involved in the correct targeting of the protein to the secretory granules.

More recently, Abdul-Latif *et al.*, reported a novel IVS4+5 G>C transversion in *GH1* of members of a highly inbred Bedouin kindred with the clinical features of type IB IGHD[22]. Through a similar mechanism, as described by Cogan *et al.*, the transversion does generate a different protein by means of activating a cryptic splice site[21]. Interestingly, 48.5% of heterozygous carriers presented with short stature (–1.7 SD) in spite of normal circulating levels of GH[23].

3.1.3 IGHD type II (IGHD II; OMIM #173100)

IGHD II presents clinical characteristics and diagnostic criteria similar as for patients with IGHD IB[24], but it is associated with an autosomal dominant mode of inheritance. Genetic linkage studies demonstrated cosegregation of IGHD II with *GH1* in the majority of the families, while *GHRH* was excluded by linkage analysis in all of the families studied[18]. Most of the IGHD II cases described, involved *GH1* mutations that cause aberrant splicing of *GH1* transcripts[8,25], affecting sequences necessary for normal mRNA processing ("splice enhancers") or the secondary structure of the heteronuclear RNA. All of these intronic mutations produce the same effect on the post-transcriptional processing of the RNA and result in the loss of exon 3 of *GH1* in the mature mRNA. The resulting mutant GH protein is a previously described 17.5-kDa protein that lacks amino acids 32–71, including a cysteine residue. Although the dominant negative effect of these mutations has not yet been fully defined in humans, it has been shown that in transgenic mice, overproduction of the 17.5-kDa isoform, prevents maturation of the GH secretory vesicles, leading to somatotrophs loss and causing anterior pituitary hypoplasia[25].

More recently, three heterozygous missense mutations of *GH1* have been described that result in a milder IGHD II phenotype than that caused by exon 3 skipping[26,27]. All three affect conserved residues, which apparently are critical for the molecular mechanism of GH secretion. Subtle mutations in *GH1* have been regarded as a comparatively rare cause of short stature. Miller *et al.* postulated that this bias might be due to the patient selection criteria usually adopted for *GH1* mutational screening rather than to the inherent mutational properties of the gene[28]. They screened *GH1* in 41 Caucasian individuals selected for short stature, reduced height velocity, and bone age delay; a group of 22 individuals with short stature and idiopathic GH deficiency, and a group of 154 controls. Twenty-four novel *GH1* mutations were identified. Signal transduction activity was assessed using *in vitro* expressed variants coding for 13 of the novel missense mutations. Reduced ability to activate the postreceptor JAK/STAT signaling pathway (see Sections 3 and 3.1 of this chapter) was found in six of these alterations.

A total of 15 novel *GH1* mutations were considered to be of probable phenotypic significance. Thus these lesions are more prevalent than previously recognized and although most of them may be insufficient on their own to account for the observed clinical phenotype, they are, nevertheless, likely to play a contributory role in the etiology of short stature.

3.1.4 IGHD type II (OMIM #307200)

Only a few families with IGHD with an X-linked recessive pattern of inheritance have been described. All of the affected males presented with hypogammaglobulinemia[29,30]. Exogenous GH treatment to these patients increased β lymphocytes and elevated plasma immunoglobulin levels[31]. Genetic analysis of various affected families suggested that the combination of X-linked agammaglobulinemia (XLA) and isolated GH deficiency could be due to an alteration of the *XLA* gene (Bruton tyrosine kinase, *BTK*) on Xq21.3–q22 and/or of a contiguous locus that is probably involved in the expression of GH[32]. However, point mutations in *BTK* have been demonstrated to be the sole cause of the immunodeficiency and GH deficit in at least two families[33,34].

Other forms of X-linked IGHD probably exist and this could explain the preponderance of males in relation to females with IGHD. Current evidence[32,35] suggests that there are various loci on the X chromosome implicated in GH regulation: Xp22.3 and Xq13.3–q21.2.

3.2 *GH1* promoter and locus control region (LCR)

The proximal promoter region of *GH1* is highly polymorphic containing at least 16 single nucleotide polymorphisms (SNPs)[36,37]. Fifteen SNPs were shown to be arranged in 40 different haplotypes[38] (Fig. 4A). Functional analysis showed that 12 more common haplotypes were associated with a significantly reduced level of reporter gene expression *in vitro* whereas 10 rarer haplotypes were associated with significantly increased level. Individual SNPs contributed to promoter strength in a highly interactive and non-additive fashion. Haplotype partitioning was successful in identifying six SNPs as major determinants of *GH1* expression. An association between adult height and the mean *in vitro* expression value corresponding to an individual's *GH1* promoter haplotype combination was noted, although only 3.3% of the variance of adult height was found to be explicable by this parameter[38].

The expression of the human *GH1* is also influenced by a locus control region (LCR) located between 14.5 kb and 32 kb upstream of *GH1*[39]. The

LCR contains multiple DNase I hypersensitive sites and is required for the activation of the *GH* cluster genes in both pituitary and placenta[40,41]. Two DNase hypersensitive sites (I and II) containing binding sites for the pituitary-specific transcription factor POU1F1 are responsible for the high level of somatotroph-specific expression of the *GH1*[42-45]. Horan *et al.*, identified three SNPs within sites I and II of the LCR (Fig. 4B), and ascribed these to three distinct LCR haplotypes[38]. Functional analysis using a series of LCR-*GH1* proximal promoter constructs demonstrated that the LCR enhanced proximal promoter activity by 2.8-fold depending upon the proximal promoter haplotype, and that the activity of the proximal promoter haplotype was also differentially enhanced by different LCR haplotypes. Thus the genetic basis of inter-individual differences in *GH1* gene expression appears to be much more complex than initially thought.

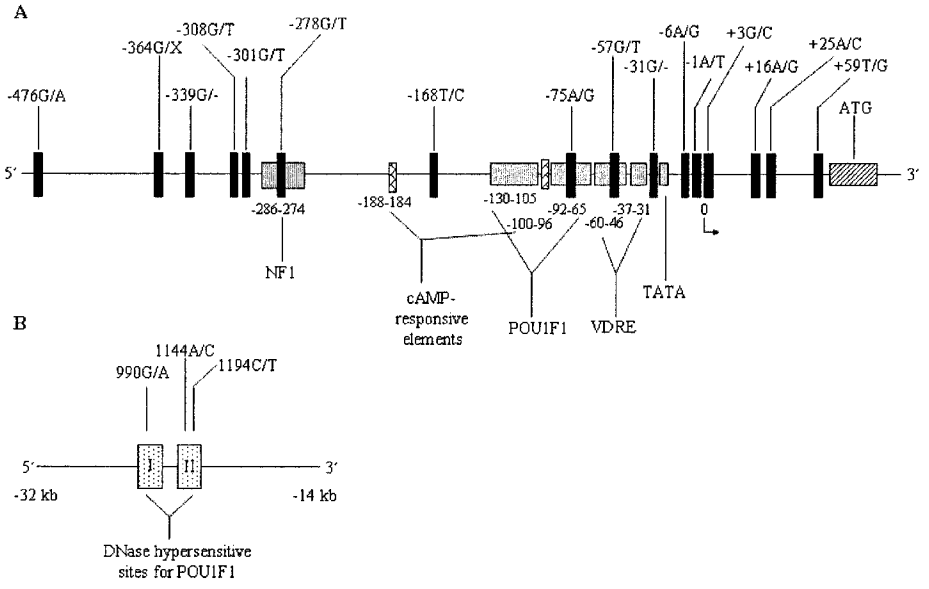

Figure 4. Location of the 16 reported SNPs in the *GH1* promoter **(A)** and the three SNPs in the LCR **(B)**. **(A)** The positions of the binding sites for the transcription factors: nuclear factor 1 (NF1), POU1F1, and vitamin D receptor (VDRE) as well as the location of the cAMP-responsive elements, the TATA box, the transcriptional start site (denoted by an arrow), and the translational initiation codon (ATG) are also shown. The hatched box represents exon 1. **(B)** Positions of the three SNPs in the LCR relative to the two DNase hypersensitive sites I and II, required for the pituitary-specific expression of *GH1*[43]. A, Adenine; C, cytosine; G, guanine; T, thymine.

3.3 Biologically inactive GH: Kowarski syndrome (OMIM # 262650)

Kowarski *et al.*, described two unrelated boys with growth retardation, normal immunoreactive GH after stimulation, and low levels of IGF-I[46]. They deduced that the children had biologically inactive GH. The short stature of Kowarski syndrome is caused by biologically inactive GH (OMIM # 262650) and is characterized by lack of GH action despite high immunoassayable GH levels in serum and marked catch-up growth to exogenous GH administration. In 1996, Takahashi *et al.*, reported a patient with short stature and low GH activity[47]. Isoelectric focusing of the proband's serum revealed the presence of an additional peak. Sequencing of *GH1* revealed a heterozygous C>T transition that resulted in the amino acid alteration R77C. Further studies demonstrated that the child's GH not only could not activate the GH receptor (GHR) but also inhibited the action of wild type GH because of its greater affinity for GHR and GH binding proteins (GHBPs). Takashi *et al.*, later described another case, where the girl's height was 3.6 SD below the mean for age and sex[48]. Bone age was delayed by 1.5 years. She had a prominent forehead and a hypoplastic nasal bridge with normal body proportions. Results of biochemical studies showed the lack of GH action despite high immunoassayable GH levels in serum and marked catch up growth to exogenous GH administration. The studies were compatible with the theory of the production of bioinactive GH, which prevented dimerization of the GH receptor and thus affecting the signal transduction of GH. The patient was shown to be heterozygous for an A>G substitution in exon 4 of *GH1* resulting in the amino acid change, D112G.

In a recent report, 74 children with familial short stature were screened for the presence of mutations in *GH1*[49]. The functional characterization of one novel variant, I179M, identified in a Spanish patient, showed normal misfolding and secretion patterns as well as normal activation of STAT5, in the face of a reduced ERK activation (approximately 50%). It should be noted that the I179M variant did not cosegregate with the short stature phenotype in the family, strongly suggesting that this variant is on its own insufficient to fully account for the observed phenotype.

3.4 Combined pituitary hormone deficiency (CPHD; OMIM # 262600)

Combined pituitary hormone deficiency, previously also known as panhypopituitarism, is characterized by a phenotype of proportional undergrowth in the presence of a deficiency in one or more of the trophic pituitary hormones [thyroid-stimulating hormone (TSH), prolactin (PRL),

adrenocorticotropic hormone (ACTH), follicle-stimulating hormone (FSH) and luteinizing hormone (LH)] in addition to GH deficiency. At least five different loci responsible for this hereditary disease have been established (Table 3). All of them encode pituitary-specific transcription factors that are involved in the activation and control of the embryonic development and cell lineage specification of the anterior pituitary (Fig. 5).

Gene involved (chromosome)	Mode of inheritance	Affected pituitary hormones	Other clinical characteristics	Most frequent mutations
POU1F1 *(PIT1)* (3p11)	AR or AD	GH, PRL, TSH	Short stature, possible hypoplasia of the pituitary gland; undetectable GH and PRL	DNA binding domain mutations (AR) or homeodomain mutations (AD)
PROP1 (5q)	AD or AR	GH, PRL, TSH, LH, FSH (ACTH)*	Short stature, possible hypo- or hyperplasia of the pituitary gland; undetectable LH and FSH	DNA binding domain mutations
LHX3 (9q34)	AR	GH, PRL, TSH, LH, FSH	Short stature, "stiff neck"; possible hypo- or hyperplasia of the pituitary gland	Missense mutations at conserved residues; homozygous deletions
LHX4 *(1q25)*	AD	GH, PRL, TSH, LH, FSH, ACTH	Short stature, defects in the *sella turcica*	Missense mutations at conserved residues
HESX1 *(3p21.2-21.1)*	AD	GH, variable for the other pituitary hormones	Short stature, congenital optical atrophy, septo-optic dysplasia; anterior pituitary hypoplasia; *diabetes insípida*	DNA binding domain mutations affecting conserved residues

Table 3. Combined pituitary hormone deficiency (CPHD). * Observed approximately in one third of affected patients.

3.4.1 POU1F1 (POU domain, class 1, transcription factor 1; OMIM + 173110)

Dwarf *Snell* and *Jackson* dwarf mice present an autosomal recessive phenotype associated with specific deficits in GH, PRL and, occasionally TSH. Both mutant mice are caused by different mutations in *Pit1* (Pituitary transcription factor 1). The similarity of the phenotype with some forms of CPHD in humans led to the analysis of the homologous gene, *i.e., POU1F1* (POU domain, class 1, transcription factor 1, previously known as *PIT1,* pituitary specific transcription factor 1). The human *POU1F1* (Fig. 6) is located on chromosome 3p11 and consists of 6 exons and 5 introns[55]. It encodes a 290-amino-acid, 33-kDa homeobox protein that contains two specific DNA binding domains, the *POU*-specific domain and the *POU* homeodomain. Through the DNA binding domains, *POU1F1* binds to

specific sequences located in the promoter[56] and distal locus control region[45] of the human *GH1* (Fig. 4). Binding of POU1F1 as a dimer to the two *cis*-acting elements present in the promoter of the *GH1* seems to be essential for driving *GH1* expression in the pituitary. Mutations of the functional domains of *POU1F1* may be inherited as dominant or recessive traits. They are the cause of a combined deficiency of GH, TSH, and PRL because the specific activation of the *GH*, *PRL*, and *β-TSH* coding genes requires binding of POU1F1 to the cis-acting elements present in the promoter region of *GH1*, *PRL* and *β-TSH* coding genes[6,57-59].

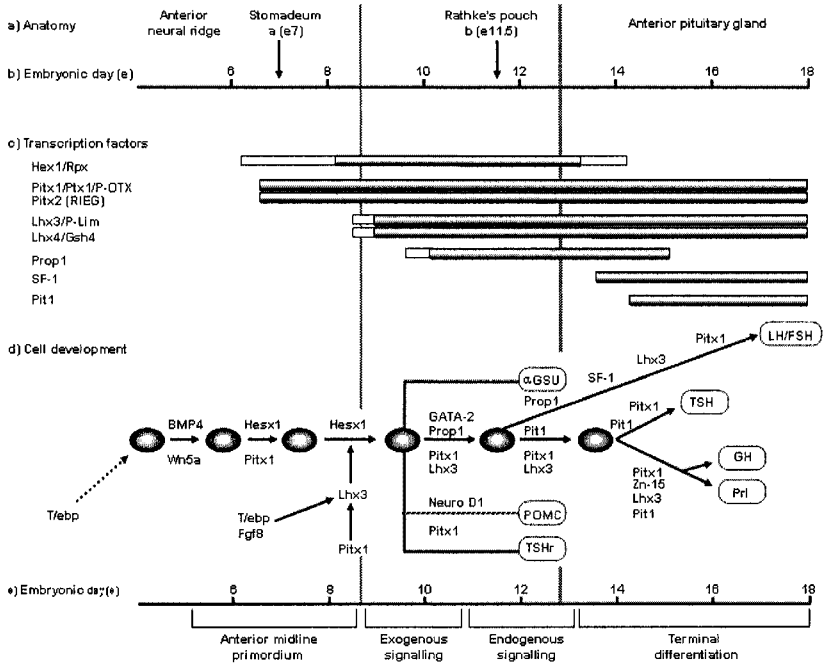

Figure 5. Transcription factors involved in the ontogenic development of the mouse anterior pituitary cell lineage. The finely tuned spatio-temporal expression pattern of pituitary specific transcription factors drives the cell-lineage specification during ontogenic development of the anterior pituitary. Hesx1 is first expressed and plays a role in the formation of the anterior neuroectoderm. Prop1 is expressed in somatotrophs, lactotrophs, and thyrotrophs during embryonic development and seems to be required for the extinction of Hesx1 expression[50] and to promote Pit1 expression, required to promote the expression of *GH1*, *PRL* and *β-TSH* coding genes. Lhx3 is expressed from early developmental stages in the mouse pituitary, and its expression between embryonic day 10.5 (e10.5) and e12.5 seems to be critical for the posterior differentiation of thyrotrophs, lactotrophs, somatotrophs, and gonadotrophs. Lhx4 expression becomes restricted to the future anterior lobe of the pituitary where it seems to be supportive, but not essential, for specification of gonadotroph cells and to the specification, expansion, and terminal differentiation of the other pituitary cell lineages. (Adapted from PE Mullis[51]).

Several recessive and at least three dominant mutations in the human *POU1F1* have been identified in sporadic cases and in multiple families with CPHD[50] (Fig. 6), which cause distinct phenotypes depending on the type of mutation. Production of a truncated protein in homozygosity due to a R172X mutation is associated with a predominant TSH deficiency and congenital cretinism[54,60]. A missense mutation in the POU specific domain, R158P, either in homozygosity or heterozygosity in combination with a null allele, results in the absence of GH and PRL secretion, partial TSH deficiency, and a normal pituitary size[54]. Finally, a *de novo* heterozygotic missense mutation has been described in a patient with panhypopituitary dwarfism[61]. This mutation is located further down from the DNA binding domain (R271W) and the mutant protein appears to have a dominant negative effect that inhibits the DNA binding of the normal protein. The discovery of the same mutation in another Japanese family, in which various carriers had no pathological phenotype, revealed the monoallelic expression of *POU1F1* through genomic imprinting in nonaffected carriers[62].

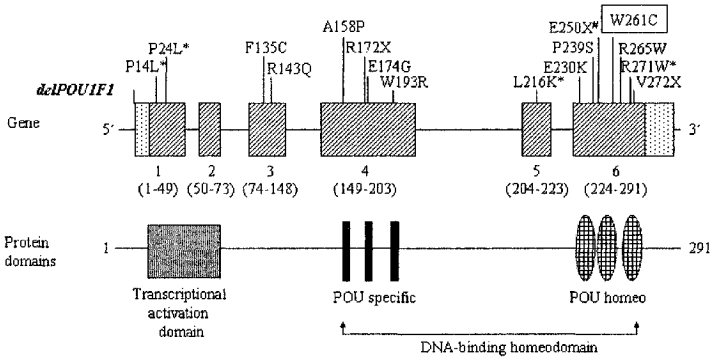

Figure 6. Schematic representation of *POU1F1*. Exons 1–6 are shown as striped boxes for coding sequence and spotted boxes for noncoding regions. Mutations, noted at their locations, without an asterisk represent recessive mutations and those with an asterisk represent dominant mutations. Recessive mutations produce varying degrees of loss of DNA-binding and/or transcriptional activation functions. All dominant mutations affect the POU specific domain[52]. Mutation W261C is boxed as it represents the *Snell* dwarf mouse mutation and has not been found in humans[53]. A hash is located against the mutation E250X as it is due to both a missense mutation at codon 250 and a 1 ^{1}bp deletion at nucleotide 747, which causes a missense mutation at codon 249. DelPOU1F1 is a deletion of the entire gene[54]. The protein domains showing the transcriptional activation domain and the homeodomain are shown in the lower panel.

* Amino acid abbreviations: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; L, leucine; M, methionine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; W, tryptophan; X, termination.

3.4.2 PROP1 (Prophet of Pit1, paired-like homeodomain transcription factor; OMIM + 601538)

The *Ames* dwarf mouse presents with a phenotype similar to that expressed by the *Snell* dwarf. This similarity helped to define another locus involved in dwarfism, *i.e.*, the *Prop1* locus. Localization of the *Prop1* in the *Ames* mouse by Sornson *et al.*[53], led to the localization of the human ortholog on the long arm of chromosome 5 (5q). *PROP1* expression precedes and is a requisite for the expression of *POU1F1*. The human *PROP1* gene spans approximately 3 kb and consists of three exons encoding a 223 amino acid protein with a central DNA, paired-like, binding domain and a transcriptional activation domain (Fig. 7).

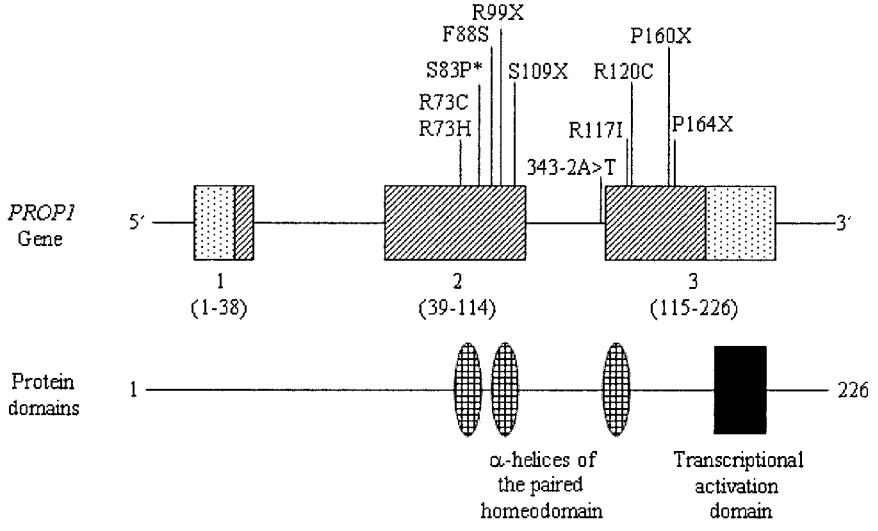

Figure 7. Schematic representation of *PROP1*. Coding sequences of exons 1–3 are represented by shaded areas, while noncoding sequences are shown as spotted areas. Mutations are noted at their corresponding locations. Mutation S83P represents the *Ames* mouse dwarf mutation and has not been found in humans[53]. The protein domains showing the α-helices of the paired homeodomain and the transactivation domain are shown in the lower panel.

The transcription factor PROP1 is expressed in the somatotrophs, lactotrophs, and thyrotrophs during embryonic development. Its expression seems to be required for the extinction of *HESX1* expression[50], another pituitary transcription factor involved in the etiopathogenesis of CPHD (see later). PROP1 binds as a dimer to promoter P3 elements (palindromic

TAAT(NNN)ATTA sequences) localized in the 5′-UTR of *POU1F1*. Several mutations in the human *PROP1* associated to the CPHD phenotype have been identified in the recent years[50,63] (Fig 7). The hormonal phenotype (Table 3) is variable, including deficiencies of GH, TSH, PRL, LH, and FSH. The hormone deficiencies emerge gradually during postnatal development in a typical order rather than being uniformly present at once[63]. In some cases, affected patients may not produce LH and FSH in sufficient amounts to enter puberty spontaneously, while in other cases the gonadotropin secretion gradually decreases with increasing age without affecting the spontaneous puberty switch-on[64]. ACTH may be also affected with increasing age[65,66]. Most of the recessively inherited cases of CPHD in Europe are due to *PROP1* mutations. The allelic variants described include two simple amino acid substitutions, R120C and F117I, and a deletion of two basepairs in exon 2 (301delAG) that produces a frameshift reading error from codon 101 with an early stop codon appearing at 109[64]. The 301delAG mutation could be the most frequent cause of familial combined pituitary hormone deficiency[67].

3.4.3 LHX3 (LIM-homeobox-3; OMIM *600577) and LHX4 (LIM-homeobox-4; OMIM*602146)

LHX3 and *LHX4* encode two pituitary specific LIM-type homeodomain transcription factors that play a key role in the determination of the pituitary cell lineages. In the mouse, both *Lhx3* and *Lhx4* are expressed from early embryological stages in Rathke's pouch (Fig. 5). During subsequent developmental stages, *Lhx3* expression is detectable at high levels before the initial detection of the α-glycoprotein subunit (α-GSU), localized to the anterior and intermediate lobes of the pituitary gland and persists in the adult pituitary, where it is expected to still play a functional role during adult life.

Human *LHX3* spans 7.2 kb, consists of 7 exons and is located in the subtelomeric region of chromosome 9 (9q34.2-34.3, Ref 68). A homozygous missense mutation (Y116C) in the LIM2 domain of *LHX3* that impairs the transcriptional activation function, and an intragenic 23 amino acid deletion that includes the DNA binding domain, have been identified as the molecular cause of the disease in some CPHD patients[69]. In these cases, additionally to the combined hormonal deficit of GH, TSH, PRL, FSH, and LH, a rigid cervical spine leading to a limited head rotation was the clinical hallmark of the recessively inherited disorder[69].

In contrast to the sustained expression pattern of *Lhx3* during developmental of the mouse pituitary gland, *Lhx4* expression becomes restricted to the future anterior lobe of the pituitary. *Lhx3*$^{-/-}$ and *Lhx4*$^{-/-}$ mice studies have shown that the formation of a definitive pouch is dependent of

both *Lhx3* and *Lhx4*[70,71]. During the next step, *i.e.*, the actual formation of the pituitary gland, the expression of *Lhx3* is essential. Later, together with *Lhx3*, *Lhx4* expression seems to be supportive, but not essential, for specification of gonadotroph cells and to the specification, expansion and terminal differentiation of the pituitary cell lineages.

LHX4 is located on the long arm of chromosome 1 (1q25). It spans more than 45 kb, and is composed of 6 exons. The protein contains two LIM-like domains (encoded by exons 2 and 3, respectively) and one homeodomain, encoded by exons 4 and 5[72]. A heterozygous intronic point mutation in the splice acceptor preceding exon 5 (G>C) was recently identified[72] in a family with a phenotype of combined GH, TSH, and ACTH deficiency, with a small sella turcica, hypoplastic anterior hypophysis, and ectopic posterior hypophysis. The mutation in the splice site acceptor results in the activation of two cryptic splice-acceptor sites present in exon 5, giving origin to two different shorter transcripts of 1252 and 1247 amino acids (vs. 1264 in the wild-type), respectively. Both alternative transcripts lack conserved residues which are essential components of the homeodomain.

3.4.4 HESX1 (homeobox 1 expressed in embryonic stem cells; OMIM: *601802)

The *Hesx1* gene, originally named *Rpx* for Rathke pouch homeobox, was originally identified in mouse[73]. Dattani *et al.*, generated a *Hesx1* null mutant mouse and these mice exhibited variable anterior CNS defects and pituitary dysplasia[74]. Mutants have a reduced prosencephalon, either anophthalmia or microphthalmia, defective olfactory development, and bifurcations in the Rathke pouch. Neonates exhibit abnormalities in the corpus callosum, the anterior and hippocampal commissures, and the septum pellucidum. Thus the *Hesx1* knockout mouse phenotype is comparable to the phenotype shown in patients with septo-optic dysplasia (SOD; OMIM #182230), initially named as Morsier syndrome. The term septo-optic dysplasia was coined in 1956 by de Morsier[75] who pointed out the association of optic nerve hypoplasia and absence of the septum pellucidum. This syndrome is characterized by the triad of pituitary hypoplasia, optic nerve hypoplasia and agenesis of midline brain structures, including the corpus callosum and septum pellucidum (Fig. 8). The phenotype is highly variable, with 62% of affected individuals having hypopituitarism and 30% having all three manifestations[76-78].

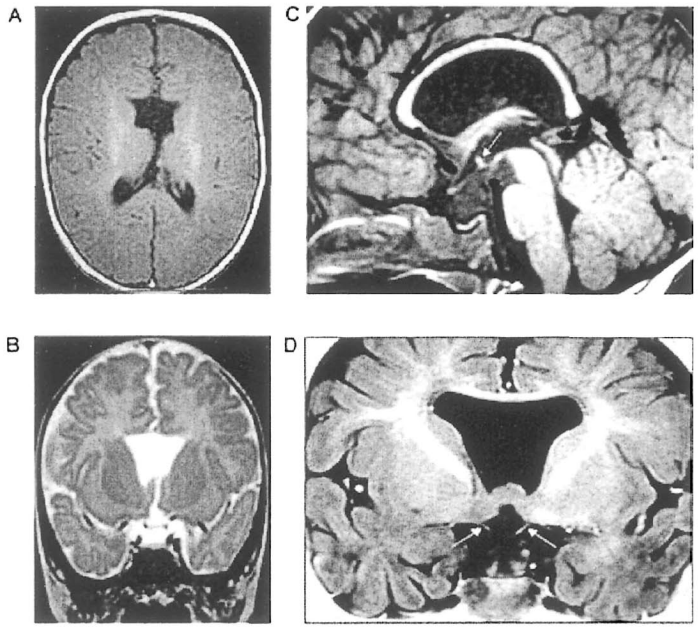

Figure 8. **(A,B)** Axial (T1) magnetic resonance image (MRI) of a patient with septo-optic dysplasia (SOD). Squared ventricular cavity due to the absence of the septum. **(C)** MRI of the medial line section of the sagittal T1. Ectopic posterior pituitary (indicated with an arrow) and hypoplasia of the anterior pituitary. **(D)** MRI of the coronal T1. Hypoplasia of the optic nerves (indicated with arrows).

Owing to this similarity, Dattani *et al.* screened the human homologue, *HESX1* (OMIM 601802) in familial SOD patients[74]. *HESXI* spans 1.7 kb and contains four coding exons. They demonstrated homozygosity for a missense mutation, R160C, in the homeodomain of *HESX1* in a brother and sister with agenesis of the corpus callosum and CPHD. The mutation destroyed the ability of HESX1 to bind target DNA. The protein has two functional domains: the engrailed homology repressor domain at the N-terminus, which functions as a promoter-specific repressor, and a homeodomain in the C-terminal, which functions as a transcription factor binding domain[79]. Different studies have since shown that *HESX1* mutations are rare in sporadic SOD patients and that there is incomplete penetrance[79,80]. The *HESX1* mutations found to date are listed in Table 4. The degree of pituitary deficiency varies from an isolated deficit in GH to situations of panhypopituitarism. Diabetes insipidus is present in more than half of the cases. The alteration appears to be in the hypothalamus. This syndrome is

almost always sporadic, although some family cases have been reported which show an autosomal recessive pattern of inheritance.

Mutation (amino acid)	Mutation (nucleotide)	Exon	State	Familial/ Sporadic	Functional domain	Functional consequences	MRI	Ref.
Q6H	18G>C	1	Htz	S	-	Unknown	AP hypoplasia, ectopic PP	80
I26T	77T>C	1	Hz	F	Engrailed homology repressor domain (eh1)	Impaired ability to recruit mammalian Groucho homolog/transducin-like enhancer of split-1 (Gro/TLE1), a crucial corepressor for HESX1, leading to partial loss of repression	AP hypoplasia, ectopic PP, severely hypoplastic AP lobe	81
Fs104, stop codon at 105	306/307 Ins AG	2	Htz	S	Truncated mutant,lacking homeodomain	Lack homeodomain (DNA binding domain)	Left ON hypoplasia, AP hypoplasia, ectopic PP	82
N125S*	374A>G	3	Htz	S	Homeodomain	Slightly elevated DNA binding		79
R160C	478C>T	4	Hz	F	Homeodomain	Loss of *in vitro* DNA binding	Agenesis of CC, ON hypoplasia	74
S170L	509C>T	4	Htz	F	*RESQFL* motif which is unique to *HESXI.*	Reduced DNA binding	Bilateral ON hypoplasia in one sibling and normal in other	80
					Immediately C-terminal to homeodomain		AP hypoplasia, undescended PP	79
T181A	541A>G	4	Htz	S	-	Unknown	AP hypoplasia	80
Fs175, HESX1 terminates at one aa further than Wt	1684delG	4	Htz	S	-	Increased DNA binding which in turn causes increased repression of PROP1-dependent gene activity	Absent CC, thin ON, small AP gland and absence of posterior bright spot.	83

Table 4. Mutations in the human *HESX1* gene. AP, anterior pituitary; aa, amino acid; CC, corpus callosum; ON, optic nerve; PP, posterior pituitary. *N125S mutation occurs at a high frequency in the Afro-Carribean population and may therefore reflect a population-specific polymorphism[79].

4. GH INSENSITIVITY SYNDROME (LARON SYNDROME, GHIS; OMIM #262500)

In 1966, Laron described the cases of three siblings with the clinical and biochemical characteristics of GH deficiency, but with extremely elevated levels of circulating GH[84]. The syndrome was named Laron syndrome but it

is now also referred to as the syndrome of primary GH resistance or primary GH insensitivity. In the following 2 years, the same authors diagnosed 22 patients with a similar clinical picture. These patients were of eastern Jewish origin[85].

Laron syndrome is a rare autosomal recessive condition[86] associated with postnatal growth failure leading to extreme short stature, midfacial hypoplasia, truncal obesity, and hypoglycemia. If these patients do not receive treatment with recombinant GH, the adult height remains between 119 and 142 cm in males and 108 and 136 cm in females[87]. Patients have increased GH levels, associated with a deficiency of IGF-I and IGFBP-3[88,89]. These features commonly result from dysfunction of the GH receptor (GHR) and consequent failure of signal transduction pathways.

In 1984, Laron syndrome was shown to be due to mutations in *GHR* resulting in a resistance to GH[90]. The *GHR* is located on chromosome 5p13-p12[91]. It spans 87 kb and is comprised of 10 exons. *GHR* encodes a receptor of 620 amino acids and a signal sequence of 18 amino acids (encoded by exon 2). Exons 3 to 7 encode for the extracellular domain of 246 amino acids. Exon 8 corresponds to the transmembrane domain of 24 amino acids. Finally, exons 9 and 10 correspond to the intracellular domain made up of 350 amino acids (Fig. 9A).

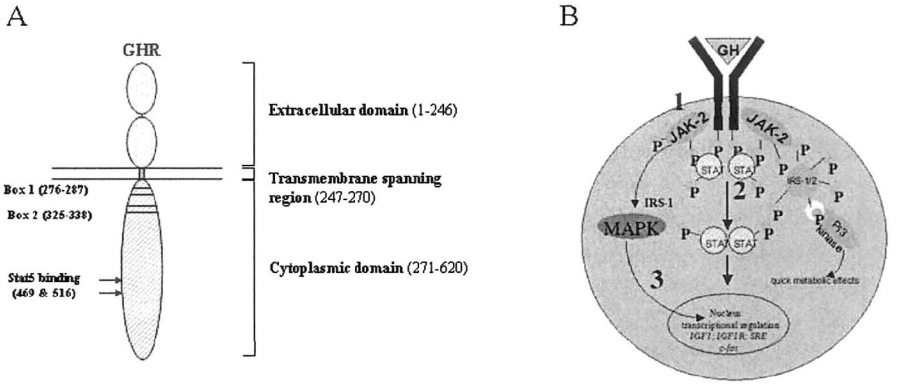

Figure 9. **(A)** Schematic outline of the GH receptor showing the extracellular, transmembrane and cytoplasmic domains. **(B)** The post-receptor intracellular signaling cascade: 1, Activation of cytosolic Janus kinase 2 (JAK-2); 2, activation of the signal transducer and activator of transcription proteins (STAT), STAT1, STAT5, and the mitogen activating protein kinase (MAPK) pathway including extracellular regulated kinase 1 and 2 (ERK1/2), p38, and c-Jun N-terminal kinase (JNK); 3, transcriptional regulation of target genes.

To date, several patients have been diagnosed. Most patients with GHIS have missense, nonsense and splice site mutations in *GHR* exons 4–7, encoding the extracellular domain of the GHR[88,89,92] (Human Gene Mutation Database, www.jgmd.org). These mutations usually result in low or absent serum GH binding protein (GHBP) and thus, failure of GH to bind and signal at GH responsive tissues, associated to a deficiency of IGF-I and IGFBP-3[89,93,94]. The relatively few mutations described in the transmembrane or intracellular domain of the GHR result in truncated GHRs lacking most of the intracellular domain of the receptor (dominant negative mutations) or consist of compound heterozygous mutations involving the cytoplasmic domain[95-99]. A few deletions have also been reported[99,100].

Recently, Laron reviewed the treatment with exogenous recombinant biosynthetic IGF-I and a follow-up of the 60 patients from the original Israeli cohort[92]. Although stimulation of linear growth is observed after the start of the treatment, its effects on growth velocity was not as intense as that observed in GH-deficient patients. Some of the undesired consequences of the exogenous IGF-I treatment could be avoided if the treatment would be initiated at birth or in infancy, but still nowadays it remains the exception with only a small number of children on time-limited clinical trials.

4.1 Defective post-receptor signal transduction

The GHR is a member of the family of cytokine receptors. GHR lacks intrinsic kinase activity and relies on the regulation and activation of cytosolic Janus kinase 2 (JAK-2). A single GH molecule binds two GHR molecules inducing receptor dimerization and hence activation[101]. Binding of GH to the GHR results in a conformational change in the receptor dimer and transphosphorylation of associated JAK-2[102].

JAK-2 is constitutively associated with the receptor at the proline-rich Box1 site (amino acids 276–287), and it is thought that ligand binding may stabilize the preformed receptor-JAK-2 complex[102,103]. Box 2 (amino acids 325–338) of the GHR is though to be required for full activation of JAK-2 by GH[103,104]. After GH stimulation, phosphorylated JAK-2 has been reported to activate the signal transducer and activator of transcription proteins (STAT), STAT1, STAT5, as well as the mitogen activating protein kinase (MAPK) pathway, including extracellular regulated kinase 1 and 2 (ERK1/2), p38, and c-Jun N-terminal kinase (JNK[105-107], Fig. 9B). Activated STAT5 is translocated to the nucleus where the transcription of GH-responsive genes such as *IGF-I* and *IGFBP-3* among others, is modulated[108-110] (Fig. 9B). STAT5 requires anchorage to tyrosine 469 and/or 516 of the GHR for activation[111,112], whereas STAT1, STAT3, and MAPK are activated via JAK2, independently of any direct association with the GHR.

Recently, Kofoed *et al.*, described a patient with the clinical and biochemical characteristics of GHIS but with no mutation in the *GHR*[113]. They found that signal transduction mediated by the GHR occurs by means of at least three well established pathways: the signaling module involving MAPK, ERK1, and 2, STAT, and the phosphatidylinositol-3 kinase B signaling module. After analyzing these signalling pathways in fibroblast cell lines derived from the patient, they found that signal transduction from the GHR to ERK1 and ERK2 was normal. However, GH-induced signalling through the STAT pathways (STAT1, STAT3, STAT5a, and STAT5b) was aberrant in the patient derived cells. STAT1 phosphorylation was increasingly induced (8–10 times) by GH in the patient's fibroblasts as compared to normal fibroblasts. The combined phenotype found in the patient, GHIS and immunodeficiency, was consistent with the presence of a defect in the JAK/STAT system. A missense mutation in *STAT5b* (OMIM *604260) in homozygosis, A630P, was indeed identified. The parents were shown to be heterozygous for this change.

STAT5b is localized to chromosome 17q11.2 and consists of 19 exons. The domains of the encoded protein are represented schematically in Fig. 10. The A630P mutation occurs in exon 15 which encodes for the src homology (SH2) domain of STAT5b.

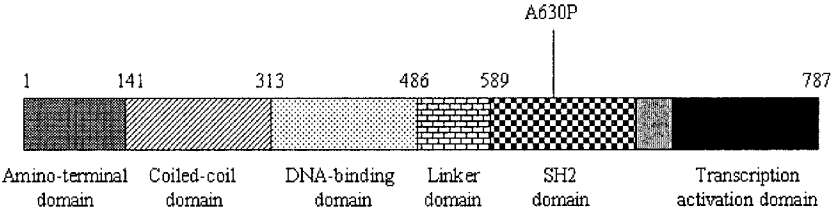

Figure 10. Domains of the STAT5b protein. The location of the mutation identified in the patient with GH insensitivity syndrome is marked in the SH2 domain[113]. Numbers indicate the amino acid positions.

How GHIS is classified is still debatable. Do two types of Laron syndrome, type 1 and type 2, caused by mutations in *GHR* and *STAT5* respectively, exist or is Laron syndrome distinguishable from GHIS as a result of mutations in *STAT5b*? To date only one patient has been found to carry a *STAT5b* mutation; thus further patients need to be screened until a detailed clinical comparison can be undertaken.

5. ANOMALIES IN THE IGF-I AND IGF-I RECEPTOR GENES

5.1 IGF-I gene (*IGF1*) anomalies

As outlined in section 1, GHIS can be also due to a primary resistance to the IGF-I caused by defects in the IGF-I receptor or downstream of the receptor, in the face of normal or even elevated IGF-I levels. The few cases of IGF-I resistance reported up to now are discussed below.

The human *IGFI* gene spans more than 80 kb and consists of six exons and five introns (Fig. 11). *IGFI* has been mapped to the human chromosome 12. As a result of alternative splicing of the primary gene transcript, different IGF-I mRNAs are produced that encode protein precursors with different C-termini, called E domains (Fig. 11). The human IGF-IA protein contains a 35-residue E domain, and the IGF-IB transcript a 77-amino-acid C-terminal extension[118,119].

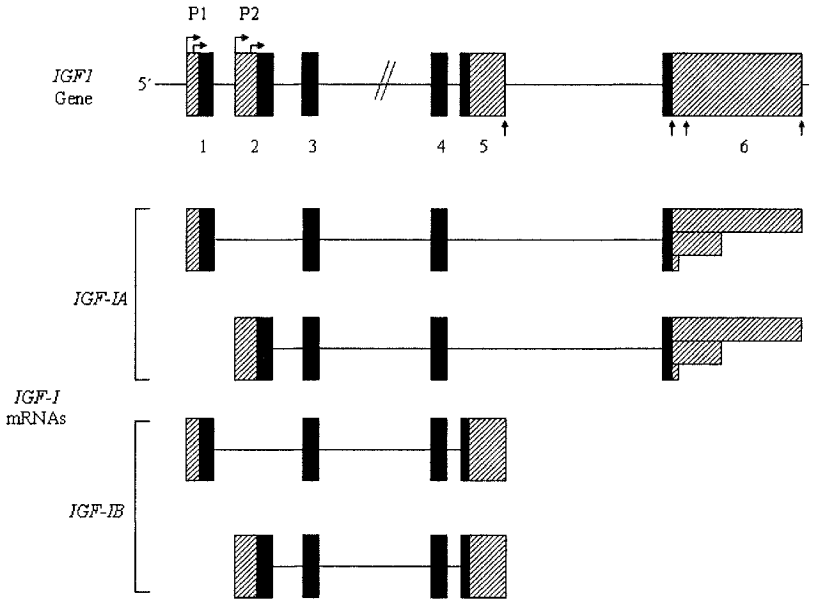

Figure 11. Structure and expression of the human *IGFI*. Structures of different human *IGFIA* and *-IB* transcripts are displayed[114-118]. Exons are numbered and indicated by boxes, with coding regions in black and noncoding segments in stripes. Promoters are shown by the letter P, transcription start sites by arrows and locations of differental polyadenylation at the 3' end by arrows in the gene and by boxes of different lengths in the mRNAs.

Woods *et al.*, described a male, son of consanguineous parents, with extreme short stature, in whom a homozygous deletion of exons 4 and 5 of *IGF1* (OMIM *147440) was demonstrated[120]. The patient had marked mental retardation, small cranial perimeter, acromicria, hypogonadism, delayed motor development, sensorineural deafness (see also Chapter 9 of this book), hypoglycemia during infancy, elevated serum GH levels, and low serum IGF-I levels that did not increase with GH administration. In 2000, Woods *et al.*, reported the results of 1-year recombinant human IGF-I therapy on body composition, bone mineral density, insulin sensitivity, and linear growth in this patient[120]. They concluded that recombinant IGF-I therapy increased bone mineral density (BMD) by 17% and bone mineral apparent density by 7%, indicating that IGF-I has a greater effect on bone growth than bone mineralization. Bone turnover markers and growth velocity also increased on recombinant human IGF-I treatment.

Rasmussen *et al.*, considered *IGF1* and the IGF-I receptor gene (*IGF1R)* as candidates for low birth weight, insulin resistance and type 2 diabetes, but they found no sequence alterations resulting in amino acid substitutions in the two genes in 82 Danish families with type 2 diabetes[121]. Arends *et al.*, then hypothesized that minor genetic variation in *IGF1* could influence pre- and postnatal growth[122]. Three microsatellite markers located in *IGF1* were analyzed in 124 short children born small for gestational age and their parents. Two polymorphic markers showed association with IGF-I levels and short stature and small head circumference. Thus the authors concluded that since low IGF-I levels are associated with type 2 diabetes and cardiovascular disease, they proposed that the *IGF-I* may provide a link between low birth weight and such diseases in later life.

5.2 The Pygmy phenotype

Pygmies show partial GH resistance, which has been suggested to be mediated either at the level of the GH receptor[123,124] or at the level of the IGF-I receptor[125,126]. The GHR has been implicated based on the low levels of GHBP in the plasma of pygmies from Africa and Papua-New Guinea[123,124]. The plasma level of GHBP, the circulating ectodomain of the GHR, is believed to reflect GHR concentrations in target tissues. The concept of IGF-I resistance has been based on the lack of a proliferative response of immortalized lymphocytes derived from African Efe pygmies to IGF-I, and decreased *IGFIR* expression and function in these cells[125,126]. Both GH resistance and IGF-I resistance may lead to short stature, and they are not mutually exclusive. Davila *et al.*, more recently studied two pygmy populations from the Philippines (Aeta and Mamanwa people) that are

unrelated to the African pygmies[127]. Serum GHBP and IGF-I levels were significantly decreased in both pygmy populations, compared to normal-statured Philippino controls. The results, together with previous observations in African and New Guinean pygmies, indicated that short stature is associated with low serum GHBP levels in pygmy populations of diverse origins and in different parts of the world. This strengthens the tentative postulate that the GHBP/GHR system plays an important role in the genetic and perhaps nutritional determination of adult stature in humans. Molecular genetic studies of *GHR* in various pygmy populations may shed further light on the mystery of pygmy short stature.

5.3 IGF-I resistance

There are very few references of patients with short stature that could be included in this category. The first clinical reported case was of a girl with severe growth retardation and elevated IGF-I levels[128]. Other similar cases have been described[129,130]. Further studies of fibroblasts derived from this patient reported by Heath-Monnig *et al.*, were undertaken[129]. They concluded that the resistance to IGF-I action in the patient's fibroblasts was caused by an abnormal production and/or cell association of IGF binding proteins[131]. However, a genetic basis could not been demonstrated in any of these cases.

In 1992, the human gene for the IGF-I receptor, *IGF1R,* was identified[132], (OMIM *147370). It contains 21 exons and spans approximately 100 kb on chromosome 15q25–q26[133].

Approximately 10% of infants with intrauterine growth retardation remain small and the causes of their growth deficits are often unclear. Abuzzahab *et al.* screened for mutations in *IGF1R* in 42 patients with unexplained intrauterine growth retardation and subsequent short stature[134]. They identified a compound heterozygote who had two point mutations in exon 2 of *IGF1R,* that resulted in the amino acid substitution R108Q in one allele and K115N in the other allele. Fibroblasts cultured from the patient showed decreased IGF-I receptor function. The child also had signs of a nonverbal learning disorder, obsessive tendencies and social phobias. Experimental evidence that the IGFs are necessary for normal brain development and function suggest that these mutations may also affect brain growth and neurological development[135] (see also Chapter 8 in this book), the clinical features described in the patient of Abuzzahab *et al.,* may reflect the state of partial resistance to IGF-I[134].

In a second cohort of 50 children with short stature who had elevated circulating IGF-I concentrations, Abuzzahab *et al.*, identified one boy with an *R59X* mutation that reduced the number of IGF-I receptors on fibroblast

cultures[134]. The boy also had microcephaly at birth that persisted, and he had mild retardation of motor development and speech. The patient displayed abnormal facial features, short fingers, clinodactily, wide-set nipples, and pectus excavatum. Both children had intrauterine growth retardation and poor postnatal growth but the phenotypes were not identical.

Thus mutations in *IGF1R* that lead to abnormalities in the function or number of IGF-I receptors may also retard intrauterine and subsequent growth in humans. The identification and understanding of further *IGF1R* mutations and their associated phenotypes may provide insights into both growth retardation and the IGF system in humans.

5.4 IGF-I acid-labile subunit (IGFALS; OMIM +601489)

In vivo, IGF-I and IGF-II are always complexed to one of a family of six IGF-binding proteins, IGFBP1-6 (see Chapter 3 in this book). Prenatally, binary IGFBP–IGF complexes of 50 kDa predominate in the serum, with IGFBP-2 being the most frequently occurring IGFBP moiety. In juveniles and adults, however, 80–85% of serum IGFs are found in a 150-kDa ternary complex composed of one molecule each of IGF, IGFBP3, and a protein that is found only in the serum, the acid-labile subunit (ALS) (OMIM 601489)[136]. ALS is a glycoprotein that is mainly synthesized in the liver and is stimulated by GH[137,138]. This subunit stabilizes the IGF-I and IGFBP-3 complex, reduces the passage of IGF-I to the extravascular compartment and extends its life[139].

The *IGFALS* gene, located on chromosome 16, consists of two exons. The mature ALS protein consists of 578 amino acids preceded by a 27 amino acid signal sequence[140]. The amino acid sequence of ALS is largely composed of 18–20 leucine-rich repeats of 24 amino acids which participate in protein–protein interactions.

An animal model where the *Igfals* gene had been inactivated (the *Igfals*-knockout mouse) resulted in the offspring having a small growth deficit[141]. This modest phenotype was observed despite reductions of 62% and 88% in the concentrations of IGF-I and IGFBP-3, respectively. Therefore, the authors concluded that ALS was required for postnatal accumulation of IGF-I and IGFBP-3 but, consistent with findings supporting a predominant role for locally produced IGF-I, was not critical for growth.

Recently, a mutation was reported in *IGFALS* in a 17-year-old boy who had a delayed onset of puberty, slow pubertal progress, and yet minimal slowing of his linear growth[142]. ALS was undetectable in the serum before and after stimulation with GH. A frameshift mutation was due to a deletion of a guanine at nucleotide 1338 (1338delG) which resulted in the appearance

of a premature stop codon at position 120 (E35FsX120) of the precursor form of ALS. If the protein was expressed, secreted, and stable in the circulation, this mutant ALS protein would retain only seven amino-terminal amino acids after the cleavage of the signal peptide and would therefore lack the IGFBP-3 binding domain. The patient was thought to be homozygous for the mutation but parental DNA was not available. Although they were unable to prove that there was a relation between the ALS deficiency and the patient's clinical condition, many of the clinical and biochemical features were similar to the *Igfals* knockout mouse. Thus, it can be speculated that the lack of ALS might be involved in a subtle impairment of linear growth, a delay in the onset and slow progresses of puberty, and a certain degree of insulin resistance. The ALS deficiency caused by inactivation of the *IGFALS* gene is associated with a severe disruption in the GH–IGF-I axis. These recent findings support the hypothesis that the circulating total IGF-I level might not be the major mediator of the growth promoting actions of GH. The main roles of circulating IGF-I may be the feedback control of GH secretion and the regulation of carbohydrate metabolism through the facilitation of insulin action[143]. Further investigation may reveal whether this disorder represents a rare case of ALS deficiency caused by a rare molecular defect or whether mutations in *IGFALS* might be involved in other cases of delayed growth and pubertal development in children.

6. ALTERATIONS IN SEX CHROMOSOMES

6.1 Mutations in *SHOX* (Short stature homeobox-containing gene; OMIM *312865)

In 1997, two groups isolated a candidate gene for short stature, *SHOX*[144,145]. *SHOX* is located in the pseudoautosomal region (*PAR1*) of the short arm of the X and Y chromosomes[144,145] and thus it is present as two copies in both females and males. *SHOX* encodes a homeodomain protein that has been shown to act as a transcription factor. Rao *et al.*, demonstrated that an individual diagnosed with idiopathic short stature (ISS), carried a mutation in one copy of *SHOX* that cosegregated with the short stature phenotype in the family[144]. They also proposed that *SHOX* was also responsible for the short stature phenotype associated with Turner syndrome (45, XO). Shortly after, mutations and deletions in *SHOX* were identified in families with Léri-Weill dyschondrosteosis[146,147] (LWD, OMIM #127300), a skeletal dysplasia with short stature, which suggested that the same gene is also involved in the skeletal anomalies observed in Turner syndrome. It was

also shown that the more severe form of LWD, Langer mesomelic dysplasia (LMD) was due to the homozygosity of the *SHOX* defect[147] (OMIM #249700). Molecular cytogenetic studies showed that *SHOX* dosage was reduced in Turner syndrome, suggesting that haploinsufficiency of SHOX is responsible for not only the short stature in Turner syndrome but also for the skeletal abnormalities[148,149].

SHOX spans 40 kb of genomic DNA in the 2.6 Mb PAR1 region, and is composed of six coding codons[144,145]. In Turner syndrome the majority of cases are the result of a lack of one X-chromosome (45, XO), thus lacking one copy of *SHOX*. In the other syndromes, the majority of defects found are also deletions of *SHOX*. Most of the single base pair mutations are nonsense mutations that cluster in the homeobox domain and the DNA binding domain of *SHOX* (*SHOX* database: *http://www.shox.uni-hd.de/*).

At the molecular level, there are two alternative splice variants of *SHOX*, *SHOXa* and *SHOXb*. *SHOXa* is the transcriptionally active form. It contains a SH3 binding site and sites for phosphorylation which are absent in *SHOXb*[144,145]. *SHOXa* is widely expressed in different tissues from embryogenesis to adolescence. *In situ* hybridization studies of *SHOX* expression during human embryogenesis have shown that expression occurs in the mid-limb regions; radius, ulna, elbow, wrist, tibia, fibula, knee, and ankle[150] and in the first and second pharyngeal arches. In contrast, the *SHOXb* isoform has a more restricted expression pattern, with the highest level in bone marrow fibroblasts, providing additional evidence for its role in linear growth[144]. Its temporal and spatial expression patterns have led to suggestions that SHOX acts as a key regulator of skeletal development in the middle portions of the long limbs. Recently SHOX was shown to be expressed in hypertrophic/apoptotic chondrocytes of the growth plate, strongly suggesting that the protein plays a direct role in regulating the differentiation of these cells[151]. They also demonstrated that *SHOX* expression leads to cell cycle arrest and apoptosis in osteogenic stable cell lines, primary oral fibroblasts, and primary chondrocytes. They provided evidence that mutations that delete the homeodomain of SHOX (*ie.*, C-terminal truncations) failed to display these activities.

Currently, the only treatment for these disorders involving SHOX is GH therapy. Although growth velocity is increased in some patients, the underlying molecular mechanism remains unclear. One important question is: where does SHOX lie in the GH–IGF-I axis? Are there other treatments more suitable for this group of disorders? Further clinical and basic investigations will hopefully help to answer some of these points.

6.2 Panhypopituitarism

In a single family in which the males were deficient in GH, an in-frame duplication of 33 bp encoding for 11 alanines in a polyalanine tract of *SOX3* (OMIM *313430) was identified[152]. The consequences of this mutation on the function of the protein are not known. The mammalian genome contains a family of genes that are related to *SRY*, the testis-determining gene. The homology is restricted to the region of *SRY* that encodes a DNA-binding motif of the *HMG*-box class (the DNA-binding domain is called *HMG* for high mobility group). These genes have been named SOX, for *SRY*-related *HMG*-box. *SOX3* is a single exon gene on the X chromosome[152] (Xq26.3). Conditional disruption of *Sox3* in mice now suggests that anterior pituitary development depends on *Sox3* expression in the overlying neural ectoderm, which establishes midline structures and regulates production of inductive BMP and FGF signals. Concentrations of pituitary GH, FSH, LH and TSH were lower in 2-month old *Sox3* mutant mice.

The hypopituitary phenotype of the *Sox3* null mice resembles that described for humans with an 11-mer polyalanine tract expansion in *SOX3*. Another group of individuals with X-linked hypopituitarism have duplications of Xp26 encompassing *SOX3* have mental retardation and hypopituitarism, and their phenotype is probably due to overexpression of this gene[153]. As these individuals do not have craniofacial defects, the polyalanine tract expansion is probably a loss-of-function mutation. Thus, if both gain- and loss-of-function mutations can lead to hypopituitarism, then pituitary development must be very sensitive to *SOX3* dosage. Analysis of *Sox3* gain-of-function mice and analysis of additional humans with *SOX3* alterations will be particularly informative in unravelling the developmental functions of this gene and its contribution to pituitary dysfunction in humans.

7. INTRAUTERINE GROWTH RETARDATION

Intrauterine growth retardation has multifactorial causes that are very complex, including nutritional problems, toxic agents, deficiencies confined to the placenta, chromosome anomalies and other genetic alterations. Primordial dwarfism constitutes a delay in growth of prenatal origin that continues during the postnatal period and that can be divided into two large groups, depending on whether it is associated or not with microcephaly. One specific form of primordial dwarfism without microcephaly is Silver-Russell syndrome (SRS; OMIM #180860). This syndrome is associated with a specific craniofacial phenotype, including triangular face, prominent ears,

possible asymmetry of limbs, and clinodactily of the fifth finger. The cause of this syndrome is probably heterogeneous and the majority of the cases may actually be caused by dominant *de novo* mutations.

The first real indication of the molecular bases of this syndrome was the observation of uniparental maternal disomy of chromosome 7 in an individual homozygous for a mutation that causes cystic fibrosis where only the mother was a carrier[154]. This patient, as well as others described afterwards, had intrauterine growth retardation and postnatal growth retardation that was not justified by the cystic fibrosis. Uniparental maternal disomy of chromosome 7 was later found in 10% of the patients with primordial dwarfism belonging to the group of SRS[155]. Hence, the results indicated the existence of one or more genes on chromosome 7 that regulate growth and undergo gamete imprinting. These could be either genes that stimulate growth and are expressed only by the chromosome of paternal origin or growth inhibitory genes that are expressed only by the maternal chromosome. The finding of one familial case of SRS, a mother and daughter with a tandem duplication in the chromosomal 7p13–p11.2 region, has allowed the critical region of chromosome 7 to be defined to a region that contains the genes for *IGFBP1*, *IGFBP3,* and growth factor receptor-binding protein 10 (*GRB10)*[156]. Subsequently, another patient with a similar duplication has been described. Molecular characterization of this patient demonstrated that the above mentioned genes were included and the duplicated region was of maternal origin[157]. Therefore, there must be one or more growth inhibiting genes in this region of chromosome 7 that are exclusively expressed from the maternal chromosome. Increased expression of this gene (or genes) either by maternal uniparental disomy or maternal duplications of the critical region will cause growth retardation. The *GRB10* gene is a possible candidate for several reasons: (1) It is located in the critical region of the described duplications. (2) The homologue in mice (the *meg1/Grb10* gene located on chromosome 11 is expressed exclusively by the maternal chromosome. (3) It is probably implicated in the growth defects that are observed in mice with duplication of chromosome 11 and reciprocal deficiency[158]. The known function of the GRB10 protein is also suggestive of a role in growth control. GRB10 binds the insulin receptor and IGF-IR via an SH2 domain and inhibits the tyrosine kinase activity associated with the receptor that is implicated with the growth promoting actions of insulin, IGF-I and IGF-II[159]. Yoshihashi *et al.*, performed mutation analysis of the *GRB10* gene in 58 unrelated patients with SRS and identified a P95S substitution within the N-terminal domain in two of the patients[160]. Evidence creating uncertainty about the role of *GRB10* in SRS has been reported[161-163]. Doubt was cast by the absence of mutations detected by sequencing *GRB10* in classic SRS patients, in whom major structural chromosomal

abnormalities and maternal uniparental disomy had previously been excluded.

8. FINAL REMARKS

The many advances in molecular biology and genetics over the last decade have facilitated the studies of the GH/IGF-I axis in humans and mice yielding great insights into pituitary development and control. The continued identification and characterization of novel factors involved in this complex growth cascade will lead to a better understanding of the GH/IGF-I axis and may provide new therapies for patients affected for these disorders.

All of the classic Mendelian patterns of inheritance have been demonstrated in human undergrowth (autosomal, X-linked, pseudoautosomal dominant). In fact, mutations in most of these genes have been implicated as the cause of abnormal and altered growth patterns from intrauterine life to childhood and puberty. In addition to the genetic component in familial cases, a high proportion of the sporadic cases may be also due to genetic abnormalities. The physiological bases of the GH/IGF-I axis are very complex as revealed by the increasing number of different genes that have been related with the etiopathogenesis of proportional short stature. Indeed, either GH deficiency or GH resistance, as well as IGF-I deficiency and IGF-I resistance can all produce a similar human phenotype due to the absolute or relative lack of IGF-I. The genes involved include the pituitary transcription factors *POU1F1*, *PROP1*, *LHX3, LHX4,* and *HESX1*, and genes directly or indirectly related with GH expression, release and signal transduction like *GH1, GHRHR*, *GHR*, *STAT5b*, *IGF1*, *IGF1R*, *IGFALS*, *SHOX*, *BTK*, and *SOX3*.

Furthermore, some potential candidates including *IGFBP-3* and *GRB10*, in particular in patients with proportional undergrowth of prenatal origin should be considered. Hence, the molecular studies in patients with prenatal abnormalities in growth provide a very exciting field of research at present and in the near future. In addition, the molecular bases of short stature due to gene abnormalities on both the X and Y chromosomes, is now under intense investigation, since the mechanisms that regulate growth are not well understood or remain completely unknown.

Therefore, the clinician should further investigate children with a phenotype of IGF-I deficiency, not only from an auxological, biochemical and radiological point of view, but also using appropriate molecular techniques.

In conclusion, the advances in molecular biology and genetics are rapidly unravelling the complex pathology of human growth. The number of disorders with a known molecular genetic basis is dramatically increasing, and the list will continue to grow in the near future. Therefore, it is relevant for physicians and scientists to be aware of all advances and novel techniques available for achieving a better diagnosis, appropriate family counselling and patient care. Although our knowledge has advanced rapidly in this field, an international consensus or clinical guidelines for a specific molecular diagnosis of proportional short stature remains to be established.

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Chapter 15

FUTURE PERSPECTIVES:

From stem cells and IGF biology to the clinic

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Key words: Cancer; cell differentiation; growth; regeneration; stem cell renewal; transplantation.

1. INTRODUCTION

From the previous chapters, it appears that insulin-like growth factors (IGFs) control the growth and the development of different organs showing the role of IGFs as master regulators during organogenesis. These chapters review the actual knowledge of IGF functions and discuss their roles during the building of our body and its maintenance, as well as in different pathologies. Here, I would like to show how the study of stem cells can help to dissect some IGF functions and how the knowledge of stem cells and their regulators may help in making clinical applications possible in the long term. IGF-I has pleiotropic functions during embryogenesis and adulthood; therefore, it is necessary to develop systems that allow to dissect the function of IGF-I, cell by cell phenotype, and identify the mechanism of IGF-I in each of these cell systems to understand how organogenesis and body growth take place. In this chapter I present various concepts rather than provide a review of the literature. Nonetheless, representative data and reviews will help to lead the reader through these concepts. The role of IGF-I in different stem cell systems is discussed in light of the concepts emerging from the study of stem cells. Indeed, when someone has to work with stem cells, he or she has to understand what a stem cell is and what the other cells

are. The protocols that allow the study of stem cells also bring new tools for understanding the function of genetic and epigenetic factors, such as IGF-I. But first, what is a stem cell?

2. STEM CELL DEFINITIONS

All stem cells are derived from more primitive ones, the embryonic stem cells (ES cells) that are located in the inner cell mass of the blastocystes. The ES cells are pluripotent: they give rise to all cell phenotypes of the body[1]. It is believed that ES cells generate more specialized stem cells (organ-specific fetal stem cells) that will be the origin of each organ that composes the body[2,3]. During development, the fetal stem cells give rise to all cells that will form an organ: each organ has its specific stem cell that will initiate the formation of said organ. Such a phenomenon implies that the stem cells have to proliferate extensively. This is the most important characteristic of a stem cell: its enormous capacity to proliferate. The control of stem cell proliferation and renewal is discussed in detail later. Depending on the organ, a specific stem cell type can generate all the cell types of the organ. For instance, the neural stem cells generate successively all neural cells during brain formation, *i.e.*, neurons, astrocytes, and finally, oligodendrocytes. Thus, each neural stem cell is multipotent and can generate all the mentioned cell phenotypes[4]. Multipotentiality is the second characteristic. Nonetheless, in certain organs, such as the skin, you can find stem cells that produce only one cell type, for instance the melanocytes[5]. Organ-specific stem cells persist after birth and during adulthood, where they participate to renew a tissue (skin, bowels), to add new cells (neurons in the olfactory bulb) or to repair a damaged organ. When a bone is broken, or the skin cut, the local stem cells generate cells that will allow the bone to mend and the wounded skin to heal[6]. Tissue renewal and repair is the third characteristic of stem cells.

In summary, a stem cell is able to renew and generate the quantity of cells necessary to build the organ from where the stem cell is issued, to produce the specific cells of the organ, and participate to the repair of the wounded organ (Fig. 1).

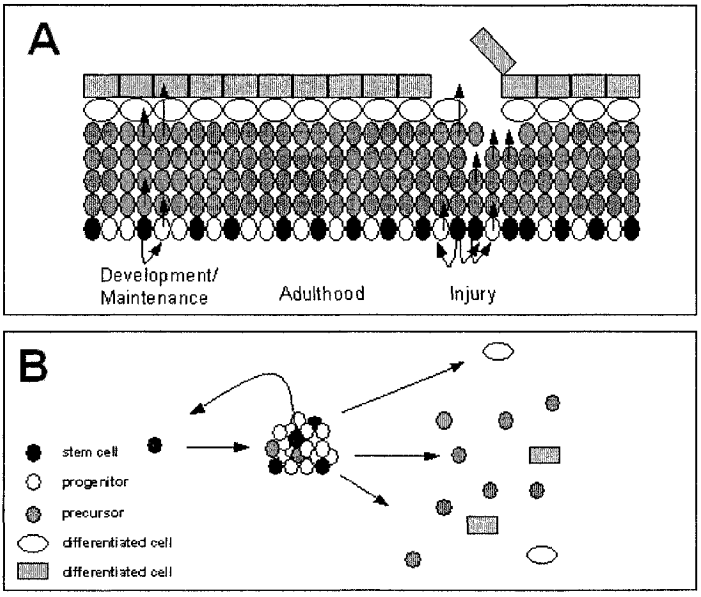

Figure 1. Stem cells renew and produce all the cell phenotypes of an organ and participate in its repair. **(A)** *In situ*, stem cells (black circles) generate transient amplifying progenitors (white circles) that produce precursors (gray circles). Then, the precursors give rise to differentiated cells (white ellipse and gray rectangle). During adulthood, such phenomenon can occur throughout life (skin, small intestine epithelium) or the stem cell can be quiescent or have a slow turnover (brain). After injury, in many tissues the stem cells are activated and generate cells necessary for healing. **(B)** In vitro, a stem cell should be capable of generating progenitors and precursors, as well as itself (backwards arrow) showing by this property its renewal capacity. Differentiation induction of the precursors leads to the formation of the differentiated cells that compose the organ from which the stem cell is derived.

3. STEM CELL IDENTIFICATION

To work with stem cells, we would like to be able to precisely identify the stem cells in order to follow their proliferation and the generation of cells committed to a specific phenotype. However, only a few stem cells can be recognized by one or several markers, as is the case for the hematopoietic stem cells[7,8] or the embryonic stem cells[1]. In general, only clonal analysis may provide the means to identify that the cell growing in your dish is a stem cell. Clonal analysis means that a single cell is studied individually, without interference from the other cells. By understanding how one can characterize a stem cell, we will see that the same protocol

may be applied to study the function of genes or environmental factors in stem cells (growth factors, metabolites, oxygen, etc.).

The analysis of stem cell characteristics occurs in several steps. First, a piece of tissue is dissected from an organ in an area of interest, for instance, the germinative zone of the developing striatum in a newborn brain. Obviously, the dissection allows one to select the area of interest to be investigated, but this approach does not result in a pure cell population. In this blending of cell types, differentiated cells (such as neurons and glial cells), precursors,* progenitors[*9]*, and stem cells are mixed together. The culture condition may help to enrich a specific population, but cellular heterogeneity remains. The first goal is to determine if a population of highly proliferative cells exists. The tissue is dissociated and cells are plated at clonal density in the presence of mitogens to determine which cells are proliferating. Only a small percentage of cells give rise to large colonies. These colonies can then be isolated and propagated to test their potential of proliferation. In fact, we expect that a stem cell can produce the number of the cells necessary to compose an organ[10]. However, when the stem cell divides, it will not necessarily give rise to another stem cell, but can also produce other cell types. In the case where the generation of two daughter stem cells occurs, we say that the stem cell has undergone a symmetric division, and an asymmetric division when one daughter stem cell and another cell type are generated (Fig. 2).

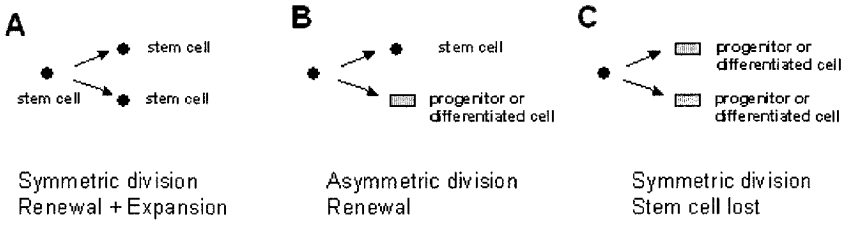

Figure 2. Stem cells, symmetric and asymmetric cell division. **(A)** One stem cell gives rise to two stem cells after a cell division: symmetric division (both cells are identical). In this case, we observe an expansion of the stem cell number. **(B)** One stem cell generates one stem cell and one progenitor or differentiated cell: asymmetric division (both cells are different). It is important to emphasize that the stem cell is always present, it renews itself. **(C)** One stem cell produces two non-stem cells. The stem cell is lost.

* Progenitors are cells that divide for a long period of time, but with a limited renewal capacity; precursor cells undergo only few cell cycles and are restricted in their fate.

Thus, we can deduce the existence of a stem cell only retrospectively. Moreover, when the stem cell has started to form a colony, this colony is composed of a heterogeneous population of cells, maybe less heterogeneous than *in vivo*, but we have to assume that the stem cell has generated another cell phenotype unlike itself (Fig. 3). In consequence, to test the action of a gene or of a factor on stem cells, we need to target our study to this first cell that is at the origin of the colony. Nonetheless, culture conditions may help to enrich the colony in stem cells. For instance at birth, neural stem cells represent approximately 1% of the total cell population in the germinative area (only a small percentage of these cells can form large colonies that can be propagated[11-13]), but a 17-fold increase of the stem cell percentage can be achieved by cell culture in defined conditions (Zencak *et al.*, submitted). How can we determine if a stem cell generates other stem cells? By subcloning the original colony it is possible to determine how many new clones are able to generate new large colonies with a great capacity of proliferation and differentiation: these new colonies are also derived from new stem cells (Fig. 3).

Parallel to the assessment of cell renewal potential, it is necessary to reveal whether the proliferating cell is able to generate the cells composing the organ. After cell expansion, to reveal the multipotentiality of the supposed stem cell, the derived progeny are induced to differentiate. The differentiation process requires conditions allowing the formation of all the cells composing the organ. For instance, for neural stem cells standard culture conditions allows the induction of the differentiation of neurons, astrocytes, and oligodendrocytes[4,14-19]. Depending on the stem cells and the phenotype to be generated, a unique culture condition is not sufficient to reveal the multipotentiality of the stem cell. In consequence, it is necessary to provide enough cells from a clone to test the same clone in different conditions to reveal the cell phenotypes.

Nonetheless after this *in vitro* characterization, the first thing to assess is to show that what we are studying *in vitro* is related to what happens *in vivo* (see Section 4.3).

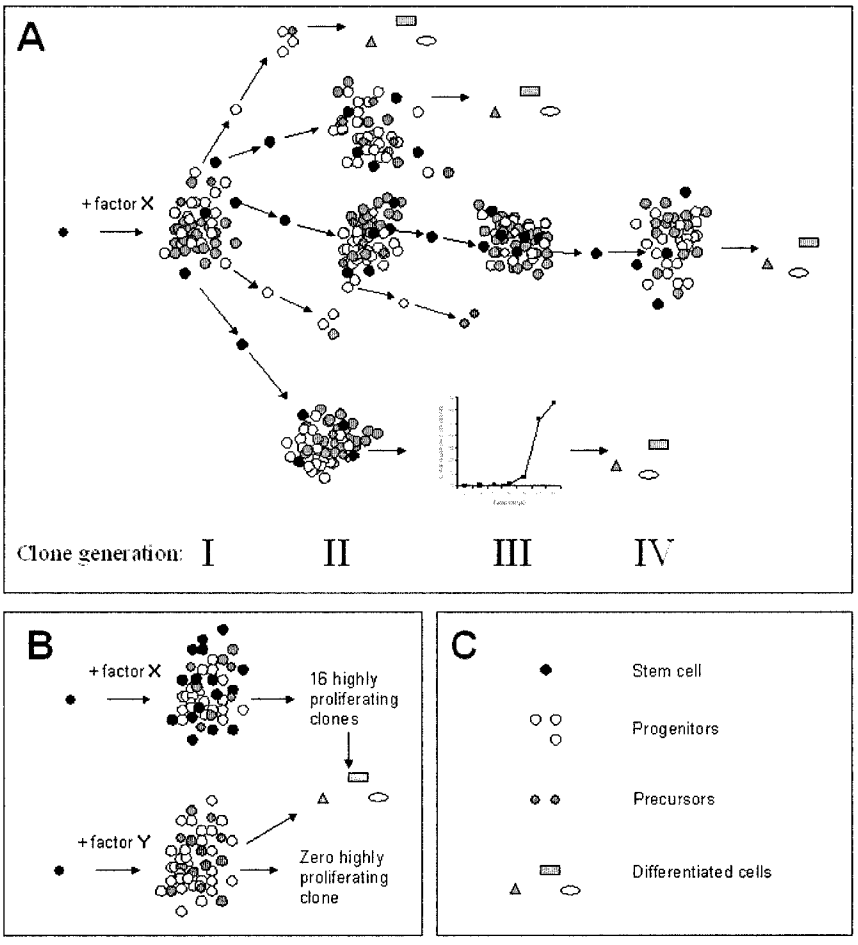

Figure 3. Clonal analysis of stem cell action. **(A)** Under the stimulation of a factor X, a single stem cell gives rise to a large clone (clone generation I) containing progenitors and stem cells (see **(C)** for symbols). To know if this clone contains stem cells, it is necessary to isolate single cells from the clone and to stimulate its proliferation: a stem cell (black circle) will generate a new clone (clone generation II), whereas a precursor (white circle) will give rise only to a limited number of cells. Clones from generation II can be subsequently subcloned to test for the presence of a stem cell. Such procedure can be renewed several times (clone generation III and IV, and subsequent if necessary). A single clone can also be expanded to determine the expansion potency of the cell at the origin of the clone (bottom clone + graph). Each clone has to be tested to reveal its differentiation potential (arrow with gray symbols and white ellipse). **(B)** The actions of the factors X and Y are tested on two stem cells (supposed identical). Factor X favors the expansion of the stem cells (black circles), whereas factor Y stimulates the proliferation of the stem cells leading to the generation of progenitors, but without stem cells. Both clones are multipotent, but only the clones generated by factor X can renew. In conclusion, factor X stimulates the renewal of the tested stem cell, whereas factor Y does not. Using the progeny derived from the stem cell, different factors can also be tested to evaluate their capacity to induce cell differentiation. **(C)** Symbol nomenclature.

4. ADVANTAGES OF USING STEM CELLS

4.1 An unlimited source

One of the main advantages in using stem cells, is the large amount of cells that can be used for one study. For instance, the EGM and the EGL (the two germinative zones of the developing striatum in the brain) of 10 embryos at E14 give rise to around 20 million cells. These cells can be used directly (without induction of growth) to study neuronal differentiation for instance. If neural stem cells are isolated at this time, 3 weeks later, equivalent to the period of gestation, the same amount of cells can produce 2.5 billion striatal cells, corresponding to 1200 embryos. The advantage of such system is obvious. Moreover, these cells can be passed during months and frozen anytime to be used at a more opportune time. Nonetheless, it is necessary to control or not whether expanded cells maintain a normal karyotype and remain dependent on the presence of exogenous mitogens to stimulate their proliferation. In parallel, the main problem is to characterize the system and show that what you are looking at *in vitro* with these cells is relevant to what happens during *in vivo* development (see later). Nonetheless, the capacity for renovation is huge for skin stem cells, neural stem cells, and mesenchymal stem cells, for instance. Such cell culture systems may help make it possible to perform large scale screening of the function of hundreds or thousands of molecules or to screen gene expression in a given situation as described earlier. Other advantages reside in the possibility to generate a large cell number for transplantation studies (see Section 9).

4.2 Controls of cell state: drug and gene function screening

What is the advantage of using stem cells in comparison to primary cells from fetal tissue, for instance? By manipulating the stem cells *in vitro*, different cell populations can be separately studied, from the stem cell to the differentiated cell. Each step of cell development can be investigated using defined culture conditions that can help to synchronize cells in a specific cell stage. In consequence, the role and the mechanism of action of intrinsic and extrinsic factors in a population of cells can be more easily determined.

To study the function of a gene or the action of a factor specifically on stem cells, two approaches have to be considered. Either the possibility exists to isolate certain stem cells such as hematopoietic stem cells[20] or muscle stem cells[21,22] by specific markers, or it is first necessary to develop

a method to study the characteristics of the stem cell. In the first case, the analysis is facilitated by the fact that the cell population is homogeneous. In the second case, the use of clonal analysis, as described in Section 3 and in Fig. 3, could be the solution. In both cases, to reveal whether a factor enhances stem cell division, for instance, stem cell subcloning protocols allow the investigator to analyze whether the tested factor produces more new colonies when added to the incubation media during the proliferation of the first clone (Fig. 3B). To prove that this factor acts directly on stem cells, it is necessary to work at clonal density to prevent the influence of the neighboring cells. To test an inhibitor, a similar approach can be undertaken. In this case, we expect that no new colony will be formed.

An example of the utility of this approach is that several research groups think that glial scar formation during spinal cord injury impairs the regrowth of cortico-spinal fibers that control motricity. Certain researchers believed that the scar formation is derived from activated stem cells in the spinal cord stimulated by the injury[23]. To show that stem cells and the glial scar have a deleterious action, one of the goals is to prevent neural stem cell proliferation. The protocol of clonal analysis described earlier may help to identify an inhibitor of spinal cord stem cell proliferation. The screening of molecules preventing such proliferation appears to be of prime importance and the methods described here have already been used to study the actions of factors that stimulate stem cell proliferation[11,13,24-30] and may serve to identify therapeutic agents.

Other domains also take advantage of stem cell investigation. Studies of hematopoiesis and muscle cell differentiation processes are very well documented, because the culture conditions of these cell systems allow the precise identification of the different stages of development of each cell investigated. This approach leads to the determination of the function and the mechanism of action of different factors or genes on stem cells during hematopoiesis[31] and myogenesis[32]. Nonetheless, important progress has also been recently obtained in the study of stem cell based neurogenesis[33-35], bone formation[36], skin epithelium formation[6], and so forth. The role of IGFs in these systems is discussed in the sections that follow.

The study of the induction of differentiation may also be approached by similar protocols. Indeed, several stem cell culture conditions produced an enriched population of nondifferentiated cells, allowing the study of factors generating differentiated cells, *i.e.*, the passage of a nondifferentiated into a specific cell phenotype. The appearance of differentiated cells in the culture could be due to different actions of factors in the medium, one committing the cells to the neuronal fate[16], the other inducing the differentiation[14,15], and finally the third one acting as a survival factor. Using such approach with stem cells, it was shown that IGFs control the differentiation of a multitude

of different cell types. From this book, as well as from different articles and reviews, it is evident that the IGF family controls the cell differentiation process in bone[36,37]; muscle[38], blood[39,40], brain[15,27,28,41], heart[42], gut[43], and so forth. It appears that IGF-I has a pleiotropic function during the organogenesis of many tissues. Other factors, such as fibroblast growth factor (FGF)-2, have similar pleiotropic functions during cell proliferation and commitment. *In vitro* cell differentiation studies based on stem cell cultures system will surely help to dissect the function and the respective mechanism of each factor during development. The understanding of how one molecule can control different cell functions just after a cell division or cell commitment will surely help to understand how we can manipulate these systems in pathogenic conditions or for tissue regeneration.

4.3 *In vitro* stem cells reinitialize different steps of development

In vitro stem cell studies have an interest in developmental biology only if the stem cell behavior *in vitro* reflects a function that can or does happen during embryogenesis. Fortunately, several studies show similarities between cell fate induction *in vitro* and what occurs naturally *in vivo*. Some examples are described below for different stem cell systems, but we need to always keep in mind that we never know to which extent we "push" the system *in vitro*. Indeed, the concentration of several factors added in the growth medium are often supraphysiologic [*e.g.*, epidermal growth factor (EGF), insulin]. In this context, it is important to try to work with physiological concentrations of the growth factor to better understand the role of each factor avoiding the unknown nonspecific activation that high factor concentration can produce. The replacement of high concentrations of insulin by physiological levels of IGF-I and/or insulin and/or IGF-II should allow us to better understand the role of each of these factors and to identify whether their actions are sufficient for replacing insulin action.

Several studies with neural stem cells have shown that different steps of *in vitro* neural stem cell based neurogenesis parallel the steps observed during embryogenesis. For instance, *in vitro* neural stem cells first acquire the capacity to respond to FGF-2 stimulation, and then to EGF[3,13,24]. These experiments were confirmed by similar observations in studies of neural stem cells derived from EGF and FGF-2 knockout mice[13]. Zhu *et al.*[19] have shown that neural stem cells, isolated at different times of development, generate preferentially the cell phenotype produced during the time when these stem cells were isolated: indeed, stem cells isolated early produced more neurons, and NSC isolated after birth produced more

oligodendrocytes, as observed *in vivo*. The most convincing demonstration that NSCs maintain the capacity *in vitro* to mimic normal development was shown by transplantation studies. When NSCs are transplanted into a developing brain, cells generated from the neural stem cells can differentiate into various neuronal and glial phenotypes[44-48] and can integrate into a neuronal circuitry[49].

Similar results were obtained using hematopoietic stem cells. The study of HSCs is facilitated by the possibility of isolating stem cells depending on the expression of specific markers expressed on the cell surface[8]. The most convincing demonstration of renewal, multipotentiality, and the maintenance of a normal HSC program was achieved by the transplantation of a single HSC in mortally irradiated mice. The HSC was capable of homing the bone marrow and repopulating all the blood cells[50]. This procedure can be repeated several times and shows the fantastic potential of the HSC cells. In fact, for researchers in all other stem cell fields, this protocol is the gold standard to demonstrate the stem cell identity and is the goal that each researcher would like to reach for his or her own specific field of research.

The ideal condition of testing stem cells would be to compare the engrafting potential of stem cells isolated directly from the body and after cell culture expansion. But this approach is often impossible owing to a lack of stem cell markers in several cell systems. Nonetheless, several stem cells can have an *in vitro* behavior that corresponds to their normal function *in vivo*: muscle stem cells can differentiate and fuse to form active contractile skeletal muscle cells[32], cultured mesenchymal stem cells can participate in cartilage repair[51], limbal stem cell of the cornea can be expanded *in vitro* to form a cornea for transplantation[52], and epithelial skin stem cells can reconstitute a dermis for a person burned over a large area[6].

5. STEM CELL RENEWAL AND IGFS

Stem cell renewal is due to different factors and genes that control several functions of the stem cell. Indeed, to survive, for long term renewal, a stem cell needs to remain nondifferentiated, proliferate, and to protect against the loss of the chromosome extremities (a degradation process occurring at each cell division[53,54]) by maintaining the presence of telomeres. Moreover, a stem cell has to be quiescent when necessary. It appears that self-renewal implicates a multitude of controls in a stem cell to proliferate adequately. Here, several examples of the role of IGFs on different stem cells systems are described to show the importance of IGFs in the control of stem cell renewal.

The first studies implicating IGFs in sustaining long term cell proliferation were undertaken on fibroblasts and placenta cells. In absence of serum, IGF-I has little effect on fibroblast division. However, in the presence of human hypopituitary serum, IGF-I strongly stimulates fibroblast proliferation. This effect is reinforced by dexamethasone[55]. Interestingly, GH can directly stimulate fibroblast division via the induction of IGF-I secretion that acts in an autocrine/paracrine mode[56]. This study supports the idea that GH can directly target certain cells without acting through the liver[37].

Recent studies have shown that the IGF family, including the IGFBPs, stimulate the proliferation of primitive hematopoietic progenitor cells and stem cells. It is important to keep in mind that hematopoietic stem cell renewal was demonstrated by *in vivo* transplantation, because no factors allowed their expansion *in vitro*. In this context, any induction of cell division in hematopoietic stem cells is an important advance for understanding the cell renewal mechanism in these cells. Interestingly, during development CD3 liver cells secrete IGF-II when hematopoietic stem cells reside in this organ[57]. Moreover, coculture of CD3 cells with hematopoietic stem cells supports the expansion of the stem cells. The use of antibodies to block IGF-II prevents the supporting effect of liver cells showing that IGF-II may participate in the control of hematopoietic stem cell proliferation. A supporting effect of cells surrounding the primitive hematopoietic $CD34^+CD38^-$ cells was also identified in human endothelial cells[58]. Interestingly, in this paradigm IGFBP-3 was shown to stimulate the proliferation of $CD34^+CD38^-$ cells.

A role of the IGF family in the hematopoiesis control of early progenitors was also identified in myeloid progenitor cells in which IGF-I stimulated DNA synthesis and reduced apoptosis[59]. Enhanced proliferation of myeloid cell lines by IGF-I was also documented[60]. Interestingly, GH itself can have a direct action on B cells of normal subjects or on leukemic cell lines[61]. Moreover, GH is synthesized by these cells and can act via an autocrine mode. Taken together, these data show that different members of the growth hormone-insulin-like growth factor axis control early stages of hematopoiesis. IGF-I also inhibits apoptosis of erythroblasts and megakaryoblasts, and has a slight effect on their proliferation[39]. These data and those presented in various chapters of this book show that IGF related proteins act on proliferation and apoptosis of different blood cells. Like in other systems, several factors control the renewal of the hematopoietic stem cells[62,63]. In this context, the IGF family may play an important role shared with these factors.

Using muscle stem cells, Deasy *et al.*[22] have shown that IGF-I, in the presence of serum and chick embryo extract, stimulates proliferation. IGF-I

was also shown to stimulate by two-fold the proliferation of porcine embryonic myogenic cells[64]. These cells also synthesize IGFBP-3, suggesting a regulatory role of this protein. Indeed, overexpression of IGFBP-3 by baculovirus prevents IGF-I induced proliferation. These examples, and those presented in Chapter 10, reveal that the production of muscle cells, that compose a great portion of our body, is also regulated by the IGF family.

IGF-I also plays an important role in the control of neural stem cell proliferation. Several factors have been shown to stimulate or control neural stem cell division, such as EGF[4], FGF-2[13,18,65], cystatin-C[26], sonic hedgehog[66,67], ciliary neurotrophic factor (CNTF)[25], leukemia inhibitory factor (LIF)[68], and probably delta[69]. The exact link between these factors is still unclear. Nonetheless, the *in vitro* study of neurogenesis based on stem cells allows us to start to identify the function of IGF-I in neural stem cells. It was shown that the mitogens EGF and FGF-2 cannot induce neural stem cell proliferation without the presence of IGF-I[11]. Moreover, this study revealed that FGF-2 acts as a survival factor, whereas IGF-I or EGF did not have this action alone, but only in combination.

It appears that each factor has a specific role in the control of neural stem cell proliferation. A collaboration between these factors was also described for stem cells derived from the olfactory bulb[28]. Nonetheless, the fact that mice knocked out for the *IgfI* or the *IgfIR* genes have a smaller brain, but still have a brain, shows that IGF-I is not necessary to maintain the population of neural stem cells, but may act on the neural stem cell number. It would be interesting to quantify the number of neural stem cells in these knockout versus wild type mice, in order to define whether endogenous IGF-I controls the neural stem cell number during development. Interestingly, in the absence of IGF-I (*IgfI* knockout mice) neural stem cells of the olfactory bulb maintain the capacity to proliferate normally in the presence of EGF, FGF-2, and high concentrations of insulin[28]. In consequence, other factors may have stimulated neural stem cell proliferation during the development of the *IgfI* knocked out brain such as those listed above, or pro-insulin which was detected in olfactory bulb progenitor cells[28]. Moreover, pro-insulin was shown to stimulate the proliferation of these progenitors[28] suggesting that it may play a role in the control of the neural stem cell number. On the other hand, it was also shown that injections of high doses of insulin (2 μg) into the newborn chick eye stimulate the proliferation of retinal progenitor cells[70]. In this case, it remains to determine whether this dose of insulin acts via the insulin or the IGF-I receptors.

Mammary gland cell proliferation is also controlled by IGFs and the IGFBPs[71,72]. Indeed, transgenic mice expressing IGF-II have a decreased

number of apoptotic cells in the mammary epithelial cells[73]. Moreover, the involution of the mammary gland after lactation is inhibited by the overexpression of IGF-I in transgenic mice[74]. IGF-I is expressed in the terminal end buds, which builds the gland during puberty[75] and allows the maintenance of the proliferation of the mammary epithelial cells *in vitro*[76]. These data show that IGF-I is a major actor in mammary gland morphogenesis.

IGF-I can also act on the most primitive cell of the body, the pluripotent zygote. *In vitro* IGF-I enhances the survival of rabbit and mouse embryos[77,78]. Moreover, IGF-I promotes rabbit blastocyst formation[77]. The establishment of an embryonic stem cell line (derived from the inner cell mass of blastocysts) is facilitated when IGF-I is present. Such effect is reinforced when IGFBP-1 is added[79]. Embryonic stem cells usually need serum or a feeder layer of cells to grow. In a recent study, it was shown that monkey embryonic stem cell proliferation can be induced by using a serum-free medium composed of defined factors, such as IGF-I, transforming growth factor (TGF)α, FGF-1, estradiol, and progesterone[80].

Taken together, it seems that IGF-I acts throughout all developmental stages and on numerous stem cells, if not all. Several other *in vitro* stem cell systems use high insulin concentration in the medium to stimulate cell proliferation. In fact, it remains to be determined what is the function of insulin in these cultures and whether insulin acts through the IGF-I receptors or through the insulin receptor. Indeed, it is also possible that insulin plays a direct role on stem cells as suggested by the study of pro-insulin on the proliferation of olfactory bulb progenitors[28]. When all stem cells have been investigated to determine the potential role of IGFs, we will have an overall idea of the role of IGFs on stem cells. Do IGFs have an identical role between all stem cells or do they control stem cell renewal with specific function depending on the stem cell type that they target? This understanding will help to design strategies to specifically manipulate a stem cell population either to promote tissue repair (see Section 10) or to prevent tumor formation (see Section 8) .

6. IGF-I, STEM CELLS, AND BODY GROWTH

Knock-out of the *IGF-I* gene or deletion of the *IGF-II* gene[81,82] results in a reduction in the size of the transgenic animals. Moreover, the combination of two gene deletions provokes a more severe growth deficit. In all transgenic animals, the gross morphology of the majority of the organs seems to be affected in the same proportional manner (even if certain cell types are more affected by this deficiency). Indeed, the size of the legs, the

head, including the brain and the eyes, and the chest are reduced in the same proportion. The heart and the liver also reside normally in the body. In view of the redundant actions of IGFs on different stem cells, one hypothesis of the control of body size by IGFs could be an action of IGFs on stem cells, with a subsequent possibility that IGFs also control cell proliferation of the down stream precursors and their differentiation.

The growth of several organs is organized by the generation of columns of committed and differentiated cells derived from the stem cells[83] (Fig. 4) or cells derived from stem cells organized in clusters. This approach is simplistic because it is known that in certain organs, such as the brain, several cells migrate extensively before reaching their target[84]. Nonetheless, the model proposed here allows one to draw a general hypothesis that can be further ameliorated or denied.

Growth in columns occurs in the skin[83], the small intestine[85], several regions of the brain (for instance the cortex, the retina), the bones[36], and so forth. The difference in size between the human cortex and the mouse cortex is not related to the thickness of the tissue, but to its extension. Moreover, the overexpression of β-catenin in mouse cortical neural progenitor cells leads to an expansion of neural progenitors and, consequently, to a dramatically increased surface of the cortex with an approximately normal pattern of neuron location[86]. This study (and those cited within) show that the number of cortical columns is related to the number of progenitor cells. Also, when we compare certain organs between a short and a tall person, the skin and the cortex, for instance, one can easily determine the origin (tall vs. short) of the organ if you consider the surface of the organ, whereas the cell layers existing in these organs will be similar in cell number and cell size.

These observations show that several organs grow by adding successive columns of cells laterally, rather than by multiplying the number of cells in the thickness of the tissue. Obviously, for organs like muscles, the size of the muscle fibers will be increased in tall people and the papers discussed in this book demonstrate clearly that GH and IGF-I also control the cell size. But here, I would like to point out that growth regulation can also occur in a horizontal/lateral plan. If IGF-I controls the survival or the proliferation of stem cells, an increased concentration of IGF-I would results in a greater pool of stem cells. If a future organ contains more stem cells and the environment favors the normal development of these cells, we can expect that this germinative zone will give rise to more columns of cells, rendering this organ larger in comparison to an organ that has fewer stem cells (Fig. 4). This could be possible only if the environment provides factors allowing the normal development of the cells derived from the stem cells.

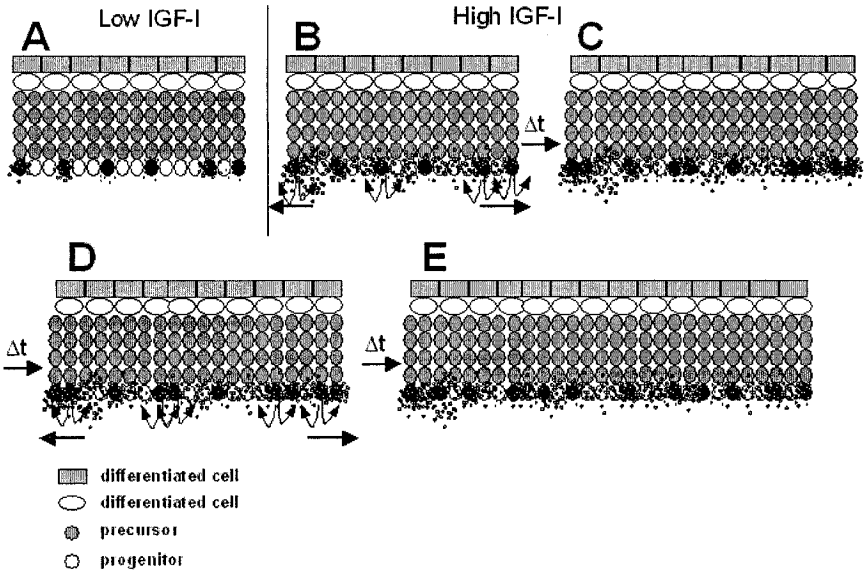

Figure 4. Stem cells and the control of organ growth size. **(A)** At low IGF concentrations, stem cells do not expand, but generate the progenitor cells necessary to build the organ. **(B and C)** At high IGF concentrations, stem cells start to divide and expand, resulting in an increased number of cells in the horizontal plan. **(D** and **E)** Each new stem cell generates new progenitors that will add new column of cells increasing the lateral growth of the organ.

This hypothesis would explain the difference in body size between individuals and animals with different levels of IGF-I ranging from zero to physiological concentrations. Supposing that IGF-I has a direct action on stem cells (we supposed that all stem cells can be influenced by IGF-I; nonetheless other experiments are needed to evaluate if it is really the case) and on several other committed cells as evidenced by the pleiotropic actions of IGFs on precursor proliferation and cell differentiation, the cells generated in columns will survive and remain to build the organ. Increased organ growth can also be stimulated through stem cells to produce an increased number of progenitors, but not via a higher number of stem cells. The results would be a larger column of cells that generate more differentiated cells. For example, the increased size of a bone is believed to result from the following mechanism. GH stimulates the local production of IGF-I from the growth plate, leading to the acceleration of stem cell and progenitor proliferation[36,87] and the expansion of the proliferating cells[88]. Then, IGFs and IGFBPs participate in the differentiation of the chondrocytes (for review see refs. 36,88). To prove such a theory, implicating stem cells and IGFs in the control of organ size, a larger organ should have more stem cells than a

smaller one, with the percentage of stem cells in the studied organ remaining the same. Such experiments remain to be undertaken, as does the identification of the exact role of IGFs on different stem cells.

The importance of IGF in the generation and survival of stem cells and the derived progeny leads one to ask the question of the source of IGFs and how the concentration of IGF influences the number of stem cells. Recent studies on transgenic mice that fail to express IGF-I in the liver have shown that hepatic IGF-I is not responsible for the normal development and growth of an animal[89,90]. Indeed, these transgenic mice have an identical growth compared to wild-type animals. Only the spleen shows a reduced size. A new theory proposes that the growth is in part due to the production of IGF-I by an autocrine/paracrine mode of several cell types contained in different organs of the body[37]. Indeed, numerous studies revealed that stem cells or differentiated cells produce IGF-I and/or IGF-II in an autocrine manner to sustain their proliferation or differentiation (this book and references[11,27,37,41,71,91,92]. Supposing that stem cells secrete a low amount of IGF-I, this concentration will influence only the fate of said stem cell. In this case, IGF-I has an autocrine action, whereas higher amounts of IGF-I will also reach cells located in the neighborhood, provoking autocrine and paracrine actions of IGF-I. (The paracrine action of IGF-I on body growth and the respective role of growth hormone is developed in details in ref. 37).

The IGF-I released from all the stem cells in a region will have an important cumulative effect, potentiating the actions of IGF-I leading to the proliferation of stem cells. Supporting this hypothesis, the number of stem cells entering the cell cycle *in vitro* is IGF-I dose dependent and neural stem cells secrete IGF-I[93]. A small elevation in IGF-I concentration, between 0.3 nM and 1 nM, produces a significant increase in proliferating stem cell number. Consequently, in a local environment the stem cells will be exposed to the same amount of IGF-I throughout the said area, leading to coordinate growth of the organs (Fig. 4). The stem cells will either expand (*e.g.*, brain, skin) or generate more precursors (bone). The arrest of cell proliferation will be controlled by the maturation of different cell types and circulating hormones (for instance see ref. 36).

The dissection of such a mechanism should help to target the cells that are necessary to adequately stimulate body growth. Until this is known, the respective roles of GH, IGFs, and insulin in the control of body growth will be continuously under debate. The understanding of which cell types are the target of these factors will surely help to dissect these mechanisms and help to further our understanding of normal and pathological growth processes.

7. IGFS AND CANCER STEM CELLS

Stem cells have an almost unlimited capacity to renew, as cancer cells do. The difference between these two cells resides in the fact that cancer cells have lost their capacity to exit the cell cycle under the influence of normal environmental stimuli. How immortalization leads to oncogenesis is still obscure. Reya *et al.*[94] described that several cancer cells share various characteristics of stem cells and named the cells at the origin of cancer, cancer stem cells. They consider a cancerous tumor to be an abnormal organ that is built with cancer stem cells, but also with precursors and differentiated cells. In fact, the cancer stem cell can renew itself as does as a stem cell and generate all the cells composing an organ. It appears that during the proliferation process of the cancer stem cells, certain signals can drive the cancer stem cells to generate postmitotic cells showing that the proliferation of a cancer stem cell is not blocked to an irreversible process of cell division.

By understanding how normal stem cells and cancer stem cells renew, we may understand how to interfere with cancer cell division. Certain mutations lead to oncogenesis, however how this process of oncogenesis occurs after the mutation remains unsolved in many cases. Many genes can be upregulated by just one mutation, resulting in a new homeostasis of cell cycle regulation. Like stem cells, cancer stem cell division is controlled by several intrinsic and extrinsic factors, each one playing a specific role. We have previously seen that IGFs control the proliferation of several stem cells and other precursors. It is therefore not surprising to observe that IGF-I contributes to the development of numerous tumors (see Chapter 13) such as leukemia[40], meduloblastoma[95], breast cancer[71], hepatocellular carcinoma[96], and in several other cancers reviewed in ref. 97. It would be important to determine whether IGF-I stimulates the proliferation of the cancer stem cell or whether it acts on downstream progenitors. Such knowledge will help to further understand cancer stem cell renewal and how we can attempt to target specific mechanisms of the cancer stem cells by specific agents to prevent their proliferation. Indeed, if chemotherapy acts only on cancer progenitors and not on cancer stem cells, we would succeed in decreasing the size of the tumor, but could expect a second event of tumor formation, because the cancer stem cells were not eliminated. In this context, the dissection of the IGF-I role on tumor formation is of prime importance.

8. CELL TRANSPLANTATION

Cell transplantation is already used with recognized success in medicine to repopulate the hematopoietic system[31]. This approach also seems to be promising for certain organs that have fully or partially lost their function, like the pancreas[98] and the brain[99-101]. All encouraging data harvested from experiments done on these organs, with some success in the clinic, were obtained with cells derived directly from fetal tissues. One of the major problems is the quantity of cells that can be generated. For instance, depending on the hospital center, it is necessary to have one to five fetuses to transplant mesencephalic cells in one hemisphere of one Parkinson patient[102]. This approach has shown some success and some limitations (discussed in ref. 103).

One of these limits is obviously the number of cells that are available to transplant thousands of patients (number necessary for a region as large as Switzerland) and another limitation is the lack of external control of cell fate after transplantation. Indeed, in certain rare cases, the graft developed into a cyst[104] or was too efficient and produced aberrant motor behavior such as dyskenesia[103]. It appears that we need a cell source that can be both greatly amplified and genetically modified to eliminate uncontrolled cells and to prevent side effects. Stem cells appear to be a source of choice if we understand how to generate the desired cell phenotype that we want to transplant.

Knowing IGF's functions in the different stem cell systems that could be used for cell transplantation is of prime importance for the development of such therapy. Indeed, if IGFs are necessary for stem cell renewal and the differentiation of various cells, as described in Sections 5 and 6, the understanding of their mechanisms of action may help to generate the adequate cell phenotype for a defined transplantation paradigm. Moreover, recent studies have shown that the combination of IGF-I delivery with hematopoietic stem cell transplantation enhances graft integration[105] showing that IGF-I can also play a role in the dynamic process of cell engrafting.

9. REGENERATION

Comprehension of how a stem cell can be stimulated *in vitro* and how the environment drives the related progeny to adopt a specific cell fate opens new approaches for tissue regeneration *in vivo*. Indeed, almost every organ contains stem cells during adulthood that can be recruited to repair a wounded tissue area. In consequence, it is potentially possible to mobilize the endogenous stem cells by exogenous factors to rebuild a lesioned area.

This approach has had some important and encouraging success in different animal models, with the most recent impressive result being the regeneration of skeletal muscles of old mice by Notch activation[106]. Interestingly, in this study it was shown that the degeneration of muscle during aging was due to a depletion of the Notch ligand, delta, in satellite cells and not to an intrinsic impairment of these cells, bringing new hope for other tissue regeneration.

In this context, IGF-I was also shown to be synthesized in injured skeletal muscles[107]. Moreover after motoneuron axotomy in transgenic mice expressing IGF-I, the regeneration of muscle is enhanced in comparison to wild type animals[108]. The number of satellite cells and of Sca-1 multipotent stem cells derived from the bone marrow located in skeletal muscles is increased in these IGF-I-transgenic animals, suggesting a better regeneration process. In addition, the gene transfer of IGF-I cDNA by viral vectors into the muscle of young mice, or by transgenesis with a specific IGF-I expression in muscles, prevents the loss of muscular function due to aging[109,110]. Indeed at old age, IGF-I treated (during youth) animals maintain muscular strength and mass at a level comparable to young mice.

Concerning peripheral neurons, several studies have shown that ectopic IGF-I adjunction leads to improvement of neuron regeneration after nerve lesions (for review see Ishii *et al.*, 1994). In the same study using motoneuron axotomy, IGF-I transgenic mice show a quicker recovery and better nerve conduction velocity in comparison to controls[108]. In the central nervous system, it was shown that IGF-I stimulates neurogenesis in the hippocampal region[111] and that other factors can mobilize neural stem cells residing around the lateral ventricles in the adult mouse brain like EGF[112]. This mobilization leads to the generation of new neurons[111,112]. Interestingly, the infusion of a high dose of insulin with FGF-2 in the chick eye induces the formation of new neurons in the periphery of the retina[70]. These experiments show that different regions of the CNS can generate new neurons. The combination of factors controlling stem cell renewal and generation of neurons, such as IGF-I, should help to reconstitute a damaged area of brain after stroke or ischemia or during a process that induces neural degeneration such as Parkinson's and Huntington's diseases.

As seen above with muscle cells, a denervated tissue is often more sensitive to degenerative processes. Such a phenomenon also occurs in the rat cornea when the trigeminal nerve is sectioned[113]. Indeed, when the corneal epithelium is removed 2 weeks after trigeminal denervation, the healing is delayed and incomplete in comparison to control animals[114]. It was previously shown that IGF-I and substance P synergize *in vivo* to enhance the closure of the corneal wound[115]. This positive effect was also observed when both substances were administrated on the wounded denervated

cornea[114]. These experiments demonstrate that IGF-I can accelerate and restore normal healing after corneal injury.

An increased healing rate was also observed in fractured bones in the presence of IGF-I[116,117]. Studies of the potential intracellular pathways used by IGF-I revealed that IRS-1 is involved in such phenomenon[118]. Indeed, deletion of the *IRS-1* gene leads to impairment of fracture healing, even 10 days post-surgery, whereas bone was well healed after 3 weeks in wild type animals. Overexpression of IRS-1 in the lesioned area of bone in *IRS-1*$^{-/-}$ mice resulted in a recovery of bone healing. Understanding of the mechanisms mediating IGF-I's action on bone healing, as described here, will certainly help to design precise strategies to assure union of fractured bones in problematic lesions.

Burns are a dramatic injury that affect skin, but also organs located deeper in the body, such as muscles. IGF-I was shown to improve tissue recovery after thermal injury[119]; however, IGF-I has side effects in such treatments, probably due to the high concentrations of IGF-I used. In order to bypass the problem of high concentration of IGF-I, Jeschke *et al.* transferred IGF-I cDNA to burned rats by subcutaneous injections of liposomes containing IGF-I cDNA[119]. Interestingly, IGF-I expression induced not only skin re-epithelialization, but also preserved muscle protein content, as well as body weight. These experiments show that IGF-I also has a beneficial role on skin and muscle recovery after thermal injury, but also suggest that its action is tightly linked and limited by the local concentration of IGF-I in the wounded area.

Taken together, these data show that GH and IGF related factors play a role not only during development, but can also help to recapitulate certain stages of this development in injured tissue in order to regenerate this tissue, giving new hope for regenerative medicine.

10. CONCLUDING REMARKS

The respective roles of GH and IGFs are still unclear for several cell systems, but with the availability of stem cells for almost all organs, the dissection of the functions and the mechanism of these growth factors will surely be solved, creating great hopes for the development of regenerative medicine or to target cancer stem cells as described above. Such knowledge will also help to adequately stimulate growth in children with severe growth deficiency without excessively stimulating the whole body. This is of great importance, because several studies have shown that the loss of GHR (GHR deficient mice) or of IGF-I functions (*IGF-I-R*$^{+/-}$ mice) leads to an increased lifespan[120,121] probably due to a decreased susceptibility to oxidative damage

generated in stressful conditions[121]. Sustaining these data, the overexpression of GH reduces lifespan[122], suggesting that inadequate dosages of GH might have long-term side effects. The understanding of the GH-IGF axis is not only of great interest for medicine, but also allows us to contemplate the limits of the body's lifespan and the balance between the benefits and side effects of the actions of IGF.

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