J Musculoskelet Neuronal Interact 2015; 15(1):1-9

Review Article

Hylonome

Muscle-bone interactions during fracture healing

K.M. Davis[1]*, K.S. Griffin[1]*, T-M.G. Chu[2], J.C. Wenke[3], B.T. Corona[3], T.O. McKinley[1], M.A. Kacena[1]

[1]Department of Orthopaedic Surgery, Indiana University School of Medicine, Indianapolis, IN;
[2]Department of Restorative Dentistry, Indiana University School of Dentistry, Indianapolis, IN;
[3]Extremity Trauma & Regenerative Medicine Task Area, United States Army Institute of Surgical Research, San Antonio, TX
*contributed equally to this work

Abstract

Although it is generally accepted that the rate and strength of fracture healing is intimately linked to the integrity of surrounding soft tissues, the contribution of muscle has largely been viewed as a vascular supply for oxygen and nutrient exchange. However, more is becoming known about the cellular and paracrine contributions of muscle to the fracture healing process. Research has shown that muscle is capable of supplying osteoprogenitor cells in cases where the periosteum is insufficient, and the muscular osteoprogenitors possess similar osteogenic potential to those derived from the periosteum. Muscle's secrotome includes proteins capable of inhibiting or enhancing osteogenesis and myogenesis following musculoskeletal injury and can be garnered for therapeutic use in patients with traumatic musculoskeletal injuries. In this review, we will highlight the current knowledge on muscle-bone interaction in the context of fracture healing as well as concisely present the current models to study such interactions.

Keywords: Muscle, Bone, Fracture, Mesenchymal Stem Cells, Paracrine

Introduction

In the orthopaedic field, the muscle-bone relationship is of utmost importance as surgeons must often battle increased complications, morbidity, and delayed fracture healing in cases with extensive soft tissue damage resulting from high energy trauma. The Gustilo-Anderson open fracture classification scale, which has been commonly used for nearly 4 decades, classifies severity almost solely on soft tissue (primarily muscle) injury, and the complication rate is much higher in fractures with soft tissue damage[1]. Although it has long been accepted that intact surrounding soft tissues are important in the fracture healing process, the underlying mechanisms have not been fully elucidated. However, basic science and translational research have made advances in the understanding of how muscle injuries impede fracture healing.

The authors have no conflict of interest.

Corresponding author: Melissa Kacena, Ph.D., August M. Watanabe Translational Scholar, Showalter Scholar, Associate Professor of Orthopaedic Surgery, Indiana University School of Medicine, 1120 South Drive FH 115, Indianapolis, IN 46202, United States
E-mail: mkacena@iupui.edu

Edited by: M. Hamrick
Accepted 8 December 2014

To understand muscle's potential role in fracture repair, a comprehension of the repair process is necessary. In brief, fracture repair consists of three chronological and overlapping phases: a reactive phase, a reparative phase, and a remodeling phase. The reactive phase peaks within the first 24-48 hours and lasts less than 1 week. During this phase, endothelial damage to the vasculature causes a hematoma, drawing in inflammatory cells (lymphocytes, polymorphonuclear cells, monocytes) and fibroblasts to form granulation tissue[2]. The granulation tissue is important for vascular ingrowth as well as the recruitment of mesenchymal stem cells (MSCs). The inflammatory cells release cytokines such as TNF-α, IL-1, IL-6, IL-11, and IL-18 to induce osteogenic differentiation of MSCs as well as promote angiogenesis[3]. The reparative phase begins within a few days after fracture and lasts several weeks. Pluripotent mesenchymal cells, dependent on local strain and oxygen tension, differentiate into fibroblasts, chondroblasts, or osteoblasts. Healing can occur through intramembranous ossification alone (direct healing) or a combination of intramembranous and endochondral ossification (indirect healing), depending on the degree of mechanical stability[4]. In endochondral ossification, a fibrocartilage callus forms and is subsequently replaced by a bony callus with woven bone deposition. In intramembranous ossification, lamellar bone regeneration occurs without the need for remodeling, but it requires stable fixation[2]. Thus, the ossification process is dependent on

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01 MAR 2015	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Muscle-bone interactions during fracture healing	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
Davis K. M., Griffin K. S., Chu T. G., Wenke J. C., Corona B. T., McKinley T. O., Kacena M. A.,	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **United States Army Institute of Surgica; Research, JBSA Fort Sam Houston, TX 78234**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**SAR**	**9**	

the stability of the fracture site. During the remodeling phase, the woven bone is replaced with lamellar bone, and the bone is gradually remodeled under mechanical stress to its original contour. This phase can last for several years[2,5].

Vascularization and fracture healing

The importance of vascularization in osteogenesis cannot be overemphasized, as a nearby vascular supply is required for both normal development and bone regeneration[6-9]. Indeed, an early step in the fracture healing process is the formation of granulation tissue consisting of connective tissue and small blood vessels[10,11], reinforcing the importance of vascularization in healing. Surrounding soft tissues at the fracture site primarily have been considered an important vascular source[12] to deliver oxygen[13], nutrients[13], and potential osteoprogenitor cells to the injured area[14,15]. In the surrounding soft tissue are MSCs and pericytes, which are crucial for angiogenesis in the wounded tissue[16,17]. In the clinical arena, the rate of non-union is 4 times higher in cases with reduced vascular function[18], and in animal fracture models that disrupt angiogenesis, bone formation is hindered through the suppression of osteoblast proliferation[18-20]. Muscle flap coverage has been shown to increase bone blood flow and the rate of osteotomy union compared to skin tissue coverage, supporting the vascular role of muscle in bone regeneration[21-23].

Although vascularization has been shown to be critical for regeneration, there has been evidence of nearly equal vascularization in healed bone and non-unions in animal studies as well as in human patients[20,24-26]. In a murine open tibial fracture model, Harry et al. observed faster fracture healing in musculocutaneous compared to fasciocutaneous flaps, despite the musculocutaneous flaps having decreased vascularization[27]. These studies point to a more extensive role of muscle in the repair process than solely as a vascular supply.

Osteoprogenitors derived from muscle

The relationship between muscle and bone has been observed for decades and continues to be elucidated. Urist first deduced muscle's ability to induce bone formation in 1965 when decalcified bone implanted into muscle resulted in new bone formation[28,29]. In fracture healing studies in multiple species, callus formation tends to be the largest and most dense at the interface between bone and muscle[30], suggesting that muscle contributes to callus formation or provides a suitable environment for its occurrence.

Muscle is also a common site for ectopic bone formation following physical trauma[31], orthopaedic surgery[32], or due to disease like fibrodysplasia ossificans progressiva, which has been identified to be a result of a mutation in a gene encoding a bone morphogenetic protein (BMP) receptor[33]. BMPs, a group of growth factors involved in tissue architecture throughout the body, are of particular importance to bone formation as they induce osteoblast differentiation.

In the presence of BMPs, cells derived from muscle are capable of differentiating into cells expressing bone markers[34-37]. That muscle-derived cells capable of displaying osteogenic potential under proper conditions could partly explain the importance of muscle in fracture healing aside from their role in vascularization. In addition, muscle may be able to influence bone in a manner unlike any other tissue. When both muscle and fat are activated by exposure to a BMP-2 encoded adenovirus, the "gene-activated" muscle results in more consistent bone regeneration than the "gene-activated" fat[38]. Furthermore, when muscle-derived stem cells (MDSCs) are recruited and driven to osteogenic differentiation by BMPs, they display an osteogenic potential that is equivalent to those derived from bone marrow[39]. Lineage-traced MDSCs in a fracture healing model have been found to alter gene expression to give rise to chondrocytes, up-regulating chondrogenic markers Sox9 and Nkx3.2 and down-regulating the muscle marker Pax3[36]. These studies provide evidence that, in the appropriate environmental conditions, muscle can supply osteoprogenitor cells required for the fracture repair process.

It should be noted, however, that MDSCs are not the sole osteoprogenitor cells derived from muscle. C2C12 myoblasts infected with a retroviral vector have been found to overexpress osteoactivin (OA) and transdifferentiate into osteoblasts and express bone-specific markers[40]. Muscle-derived stromal cells, when administered TNF-α at low concentrations, are also capable of undergoing recruitment and osteogenic differentiation[41]. Muscle satellite cells were originally believed to be muscle stem cells restricted to the myogenic lineage[42], but the osteogenic potential of these cells has been observed under several conditions. Satellite cell-derived myoblasts have been shown to differentiate into osteocytes following treatment with BMPs[43], into osteoblasts *in vivo* and *in vitro* in the presence of platelet-rich plasma[44], and the osteogenic potential of satellite cells can increase in response to cutaneous burn trauma[45]. Satellite cells have been observed to express both myoblastic (Pax7, MyoD) and osteoblastic (alkaline phosphatase, Runx2) markers and are capable of differentiating into osteoblasts spontaneously[46].

The abundance of potential osteogenic cells derived from muscle could have applications in the future in tissue engineering techniques, particularly in cases where the bone marrow or periosteum is compromised. It has been commonly believed that in fractures in which the periosteum is intact, repair occurs largely through endochondral ossification driven by a periosteal supply of cells[10,47-50]. Indeed, in open fractures with a stripped periosteum, Liu et al. found that myogenic cells of the MyoD-lineage contributed to fracture repair, but MyoD-expressing cells were not incorporated into the callus in the case of a closed fracture with intact periosteum[51]. Such a study demonstrates that myogenic cells can be activated to serve as a secondary supply of cells when the periosteal supply becomes compromised[52,53]. These recent findings of muscle's ability to augment the periosteal supply of osteoprogenitor cells provide insight into the clinical observations of prolonged recovery time and increased morbidity that is especially seen associated with high energy fractures with substantial soft tissue damage.

Muscle-bone paracrine interactions in bone repair

Only within the past two decades has the muscle secretome been identified and explored. With the recent advent of improved characterization instruments, the muscle secretome has rapidly expanded to over 200 proteins[54]. Muscle secreted proteins important in muscle-bone interactions include, but are not limited to: myostatin, BMPs, secreted protein acidic and rich in cysteine (SPARC or osteonectin), interleukin (IL)-1, IL-4, IL-6, tumor necrosis factor (TNF)α, and insulin-like growth factor (IGF)-1[41,54-56]. Many of the muscle derived factors have previously been described to play a role in muscle-bone interactions without addressing the interactions specifically during fracture repair. Importantly, the presence of inflammation differentiates fracture repair from bone formation during development. That is, fracture healing is initiated by an inflammatory cascade, which is mediated by a number of factors, including but not limited to: neutrophils, macrophages, lymphocytes, and various inflammatory cytokines (i.e., IL-1, IL-6, TNFα)[2,57-59]. Mounting and maintaining an appropriate inflammatory response in early fracture healing is critical for adequate repair and multiple studies have demonstrated that interference with the inflammatory process can either impair[60,61] or improve[62] fracture healing. This review focuses primarily on four factors known to be involved in muscular injury and fracture repair and are therefore likely to contribute to muscle-bone interactions in the presence of inflammation.

Insulin-like growth factor-1

IGF-1 is recognized as a key myokine that may direct local fracture healing[63]. IGF-1 is expressed by maturing osteoblasts in culture[64] and expression has been localized using *in situ* hybridization to osteoblasts during phases of matrix formation and remodeling in fractured human bone[65]. Further signifying the importance of IGF-1 to fracture healing, delivery of IGF-1 to ovine bone defects promotes accelerated bone formation[66,67]. The association of low systemic levels of IGF-1 with osteoporosis[68,69] suggests that local production of IGF-1 by nearby skeletal muscle tissue may support bone healing. Given that skeletal muscle up-regulates expression of IGF-1 in response to injury[70-72], the context of fractures involving muscle trauma specifically highlight this possibility. Overexpression of IGF-1 in skeletal muscle can result in increased systemic concentrations evidencing the capacity of skeletal muscle as a paracrine organ to support nearby bone healing[73]. IGF-1 plays a role in muscle fiber repair and regenerative processes via a number of mechanisms to include increasing protein synthesis via PI3-AKT-mTOR pathway and by activating and promoting proliferation of satellite cells[74,75]. Perhaps most interesting in the context of complex musculoskeletal injury is the anti-inflammatory (i.e., inhibition of NF-κB) role of IGF-1 in muscle[76,77] and bone[67].

Myostatin

Perhaps the most well-known muscle derived protein, myostatin, has been implicated to play a significant, albeit inhibitory, role in fracture repair. Myostatin is a member of the TGF-β superfamily, negatively regulating muscle growth, development, and regeneration[78,79]. Its negative trophic influence has been supported in myostatin null mice that demonstrate increased bone strength and increased bone mineral density[80-82]. Furthermore, myostatin inhibition by decoy receptors increases musculoskeletal mass[83]. Interestingly however, expression of myostatin is elevated with significant musculoskeletal injury, specifically in the early part of bone repair[84,85]. Due to its negative role in musculoskeletal development, interventions were targeted toward inhibiting myostatin after skeletal injury. Small molecule inhibition of myostatin following orthopaedic trauma has been demonstrated to improve muscle regeneration and fracture healing[79,85,86]. These data suggest that inhibition of myostatin may be a plausible intervention to improve fracture healing outcomes in patients with significant musculoskeletal injuries. However, the conundrum of elevated myostatin after musculoskeletal injury remains poorly understood.

Bone morphogenetic proteins

Generally speaking, BMPs are growth factors for various skeletal tissues and are required for skeletal development. Conditional knockout mice deficient in BMPs displayed a wide range of skeletal defects[87,88]. There are 7 members of the BMP family, of which BMPs 2-7 belong to the TGFβ superfamily[89]. Multiple BMPs have been demonstrated to promote osteoblastic differentiation of bone marrow stromal cells[90,91]. Specifically, BMP-2 and BMP-7 are FDA approved for use in clinical musculoskeletal therapeutics due to their role in osteoblast differentiation and musculoskeletal repair. Unfortunately, concerns have arisen regarding the multiple side effects and off-label usage of BMPs including a recent link to oncogenic side effects with use of BMP-2[92,93]. More novel approaches to utilization of BMP-2 in fracture healing includes modified muscle cells that secrete BMP-2. Critical size rat femoral defects underwent quicker bridging and restored mechanical strength when receiving activated muscle secreted BMP-2[38]. Though not a member of the TGFβ superfamily and not used in the clinical setting currently, BMP-1 is secreted by muscle and may play a role in fracture healing. BMP-1, specifically, is a protease secreted by muscle that cleaves procollagen[94]. In patients with traumatic blast injuries, both BMP-1 protein and mRNA levels were elevated[95], suggesting a significant role for BMP-1 in musculoskeletal repair. Therefore, better understanding of the roles of muscle derived BMPs in skeletal tissue regeneration is warranted to improve musculoskeletal repair in patients who suffer extensive traumatic injuries.

SPARC or osteonectin

Osteonectin is a phosphorylated glycoprotein present in developing bone in many animal species[96]. Osteonectin is suggested to serve multiple functions in the developing bone

matrix, including collagen organization, osteoblast growth and proliferation, and matrix mineralization[97]. Mice deficient in osteonectin display osteopenia and decreased bone mineral content[98]. Importantly, osteonectin is secreted by injured and regenerating myotubes and muscle fibers[99]. Osteonectin expression by these sources is dependent on injury severity, suggesting that more severe musculoskeletal injuries result in greater ostonectin expression[99]. Longitudinal studies of fracture healing show detectable osteonectin transcripts throughout the healing phase[100,101], most notably from days 9 to 15[102]. These studies provide evidence for the significant role osteonectin plays in bone regeneration and suggest muscle may be a source of osteonectin during musculoskeletal repair.

Mechanical muscle-bone interactions

It would be remiss to forego some discussion of the mechanical influences involved in muscle bone interactions. The cellular mechanisms by which mechanical strain affects bone are largely uncharacterized, but some data suggest it is due in part to gap junctions in bone formed by connexin43[103,104]. Though characterization of mechanically induced cellular mechanisms remains limited, multiple studies have pointed to the importance of muscle's mechanical interactions on bone health[105]. Disuse atrophy via denervation or immobilization has been shown to decrease bone integrity in animal models[106-108]. Furthermore, multiple studies have demonstrated that muscle paralysis induced by administration of botulinum toxin impairs bone quality and/or fracture healing[109-113]. Further research into the cellular mechanisms of the mechanical influence of muscle is warranted to better understand how bone can be further modified by muscle during the healing process.

Muscle in fracture healing - current models

Murine

Multiple murine studies have been conducted to examine the extent to which muscle enhances bone repair after significant musculoskeletal injury. Zacks and Sheff[114] conducted early sentinel research addressing the potential for muscle to contribute to bone regeneration in 1982. Zacks and Sheff utilized experimental groups where after limb muscle resection, isotopic or heterotopic minced muscle implants were placed immediately adjacent to the periosteum. Their control groups consisted of liver minced implant or no implant. They concluded that isotopic and heterotopic minced muscle preparations implanted adjacent to the periosteum could directly induce new bone formation *in situ* as demonstrated by the formation of exostoses and metaplastic nodules in the minced muscle implants[114]. The work of Zacks and Sheff confirmed the importance of studying the trophic influence of muscle on bone.

As previously mentioned, Harry et al. conducted a murine study addressing the importance of muscle in open tibial fracture repair[27]. The authors demonstrated that musculocutaneous flaps performed superior to the fasciocutaneous flaps, though the fasciocutaneous flaps provided more angiogenic capacity. Therefore, the osteogenic capability of muscle is greater than that of cutaneous flaps and extends beyond simply angiogenesis.

Rattus

Multiple studies have also been conducted utilizing rat models to assess bone healing in light of soft tissue injuries. A study by Hao et al.[109] evaluated the effect of muscle atrophy and paralysis on femoral fracture healing. Atrophy of the quadriceps muscle, induced by administration of botulinum A toxin, negatively impacted the healing capacity of femoral fractures in rats. Utvag et al. conducted three critical studies[115-117] assessing the role of periosteum or surrounding soft tissue in bone healing. In 1998 Utvag et al.[115] demonstrated that fracture healing was impaired when periosteal tissue was mechanically removed from interacting with surrounding muscle. Additionally, Utvag et al. showed that significant muscle injury and absence of muscle by resection, or by traumatic injury in the clinical setting, significantly compromised the regeneration potential of non-augmented healing bone[116,117]. The importance of muscle for bone healing was further confirmed by the work of Willett et al. that demonstrated that volumetric muscle loss (VML) also impairs the effectiveness of BMP-2 in the healing of a critical size bone defect[118]. Taken together, it is clear that frank loss of muscle tissue (VML) is a significant comorbidity to poor bone healing outcomes.

Humans

Since the mid 1970s, open fractures have been graded clinically according to the Gustilo-Anderson classification scale[1,119], which is largely based on the severity of soft tissue injury associated with open fractures. Gustilo and Anderson identified 3 types of fractures: Type I - open fracture with a wound <1 cm and clean; Type 2 - open fracture with a wound >1 cm without extensive soft tissue damage; and Type 3 - open fracture with extensive soft tissue damage[119]. Type 3 fractures were later subdivided into 3 subcategories[1]. The Gustilo Anderson classification makes it evident that soft tissue injury plays a significant role in the musculoskeletal repair process in the clinical setting. Specifically, open fractures (Type 3) with extensive soft tissue injury demonstrate greater complication rates than open fractures without soft tissue injury (Types 2 & 3)[120,121].

Similar to the results observed from animal studies, substantial clinical data exist characterizing the importance of muscle integrity in bone repair. A multitude of studies have demonstrated soft tissue damage associated with fractures impairs the ability of bone to repair properly[122,123], while the quality of the muscle bed is essential for appropriate bone formation and bone healing[30,51].

Similar to the murine study conducted by Harry et al.[27], Gopal et al.[124] specifically examined the treatment of open tibial fractures with fasciocutaneous flaps versus muscle flaps in humans. The results of their study were then later confirmed by Harry et al. in the mouse model, with both groups concluding that muscle flaps are superior in bone healing. Even in clinical

practice, the gold standard of treating critical size defects or extensive fractures includes soft tissue coverage, supporting the significance of muscle-bone interactions during bone healing.

A more recent meta-analysis by Reverte et al.[125] analyzed 16 studies addressing the union rate and time to fracture union in patients with tibial fractures and associated compartment syndromes. Reverte et al. demonstrated that tibial fractures with associated soft tissue injury significantly impaired fracture healing. The rate of delayed union or non-union in tibial fractures with associated compartment syndrome was 55% compared to only 18% in patients with tibial fracture without associated compartment syndrome[125]. This study points to the importance of soft tissue integrity in the quality of fracture healing.

Conclusion

Taken together, these studies illustrate the importance of muscle-bone interactions in bone regeneration. Exact mechanisms by which muscle is responsible for bone formation in the healing process are not well elucidated. Most of the current literature is limited to qualitative findings of muscle's role in bone healing. Therefore, more rigorous models with aims directed toward identification and quantification of muscle-derived effectors of bone regeneration are required. Identifying and characterizing the muscle-derived factors responsible for bone healing may provide opportunities to develop therapies to augment normal physiologic mechanisms underlying bone regeneration.

Current strategies, such as the use of BMPs, in fracture healing have recently been thought of as having more limited benefit due to the more robust understanding of detrimental side effects. This review outlines some potential targets for therapeutic development, including stimulation of MDSCs, inhibition of myostatin, or administering or enhancing the targeted expression of osteonectin. Future studies addressing muscle factors associated with bone healing may provide insight into these mechanisms necessary to promote bone regeneration. Soft tissue integrity is crucial to appropriate bone regeneration, but our understanding of the mechanisms is limited at the present time. A better understanding of muscle's effect on fracture healing at the cellular and molecular levels will open translational opportunities to incorporate the findings into clinics and operating rooms abroad.

Acknowledgements

This work was supported by the Medical Student Affairs Summer Research Program in Academic Medicine, Indiana University School of Medicine funded in part by NIH grant HL110854 (KMD) and the Department of Orthopaedic Surgery, Indiana University School of Medicine (MAK, TOM). In addition, research reported in this publication was supported in part by the following grants: NIH NIAMS R01 AR060863 (MAK), USAMRMC OR120080 (MAK, T-MGC), an Indiana University Health Values Grant (MAK), an Indiana Clinical and Translational Sciences Institute Grant partially supported by NIH UL1TR001108 (MAK, T-MGC), and an Indiana University Collaborative Research Grant (MAK, T-MGC).The content of this manuscript is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

References

1. Gustilo RB, Mendoza RM, Williams DN. Problems in the management of type III (severe) open fractures: a new classification of type III open fractures. J Trauma 1984;24:742-6.
2. Marsell R, Einhorn TA. The biology of fracture healing. Injury 2011;42:551-5.
3. Gerstenfeld LC, Cullinane DM, Barnes GL, Graves DT, Einhorn TA. Fracture healing as a post-natal developmental process: molecular, spatial, and temporal aspects of its regulation. J Cell Biochem 2003;88:873-84.
4. Gerstenfeld LC, Alkhiary YM, Krall EA, et al. Three-dimensional reconstruction of fracture callus morphogenesis. J Histochem Cytochem 2006;54:1215-28.
5. Claes L, Recknagel S, Ignatius A. Fracture healing under healthy and inflammatory conditions. Nat Rev Rheumatol 2012;8:133-43.
6. Schipani E, Maes C, Carmeliet G, Semenza GL. Regulation of osteogenesis-angiogenesis coupling by HIFs and VEGF. J Bone Miner Res 2009;24:1347-53.
7. Glowacki J. Angiogenesis in fracture repair. Clin Orthop Relat Res 1998:S82-9.
8. Zelzer E, Olsen BR. Multiple roles of vascular endothelial growth factor (VEGF) in skeletal development, growth, and repair. Curr Top Dev Biol 2005;65:169-87.
9. Kristensen HB, Andersen TL, Marcussen N, Rolighed L, Delaisse JM. Increased presence of capillaries next to remodeling sites in adult human cancellous bone. J Bone Miner Res 2013;28:574-85.
10. Colnot C, Zhang X, Knothe Tate ML. Current insights on the regenerative potential of the periosteum: molecular, cellular, and endogenous engineering approaches. J Orthop Res 2012;30:1869-78.
11. Zuscik MJ, Hilton MJ, Zhang X, Chen D, O'Keefe RJ. Regulation of chondrogenesis and chondrocyte differentiation by stress. J Clin Invest 2008;118:429-38.
12. Rhinelander FW. Tibial blood supply in relation to fracture healing. Clin Orthop Relat Res 1974:34-81.
13. Laroche M. Intraosseous circulation from physiology to disease. Joint Bone Spine 2002;69:262-9.
14. Brighton CT, Lorich DG, Kupcha R, Reilly TM, Jones AR, Woodbury RA, 2nd. The pericyte as a possible osteoblast progenitor cell. Clin Orthop Relat Res 1992:287-99.
15. Diaz-Flores L, Gutierrez R, Lopez-Alonso A, Gonzalez R, Varela H. Pericytes as a supplementary source of osteoblasts in periosteal osteogenesis. Clin Orthop Relat Res 1992;(275):280-6.
16. Mills SJ, Cowin AJ, Kaur P. Pericytes, mesenchymal stem cells and the wound healing process. Cells 2013;2:621-34.
17. Kumar A, Salimath BP, Stark GB, Finkenzeller G.

Platelet-derived growth factor receptor signaling is not involved in osteogenic differentiation of human mesenchymal stem cells. Tissue Eng Part A 2010;16:983-93.
18. Lu C, Miclau T, Hu D, Marcucio RS. Ischemia leads to delayed union during fracture healing: a mouse model. J Orthop Res 2007;25:51-61.
19. Green N, French S, Rodriquez G, Hays M, Fingerhut A. Radiation-induced delayed union of fractures. Radiology 1969;93:635-41.
20. Liu R, Schindeler A, Little DG. The potential role of muscle in bone repair. J Musculoskelet Neuronal Interact 2010;10:71-6.
21. Richards RR, Schemitsch EH. Effect of muscle flap coverage on bone blood flow following devascularization of a segment of tibia: an experimental investigation in the dog. J Orthop Res 1989;7:550-8.
22. Richards RR, McKee MD, Paitich CB, Anderson GI, Bertoia JT. A comparison of the effects of skin coverage and muscle flap coverage on the early strength of union at the site of osteotomy after devascularization of a segment of canine tibia. J Bone Joint Surg Am 1991; 73:1323-30.
23. Richards RR, Orsini EC, Mahoney JL, Verschuren R. The influence of muscle flap coverage on the repair of devascularized tibial cortex: an experimental investigation in the dog. Plast Reconstr Surg 1987;79:946-58.
24. Choi P, Ogilvie C, Thompson Z, Miclau T, Helms JA. Cellular and molecular characterization of a murine nonunion model. J Orthop Res 2004;22:1100-7.
25. Brownlow HC, Reed A, Simpson AH. The vascularity of atrophic non-unions. Injury 2002;33:145-50.
26. Reed AA, Joyner CJ, Brownlow HC, Simpson AH. Human atrophic fracture non-unions are not avascular. J Orthop Res 2002;20:593-9.
27. Harry LE, Sandison A, Paleolog EM, Hansen U, Pearse MF, Nanchahal J. Comparison of the healing of open tibial fractures covered with either muscle or fasciocutaneous tissue in a murine model. J Orthop Res 2008;26:1238-44.
28. Urist MR. Bone: formation by autoinduction. Science 1965;150:893-9.
29. Chan JK, Harry L, Williams G, Nanchahal J. Soft-tissue reconstruction of open fractures of the lower limb: muscle versus fasciocutaneous flaps. Plast Reconstr Surg 2012;130:284e-95e.
30. Stein H, Perren SM, Cordey J, Kenwright J, Mosheiff R, Francis MJ. The muscle bed--a crucial factor for fracture healing: a physiological concept. Orthopedics 2002; 25:1379-83.
31. Davis TA, Lazdun Y, Potter BK, Forsberg JA. Ectopic bone formation in severely combat-injured orthopedic patients - a hematopoietic niche. Bone 2013;56:119-26.
32. DeLee J, Ferrari A, Charnley J. Ectopic bone formation following low friction arthroplasty of the hip. Clin Orthop Relat Res 1976;(121):53-9..
33. Kaplan FS, Lounev VY, Wang H, Pignolo RJ, Shore EM. Fibrodysplasia ossificans progressiva: a blueprint for metamorphosis. Ann N Y Acad Sci 2011;1237:5-10.
34. Kato S, Sangadala S, Tomita K, Titus L, Boden SD. A synthetic compound that potentiates bone morphogenetic protein-2-induced transdifferentiation of myoblasts into the osteoblastic phenotype. Mol Cell Biochem 2011; 349:97-106.
35. Wong E, Sangadala S, Boden SD, et al. A novel low-molecular-weight compound enhances ectopic bone formation and fracture repair. J Bone Joint Surg Am 2013; 95:454-61.
36. Cairns DM, Liu R, Sen M, et al. Interplay of Nkx3.2, Sox9 and Pax3 regulates chondrogenic differentiation of muscle progenitor cells. PLoS One 2012;7:e39642.
37. Lee JY, Qu-Petersen Z, Cao B, et al. Clonal isolation of muscle-derived cells capable of enhancing muscle regeneration and bone healing. J Cell Biol 2000;150:1085-100.
38. Evans CH, Liu FJ, Glatt V, et al. Use of genetically modified muscle and fat grafts to repair defects in bone and cartilage. Eur Cell Mater 2009;18:96-111.
39. Gao X, Usas A, Tang Y, et al. A comparison of bone regeneration with human mesenchymal stem cells and muscle-derived stem cells and the critical role of BMP. Biomaterials 2014;35:6859-70.
40. Sondag GR, Salihoglu S, Lababidi SL, et al. Osteoactivin induces transdifferentiation of C2C12 myoblasts into osteoblasts. J Cell Physiol 2014;229:955-66.
41. Glass GE, Chan JK, Freidin A, Feldmann M, Horwood NJ, Nanchahal J. TNF-alpha promotes fracture repair by augmenting the recruitment and differentiation of muscle-derived stromal cells. Proc Natl Acad Sci U S A 2011; 108:1585-90.
42. Jankowski RJ, Deasy BM, Huard J. Muscle-derived stem cells. Gene Ther 2002;9:642-7.
43. Asakura A, Komaki M, Rudnicki M. Muscle satellite cells are multipotential stem cells that exhibit myogenic, osteogenic, and adipogenic differentiation. Differentiation 2001;68:245-53.
44. Huang S, Jia S, Liu G, Fang D, Zhang D. Osteogenic differentiation of muscle satellite cells induced by platelet-rich plasma encapsulated in three-dimensional alginate scaffold. Oral Surg Oral Med Oral Pathol Oral Radiol 2012;114:S32-40.
45. Wu X, Rathbone CR. Satellite cell functional alterations following cutaneous burn in rats include an increase in their osteogenic potential. J Surg Res 2013;184:e9-16.
46. Hashimoto N, Kiyono T, Wada MR, et al. Osteogenic properties of human myogenic progenitor cells. Mech Dev 2008;125:257-69.
47. Malizos KN, Papatheodorou LK. The healing potential of the periosteum molecular aspects. Injury 2005;36 Suppl 3:S13-9.
48. Dwek JR. The periosteum: what is it, where is it, and what mimics it in its absence? Skeletal Radiol 2010;39:319-23.
49. Zhang X, Xie C, Lin AS, et al. Periosteal progenitor cell fate in segmental cortical bone graft transplantations: im-

plications for functional tissue engineering. J Bone Miner Res 2005;20:2124-37.
50. Hutmacher DW, Sittinger M. Periosteal cells in bone tissue engineering. Tissue Eng 2003;9 Suppl 1:S45-64.
51. Liu R, Birke O, Morse A, et al. Myogenic progenitors contribute to open but not closed fracture repair. BMC Musculoskelet Disord 2011;12:288.
52. Kaufman H, Reznick A, Stein H, Barak M, Maor G. The biological basis of the bone-muscle inter-relationship in the algorithm of fracture healing. Orthopedics 2008; 31:751.
53. Utvag SE, Grundnes O, Reikeras O. Early muscle-periosteal lesion inhibits fracture healing in rats. Acta Orthop Scand 1999;70:62-6.
54. Bortoluzzi S, Scannapieco P, Cestaro A, Danieli GA, Schiaffino S. Computational reconstruction of the human skeletal muscle secretome. Proteins 2006;62:776-92.
55. Norheim F, Raastad T, Thiede B, Rustan AC, Drevon CA, Haugen F. Proteomic identification of secreted proteins from human skeletal muscle cells and expression in response to strength training. Am J Physiol Endocrinol Metab 2011;301:E1013-21.
56. Seale P, Bjork B, Yang W, et al. PRDM16 controls a brown fat/skeletal muscle switch. Nature 2008;454:961-7.
57. Kon T, Cho TJ, Aizawa T, et al. Expression of osteoprotegerin, receptor activator of NF-kappaB ligand (osteoprotegerin ligand) and related proinflammatory cytokines during fracture healing. J Bone Miner Res 2001;16:1004-14.
58. Ai-Aql ZS, Alagl AS, Graves DT, Gerstenfeld LC, Einhorn TA. Molecular mechanisms controlling bone formation during fracture healing and distraction osteogenesis. J Dent Res 2008;87:107-18.
59. Mountziaris PM, Mikos AG. Modulation of the inflammatory response for enhanced bone tissue regeneration. Tissue Eng Part B Rev 2008;14:179-86.
60. Park S, Silva M, Bahk W, McKellop H, Lieberman J. Effect of repeated irrigation and debridement on fracture healing in an animal model. J Orthop Res 2002;20:1197-204.
61. Grundnes O, Reikeras O. The importance of the hematoma for fracture healing in rats. Acta Orthop Scand 1993;64:340-2.
62. Glass GE, Chan JK, Freidin A, Feldmann M, Horwood NJ, Nanchahal J. TNF-α promotes fracture repair by augmenting the recruitment and differentiation of muscle-derived stromal cells. Proceedings of the National Academy of Sciences 2011;108:1585-90.
63. Hamrick MW. A role for myokines in muscle-bone interactions. Exerc Sport Sci Rev 2011;39:43-7.
64. Kalajzic I, Staal A, Yang WP, et al. Expression profile of osteoblast lineage at defined stages of differentiation. J Biol Chem 2005;280:24618-26.
65. Andrew JG, Hoyland J, Freemont AJ, Marsh D. Insulin-like growth factor gene expression in human fracture callus. Calcif Tissue Int 1993;53:97-102.
66. Luginbuehl V, Zoidis E, Meinel L, von Rechenberg B, Gander B, Merkle HP. Impact of IGF-I release kinetics on bone healing: a preliminary study in sheep. Eur J Pharm Biopharm 2013;85:99-106.
67. Meinel L, Zoidis E, Zapf J, et al. Localized insulin-like growth factor I delivery to enhance new bone formation. Bone 2003;33:660-72.
68. Ljunghall S, Johansson AG, Burman P, Kampe O, Lindh E, Karlsson FA. Low plasma levels of insulin-like growth factor 1 (IGF-1) in male patients with idiopathic osteoporosis. J Intern Med 1992;232:59-64.
69. Liu JM, Zhao HY, Ning G, et al. IGF-1 as an early marker for low bone mass or osteoporosis in premenopausal and postmenopausal women. J Bone Miner Metab 2008; 26:159-64.
70. Hill M, Goldspink G. Expression and splicing of the insulin-like growth factor gene in rodent muscle is associated with muscle satellite (stem) cell activation following local tissue damage. J Physiol 2003;549:409-18.
71. Marsh DR, Criswell DS, Hamilton MT, Booth FW. Association of insulin-like growth factor mRNA expressions with muscle regeneration in young, adult, and old rats. Am J Physiol 1997;273:R353-8.
72. Hammers DW, Merritt EK, Matheny RW, Jr., et al. Functional deficits and insulin-like growth factor-I gene expression following tourniquet-induced injury of skeletal muscle in young and old rats. J Appl Physiol (1985) 2008;105:1274-81.
73. Shavlakadze T, Winn N, Rosenthal N, Grounds MD. Reconciling data from transgenic mice that overexpress IGF-I specifically in skeletal muscle. Growth Horm IGF Res 2005;15:4-18.
74. Machida S, Booth FW. Insulin-like growth factor 1 and muscle growth: implication for satellite cell proliferation. Proc Nutr Soc 2004;63:337-40.
75. Han B, Tong J, Zhu MJ, Ma C, Du M. Insulin-like growth factor-1 (IGF-1) and leucine activate pig myogenic satellite cells through mammalian target of rapamycin (mTOR) pathway. Mol Reprod Dev 2008;75:810-7.
76. Mourkioti F, Rosenthal N. IGF-1, inflammation and stem cells: interactions during muscle regeneration. Trends Immunol 2005;26:535-42.
77. Lee WJ. IGF-I Exerts an Anti-inflammatory Effect on Skeletal Muscle Cells through Down-regulation of TLR4 Signaling. Immune Netw 2011;11:223-6.
78. Elkasrawy M, Fulzele S, Bowser M, Wenger K, Hamrick M. Myostatin (GDF-8) inhibits chondrogenesis and chondrocyte proliferation *in vitro* by suppressing Sox-9 expression. Growth Factors 2011;29:253-62.
79. Hamrick MW, Arounleut P, Kellum E, Cain M, Immel D, Liang LF. Recombinant myostatin (GDF-8) propeptide enhances the repair and regeneration of both muscle and bone in a model of deep penetrant musculoskeletal injury. J Trauma 2010;69:579-83.
80. Hamrick MW, McPherron AC, Lovejoy CO, Hudson J. Femoral morphology and cross-sectional geometry of adult myostatin-deficient mice. Bone 2000;27:343-9.
81. Hamrick MW, McPherron AC, Lovejoy CO. Bone min-

eral content and density in the humerus of adult myostatin-deficient mice. Calcif Tissue Int 2002;71:63-8.
82. Hamrick MW. Increased bone mineral density in the femora of GDF8 knockout mice. Anat Rec A Discov Mol Cell Evol Biol 2003;272:388-91.
83. Bialek P, Parkington J, Li X, et al. A myostatin and activin decoy receptor enhances bone formation in mice. Bone 2014;60:162-71.
84. Elkasrawy M, Immel D, Wen X, Liu X, Liang LF, Hamrick MW. Immunolocalization of myostatin (GDF-8) following musculoskeletal injury and the effects of exogenous myostatin on muscle and bone healing. J Histochem Cytochem 2012;60:22-30.
85. Elkasrawy MN, Hamrick MW. Myostatin (GDF-8) as a key factor linking muscle mass and bone structure. J Musculoskelet Neuronal Interact 2010;10:56-63.
86. Kellum E, Starr H, Arounleut P, et al. Myostatin (GDF-8) deficiency increases fracture callus size, Sox-5 expression, and callus bone volume. Bone 2009;44:17-23.
87. Gazzerro E, Canalis E. Bone morphogenetic proteins and their antagonists. Rev Endocr Metab Disord 2006;7:51-65.
88. Chen D, Zhao M, Mundy GR. Bone morphogenetic proteins. Growth Factors 2004;22:233-41.
89. Wozney JM. Bone Morphogenetic Proteins. Progress in Growth Factor Research 1989;1:267-80.
90. Yamaguchi A, Ishizuya T, Kintou N, et al. Effects of BMP-2, BMP-4, and BMP-6 on osteoblastic differentiation of bone marrow-derived stromal cell lines, ST2 and MC3T3-G2/PA6. Biochem Biophys Res Commun 1996;220:366-71.
91. Lavery K, Swain P, Falb D, Alaoui-Ismaili MH. BMP-2/4 and BMP-6/7 differentially utilize cell surface receptors to induce osteoblastic differentiation of human bone marrow-derived mesenchymal stem cells. J Biol Chem 2008;283:20948-58.
92. Carragee EJ, Hurwitz EL, Weiner BK. A critical review of recombinant human bone morphogenetic protein-2 trials in spinal surgery: emerging safety concerns and lessons learned. Spine J 2011;11:471-91.
93. Carragee EJ, Chu G, Rohatgi R, et al. Cancer Risk After Use of Recombinant Bone Morphogenetic Protein-2 for Spinal Arthrodesis. J Bone Joint Surg Am 2013;95:1537-45.
94. Kessler E, Takahara K, Biniaminov L, Brusel M, Greenspan DS. Bone morphogenetic protein-1: the type I procollagen C-proteinase. Science 1996;271:360-2.
95. Jackson WM, Aragon AB, Onodera J, et al. Cytokine expression in muscle following traumatic injury. J Orthop Res 2011;29:1613-20.
96. Villarreal XC, Mann KG, Long GL. Structure of human osteonectin based upon analysis of cDNA and genomic sequences. Biochemistry 1989;28:6483-91.
97. Brekken RA, Sage EH. SPARC, a matricellular protein: at the crossroads of cell-matrix communication. Matrix Biol 2001;19:816-27.
98. Delany AM, Amling M, Priemel M, Howe C, Baron R, Canalis E. Osteopenia and decreased bone formation in osteonectin-deficient mice. J Clin Invest 2000;105:1325.
99. Jorgensen LH, Petersson SJ, Sellathurai J, et al. Secreted protein acidic and rich in cysteine (SPARC) in human skeletal muscle. J Histochem Cytochem 2009;57:29-39.
100. Yamazaki M, Majeska R, Moriya J. Role of osteonectin during fracture healing. Trans Orthop Res Soc 1997; 22:254.
101. Hiltunen A, Metsaranta M, Virolainen P, Aro HT, Vuorio E. Retarded chondrogenesis in transgenic mice with a type II collagen defect results in fracture healing abnormalities. Dev Dyn 1994;200:340-9.
102. Jingushi S, Joyce ME, Bolander ME. Genetic expression of extracellular matrix proteins correlates with histologic changes during fracture repair. J Bone Miner Res 1992; 7:1045-55.
103. Grimston SK, Goldberg DB, Watkins M, Brodt MD, Silva MJ, Civitelli R. Connexin43 deficiency reduces the sensitivity of cortical bone to the effects of muscle paralysis. J Bone Miner Res 2011;26:2151-60.
104. Grimston SK, Brodt MD, Silva MJ, Civitelli R. Attenuated response to *in vivo* mechanical loading in mice with conditional osteoblast ablation of the connexin43 gene (Gja1). J Bone Miner Res 2008;23:879-86.
105. Gross TS, Poliachik SL, Prasad J, Bain SD. The effect of muscle dysfunction on bone mass and morphology. J Musculoskelet Neuronal Interact 2010;10:25-34.
106. Ijiri K, Ma YF, Jee WS, Akamine T, Liang X. Adaptation of non-growing former epiphysis and metaphyseal trabecular bones to aging and immobilization in rat. Bone 1995;17:207S-12S.
107. Tuukkanen J, Wallmark B, Jalovaara P, Takala T, Sjogren S, Vaananen K. Changes induced in growing rat bone by immobilization and remobilization. Bone 1991;12:113-8.
108. Liu D, Zhao CQ, Li H, Jiang SD, Jiang LS, Dai LY. Effects of spinal cord injury and hindlimb immobilization on sublesional and supralesional bones in young growing rats. Bone 2008;43:119-25.
109. Hao Y, Ma Y, Wang X, Jin F, Ge S. Short-term muscle atrophy caused by botulinum toxin-A local injection impairs fracture healing in the rat femur. J Orthop Res 2012; 30:574-80.
110. Poliachik SL, Bain SD, Threet D, Huber P, Gross TS. Transient muscle paralysis disrupts bone homeostasis by rapid degradation of bone morphology. Bone 2010;46:18-23.
111. Thomsen JS, Christensen LL, Vegger JB, Nyengaard JR, Bruel A. Loss of bone strength is dependent on skeletal site in disuse osteoporosis in rats. Calcif Tissue Int 2012;90:294-306.
112. Ellman R, Grasso DJ, van Vliet M, et al. Combined effects of botulinum toxin injection and hind limb unloading on bone and muscle. Calcif Tissue Int 2014; 94:327-37.
113. Aliprantis AO, Stolina M, Kostenuik PJ, et al. Transient muscle paralysis degrades bone via rapid osteoclastogenesis. FASEB J 2012;26:1110-8.
114. Zachs SI, Sheff MF. Periosteal and metaplastic bone for-

mation in mouse minced muscle regeneration. Lab Invest 1982;46:405-12.
115. Utvag SE, Grundnes O, Reikeras O. Effects of lesion between bone, periosteum and muscle on fracture healing in rats. Acta Orthop Scand 1998;69:177-80.
116. Utvag SE, Grundnes O, Rindal DB, Reikeras O. Influence of extensive muscle injury on fracture healing in rat tibia. J Orthop Trauma 2003;17:430-5.
117. Utvag SE, Iversen KB, Grundnes O, Reikeras O. Poor muscle coverage delays fracture healing in rats. Acta Orthop Scand 2002;73:471-4.
118. Willett NJ, Mon-Tzu LA, Uhrig BA, et al. Attenuated human bone morphogenetic protein-2-mediated bone regeneration in a rat model of composite bone and muscle injury. Tissue Eng Pt C-Meth 2013;19:316-25.
119. Gustilo R, Anderson J. Prevention of infection in the treatment of one thousand and twenty-five open fractures of long bones: retrospective and prospective analyses. J Bone Joint Surg Am 1976;58:453-8.
120. Papakostidis C, Kanakaris NK, Pretel J, Faour O, Morell DJ, Giannoudis PV. Prevalence of complications of open tibial shaft fractures stratified as per the Gustilo–Anderson classification. Injury 2011;42:1408-15.
121. Giannoudis PV, Harwood PJ, Kontakis G, et al. Long-term quality of life in trauma patients following the full spectrum of tibial injury (fasciotomy, closed fracture, grade IIIB/IIIC open fracture and amputation). Injury 2009;40:213-9.
122. Landry PS, Marino AA, Sadasivan KK, Albright JA. Effect of soft-tissue trauma on the early periosteal response of bone to injury. J Trauma 2000;48:479-83.
123. Duda GN, Taylor WR, Winkler T, et al. Biomechanical, microvascular, and cellular factors promote muscle and bone regeneration. Exerc Sport Sci Rev 2008;36:64-70.
124. Gopal S, Majumder S, Batchelor AG, Knight SL, De Boer P, Smith RM. Fix and flap: the radical orthopaedic and plastic treatment of severe open fractures of the tibia. J Bone Joint Surg Br 2000;82:959-66.
125. Reverte MM, Dimitriou R, Kanakaris NK, Giannoudis PV. What is the effect of compartment syndrome and fasciotomies on fracture healing in tibial fractures? Injury 2011;42:1402-7.