

Role of muscle-derived growth factors in bone formation

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Abstract

Muscle and bone anabolism and catabolism are tightly coupled during growth, development, and aging, yet the cellular and molecular mechanisms linking these two tissues are not well understood. Here we show that FGF-2 and IGF-1, two growth factors known to play a major role in regulating bone formation, are localized to muscle fibers along the muscle-bone interface of the mouse forelimb. Likewise, receptors for these growth factors are also abundant in periosteum adjacent to fleshy muscle attachments along the diaphysis of long bones. Growth factor levels were quantified from homogenized mouse forelimb muscles and IGF-1 was found to be the most abundant factor with FGF-2 also detected. Growth factor levels were also analyzed in conditioned medium from cultured myotubes, and IGF-1 and FGF-2 were again detected at significant levels. Mechanically wounding C2C12 myotubes increased the release of FGF-2 into conditioned medium, whereas IGF-1 was secreted at lower concentrations than FGF-2 following injury. Together these findings suggest that muscle is an important, local source of growth factors for bone tissue. Hence, the integrated growth and development of bone and muscle is likely to be regulated in part by paracrine mechanisms at the muscle-bone interface involving growth factor signaling.

Keywords: IGF-1, FGF-2, Periosteum, Muscle Injury, C2C12 Myotubes

A primary function of the vertebrate skeleton is to provide a set of bony levers to which muscles can attach in order to move and support the body. A close functional association between muscle and bone is underscored by the tight coupling of muscle and bone anabolism and catabolism during growth, development and aging. For example, peak rates of bone mineral acquisition in the skeleton are preceded by peak rates of increase in muscle mass¹, and bone loss in later life is accompanied by significant loss of muscle mass². Likewise, loss of muscle tissue with focal paralysis induces a rapid and significant loss of cortical bone³. Muscle is not only a regulator of pediatric bone gain, and perhaps a determinant of bone loss with aging, but muscle also plays a major role in bone repair⁴. As noted by Stein et al.⁵ "Muscle is perhaps the most crucial factor in the physiological process of fracture healing". Con-

sistent with an important role for muscle in driving bone formation and bone repair, fractures that are covered with relatively intact muscle heal more rapidly than fractures associated with severe muscle damage⁶. Although there is a clear connection between muscle and bone repair, the underlying mechanisms linking these two tissues are not well understood.

New bone can form nearby, and even within, a traumatized muscle. This phenomena, termed *myositis ossificans*, has been repeatedly documented both in the clinic and in animal experiments⁷. Though the molecular basis of muscle injury-induced bone formation remains unknown, important principles were revealed by experiments in which local bone growth in mice was measured after the removal of whole leg muscles⁸. New bone growth was only observed when the muscle removed was replaced with minced (e.g. injured) muscle. Liver could not substitute as a bone anabolic stimulus. Thus, the presence of muscle, and therefore of some signal derived from it, was essential for new bone formation. The authors⁸ actually considered the possibility that fibroblast growth factor, known then to be present in skeletal and cardiac muscle, might be the responsible signal, but, to our knowledge, did not further investigate this hypothesis. In this paper we describe morphology of the muscle-bone interface along the diaphysis of the radius as a model for understanding tissue interactions in the growing skeleton. Specifically, we focus on the localization of growth

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factors in this region in order to highlight the potential of muscle-derived growth factors for regulating the proliferation of cells in the periosteum. We also quantify levels of these growth factors from muscle *ex vivo*, and in conditioned medium from myotubes cultured *in vitro*, in order to identify the growth factors that are secreted at the highest concentrations from muscle cells. It is now known that muscle provides an important vascular supply for bone, as well as stem cells that can play a key role in bone repair⁹. Our results demonstrate that muscle is an important, local source of growth factors for bone, and that paracrine signaling between muscle and bone tissue is also likely to play a key role in muscle-bone interactions.

Materials and methods

Sample, histology & immunohistochemistry

Histological and immunohistochemical studies of the muscle-bone interface were performed using forelimb specimens from a sample of six male and six female CD-1 mice, 8 weeks of age, obtained from Charles River Laboratories. Mice of this age were selected for analysis because they are still growing and have not reached their adult body weight or skeletal dimensions. Mice were euthanized following IACUC approved procedures and the right forelimbs removed by severing the humerus above the elbow joint. Forelimbs were fixed for 24h in paraformaldehyde and then decalcified in EDTA, dehydrated, embedded in paraffin, and sectioned at 4-6 μm . Alternate sections were stained with hematoxylin & eosin or Van Gieson and Celestine blue to visualize detailed morphology of the muscle-bone interface. Alternate sections were also stained with antibodies to FGF-2, IGF-1, and their receptors. These factors were studied because they are well-known osteogenic factors¹⁰⁻¹² that are also expressed during muscle proliferation and regeneration¹³. For immunohistochemistry, sections were blocked in 2% goat serum and incubated with rabbit polyclonal antibodies to IGF-1 (SC-9013, Santa Cruz Biotechnology), IGF-1R α (SC-712, Santa Cruz Biotechnology), FGF-2 (SC-79, Santa Cruz Biotechnology), and FGF-R2 (ab1068-50, AbCam). Staining was performed using a biotinylated goat anti-rabbit secondary antibody, Vector ABC staining kit, and either Cy3-labeled anti-HRP, DAB or VectorRed. In a separate set of experiments, skeletal muscle from mdx, dystrophin-deficient mice were also prepared from the sample of Montgomery et al.¹⁴ and stained as described above.

ELISA assays & cell culture

For ELISA assays using skeletal muscle homogenates, triceps brachii muscles of the mice described above were rinsed once with 1X PBS, placed in 50 ml tubes with 5 ml 1X PBS, and homogenized on ice. Homogenates were frozen at -20C overnight, thawed, and centrifuged for 5 min at 5000xG following the recommended manufacturer procedure. Supernatant was removed, aliquoted, and stored at -20C. ELISA assays were performed using kits for FGF2 (DFB50, R&D Systems) and IGF-I (MG100, R&D Systems) following manufacturer specifications. C2C12 cells were obtained from the

American Type Culture Collection (ATCC Number CRI-1772). Cells were cultured in differentiating medium (DMEM+10% horse serum) for 10 days. Conditioned medium was removed at 4 and 10 days for ELISA assays using the kits for FGF2 and IGF-1 noted above.

For analysis of muscle injury in cultured mouse C2C12 myotubes, C2C12 cells were obtained from the American Type Culture Collection (ATCC Number CRI-1772). Cells were plated in 12 well dishes at a density of 20,000 per well in DMEM+10%FBS. The next day the cells were at 30% confluency, and were treated with differentiation media (DMEM+10% horse serum) and left for 4 days until myotubules began to form. A sterile rubber policeman was used to injure the C2C12 cells in half of the wells, and conditioned media was collected 3 hours after injury. Cells in the other half of the wells were not injured. All media was centrifuged for 5 min at 1000rpm to remove cell debris from both injured and non-injured cells. ELISA assays for FGF-2 (DFB50, R&D Systems) and IGF-1 (MG100, R&D Systems) were run on conditioned medium from both injured and non-injured cells according to manufacturer specifications.

Statistical analysis

Growth factor concentrations from skeletal muscle and from cultured myotubes were compared using single-factor ANOVA with growth factor type as the factor.

Results

Localization of growth factors and their receptors at the muscle-bone interface

Sections along the mid-diaphysis of the mouse radius and ulna show that the forelimb muscles make fleshy attachments along virtually the entire periosteal surface of both the radius and ulna (Figure 1A). High magnification micrographs demonstrate that as muscle fibers reach the periosteum there is a slight increase in collagen staining (pink, asterisks; Figure 1B, C) where the plasma membrane of myocytes is adjacent to the fibrous periosteum, which is characterized by a collagen rich matrix and abundant fibroblast nuclei. It is clear that while the periosteum is relatively thick and fibrous in certain locations where it merges with muscle fibers (Figure 1B), it is only a single cell-layer thick in other locations (Figure 1C), where muscle fibers appear to attach directly onto the bone surface. Immunohistochemistry shows that the growth factor IGF-1 can be detected along the surface of myocytes, and extracellularly, along the fleshy muscle-bone interface (Figure 2A). Likewise, the IGF-1 receptor is localized to periosteum (Figure 2B). FGF-2 is also detected in myofibers along their junction with periosteum (Figure 2C), and receptors for FGF-2 are abundant in periosteum (Figure 2D).

Growth factor levels from muscle ex vivo and myotubes in vitro

ELISA assays run on mouse muscle homogenates revealed significantly ($P < 0.01$) higher levels of IGF-1 (0.32 ng/ml) in skeletal muscle compared to FGF-2 (0.15 ng/ml), suggesting that under normal conditions IGF-1 protein is more abundant

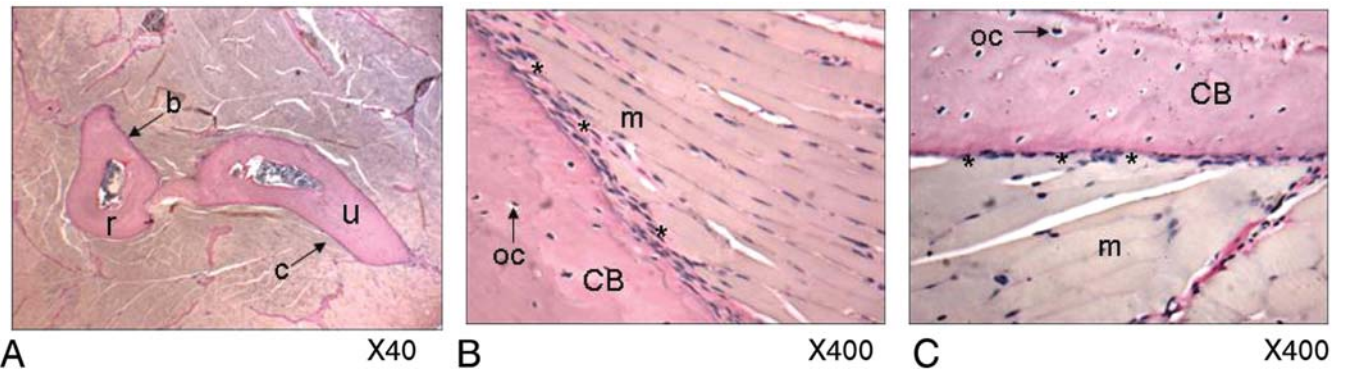


Figure 1. Van Gieson-stained sections of the mouse forelimb demonstrating microanatomy of the muscle-bone interface along the mid-diaphysis of the radius (r) and ulna (u). Arrow a indicates region of image shown in 1B and arrow b indicates region of image shown in 1C. B. Asterisks indicate relatively thick and fibrous periosteum where it merges with muscle fibers, whereas in other locations (C) the periosteum is only a single cell-layer thick and muscle fibers appear to attach directly onto the bone surface. m=skeletal muscle, oc=osteocytes, CB=cortical bone.

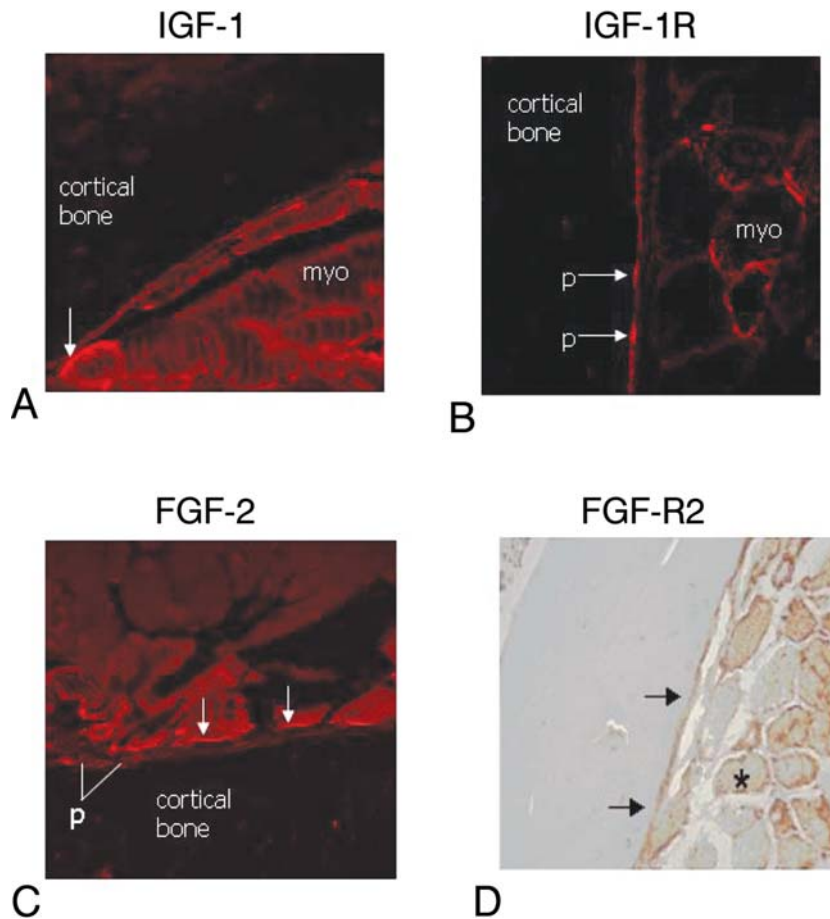


Figure 2. Immunofluorescence using antibodies to IGF-1 (A) and the IGF-1 receptor (IGF-1R; B) imaged with confocal microscopy show (arrows) IGF-1 in myofibers (myo) adjacent to the IGF-1 receptor in periosteum (p). Likewise, antibodies to FGF-2 localize this growth factor in myofibers along the muscle-bone interphase (C), and the FGF-2 receptor (FGF-R2) is abundant in muscle fibers (asterisk) and in periosteum (arrows) (D).

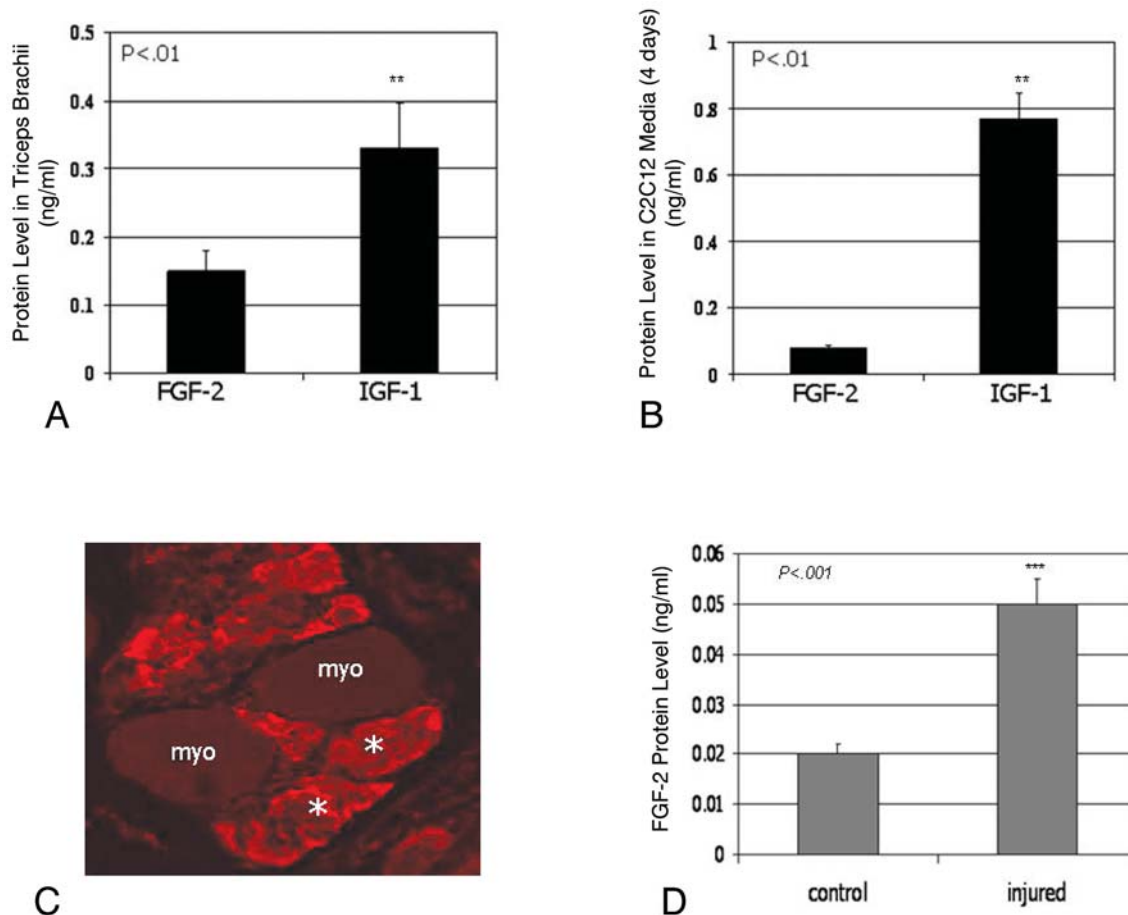


Figure 3. Protein levels of IGF-1 are significantly higher than FGF-2 levels in homogenates from forelimb muscles of normal mice (A). Protein levels of FGF-2 and IGF-1 detected using ELISA assays in conditioned medium from C2C12 cells (B). FGF-2 is abundant in injured myofibers (myo) of dystrophin-deficient mdx mice *in vivo* (asterisks) (C), and injury of mouse C2C12 myotubes increases the concentration of FGF-2 secreted into culture medium (D).

in skeletal muscle than FGF-2 (Figure 3A). Conditioned medium from mouse C2C12 myotubes was analyzed after 4 days of culture in differentiating medium using ELISA to measure levels of IGF-1 and FGF-2 secreted by muscle cells *in vitro*. FGF-2 was detected at relatively low concentrations (0.08 ng/ml) whereas IGF-1 was detected at significantly ($P < 0.01$) higher (0.77 ng/ml) levels in C2C12-conditioned medium (Figure 3B). In the second set of experiments we tested the hypothesis that muscle injury stimulates FGF-2 release. Immunostaining for FGF-2 in skeletal muscles of dystrophin-deficient, mdx mice, which show elevated levels of myofiber wounding, reveal abundant FGF-2 in injured myofibers (Figure 3C). We also tested the hypothesis that wounded myofibers release FGF-2 *in vitro* using C2C12 myotubes. Results indicate that muscle cell injury produces a significant increase in FGF-2 protein secreted into the culture medium, whereas injury actually decreases production of IGF-1, another factor produced by myocytes that can also stimulate bone formation (Figure 3D).

Discussion

In his classic *On Growth and Form* D'Arcy Thompson¹⁵, p.263 wrote "Muscle and bone are inseparably associated and connected; they are moulded one with another; they come into being together...". Indeed, muscle paralysis or the congenital absence of muscle development in utero decreases bone mineralization, bone cross-sectional diameter, and the development of long bone curvatures¹⁶. Mechanical loading is thought to be the primary mechanism by which muscle mass influences bone modeling, and a substantial body of literature supports this biomechanical relationship underlying the muscle-bone unit¹⁷. Apart from its well-known role in providing a mechanical stimulus to bone tissue, our results suggest that muscle may also influence bone metabolism by secreting local growth factors that can stimulate osteogenesis (Figure 4). This hypothesis was given additional support recently by the study of Kaufman et al.¹⁸, who demonstrated that when nitrocellulose membranes were interposed between muscle and injured bone,

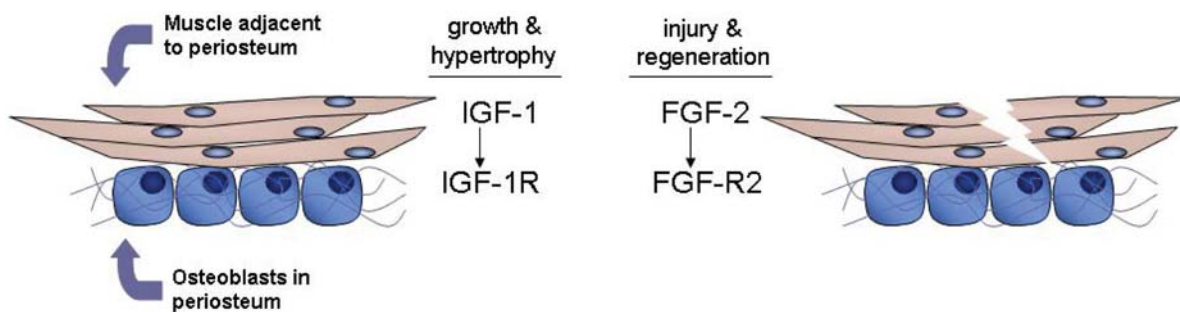


Figure 4. Generalized model showing paracrine interactions at the muscle-bone interface. Muscle growth, development, and hypertrophy lead to the secretion of IGF-1, which stimulates bone formation by osteoprogenitor cells in the periosteum that express IGF-1R. Muscle injury during strenuous exercise or traumatic extremity injury promotes the release of FGF-2, which induces bone formation and stimulates fracture healing by periosteal osteoprogenitor cells expressing FGF-R2.

bone healing was improved if pore sizes were large enough for the diffusion of growth factors. Indeed, Kaufman et al.¹⁸, concluded that “osteogenesis and bone remodeling activities induced by muscle cells are, in fact, paracrine activities of muscle-derived metabolites acting on the nearby bone”. We believe that this paracrine signaling mechanism explains a number of situations in which muscle has been observed to induce bone formation independent of mechanical loading. For example, fractures that are covered with relatively intact muscle heal more rapidly than fractures associated with more severe muscle damage⁶, muscle flaps applied to autogenous bone grafts improve healing but coverage with skin does not¹⁹, and minced muscle applied directly to intact bone tissue can stimulate bone formation but minced liver does not⁸. Likewise, muscle coverage of open tibial fractures accelerates bone healing, but coverage with fasciocutaneous tissue does not²⁰. Muscle injury also stimulates proliferation of osteoprogenitor cells in the periosteum at the muscle-bone interface²¹. It was previously suggested that in these cases muscle might function as a local source of trophic factors for bone^{8,14}, but the trophic factors emanating from muscle tissue were never specified.

It is well established that exogenous delivery of factors such as IGF-1 and FGF-2 will stimulate bone formation^{12,22}. The endogenous source of these proteins for bone is thought to be both local, as osteoblasts and chondrocytes express IGF-1 and FGF-2, and systemic, as all three factors can be detected in serum. Our data show that myofibers secrete these osteogenic factors *in vitro*, and that these same factors are localized to the muscle-bone interface *in vivo*. Furthermore, receptors for these factors are abundant in both periosteum and skeletal muscle. These results are consistent with previous studies showing that IGF-1 is abundant in wound exudates of skeletal muscle flaps²³, both IGF-1 and FGF-2 are present in extracts from crushed muscle²⁴⁻²⁶, and levels of extracellular FGF-2 and FGF-2 in serum are elevated with muscle injury^{27,28}. Local increases of FGF-2 at the muscle-bone interface during muscle regeneration would also explain why mdx mice, which have large but very weak muscles along with increased myofiber

injury and regeneration, also show greater bone diameters than normal mice¹⁴. Skeletal muscle regeneration is also associated with increased expression of IGF-1^{13,29} and IGF-1 secretion from myofibers may couple muscle growth, development, and repair with bone formation. It is certainly possible that the secretion of these factors may be altered with muscle contraction and resistance exercise, perhaps increasing local delivery of muscle-derived growth factors to bone (Figure 4). This hypothesis would be consistent with the observation that overexpression of IGF-1 in muscle increases bone size and bone mass³⁰, and that ectopic expression of IGF-1 in muscle can prevent bone loss with disuse³¹. Mechanical and biological stimuli may therefore function synergistically with one another, where growth factor secretion by muscle is one of several possible ways that mechanical signals are transduced biologically.

It is likely that other factors secreted by muscle can also significantly increase bone formation. A recent study found that proteins such as matrix metalloproteinase-2 (MMP-2), secreted protein rich in cysteine protein (SPARC, or osteonectin), and insulin-like growth factor binding protein-5 (IGFBP5) are secreted by C2C12 myotubes *in vitro*³². Each of these factors is known to influence bone metabolism. Mice lacking MMP-2 show decreased bone mineral density in the limbs³³, mice lacking SPARC develop osteopenia due to significantly decreased bone formation by osteoblasts³⁴, and IGFBP5 can stimulate bone formation *in vivo* and *in vitro*³⁵. Muscle is also a primary source of leptin³⁶, which is known to regulate both osteoblast and osteoclast differentiation³⁷, and serum leptin levels increase with increased muscle mass³⁸.

Finally, muscle cells are known to secrete transforming growth factor beta-1 (TGF β 1)³⁹, and tgf beta is a potent mitogen for osteoblasts that is generally thought to have anti-resorptive effects on bone⁴⁰. TGF β 1 does, however, contribute to the accumulation of fibrotic tissue in muscle, and TGF β 1 stimulates expression of myostatin, which suppresses muscle growth and hypertrophy³⁹. Thus, it is likely that muscle growth and hypertrophy in response to loading involve downregulation of TGF β 1, whereas muscle growth and exercise-induced

loading involve increased expression of muscle IGF-1 and decreased expression of myostatin.

A role for muscle-derived growth factors in bone metabolism raises several questions about how muscle-bone interactions may change with growth, development, and aging, and what specific regions of bone (e.g., cortical vs. trabecular) and cell populations (osteoblasts or osteoclasts) might be affected. We suggest that periosteal surfaces are most likely to be influenced by the secretion of growth factors from muscle because of the fact that periosteal tissue is closely apposed to muscle at many skeletal sites. Allen and colleagues⁴¹ have proposed that osteoprogenitor cells in periosteum differ markedly from marrow-derived osteoprogenitors in their threshold sensitivity to physical and hormonal stimuli. A future area of research is to determine how periosteal osteoprogenitors differ from marrow- and endosteal-derived stem cells in their response to muscle-derived growth factors. A second area of research is to determine how the interactions between muscle-derived growth factors and osteoprogenitor cells, particularly periosteal cells, are altered with age. It is known that muscle mass as well as periosteal cellularity and thickness all decline with age. One attractive hypothesis is that prevention of age-associated muscle wasting (sarcopenia) with treatments such as androgen receptor modulators or myostatin inhibitors might also attenuate age-related bone fragility by enhancing periosteal apposition via increased secretion of local muscle-derived growth factors. Finally, the periosteal surface is also a site of bone resorption⁴². It remains to be determined how muscle tissue might be involved in the paracrine regulation of osteoclast activity, but it is certainly possible that declines in muscle-derived factors with age or disuse could stimulate periosteal bone resorption.

The data presented here provide new insights into the functional and developmental interactions between muscle and bone at the molecular, cellular and whole-organism levels. It is well-recognized that soft-tissues such as muscle and fat influence bone mass⁴³, and while the role of fat-derived factors such as adiponectin and leptin have received considerable attention with regard to their effects on bone⁴⁴ the role of muscle as a source of osteogenic factors has received much less attention. As such, our findings have important implications for both basic and clinical science. First, the results of this study suggest that targeting muscle using novel pharmacological therapies may improve the mass and strength of both muscle and bone tissue through non-mechanical pathways. Perhaps more importantly, our findings underscore the importance of maintaining muscle mass throughout life as a means of maintaining bone density and strength, and as a strategy for preventing falls and debilitating bone fractures.

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