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Conditional deletion of IGF-I in osteocytes unexpectedly accelerates bony union of the fracture gap in mice

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ABSTRACT

This study evaluated the effects of deficient IGF-I expression in osteocytes on fracture healing. Transgenic mice with conditional knockout (cKO) of *Igf1* in osteocytes were generated by crossing *Dmp1*-Cre mice with *Igf1* flox mice. Fractures were created on the mid-shaft of tibia of 12-week-old male cKO mice and wild-type (WT) littermates by three-point bending. At 21 and 28 days post-fracture healing, the increases in cortical bone mineral density, mineral content, bone area, and thickness, as well as sub-cortical bone mineral content at the fracture site were each greater in cKO calluses than in WT calluses. There were 85% decrease in the cartilage area and >2-fold increase in the number of osteoclasts in cKO calluses at 14 days post-fracture, suggesting a more rapid remodeling of endochondral bone. The upregulation of mRNA levels of osteoblast marker genes (*cbfa1*, *alp*, *Opn*, and *Ocn*) was greater in cKO calluses than in WT calluses. μ-CT analysis suggested an accelerated bony union of the fracture gap in cKO mice. The *Sost* mRNA level was reduced by 50% and the *Bmp2* mRNA level was increased 3-fold in cKO fractures at 14 days post-fracture, but the levels of these two mRNAs in WT fractures were unchanged, suggesting that the accelerated fracture repair may in part act through the Wnt and/or BMP signaling. In conclusion, conditional deletion of *Igf1* in osteocytes not only did not impair, but unexpectedly enhanced, bony union of the fracture gap. The accelerated bony union was due in part to upregulation of the Wnt and BMP2 signaling in response to deficient osteocyte-derived IGF-I expression, which in turn favors intramembranous over endochondral bone repair.

1. Introduction

Bone has an amazing ability to regenerate itself without a scar after injuries, such as fractures. The repair of long bone fractures is mediated by endochondral bone repair, which is divided into four major overlapping stages: 1) the initial inflammatory response, 2) the formation of soft callus, 3) the formation of hard callus, and 4) the bone remodeling and bony union of the fracture gap [1]. The remodeling phase involves resorption of cartilage and conversion of the cancellous callus bone into cortical bone that is indistinguishable from native bone. This phase also includes neovascularization to re-establish blood flow to the fracture site [2]. The mechanism responsible for fracture repair is highly complex and involves actions of the various cell types (including osteoblasts, osteoclasts, chondrocytes, osteocytes, endothelial cells, and mesenchymal stem cells) through local expression of the variety of growth factors and signaling molecules that lead to coordinated development of cartilaginous callus, bony remodeling of the callus, bony union of the gap, and the eventual restoration of the bone structure and strength.

The osteocyte, which is the most abundant cell type in the cortical bone, has an extensive network of dendrites extending to and making contact with other osteocytes, periosteal and endosteal lining cells, bone surface osteoblasts and osteoclasts, and bone marrow cells through a widespread, interconnected canaliculi system [3]. This network of canaliculi allows soluble osteocyte-derived paracrine signaling molecules to migrate freely from the osteocyte to act on other bone cells [4]. Hence, the osteocyte is strategically well positioned within the bone matrix to sense physical and biochemical signals that regulate bone metabolism, remodeling, and local regeneration. There is now increasing evidence that the osteocyte and its secretory factors, such as sclerostin (*Sost*), may play key regulatory roles in fracture repair. For instance, earlier studies have suggested that surviving osteocytes at fracture sites are important for robust cellular recruitment of the various cell types and the release of osteopontin during the initial phase of fracture healing [5] and that osteocytes participate in the periosteal callus cartilage formation and bone regeneration during fracture healing [6]. More recent studies have shown that during hip fracture healing, the expression of *Sost* by osteocytes at the fracture site was downregulated [7]. Mice lacking the *Sost* gene accelerated fracture healing [8,9].

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Administration of the sclerostin neutralizing monoclonal antibody enhanced fracture repair and bone strength [10–14]. There is also evidence that conditional deletion of the connexin 43 gene in osteocytes delayed bone formation and impaired fracture repair [15].

We are interested in the regulatory role of osteocyte-derived IGF-I in fracture repair for the following reasons: first, fracture repair requires bone regeneration, and IGF-I promotes bone formation, regeneration, and fracture repair [16,17]. Second, the expression and bone cell production of IGF-I were greatly increased at the fracture site during the early healing phase [18,19]. Third, IGF-I is a key mediator of the skeletal response to PTH [20], which has shown to promote fracture healing [16,21]. Fourth, local IGF-I treatment [17,22] or systemic treatment with IGF-I-expressing mesenchymal stromal cells [23,24] promoted fracture healing in a number of animal models. Moreover, it is generally accepted that moderate axial mechanical loading enhances fracture repair by stimulating formation of periosteal callus and increases the rate of healing [25–28]. Conditional disruption of *Igf1* gene in osteocytes completely abolished the bone formation response to mechanical loading [29]. Accordingly, we anticipate that deficient expression of osteocyte-derived IGF-I would also impede the fracture repair process.

In this study, we sought to test the hypothesis that osteocyte-derived IGF-I plays an essential role in fracture repair by comparing the healing of a simple closed tibial fracture in osteocyte *Igf1* conditional knockout (cKO) mice with that in wild-type (WT) littermates. Surprisingly, this study shows that conditional deletion of *Igf1* in osteocytes not only did not impede, but in fact promoted, fracture callus remodeling and accelerated bony union of the fracture gap. These unexpected findings indicate that osteocyte-derived IGF-I may have a novel inhibitory role in fracture repair.

2. Materials and methods

2.1. Animals

All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Loma Linda University and also by the Animal Care and Use Review Office (ACURO) of the Department of the Army of the United States. In conducting research using animals, the investigators adhered to the Animal Welfare Act Regulations and other Federal statutes relating to animals and experiments involving animals and the principles set forth in the current version of the guide for *Care and Use of Laboratory Animals*, National Research Council. With the exception of μ-CT analyses that were performed at the Jerry L. Pettis Memorial VA Medical Center (Loma Linda, CA, USA), all experiments were carried out in the AALAC accredited Animal Care Facility and the laboratory space of the Department of Medicine of the Loma Linda University. Animals were housed in groups of 4 per cage under a 12-h light/dark cycle and provided water and regular rodent chow ad libitum.

Osteocyte *Igf1* cKO mice were generated by crossing *Igf1*$^{flox/flox}$ mice with *Dmp1*-Cre mice as previously described [30]. Briefly, the *Dmp1*-Cre mice (in mixed genetic background of 50% C57BL/6 and 50% CD1) were first bred with the *Igf1*$^{flox/flox}$ mice (in C57BL/6 genetic background) to generate heterozygous mice with the genetic background of *Igf1*$^{flox/-}$/*Dmp1*-Cre at F1 generation (all with a mixed genetic background of 75% C57BL/6 and 25% CD). The F1 heterozygous mice were then crossbred with each other to produce 25% homozygous cKO mice (*Igf1*$^{flox/flox}$/*Dmp1*-Cre), 50% heterozygous cKO mice (*Igf1*$^{flox/-}$/*Dmp1*-Cre), and 25% WT littermates (*Igf1*$^{-/-}$/*Dmp1*-Cre). Only male homozygous cKO mice and WT littermates were used for this study.

2.2. Closed tibial mid-shaft fractures

Standard transverse closed fractures were produced on the cortical cortex at the midshaft of the right tibiae (above the tibiofibular junction) of 12-week-old male mice by the three-point bending technique as previously described [31]. The left tibiae served as respective internal intact controls. Briefly, animals were anesthetized by isoflurane inhalation. A mid-line skin incision over the knee joint was made to gain access to the proximal tibial metaphysis, and a pilot hole was made using a 30-gauge needle at a position just medial to the patella tendon. A stainless steel pin (25-gauge) was inserted into the intramedullary space of the tibia for internal fixation and fracture stabilization. A smaller pin (27-gauge) was used for cKO mutant mice due to their 8–12% smaller bone size than WT mice [30]. The appropriate placement of the pin was confirmed by X-ray. The exposed end of the pin was cut proximally at the level of the bone. Wounds were closed with surgical sutures, and the tibia was held in a fixed position. A single complete fracture was then created by three-point bending using an Instron Mechanical tester (Norwood, MA). Fracture bones were harvested for the examination of fracture healing at 14, 21, and 28 days post-fracture, because endochondral bone repair and remodeling are maximal at these time points [2,32,33]. Early time points were not examined because we were interested in the functional aspects of the healing (i.e., callus remodeling and bony union). Fracture healing was assessed by X-ray, pQCT, μ-CT, histology, histomorphometry, and gene expression analyses.

2.3. Peripheral quantitative computed tomography (pQCT) analysis

Fractured tibiae after dissection were immediately fixed in 10% formalin overnight and were stored in phosphate buffer saline at 4 °C until analyses. The area of the tibiae corresponding to the fracture calluses was scanned and analyzed by the pQCT (STRATEC XCT). The outer and inner thresholds were set at 230 and 630 mg/cm^3 to distinguish the woven and cortical bone compartments, respectively. The scanned bone slides were analyzed with a software program (version 6.00) provided by the manufacturer.

2.4. Micro-computed tomography (μ-CT) analysis

Micro-CT analysis of fracture healing was accomplished using a Scanco *Viva*-CT 40 instrument. Scans were performed at 55 keV, and the analysis of the fracture calluses was conducted using density thresholds that resolved the higher density native cortical bone (570–1000 mg/cm^3 HA) from the lower density callus woven bone (220–570 mg/cm^3 HA). This approach might include some intramedullary trabecular bone, which is expected to be minimal at the midshaft where the fracture is produced.

2.5. Torsional bone strength measurement

The fractured tibia at 21 days post-fracture and corresponding contralateral tibia were torsion tested for torsional stiffness using an Instron 55MT1 rotary tester. Briefly, the epiphyses were cast into a dental resin that adheres to the bone and allowed each end of the tibia to be secured in the opposing jaws of the torsional tester. Torsional force was applied at 1°/s until failure. Torsional stiffness (in N-mm/°), which was used as a measure of torsional bone strength, was calculated from the linear portion of the force vs angle curve prior to the yield point. To normalize for the 8–12% smaller bone size seen in cKO mutants, the relative return of torsional bone strength to pre-injury strength of each fractured bone was shown as the ratio of torsional stiffness of the fractured bone to that of the contralateral intact bone. A ratio of 1.0 indicates the full return of its torsional strength.

2.6. Bone histology

Briefly, the isolated bone section containing the fracture callus was immersion-fixed in ice-cold 4% paraformaldehyde for 16 h. After the removal of surgical pins, specimens were decalcified in 14% EDTA for 3-4 weeks at 4 °C with changes of decalcification solution every two