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Periosteal PTHrP Regulates Cortical Bone Remodeling During Fracture Healing

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ABSTRACT

Parathyroid hormone-related protein (PTHrP) is widely expressed in the fibrous outer layer of the periosteum (PO), and the PTH/PTHrP type I receptor (PTHR1) is expressed in the inner PO cambial layer. The cambial layer gives rise to the PO osteoblasts (OBs) and osteoclasts (OCs) that model/remodel the cortical bone surface during development as well as during fracture healing. PTHrP has been implicated in the regulation of PO modeling during development, but nothing is known as regards a role of PTHrP in this location during fracture healing.

We propose that PTHrP in the fibrous layer of the PO may be a key regulatory factor in remodeling bone formation during fracture repair. We first assessed whether PTHrP expression in the fibrous PO is associated with PO osteoblast induction in the subjacent cambial PO using a tibial fracture model in PTHrP-lacZ mice. Our results revealed that both PTHrP expression and osteoblast induction in PO were induced 3 days post-fracture. We then investigated a potential functional role of PO PTHrP during fracture repair by performing tibial fracture surgery in 10-week-old CD1 control and PTHrP conditional knockout (PTHrP cKO) mice that lack PO PTHrP. We found that callus size and formation as well as woven bone mineralization in PTHrP cKO mice were impaired compared to that in CD1 mice. Concordant with these findings, functional enzyme staining revealed impaired OB formation and OC activity in the cKO mice.

We conclude that deleting PO PTHrP impairs cartilaginous callus formation, maturation and ossification as well as remodeling during fracture healing. These data are the initial genetic evidence suggesting that PO PTHrP may induce osteoblastic activity and regulate fracture healing on the cortical bone surface.

1. Introduction

Parathyroid hormone-related protein (PTHrP) is a member of the small parathyroid hormone (PTH) gene family [1,2]. Though it shares a common receptor with PTH (referred to as PTHR1), the biological specificity of PTH and PTHrP is completely different [3,4]: PTH is a classical peptide hormone, whereas PTHrP acts as an autocrine/paracrine regulatory factor. Both PTHrP and the PTHR1 are widely expressed and PTHrP has been shown to regulate a variety of processes such as mammary development, tooth eruption, the mobilization of skeletal calcium during lactation, and endochondral bone formation. Its role in endochondral bone is particularly well studied. Here, PTHrP and Indian hedgehog (Ihh) function in a classical feedback loop that regulates the rate of the chondrocyte differentiation progress that drives linear bone growth [5]. Recently, PTHrP expression has been identified in the fibrous periosteum (PO) in which it functions to model the cortical surface during development and at sites of fibrous tendon and ligament insertions with the bony cortex [6].

Even though PTHrP is widely expressed in many tissues throughout life, it was hard to detect its expression level until the generation of the PTHrP-lacZ knock-in mouse. The PTHrP-lacZ knock-in mouse is a useful and sensitive system in identifying sites of PTHrP expression as well as the regulation of gene expression in these sites [7]. This is the system that initially revealed PTHrP expression in the fibrous PO, where it appears to regulate modeling via inducing osteoclasts on the cortical surface [7,8]. Both osteoclast induction and cortical modeling fail in a conditional knock-out (cKO) mouse in which PTHrP was deleted in the fibrous PO via the scleraxis gene (Scx-Cre) [9]. It is not known whether PTHrP influences osteoblast induction or function, but the Scx-Cre cKO mouse displays a rather profound increase in endocortical bone formation and thickness; it is not known if PTHrP regulates endochondral osteoblast function in this system directly or via an osteoclast generated product.

Fracture repair involves a series of processes that regenerate and remodel the bone structure after injury [10–14]. The cells that contribute to this process are derived from the underlying cortical bone and the PO, which contains abundant mesenchymal stem cells that can differentiate into bone and cartilage. After fracture, a soft callus forms at the fracture

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site and is repaired via chondrocytes that are derived from the PO as well as the injured soft tissue at the fracture site (Fig. S1). The PO provides bone cells to the hard callus (PO new bone) that will ultimately bridge the fracture. Over several months, the entire site is remodeled into mature cortical bone. There are many signaling pathways that regulate the chondrogenesis, osteogenesis, and osteoclast formation that drive fracture repair, but a possible role of PTHrP in fracture repair has not been previously examined. Here, we used a PO-dependent open transverse tibial fracture model to study PTHrP inducibility in PTHrP-lacZ mice as well as its putative function during fracture repair in a conditional knock-out (cKO) mouse that lacks PO PTHrP [8]. In brief, whereas unstable fracture models heal via an avascular callus that recapitulates the endochondral pathway, when a long bone is fractured by open surgical transection and stabilized with intramedullary needle fixation it heals via mesenchymal stem cell (MSC)-driven osteoblast formation derived from the highly vascular PO [15–17].

It is clear that PTHrP induction of PO osteoclasts mediates the sculpting aspects of PO modeling, but it is unknown if PTHrP regulates osteoblastic induction in the PO or any other site. In this study, we used a fixed tibial fracture procedure to conduct fracture surgeries in CD1 control and PTHrP cKO mice and determined the role of PO PTHrP in cartilaginous callus formation, maturation and ossification during the fracture repair. We generated PTHrP cKO mice by conditionally deleting it using scleraxis targeting (*Scx-Cre*); *Scx* is a basic helix–loop–helix (bHLH) transcription factor that is expressed in the fascia and connective tissue that binds bones and muscle together and to each other. The mice underwent the fixed tibial fracture procedure at 10 weeks of age, with readouts at days 7, 14, 21, and 28 post-fracture. We found that PTHrP and osteoblasts are induced in parallel shortly after fracture and that deletion of PTHrP impairs cartilaginous callus formation, maturation, and ossification during fracture repair. These data are the initial genetic evidence suggesting that PO PTHrP may induce osteoblastic activity and regulate fracture healing on the cortical bone surface.

2. Materials and Methods

We bred Scx-Cre mice with $PTHrP^{lacZ/lox}$ mice to generate the PTHrP cKO mice with conditionally deleted PTHrP in the PO. The PTHrP-lacZ replacement construct served as the PTHrP-null allele in this system, providing a convenient lacZ marker of PTHrP-expression sites in the PTHrP cKO mouse. Cross-comparison of β-galactosidase (β-gal)-expressing patterns in the PTHrP-lacZ [7] and Scx-Cre/R26R mice allowed us to identify those sites in which PTHrP and Scx gene expression [18] were concordant and therefore candidate sites of interest in the cKO mouse (Fig. S2). Gender- and age-matched CD-1 (wild-type) mice served as controls. All mice were handled according to the United State Department of Agriculture guidelines and with the approval of the Yale University Animal Care and Use Committee.

2.1. Tibial Fracture Model

An open transverse tibial fracture with intramedullary needle fixation was used as the bone fracture model [19]. Ten-week-old male mice were anesthetized with ketamine (60 mg/kg) by intraperitoneal injection. The hair covering the operation sites were shaved by an electric hair shaver. The tibial fracture procedure was performed on the right hind limbs under aseptic conditions as follows: 1) a 1.5 cm incision was made in the skin on the antero-medial surface using a scalpel; 2) a 25 gauge needle was inserted into the tibia marrow cavity through the medial side of the tibial plateau at the medial side of the patellar ligament to make a pin canal; 3) the needle was removed and the marrow cavity was suitably enlarged; 4) a No. 11 surgical blade was used to transect the mid-shaft of the tibial diaphysis; 5) the 25 gauge needle was reinserted into bone marrow cavity till the narrowest point to simulate fixation, and the pin beyond tibial plateau was cut off by a wire-cutter; and 6) the wound was closed with 4.0 nylon sutures, and buprenorphine was administered in drinking water for pain relief for the first three days after the operation. The left tibia was used as a control and was only exposed by incision, and sutured without manipulating the tibia.

2.2. Radiographic and μCT Analysis

CD1 and PTHrP cKO mice were sacrificed 7, 14, 21, and 28 days post-fracture. X-ray radiography was performed in both antero-posterior and lateral views (n = 5 per time point per group) to examine the fracture pattern and the position of the fixation needle as well as the progress of fracture healing by assessment of bridging across cortices (30 kV for 8.0 s, Faxitron X-ray, Wheeling, IL). Prior to histological processing, PFA-fixed tibia tissues were evaluated by micro-CT using a Scanco μCT35 scanner (Scanco Medical AG, Switzerland) with a 55 kVp source. The samples were scanned at an isotropic resolution of 10 μm. The scanned images from each group were reconstructed at the same thresholds to allow 3-D structural rendering of each sample. The fracture site was analyzed to quantify the amount of callus mineralized volume fraction (BV/TV, %) and connectivity density (Conn.D, $1/mm^3$) (n = 5 per time point per group).

2.3. Histology and Immunohistochemistry

Mice were sacrificed and tibial samples from each group were harvested and prepared for sectioning and analysis. Toluidine blue staining was performed on non-decalcified sections to characterize the basic histological structure at each time point (n = 6 per time point per group). Samples were fixed in 4% Paraformaldehyde (PFA) on ice for 2 h, followed by decalcifying with 7% EDTA (American Bioanalytical, Natick, MA) at 4 °C for 21 days. After washing with PBS and adding magnesium (American Bioanalytical, Natick, MA), samples were processed and embedded in paraffin. Paraffin sections were stained by X-gal (5-bromo-4-chloro-2-indolyl-β-D-galacto-pyranoside), tartrate-resistant acid phosphatase (TRAP), or alkaline phosphatase (ALP) as described [6,7] using a heat step to inactivate endogenous galactosidase activity. Immunohistochemistry for receptor activator of NF-κB ligand (RANKL) used the protocol from the supplier (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as described previously [8]. All data were replicated in triplicate unless otherwise indicated. Histomorphometric measurements were performed using ImagePro Plus 4.5 software (Leeds Precision Instruments, Minneapolis, MN, USA). Total TRAP or ALP positive areas were

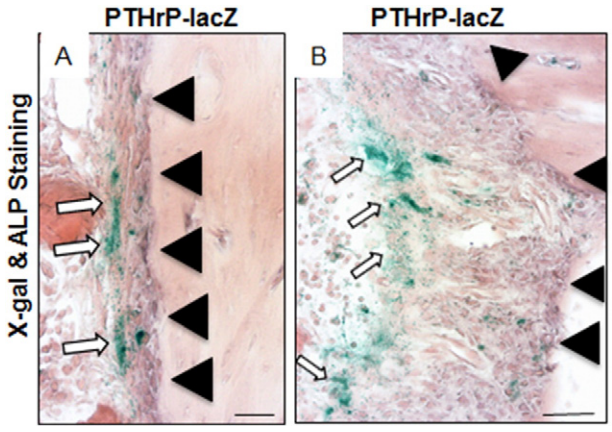

Fig. 1. The PTHrP gene was induced in mesenchymal cells after fracture. LacZ staining and ALP staining were performed 3 days post-fracture. In both images, PTHrP expression (greenish blue) was induced in the PO layer 3 days after surgery. The dark purple reflects ALP expression 3 days after fracture. Arrowheads identify ALP-positive osteoblasts/mesenchymal cells, and arrows identify PTHrP-expressing mesenchymal cells (scale bars = 100 μm in A; 50 μm in B).