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Role of IGF-I Signaling in Muscle Bone Interactions

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Abstract

Skeletal muscle and bone rely on a number of growth factors to undergo development, modulate growth, and maintain physiological strength. A major player in these actions is insulin-like growth factor I (IGF-I). However, because this growth factor can directly enhance muscle mass and bone density, it alters the state of the musculoskeletal system indirectly through mechanical crosstalk between these two organ systems. Thus, there are clearly synergistic actions of IGF-I that extend beyond the direct activity through its receptor. This review will cover the production and signaling of IGF-I as it pertains to muscle and bone, the chemical and mechanical influences that arise from IGF-I activity, and the potential for therapeutic strategies based on IGF-I.

Keywords

Insulin-like growth factor; muscle hypertrophy; bone density; regeneration; repair

The complexity of IGF-I

The liver is the major source of IGF-I, where approximately 75% of the circulating IGF-I originates. This endocrine pool is stabilized in a tertiary complex with ALS (acid-labile subunit) and IGF binding protein 3 (IGFBP3), and only a small percentage is free. In addition to the liver, most tissues express a local source of IGF-I, such that paracrine and autocrine actions of IGF-I may occur. Thus, there is a quandary in the field as to whether these sources of IGF-I are indistinguishable from each other, or if there are unique features within a given pool of IGF-I that afford greater potency, stability, or tissue specificity. Indeed, alternative splicing of the *Igf1* gene and post-translational modifications of the nascent peptides result in many proteins that arise from this single gene. Further, the

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extended protein family includes insulin and IGF-II, both of which can bind and activate IGF-I receptors, lending an additional layer of complexity to IGF actions. Finally, the IGF-I receptors themselves mix and match with the insulin receptor to form hybrid receptors, and associate with multiple intracellular docking proteins to mediate growth factor actions. Thus every step from production, to ligand binding, and to downstream signaling, can contribute to complex tissue specific activity within the IGF-I pathway.

IGF-I Production

The *Igf1* gene contains six exons, and its alternative splicing results in multiple isoforms that retain the identical sequence for mature IGF-I peptide, but also produce divergent C-terminal sequences, called the E-peptides (1–4) (Figure 1). Two classes (I and II) arise from interchangeable utilization of exons 1 and 2, respectively. These exons encode a portion of the signal peptide(s), and their use appears to be dependent on two different promoters (3). Exons 3 and 4 encode the remaining part of the signal peptide, the mature IGF-I peptide, and a portion of the E peptide. Exons 5 and/or 6 encode multiple E-peptides. Transcripts that skip exon 5 and splice exon 4 directly to exon 6 are defined as class A. Human Class B transcripts utilize only exon 5, while human class C/rodent class B is produced by the inclusion of the entire rodent exon 5 and a portion of human exon 5 via an internal splice site; in both cases the insertion causes a frame shift in the reading frame of Exon 6 (4, 5). There is strong sequence conservation across all species for the mature IGF-I peptide, as well as class A E-peptide. However, the B and C class E-peptides exhibit high variability (6). In all tissues studied, roughly 90–95% of the IGF-I transcripts are Class A. The significance of the less common splice forms has been a matter of debate, where greater potency and IGF-independent activity have been attributed to the E-peptides. In myoblasts and osteoblasts, exposure to the EB/EC peptide promotes proliferation and inhibits differentiation (7–10); however, E-peptide activity appears to require the IGF-I receptor (9), and at least for muscle, there is no functional benefit of treatments based solely on the E-peptides (11). For this review, we will focus on the most common, class A isoform, as well as the actions of the mature IGF-I growth factor for muscle and bone.

Regardless of the isoform transcribed, a pre-pro-peptide is translated, which consists of a Class I or II signal peptide directing secretion, the mature IGF-I peptide, and a C-terminal E-peptide extension (12). Following cleavage of the signal peptide, the pro-IGF-I (mature IGF-I plus an E-peptide) can be subjected to additional processing prior to secretion. This includes cleavage of the E-peptide by intracellular proteases of the pro-protein convertase family to release mature IGF-I for secretion (13), maintenance of pro-IGF-I to be secreted without cleavage (14–17), or N-glycosylation in the E-peptide of the predominant IGF-I isoform (IGF-IA) (18), followed by secretion. Hence, three forms of IGF-I protein could exist in the extracellular milieu: mature IGF-I, non-glycosylated pro-IGF-I, and glycosylated-pro-IGF-I. Figure 2 schematizes the post-translational processing steps associated with production of the IGF-I forms, and shows the divergence of these forms in liver, muscle, and the circulation.

Cell-based assays have been used to show that pro-IGF-I has improved IGF-I receptor activity compared to mature IGF-I, whereas glycosylated pro-IGF-I has significantly lower

activity (16). Further, pro-IGF-I has been attributed with greater matrix association (19). For muscle, there is a large proportion of glycosylated pro IGF-I that is retained (Figure 2), suggesting that this form also has enhanced storage capacity. Comparable studies of the forms stored in bone have not been performed to date. However, given that the liver is thought to secrete primarily mature IGF-I stabilized by ALS and IGFBP3, this provides a molecular distinction between the endocrine pool of IGF-I (made by the liver), and the autocrine/paracrine pool provided by the tissue. Whether these represent functionally separable pools of IGF-I has not been addressed.

IGF-I Receptors

For IGF-I to promote growth, it must bind to and activate receptors on the membrane surface. IGF-I acts predominantly via the IGF-I receptor (IGF-IR), a transmembrane protein consisting of two extracellular α-subunits, which contain the IGF-I binding site, and two transmembrane β-subunits that have a cluster of three tyrosine residues (Y1135, Y1131, and Y1136) (Figure 3). These undergo phosphorylation upon IGF-I binding and activation of the intrinsic kinase domain (aa 956-1256) to mediate canonical signaling pathways necessary for cell survival and growth (20–22). Tyrosine phosphorylation is needed to create multiple docking sites for a variety of endogenous substrates including members of the insulin receptor substrate (IRS) family, which associate with IGF-IR via PTB and SH2 domains, growth receptor binding protein-2 (Grb2), which binds to specific motifs in the IGF-1 receptor as well as in IRS, and the p85 subunit of phosphatidyl inositol 3 kinase (PI3K) which binds to other specific motifs within IRS. Both IRS-1 and IRS-2 are expressed in bone (23, 24) and skeletal muscle (25), and both appear to be required for the full actions of IGF-I. Their roles differ, however. Deletion of IRS-1 results in decreased bone formation and resorption (23), whereas deletion of IRS-2 results in reduced bone formation but increased bone resorption (24). In muscle, IRS-1 and IRS-2 are not functionally interchangeable and IRS-1 appears to have a major role (25). Specifically, IRS-1 is required for IGF-I mediated hypertrophy: IGF-I overexpression failed to increase skeletal muscle mass in IRS-$1^{+/-}$ mice, in contrast to the rescue of growth deficits in other tissues (26). Shc, when tyrosine phosphorylated in response to IGF-I, binds to the SH2 domain of Grb2, which in turn forms a complex with Sos, a guanine nucleotide exchange factor that mediates GDP/GTP exchange in Ras and thus activates it. Ras then activates Raf (MAPKKK), which phosphorylates and activates MEK (MAPKK), which in turn phosphorylates and activates ERK1/2 (MAPK). These are serine/threonine phosphorylations. Activated ERK enters the nucleus to phosphorylate and so activate transcription factors (e.g. elk-1 and c-jun) leading to increased cyclin D_1 and reduced $p21^{cip}$ and $p27^{kip}$ expression. The increased levels of cyclin D_1 and reduced levels of the cell cycle inhibitors $p21^{cip}$ and $p27^{kip}$ stimulate cell cycle progression from G1 to S, thus completing the pathway by which IGF-I and other growth factors promote proliferation. Activation of PI3K sets up a different pathway. PI3K phosphorylates PIP_2 to PIP_3 in the membrane, recruiting PDK1/2 to the membrane where it phosphorylates and partially activates AKT at Thr 308 (27). MTORC2, discussed below, phosphorylates AKT on Ser 473 to fully activate this molecule (28).

Activated AKT has a number of substrates important for bone and muscle. The activated AKT phosphorylates and inactivates Bad, a pro-apoptotic member of the bcl-2 family. This

blocks apoptosis. AKT phosphorylates FOXO family members, keeping them out of the nucleus, thus reducing the induction of genes such as atrogin-1 (also known as MAFbx), MuRF1, and Cbl-b, which are ubiquitin ligases involved in protein degradation. AKT increases protein synthesis via its actions on mTOR and p70S6 kinase (29, 30). mTOR exists in two distinct complexes. mTORC1 is comprised of mTOR, Raptor (the regulatory component of mTOR), mLST8/GβL (G-proteinβ subunit-like protein), and DEPTOR. This complex is rapamycin sensitive and controls protein synthesis via phosphorylation of substrates p70S6K and 4E-BP (eukaryotic initiation factor 4E-binding protein). mTORC2, which is not rapamycin sensitive, is comprised of mTOR, Rictor (rapamycin insensitive component), mLST8/GβL, mSIN1 (mammalian stress-activated protein kinase-interacting protein 1) and PROTOR (protein observed with Rictor). mTORC2, as noted above, can activate AKT. GSK-3β (glycogen synthetase kinase) is also a substrate of AKT, and when phosphorylated at Ser 9 and 21 it is inactivated. When active, GSK-3β phosphorylates β-catenin at Thr 41, Ser 37, and Ser 33, leading to its proteosomal degradation. Thus, inactivating GSK-3β potentiates wnt/β-catenin signaling and so promotes proliferation (31). Hypoxia inducible factor (HIF)α is induced by IGF-I through the PI3K/AKT/mTOR pathway, leading to increased VEGF-A production and vascular invasion in the growth plate (32). Moreover, PI3K and AKT can enter the nucleus and by phosphorylating critical transcription factors lead to increased cyclin D1 levels. Thus, the signaling pathways of IGF-I are multiple and complex, but the net result is to promote cell proliferation and differentiation while blocking protein degradation and apoptosis.

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Necessity of IGF-I: Lessons from knockout mice

A rich history examining the importance of IGF-I for organismal growth exists. In the absence of any IGF-I, growth is severely impaired, demonstrated initially by gene targeting of the *IGF-I* locus (*IGF-I*$^{-/-}$ mouse) (33, 34), and is also evident in patients with short stature with mutations in the IGF-I pathway members (35). Ablating IGF-I expression globally causes severe growth retardation, which is evident as early as 13.5 dpc (33, 34, 36). The KO mice develop smaller skeletons with a significant delay in mineralization at 14.5 dpc and onward. Despite that, all of their skeletal elements are present (37), indicating normal skeletal patterning. The skeletal defects in the KO mice are caused, at least in part, by reduced proliferation, delayed differentiation, and increased apoptosis in the growth plate (38). Most of the IGF-I KO mice die immediately after birth. Studies of the few surviving KO mice (less than 5%) show continually retarded bone growth (34, 36), indicating that IGF-I signaling is also essential for skeletal development during post-natal growth. Ablating the IGF-IR produces a similar but more severe growth retardation and skeletal defects than the IGF-I KO mice, with a phenotype comparable to the double knockout of IGF-I and IGF-IR (34). This indicates the important role of IGF-II during embryonic development. IGF-II has 67% homology to IGF-I with comparable affinity for the IGF-IR (39). In humans IGF-II circulates at three times the level of IGF-I postnatally, but in rodents its expression in the skeleton essentially disappears after birth (40).

Most of the circulating IGF-I is produced by the liver, yet ablation of this source in mice does not have a dramatic effect on general postnatal body growth (41–43), indicating that tissues can rely on other pools of IGF-I. The general consensus is that limiting liver IGF-I, or the proteins in its circulating ternary complex (IGFBP3 and ALS) does not control the growth of many of the tissues that make their own IGF-I, such as skeletal muscles, long bones, or brain, supporting the hypothesis that local IGF-I is sufficient to sustain normal growth of these tissues. One important exception is that reduced circulating IGF-I limits bone density (44), where liver-specific deletion of IGF-I in 8–55-week-old mice led to small reductions in periosteal bone growth and appendicular skeletal growth leading to thinner bones, but with no abnormalities in trabecular bone (45). This suggests that bone growth not only relies on paracrine IGF-I (46), but is also dependent on endocrine IGF-I. In the complementary experiment, boosting circulating levels of IGF-I by increasing liver production (47) or through daily injections of recombinant IGF-I increases body (and muscle) weight, supporting the premise that circulating IGF-I can enter the local tissue environment and have enhanced anabolic effects. Thus, IGF-I appears to act *directly* on muscle and bone, and potentially *indirectly* on bone via enhancing muscle mass.

Effects of Muscle IGF-I on Growth

To address the importance of muscle IGF-I, production of IGF-I from muscle was limited through muscle specific targeting of the stress protein Glucose Regulated Protein 94 (GRP94) (48, 49). GRP94 is a member of the HSP90 family of stress proteins whose expression is regulated by a variety of metabolic conditions, such as glucose tension, redox state and changes to calcium homeostasis (50). GRP94 is required for production of IGF-I and IGF-II because it binds to pro-IGF, and this interaction is thought to be required for the

proteolytic processing of the pro-form to mature IGF (48). Tissue specific ablation of GRP94 was achieved by breeding mice harboring alleles of GRP94 with loxP sites flanking the first exon of the gene (51) with mice expressing Cre recombinase driven by the muscle creatine kinase (MCK) promoter (52), generating *mGRP94*$^{-/-}$ mice. Not only did these mice exhibit reduced IGF-I content in muscle, they also had diminished circulating IGF-I. Intriguingly, the circulating IGF-I was both pro- and mature IGF-I, and pro-IGF-I was virtually eliminated from the blood in GRP94 mutant animals. Mice had smaller muscles, presumably due to the loss of local IGF-I production, and whole body growth was impaired as well. The body length growth curves also showed that *mGRP94*$^{-/-}$ mice were shorter, and consistent with this, skeletal dimensions and bone mineral density were reduced.

This study supports that muscle IGF-I is a critical factor for local and global growth. Given that ablation of liver IGF-I production did not reduce skeletal growth to the same extent as when muscle IGF-I production was inhibited, we are faced with at least two possible interpretations. First, the simplest explanation is that the loss of muscle mass as a consequence of limiting muscle IGF-I has a significant effect on bone. However, we cannot exclude the second possibility that the IGF-I produced by the muscle has different properties on bone than that produced by the liver. One strategy that could address this issue is to separate muscle IGF-I production from the muscle response to this growth factor. A mouse harboring a dominant negative IGF-IR in striated muscle (53, 54), exhibits muscle hypoplasia early in life, even though IGF-I production is normal. These mice have long but slender bones due to increased osteoclast activity (55), suggesting that it is the mechanical influence of muscle rather than the chemical production of IGF-I that alters bone growth. However, a clear interpretation is confounded by the temporal separation of the muscle and bone phenotypes, as well as the development of Type 2 diabetes in this model. Thus, a clean experiment has not been developed to resolve this issue.

Effects of Bone IGF-I on Growth

Skeletal development is a highly coordinated process, which requires initial recruitment and migration of relevant precursors to future bone sites, commitment of the precursors to chondrogenic and osteogenic lineages, and their terminal differentiation to acquire a complete set of cartilage- or bone-forming functions (56, 57). Skeletal patterning, which determines bone numbers and shapes, is controlled by progenitor recruitment and commitment, while bone growth rates mainly depend on the proliferation, survival, and differentiation of chondrogenic and osteogenic cells (56). It is the latter (ie. growth rates) that requires IGF-I signaling.

With the exception of the flat bones of the skull and the clavicle, bone formation in the embryo occurs through an endochondral process that begins when mesenchymal stem cells form clusters or condensations via adhesion molecules. Most of the cells in these condensations differentiate into chondrocytes with cells on the periphery forming a perichondrium. Cartilage increases in size through chondrocyte proliferation and secretion of a matrix enriched rich in type II collagen and aggrecan. These chondrocytes become hypertrophic and begin to synthesize type X collagen to induce mineralization of the surrounding matrix while promoting vascular invasion and initiating resorption of the

calcified cartilaginous matrix. Hypertrophic chondrocytes also stimulate the perichondrial cells to differentiate into osteoblasts, which in turn secrete a characteristic matrix that leads to the formation of a bone collar. Eventually, these hypertrophic chondrocytes are thought to undergo apoptosis, although this has not been clearly demonstrated, and the alternative hypothesis is that these cells differentiate into osteoblasts (58). To examine the role of IGF-I signaling during chondrogenesis, a chondrocyte specific knockout of IGF-IR (^{cart}IGF-1R$^{-/-}$) was generated by crossing floxed IGF-IR mice (59) with mice expressing a Cre recombinase transgene under the control of the type II collagen promoter (60). The growth of the ^{cart}IGF-1R$^{-/-}$ mice is less retarded than that of the global IGF-IR KO mice (90% of controls vs 45%, respectively), although postnatal survival was still poor. Like the global IGF-I KO mice, the skeletons showed decreased mineralization with reduced proliferation, delayed differentiation and hypertrophy, and increased apoptosis of the chondrocytes (61). To separate the requirement for IGF-I signaling in growth plate development during embryonic and postnatal growth, a conditional chondrocyte specific KO model using a tamoxifen (Tam)-inducible collagen II driven Cre recombinase (^{TamCart}IGF-IR$^{-/-}$) (62, 63) was examined at 2 weeks of age (1 week post Cre induction with tamoxifen). The ^{TamCart}IGF-IR$^{-/-}$ mice showed significant growth retardation with a body weight that was ≈70% of their control littermates, and severely disorganized growth plates due to reduced cell proliferation and delayed differentiation (61). Deletion of IGF-I from chondrocytes using a comparable Cre recombinase (64) caused a 25% reduction in growth and total bone mineral content at 2–4 weeks. However, only a 40% reduction in IGF-I expression was achieved in these cells. These results could indicate that IGF-II compensated for the loss of IGF-I during development in this model, but could also reflect the failure to fully delete IGF-I expression.

Role of IGF-I in Bone Remodeling

Throughout life, bone is constantly remodeled by cooperative actions of bone-resorbing osteoclasts and bone-forming osteoblasts (65, 66). The osteoclasts are derived from hematopoietic stem cells (HSC), while osteoblasts differentiate from bone marrow mesenchymal stem cells. Bone formation and resorption are tightly balanced to maintain structural integrity of the skeleton and to mobilize Ca^{2+} to meet systemic demands. Studies of global IGF-I KO mice (67, 68), which survived to adulthood, show decreased bone formation rates, but higher trabecular bone mass (BV/TV), indicating reduction in both osteoblastic and osteoclastic activities, and demonstrating the non-redundant role for IGF-I signaling in bone remodeling. Recent studies of cell-specific IGF-I and IGF-IR KO mice and *in vitro* cell cultures have begun to reveal detailed molecular actions of those molecules in the various populations of skeletal cells.

The osteoblastic lineage begins with mesenchymal progenitors that progress through stages of preosteoblasts (Pre-OB), mature osteoblasts (OB), and finally osteocytes (OCY). At each stage, cells express specific markers and unique regulators of cell differentiation. Osteoprogenitors and preosteoblasts express RUNX2 and osterix (OSX) -- two critical transcriptional factors that specify bone cell fates. Early stage osteoblasts produce large amounts of type I collagen as a major component of their surrounding matrix. Mature or fully differentiated osteoblasts, which express osteocalcin (OCN), acquire functions to

mineralize the collagen matrix so formed. Some mature osteoblasts are subsequently embedded in the matrix as osteocytes, which express the dentin matrix protein-1 (DMP-1) and sclerostin, a protein that inhibits osteoblast proliferation by suppressing the wnt/β-catenin signaling pathway. OCY are also an important source of RANKL by which they can control osteoclastogenesis as well. Other osteoblasts become quiescent on the bone surface to function as lining cells that may be subsequently activated by hormones such as parathyroid hormone (PTH), or otherwise die by apoptosis (69, 70).

A number of *in vitro* and *in vivo* studies have demonstrated the importance of IGF-I signaling for normal osteoblast development and function. IGF-I stimulates survival, proliferation, differentiation, and matrix production in cultured osteoblast cells (71, 72). Animals that overexpress an osteocalcin-driven IGF-I transgene exhibit higher osteocyte lacunae occupancy, increased bone formation rate (BFR), bone volume and bone mineral density (BMD), but without any change in total osteoblasts or osteoclasts. These findings suggest that osteoblast-derived IGF-I primarily exerts its anabolic effects by enhancing osteoblast function and osteocyte survival (73). There is also evidence that IGF-I promotes phosphate uptake by osteoblasts via the sodium-dependent phosphate transporter, Glvr-1, at least in the human osteoblast cell like SaOS-2 (74), which is expected to enhance bone formation. The delayed mineralization observed in global IGF-IKO indicates a role for IGF-I signaling in normal osteoblast maturation and function *in vivo*. Govoni et al (46) generated an osteoblast conditional knockout of IGF-I using Col1α2 promoter driven Cre recombinase activity. These animals had a high perinatal mortality with dwarfism and mineralization defects characteristic of the global IGF-IKO. However, data from these conditional knockouts must be interpreted with caution as the Col1α2 promoter-driven Cre recombinase used by these investigators was also highly active in muscle and other non-skeletal tissues (46). Their observations could reflect an important but as yet unexplored role for muscle derived IGF-I during skeletal development. We (75) used an osteocalcin promoter-driven Cre recombinase to delete IGF-IR from mature osteoblasts and osteocytes (^{ocn}IGF-IRKO). These mice have excellent postnatal survival with growth rates that resemble wild-type animals. Zhang et al (76) characterized the skeletal phenotype of these animals at 3 and 6 weeks. At 3 weeks the KO display reduced osteoblast and osteoclast numbers and reduced BFR compared to wild-type controls suggesting that IGF-I signaling is important in promoting osteoblast proliferation, function and survival during the early stages of postnatal bone modeling. Moreover, the loss of IGF-IR in the osteoblast also negatively affects osteoclast proliferation via mechanisms that will be discussed subsequently. However, such differences fail to persist at 6 weeks of age. At 6 weeks, excess osteoid coupled with reduced mineralization, fewer trabeculae, and lower trabecular BV/TV is the predominant phenotype. These results are consistent with our *in vitro* observations showing the marked reduction in mineralization of bone marrow stromal cell cultures from the ^{ocn}IGF-1RKO mice but with no reduction in alkaline phosphatase positive colonies(75). However, when we (Wang and Bikle, submitted) deleted IGF-IR from osteoprogenitors using an OSX promoter driven Cre recombinase (^{osx}IGF-IRKO) the phenotype was more profound, although not as severe as mice lacking IGF-IR in their chondrocytes, discussed above. These mice grew poorly, although their perinatal mortality is not high unlike the global IGF-I KO and ^{cart}IGF-IR KO. However, chondrocyte terminal differentiation is delayed, and the

development of trabecular bone is blunted. Rather than having increased BV/TV like the global IGF-I KO, their trabecular bone is osteoporotic with decreased proliferation of the osteoblasts lining the trabeculae. In culture the BMSC from these mice show decreased numbers of both alkaline phosphatase and alizarin red positive colonies, unlike BMSC from ^{ocn}IGF-IRKO, which are deficient only in mineralization. Thus ^{osx}IGF-IRKO have features of both the chondrocyte specific and osteoblast specific IGF-IRKO, but with less severity in chondrocyte proliferation than ^{cart}IGF-1RKO and an earlier defect in osteoblast proliferation and differentiation than ^{ocn}IGF-1RKO. At the other extreme Sheng et al. (77)deleted IGF-I from osteocytes using a DMP-1 promoter driven Cre recombinase (^{dmp1}IGF-IKO). These mice had a modest reduction in body weight, but their trabecular BV/TV was increased, and no reduction in OCY density was observed. Surprisingly, the ^{dmp1}IGF-IKO showed a significant reduction in growth plate length that was attributed to a decrease in the length of the hypertrophic zone with no abnormalities in the proliferative zone. Periosteal BFR, especially in the younger mice, was most impacted, and the periosteal response to mechanical loading (4 point bending) was markedly blunted (78). However, caution in interpreting these data is warranted in that the reduction in IGF-I expressing OCY was only 60%, and muscle had an equivalent reduction in IGF-I expression. Thus the failure of these mice to respond to mechanical load and the preferential decrease in periosteal BFR could reflect a contribution from the surrounding musculature.

Studies of global IGF-I KO mice showed fewer (76% of WT control) and smaller osteoclasts with fewer nuclei in the bone (38), indicating impaired osteoclastogenesis and bone resorption. These osteoclast defects could explain, at least in part, the increased trabecular BV/TV, despite a decreased bone formation rate in the global IGF-I KO mice described earlier. These osteoclast defects appear to reside at least in part in the osteoclast itself as osteoclast precursors from the global IGF-I KO mice showed a reduced ability to differentiate into mature osteoclasts (38). Moreover, co-culturing IGF-$1^{+/+}$ osteoclast precursors (spleen cells from WT mice) with IGF-$1^{-/-}$ osteoblasts (bone marrow stromal cells from IGF-I KO mice) produces significantly fewer (by ≈90%) multi-nucleated osteoclasts than co-culturing IGF-$I^{+/+}$ osteoclast precursors with IGF-$I^{+/+}$ osteoblasts, results suggesting that IGF-I from osteoblasts is required to promote osteoclastogenesis. On the other hand, co-culturing IGF-$I^{-/-}$ osteoclast precursors with IGF-$I^{+/+}$ osteoblast precursors also produces fewer (by ≈50%) multi-nucleated osteoclasts than co-culturing IGF-$I^{+/+}$ osteoclast precursors with IGF-$I^{+/+}$ osteoblasts (38), suggesting that the expression of IGF-I in osteoclast precursors is also required to fully support osteoclastogenesis. To further define the role of IGF-I signaling in osteoclast differentiation, we (Wang and Bikle, unpublished) evaluated mice in which IGF-IR was deleted from osteoclast precursors with a TRAP5b promoter driven Cre recombinase (^{trap}IGF-IR KO).These mice grow normally but have increased trabecular BV/TV and reduced osteoclast number, as seen in the global IGF-I KO mice. Similarly, ablation of IGF-IR in cultured osteoclast precursors reduces the number and size of osteoclasts and their expression of RANK, c-fms, and NFATc1 (Wang and Bikle, unpublished data). These *in vitro* and *in vivo* data demonstrate an essential role for IGF-I signaling in promoting osteoclastogenesis.

Role of IGF-1 in Muscle Growth and Repair

For skeletal muscle, IGF-I coordinates with additional growth factors to promote myoblast proliferation, differentiation, and fiber formation during normal growth as well as during regeneration after injury. Thus, IGF-I is a central therapeutic target for enhancing muscle function in aging and disease. Several strategies have been employed to boost IGF-I levels in muscle, including tissue-specific transgenic expression (79–81), viral-mediated gene transfer (82–84), and directed recombinant IGF-I delivery (85, 86). Increasing IGF-I levels can result in functional hypertrophy in young adult animals, maintenance of mass and regenerative capacity in senescent animals, and enhancement of muscle recovery to counter acute and chronic damage.

The two primary signaling pathways described above are important contributors to muscle physiology (87, 88). The PI3K/AKT pathway promotes cell survival as with many tissues (89, 90). In addition, activation of Akt is sufficient to induce hypertrophy *in vivo* via an increase in the average cross sectional area of individual muscle fibers caused by an increase in activation of protein synthesis pathways. In fact, the hypertrophy caused by active Akt is more profound that that with IGF-I stimulation (91), and led to the discovery of Fboxo40, which targets IRS-1 for degradation, and reduces the efficiency of the IGF-I signaling pathway. The other primary intracellular pathway activated by IGF-1 (Ras-Raf-ERK pathway) has been shown to increase cell proliferation in muscle cell cultures (92).

Since muscle fibers are post-mitotic, muscle repair relies on muscle satellite cells (93), a stem cell like population that is a source for replenishing the nuclear content of the muscle fibers (94). After their activation, the satellite cells divide and can undergo differentiation, fusing to damaged sites on muscle fibers, or forming new fibers, thus providing an extra boost required for the increased protein synthesis during repair (95–101) or hypertrophy (85, 102, 103). These processes have been thought to be a predominant mechanism that leads to increase in muscle mass (103, 104). Satellite cell division is enhanced by high levels of IGF-I resulting in increased cell availability for growth and repair (105), but one limitation is that satellite cells express IGF-I receptor only after activation. Whether satellite cells are necessary for muscle fiber hypertrophy has been addressed in several studies by using irradiation-induced inhibition of satellite cell activation (106, 107), emphasizing an obligatory role of these cells during the overload-dependent muscle hypertrophy process. More recently, a novel mouse strain (Pax7-DTA) was utilized, which enabled the conditional ablation of >90% of satellite cells in mature skeletal muscle (108). This study provided new evidence that satellite cells are not needed for skeletal muscle fiber hypertrophy and that fibers are capable of mounting a robust hypertrophic response to mechanical overload independently of satellite cells. However, satellite cells are necessary for both the *de novo* formation of new fibers and fiber regeneration.

Muscle repair is accompanied by a robust increase in IGF-I. In acute damage generated by cardiotoxin injection, not only do the overall levels of IGF-I increase, but there is an appearance of mature IGF-I concomitant with increased P-AKT, supporting that the presence of mature IGF-I is necessary for mediating repair. Mature IGF-I accumulation could arise either from increased production and processing by the muscle and activated

satellite cells, through enhanced delivery of mature IGF-I from the circulation, or by cleavage of the locally stored forms to release mature IGF-I. Some evidence exists that IGF-1 is produced and released by myofibers in response to increased loading or muscle damage. Specifically, resistance and/or muscle damaging exercise result in local up-regulation of IGF-1 and hypertrophy (109–112). A second bout of resistance exercise on skeletal muscle can sustain increases of Igf1 transcripts (112, 113), raising the potential for optimizing the production of IGF-I as well as the response of muscle to this growth factor through consciously timing the intervals between exercise bouts.

Even though there is an increase in locally produced IGF-1 following muscle damaging exercise, the data is equivocal with regard to whether it enters the circulation (112, 114, 115). Moreover, exogenous administration of GH or IGF-1 does not stimulate myofiber hypertrophy in the absence of mechanical loading (116, 117). Thus, even with the plethora of studies demonstrating that IGF-I drives muscle hypertrophy, where increased muscle specific expression of IGF-I through transgenesis or post-natal viral delivery increases muscle mass (refs here) the story is not consistent, suggesting that IGF-I plays a marginal role in skeletal muscle during chronic exercise (118), and that exercise-induced muscle hypertrophy is an intrinsic process independent of hormones and circulating growth factors (119). For example, contrary to the follistatin-induced muscle hypertrophy where IGF-1 signaling appears to be a critical factor in the induction of hypertrophy (120), increased mechanical load can activate the AKT/p70S6K pathway and induce muscle hypertrophy independently of a functional IGF-1 receptor (121). These findings suggested that IGF-1 may not be a limiting factor for the overloading-induced muscle hypertrophy. Thus, more studies are needed to identify the upstream mechanisms responsible for the activation of AKT-mediated signaling in response to muscle loading, and whether IGF-1 is part of those mechanisms (121).

Growth Hormone vs IGF-I

The skeletal production of IGF-I postnatally is controlled primarily by growth hormone (GH) (122) and parathyroid hormone (PTH) (123, 124), although thyroid hormone (TH) also plays a role (125–128). Glucocorticoids, on the other hand, reduce IGF-I expression in growth plates (129), likely contributing to their negative impact on skeletal growth. Regulation of IGF-I expression during embryologic development is unclear, although PTHrP is a good candidate.

GH receptors (GHR) have been demonstrated in chondrocytes (130) and osteoblasts (131), where its growth promoting actions are mediated primarily but not exclusively by IGF-I (132, 133). This is well demonstrated by the ability of IGF-I antibodies to block the growth promoting actions of GH when injected into bone (132), the markedly stunted growth of IGF-I or IGF-IR null animals despite the presumed elevated circulating GH (68), and the failure of GH to stimulate growth in IGF-I null animals (134). However, we (68) did observe a modest increase in periosteal BFR when GH was infused into global IGF-I null mice, and Lupu et al. (133) found that mice lacking both GHR and IGF-I were more profoundly growth retarded than either knockout alone. Stat 5 appears to mediate the effect of GH/GHR

in growth plate chondrocytes (135), and knockout of Stat5b results in growth retardation, narrow proliferation zones of the growth plate, and decreased IGF-I expression (136).

Potential IGF-I mediated interactions between muscle and bone

There is growing evidence that covering the fracture site with flaps of muscle is superior to using fasciocutaneous flaps with respect to fracture healing even though both tissues provide an adequate vascular supply and protection from invading microbes (137). However, it remains to be determined why muscle enhances healing, and whether the healing properties of muscle can be translated into better results with respect to fracture repair. Earlier studies in a mouse fracture model (mid shaft femur) demonstrated that insertion of a semipermeable material (Gore-tex or polytetrafluoroethylene, PTFE) that enabled gas transport but not cell migration) between the muscle layer and the fracture site markedly retarded fracture repair (138). This model was further refined by using nitrocellulose membranes that varied in pore size enabling a range of molecules from 3.5kDa to 50kDa to pass through from muscle to bone (and vice versa), but excluded cells (139). Regardless of pore size, fracture repair was impeded by the membrane. Thus it appears that cellular movement from muscle to bone (or vice versa) or direct cell-cell contact is required to facilitate fracture repair, although these experiments do not exclude the possibility that trophic factors and/or signaling molecules are also required for bone repair in response to muscle.

In the process of fracture repair, the role of stem cells capable of differentiating first into chondrocytes that form the soft callus and then into osteoblasts and osteoclasts to form and remodel the hard callus is well recognized. In fracture models utilizing bone grafts from which muscle has been dissected away, much of the fracture repair is dependent on cells within the periosteum (140, 141). The contribution of stem cells from the bone marrow and/or vascular supply appears to be of lesser importance (142). However, as noted above, when the periosteum is separated from muscle, the repair process is markedly retarded. Thus, it seems likely that there is communication between cells in muscle and periosteum that is critical for fracture repair. Identifying those cells can lead to cellular therapy especially for delayed or non-union fractures.

Muscle itself has a number of different stem-like cells with myogenic and osteogenic potential. Cells with osteogenic potential are highlighted by a phenomenon known as heterotopic ossification, in which trauma to muscle results in bone formation in the muscle generally adjacent to the surface of bone. In addition, labeled implanted myoblasts have been found to be incorporated into bone in a bone defect model (143). Similarly, cells obtained from muscle overlying a recent (3day) fracture site differentiated into bone forming cells *in* vitro (144). Taken together, these experiments suggest that muscle may provide cells to the fracture site in bone to facilitate healing. However, signals from the fracture site are needed to recruit these cells to the fracture site and stimulate their differentiation into osteoblasts. Among the potential paracrine/autocrine factors that may be involved in such signaling is IGF-I. IGF-I is the only paracrine factor that promotes both proliferation and differentiation of both osteoblasts and myoblasts (satellite cells, in particular) (88, 145, 146). We have shown that deletion of IGF-IR from either chondrocytes or osteoblasts markedly retards fracture healing (61, 147). Thus there is tantalizing evidence that muscle and bone

talk to each other in the course of their mutual repair processes by exchanging cells and paracrine signals.

Therapeutic potential of IGF-I to counter muscle and bone loss

Bone loss and muscle atrophy pose major problems for individuals immobilized or subjected to microgravity for prolonged periods of time. Little study of potential interactions between muscle and bone during unloading or reloading has been made despite their obvious proximity and parallel responses. In both cases IGF-I signaling is perturbed during the unloading process resulting in resistance to the anabolic actions of IGF-I on both muscle and bone. In particular, activation of IGF-IR is blunted during unloading, with subsequent loss of downstream signaling. On the other hand IGF-I expression is increased with muscle contraction (148), and IGF-I has been localized along the muscle-periosteal interface (149). In bone, integrins play a role in the activation of IGF-IR, as the expression of integrins is reduced during unloading. In muscle IGF-I resistance has been attributed to degradation of IRS-1 due to upregulation of ubiquitin ligases (the expression of which is inhibited by IGF-I), but it is possible that these apparently cell specific mechanisms are common to both tissues. Second, it is possible that the response of muscle to unloading and reloading contributes to the response of bone, and vice versa, providing a coordinated response to different conditions of mechanical load of the musculoskeletal unit. Although one could postulate a number of possibilities for such interactions, the potential role of IGF-I from muscle affecting bone and vice versa during the unloading/reloading process is certainly high on the list. As noted above prolonged immobilization causes major problems for the musculoskeletal system. Perhaps the most devastating example is that of spinal cord injury (SCI). SCI is well known to be accompanied by a high risk of fractures from trivial trauma such as getting out of a car. Remobilization if started early is beneficial, but the results generally leave much to be desired. Similarly, long periods of immobilization due to complicated fractures or illness are frequently accompanied by substantial loss of bone and muscle, losses which predispose the individual to further injury on remobilization. IGF-I could be explored clinically to hasten the recovery/rehabilitation efforts of individuals experiencing prolonged periods of immobilization.

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Highlights

- IGF-I has both endocrine and local production.
- IGF-I has direct growth effects on skeletal muscle and bone.
- IGF-I has indirect effects on bone via its direct actions on muscle.
- Synergy of IGF-I and mechanical crosstalk creates potential therapeutic strategies.

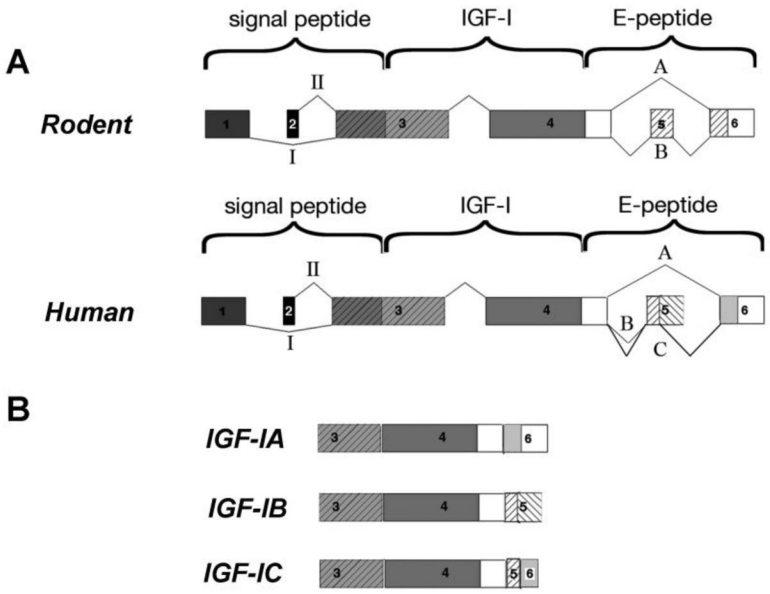

Figure 1.

Alternative splicing of the Igf1 gene in rodents and humans. A. The 6 exons in Igf1 exhibit alternative splicing at the 5′ and 3′ ends, with exons 1 or 2 plus a portion of 3 encoding two classes of signal peptides. Exons 3 and 4 are invariant, containing the sequence for mature IGF-I. The remaining sequence generates the E peptide regions. B. Variants generated by splicing of exons 4 and exon 6 are referred to as IGF-IA. In humans, retention of the entire exon 5 sequence in the absence of exon 6 is referred to as IGF-IB. Transcripts that contain exons 4, 5 and 6 are designated as IGF-IC in humans, and IGF-IB in rodents. This form is also known as MGF.

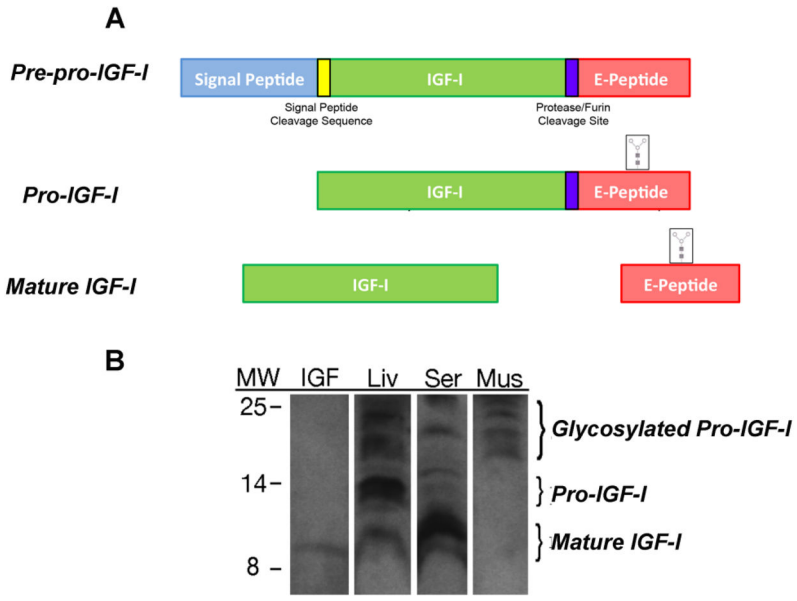

Figure 2.

Post-translational processing of IGF-I. A. Following translation of the pre-pro-peptide, which consists of a signal peptide directing secretion, the mature IGF-I peptide, and a C-terminal E-peptide extension, the signal peptide is cleaved to release pro-IGF-I (mature IGF-I plus an E-peptide). Pro-IGF-I can be subjected to cleavage of the E-peptide by intracellular proteases of the pro-protein convertase family to produce mature IGF-I for secretion, or secreted without cleavage. In addition, N-glycosylation in the E-peptide of the predominant IGF-I isoform (IGF-IA) can occur followed by secretion. B. Multiple forms of IGF-I protein exist in the extracellular milieu: mature IGF-I, non-glycosylated pro-IGF-I, and glycosylated-pro-IGF-I. Immunoblotting of lysates from liver (Liv), serum (Ser), and muscle (Mus) display the range of endogenous IGF-I species compared to recombinant mature IGF-I (IGF).

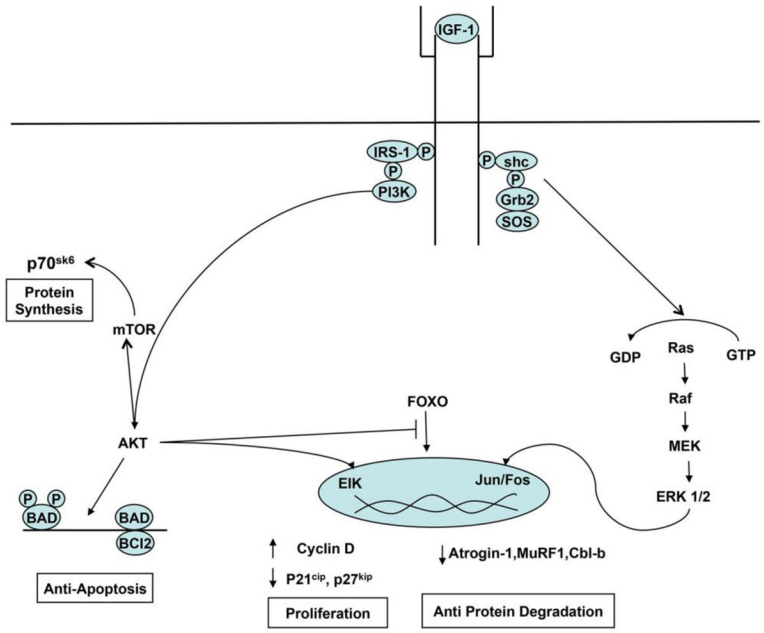

Figure 3. IGF1 signaling

The IGF1R is comprised of two α and 2 β subunits. On binding to IGF1, the cytoplasmic portion of the β subunits undergo phosphorylation at specific tyrosines, forming binding sites for a number of signaling molecules. The Shc/Grb2/SOS complex activates Ras leading to activation of the MAPK pathway. ERK1/2 phosphorylation enables these molecules to enter the nucleus to activate various transcription factors such as Jun/Fos. IRS-1 when phosphorylated can facilitate the activation of PI3K that leads in turn to PIP2 phosphorylation to PIP3, which brings PDK2 and AKT to the membrane where AKT is phosphorylated and activated. AKT has a number of substrates including BAD that when phosphorylated inactivates this proapoptotic molecule, mTOR which stimulates protein synthesis by activating $p70^{sk6}$, and FOXO which when phosphorylated is prevented from entering the nucleus and stimulating various ubiquitin ligases such as atrogin-1/MAFbx. AKT can also phosphorylate and inactivate GSK-3β, an important regulator of wnt/β-catenin signaling.