 Activation of Hedgehog Signaling during Fracture Repair Enhances Osteoblastic-Dependent Matrix Formation

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ABSTRACT: Fracture repair is a well orchestrated process involving various cell types and signaling molecules. The hedgehog signaling pathway is activated in chondrocytes during fracture repair and is known to regulate chondrogenesis however, its role in osteoblasts during injury is yet unknown. In this study we observed tibial fracture repair in mice in which hedgehog signaling was modulated through genetic alterations of the pathway activator, Smoothened. Levels of the hedgehog target genes Gli1 and Ptc1 in wildtype mice were upregulated in fracture calluses throughout the repair process. Forced activation of the hedgehog pathway in ubiquitous fashion resulted in increased matrix deposition in the fracture callus. Interestingly, inhibition in chondrocytes did not alter the fracture repair phenotype, while activation of hedgehog in osteoblasts was a requirement for normal fracture repair. In vitro, transcript levels of Gli1 and Ptc1 were elevated during osteoblastogenesis. Activation of hedgehog signaling positively affected osteoblast differentiation and mineralization as detected using alkaline phosphatase and Von Kossa staining and Alp and Col1 expression. Here we show that the hedgehog signaling pathway plays a critical role in osteoblasts during fracture repair: inhibition of the pathway in osteoblasts leads to decreased matrix at the fracture site while activation increased matrix deposition. © 2013 The Authors. Published by Wiley Periodicals, Inc. on behalf of the Orthopaedic Research Society. J Orthop Res 32:581–586, 2014.

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Fracture repair is a complex yet well orchestrated regenerative process involving numerous signaling pathways and cell types. Excluding the initial inflammatory phase, long-bone fracture repair occurs through a process which recapitulates endochondral ossification. Shortly after fracture, mesenchymal progenitor cells are recruited to the site of injury and undergo differentiation. Many of the cells localized to the site undergo chondrogenesis, bridging the injured bone with a soft callus composed of cartilaginous matrix. Upon hypertrophy of the chondrocytes, the fracture callus is calcified and osteoprogenitor cells are recruited to the site leading to osteoblastic differentiation and deposition of bone onto the calcified cartilage. The calcified cartilage is then remodeled and the fracture callus is replaced by bone, resulting in a tissue which mimics the shape, pattern, and composition prior to injury.1 Many signaling pathways are activated during fracture repair including the Hedgehog pathway. Hedgehog signaling is regulated by the constitutively active G-protein coupled receptor Smoothened (Smo). During inactivation, the cell-surface transmembrane protein Patched-1 (Ptc1) sequesters Smo and prevents signal transduction. Upon binding by one of the three hedgehog ligands, Indian, Sonic, or Desert Hedgehog (Ihh, Shh, and Dhh), Ptc1’s inhibition of Smo is removed leading to activation of the hedgehog signaling cascade. Signal transduction leads to activation of Gli transcription factors and expression of hedgehog target genes including Ptc1 and Gli1.2

Hedgehog signaling is induced during mammalian development and is involved in axial, appendicular, and facial skeletogenesis. During endochondral ossification hedgehog signaling is required early in osteoblastogenesis and plays an important role regulating chondrocyte differentiation.3,4 This pathway is involved in homeostatic osteoblast activity and in the regulation of bone remodeling.5 Hedgehog signaling is activated during the early phases of bone fracture repair, where Ihh is expressed by chondrocytes.6 The role of the hedgehog pathway in osteoblasts remains unclear.

In vitro, cell culture experiments investigating osteoblast differentiation and activity during modulated hedgehog signaling arrive to conflicting conclusions. Osteoblastic differentiation of human cell lines was unaffected or even inhibited upon activation of hedgehog signaling while in cell lines from rodent systems, activation of hedgehog signaling is shown to increase osteoblastogenesis and osteoblast activity.7–10 In vivo, removal of cilia (the site of hedgehog signaling in the cell) results in decreased bone formation and impaired osteoblast differentiation.11 Modulation of hedgehog signaling in osteoblasts was shown to be important in regulating bone turnover5 and using a bone-graft model researchers found activation of hedgehog signaling aided graft incorporation, an effect attributed to the enhanced differentiation of progenitor cells within the periosteum.12 However the impact of hedgehog signaling in osteoblasts during fracture repair has yet...
to be investigated. Given its disparate roles in various cell types and the interaction of cells during fracture repair, it is difficult to extrapolate data from in vitro or developmental studies and for application to fracture repair.

Our work here investigates the effect of hedgehog signaling in fracture repair as it pertains to osteoblasts. We use genetically modified mice to modulate hedgehog signaling ubiquitously, in chondrocytes, or in osteoblasts during fracture repair.

METHODS

Transgenic Mice

Animal protocols were approved by the animal care committee of the Toronto Centre for Phenogenomics. Hedgehog signaling was modulated using conditional alleles. Gli1(Rosa)26Soj/J (Smo+/loxP/+mice) or Smo−/− mice were used to activate hedgehog signaling. These mice contain a mutant allele encompassing a fusion product of enhanced yellow florescent protein and the constitutively active W539L point mutation of the mouse smoothened homolog (Drosophila) gene. Expression is induced upon Cre-mediated excision of the loxP-flanked STOP codon positioned at the 5′ end of this gene. Smo−/− mice (Smo−/− mice) were used to inhibit hedgehog signaling. These mice possess loxP sites on either side of exon 1 of Smo. 13 Upon Cre-mediated excision, expression of the Smo gene is nullified. Recombination was verified using PCR, and modulation of hedgehog signaling was confirmed by analyzing transcript levels of the hedgehog target genes Ptch1 and Gli1. a1(E)-Cre mice (Col1-Cre mice) express Cre recombinase in a osteoblast-specific manner using the 2.3 kb promoter fragment of Col1a1. 14 COL2-rtTA-Cre mice (Col2-Cre mice) express Cre recombinase in a chondrocyte-specific manner using the type II collagen promoter and under control of a doxycyclin inducible system. 15 The conditional alleles were activated by adding doxycyclin (5 mg/ml) to drinking water 1 week prior to and for the duration after fracture. Tg(CAC-cre/Esr1)5AMC/J mice (Esr1-Cre mice) express Cre recombinase in a ubiquitous fashion under the control of chicken beta actin promoter/enhancer. Cre recombinase is restricted to the cytoplasm, however, treatment with tamoxifen allows localization to the nucleus, leading to recombination of the target gene. Tamoxifen was administered using two intraperitoneal injections per week (100 mg/kg) beginning 1 week prior to an for the duration after fracture. Recombination has been verified using DNA and protein analysis (previously reported) 16–18 and using reverse transcription PCR (discussed below).

Generation of Fractures

Fractures were generated as previously described. 19 Briefly, 3-month old mice were anesthetized and the surgical area was shaved and disinfected. Following an incision proximal to the knee, the tibia was stabilized by inserting a 0.7 mm insect pin into the medullary cavity of the tibia and cut flush to the tibial plateau. A fracture was induced mid-shaft of the tibia using blunt scissors and the incision closed using sutures and wound clips. Analgesic (buprenorphine, 0.2 mg/kg/day) was administered for 3-days post surgery.

Evaluation of Fracture Healing

Five replicates of all samples were analyzed (n = 5). Radiographs of healing fractures were obtained using a Faxitron MX20, with the identical setting used for each animal. Isolated fracture calluses were fixed in 10% formalin for 7 days. Calluses were demineralized in 20% EDTA (pH 8.0) at room temperature. Samples were then cleared of EDTA and embedded in paraffin, cut into 5 μm sections, and stained using Safranin-O (to stain proteoglycans/cartilage red) and counter-stained with Fast Green (to stain bone green/blue). 20 Representative sections from a minimum of five fractured limbs were analyzed by computer assisted histomorphometry analysis 21 and results are presented as percent of bone deposited relative to the total fracture callus.

RT-PCR

Five replicates of all samples were analyzed (n = 5). Tissue samples harvested, immersed immediately into liquid nitrogen, and pulverized into powder prior to RNA extraction while cell culture samples were washed twice with PBS prior to RNA extraction. Total RNA was isolated using TRIzol Reagent (Invitrogen, Inc., Carlsbud, CA) according to the manufacturer’s protocol, purity and quantity of RNA was determined using spectrometric methods and three triplicates were analyzed. cDNA template was generated using random hexamers. TaqMan primers for Smo (Mm01162770_m1), Ptch1 (Mm00436026_m1), and Gli1 (Mm00494645_m1) were purchased from Applied Biosystems, Inc. (Carlsbud, CA) and samples were investigated using a 7900HT Fast Real-Time PCR System. Expression was related to the ribosomal protein 18S as a housekeeping control.

Reverse Transcription PCR

Tissue samples were harvested 7-days post fracture and immersed immediately into liquid nitrogen and pulverized into powder prior to RNA extraction. Total RNA was isolated using TRIzol Reagent (Invitrogen, Inc.) according to the manufacturer’s protocol, purity and quantity of RNA was determined using spectrometric methods. cDNA template was generated using random hexamers. Polymerase chain reaction was carried out using primers listed in table for Cre recombinase (Esr1-forward, 5′ to 3′ GCG TTC TGG CAG TAA AAA CTA TC & reverse 5′ to 3′ GTG ATA GCT GGG). Tissue from all experiments was deposited relative to the total fracture callus.

Cell Culture

Tibia of unfractured 3-month old mice were isolated and cleaned of soft tissue. AMEM culture medium (Wisent, Inc., St. Bruno, Quebec) was used to flush bone marrow from the medullary cavity, cell suspensions were passed through a 18 G needle to dissociate cell aggregates, and cells were plated at a density of 250 × 10^3/cm^2 in plating medium (AMEM, 10% FBS, 100 U/ml Pen/Strep) for 7 days. For colony forming units-fibroblastic, wells were then washed with PBS, fixed with 10% formalin, and stained with 0.25% crystal violet. For osteoblastic differentiation, osteogenic media (AMEM, 10% FBS, 100 U/ml Pen/Strep, 30 μM ascorbic acid, 10−8 M dexamethasone, 8 mM sodium phosphate) was used to induce osteoblastogenesis. After 20 days in differentiation media, wells were washed with PBS, fixed using 10% formalin, and stained for alkaline phosphatase using FastRed (Sigma, Inc., St. Louis, MO) or for mineral
using 2.5% silver nitrate (Von Kossa). Colony forming units were determined from three groups of triplicate experiments.

RESULTS
Hedgehog Target Genes are Expressed during Fracture Repair
Tibial fractures were induced in 3-month old wildtype mice and fracture calluses were harvested at 7-, 14-, and 21-days post fracture. RNA was isolated from the fracture calluses and RT-PCR was used to quantify transcript levels of the hedgehog pathway target genes *Ptch1* and *Gli1*. Throughout the fracture-repair process, the level of expression of both *Ptch1* and *Gli1* were elevated in fracture calluses relative to unfractured bone (Fig. 1A). Transcript levels in the fracture calluses reached their maxima at 14-days post fracture, subsiding thereafter. This confirms the activation of hedgehog signaling during fracture repair and establishes the time course of this activation during this process.

Hedgehog Signaling Positively Affects Fracture Repair
To investigate the effect of hedgehog signaling during fracture repair, the pathway was either activated (*SmoStab*) or inhibited (*SmoNull*) in a ubiquitous manner with recombination driven by tamoxifen-regulated activation of a conditional allele (*Esr1-Cre*). Expression of the transgene alone, or the use of tamoxifen had no effect on fracture repair or general bone phenotype. Interestingly, histological analysis from the early stages of fracture repair, 1 week following injury, a time when chondrogenesis is most active, showed no obvious phenotypic change (Supplementary Figs. 1 and 2). However, radiographic, and histological analysis at 28-days post fracture showed that fracture repair was enhanced in mice expressing the *SmoStab* conditional allele relative to the *SmoNull* allele. Fracture calluses of *Esr1-Cre;SmoStab* mice were observed to be larger than the controls while fracture calluses of *Esr1-Cre;SmoNull* mice were observed to be smaller (Fig. 1B). Histological analysis confirmed the deposition of densely packed tissue in fractures from mice expressing the *SmoStab* conditional allele, relative to fracture calluses of controls (Fig. 1C). Conversely, *SmoNull* fracture calluses contained sparse, disordered tissue. Histomorphometry data showed an increased deposition of bone tissue in the *SmoStab* fracture calluses compared to control (Fig. 1D). Taken together, these results show that there is increased matrix deposition at the fracture site when the hedgehog signaling pathway is activated during fracture repair, despite little change when hedgehog signaling is modulated in the early phases of repair (Supplementary Fig. 1 and 2).

Hedgehog Signaling in Chondrocytes is Dispensable for Fracture Healing
To determine the role of hedgehog signaling in specific cell types, we used *Col2-Cre* to drive expression of the conditional alleles in a doxycycline-dependent manner in chondrocytes during fracture repair. Expression of the transgene alone, or the use of doxycycline had no effect on fracture repair or general bone phenotype. Radiographic analysis of *Col2-Cre;SmoNull* mice indicated healing of the fracture site to be similar to that of controls (Fig. 2A). Histological analysis and histomorphometry data confirmed there to be a similar amount of tissue deposition in *Col2-Cre;SmoNull* fracture calluses and controls (Fig. 2B and C). This data indicates that while hedgehog signaling plays a critical in chondrogenesis during development, the pathway is dispensable in fracture repair.
Activation of Hedgehog Signaling in Osteoblasts Leads to Enhanced Fracture Healing

Col1-Cre was used to drive expression of the conditional alleles in an osteoblast-specific manner during fracture repair in vivo. Radiographs of fracture calluses from mice expressing the SmoStab allele showed the presence of a large, dense callus 28 days post fracture (Fig. 3A). Furthermore, histological analysis indicated the callus to be composed of dense ossified tissue with an increased amount of bone matrix deposited relative to control. Conversely, radiographs of 28-day fracture calluses from Col1-Cre;SmoNull mice indicated calluses which were smaller than those of controls. Histological analysis of the SmoNull calluses revealed the deposition of sparse tissue in response to fracture (Fig. 3B). Histomorphometric analysis confirmed the increased deposition of bone tissue in SmoStab mice while there was decreased amounts of bone tissue in the SmoNull animals (Fig. 3C).

Hedgehog Signaling in Mesenchymal Stromal Cells Leads to Enhanced Osteoblastogenesis and Osteoblast Activity

Using established osteogenic tissue culture methods, bone marrow stromal cells from 3-month old wildtype mice were differentiated into osteoblasts. RNA was isolated from undifferentiated cells or from cells differentiated after 10 or 20 days in osteogenic media and analyzed for Ptc1 and Gli1 transcript levels as indicators of hedgehog pathway activation during osteoblastic differentiation. In wildtype cultures, transcript levels of both Ptc1 and Gli1 were elevated after 10 days in osteogenic media. This data confirms the modulation of hedgehog signaling during osteoblast differentiation.

Bone marrow stromal cells from Col1-Cre;SmoStab, Col1-Cre;SmoNull, and control mice were cultured under osteogenic conditions and differentiation was monitored by staining for alkaline phosphatase and mineral deposition using Von Kossa stain. Prior to differentiation, crystal violet staining confirmed a similar number of progenitor cells (CFU fibroblastic) from these mice (Supplementary Fig. 3). Col1-Cre;SmoStab mice had enhanced alkaline phosphatase and Von Kossa staining after 20 days in differentiation culture relative to control cells while Col1-Cre;SmoNull mice had decreased staining (Fig. 4B). These findings were further verified by enumeration of colony forming units from each of the groups. CFU’s for alkaline phosphatase and Von Kossa staining were highest in cells from Col1-Cre;SmoStab mice and lowest in cells from Col1-Cre;SmoNull mice (Fig. 4C). Osteoblastic differentiation was further investigated by assaying transcript levels of osteogenic markers after 12 days in osteogenic media. Transcript levels of Col1 and Alp were elevated in cells from Col1-Cre;SmoStab mice relative to controls while cells from Col1-
Cre;SmoNull mice showed decreased levels of osteogenic transcript (Fig. 4D and E). Together this data indicates that activation of hedgehog signaling leads to enhanced osteoblast differentiation, activity, and matrix production leading to enhanced fracture repair.

DISCUSSION
Here we found that hedgehog signaling is differentially regulated during fracture repair, and positively regulates osteogenesis during this repair process. During bone development, hedgehog ligand is secreted by differentiating mesenchymal cells.22 Loss of hedgehog signaling during bone development leads to decreased bone length, loss of trabecular bone, and undermineralized long and flat bones.3,23 Here we found a similar phenotype in the fracture callus of mice lacking hedgehog signaling, showing that hedgehog signaling plays an important role in fracture repair, as well as during repair.

We show here that hedgehog signaling is differentially regulated over time in fracture healing, with peak activation at 14-days post fracture, a time in which osteoblasts are maximally activated to form new bone. Although hedgehog ligands are expressed by chondrocytes during the repair process,24–26 conditionally deleting hedgehog signaling in chondrocytes did not result in any observed fracture repair phenotype. Interestingly, early stages of fracture repair were relatively unaltered by modulation of the hedgehog signaling pathway. This is in contrast to what is observed in development, where a lack of hedgehog signaling has a profound bone phenotype. These data indicate the positive influence of hedgehog signaling during later stages of fracture repair, when osteoblasts play an important role in matrix deposition.

The temporal expression of hedgehog target genes during fracture repair, as well as the data from mice lacking hedgehog signaling suggests that the effect of hedgehog signaling regarding fracture repair is on osteoblasts, rather than on chondrocytes. Furthermore, fractures heal even in the absence of hedgehog signaling indicating that this signaling pathway modulates the quality of the repair but is not required for successful healing. This is in sharp contrast to observations in mice lacking canonical Wnt signaling in osteoblasts, in which fractures will not heal.19,27 Thus providing further support for the notion that fractures will heal as long as undifferentiated mesenchymal cells are able differentiate to osteoblasts, and that signaling pathways which modulate differentiation to osteoblasts control the amount of matrix and bone deposition at the fracture site.

Our in vitro data shows that hedgehog signaling positively regulates osteoblast differentiation. Hedgehog signaling likely plays the same role in fracture repair. This raises the possibility that agents that activate hedgehog signaling could be used to improve the quality of fracture repair. Hedgehog agonists, such as purmorphamine, could fulfill such a function.28–30

In this study we investigated the effect of hedgehog pathway modulation on long-bone fracture repair. Activation of hedgehog signaling enhanced osteoid deposition in the fracture callus while inhibition of hedgehog signaling resulted in decreased matrix deposition. This effect was most striking when studied in an osteoblast-specific manner. In vitro osteogenic cultures revealed activation of hedgehog signaling

Figure 4. Hedgehog signaling positively regulates osteoblast differentiation. Bone marrow stromal cells from wildtype mice were adhered to tissue culture plastic and differentiated to osteoblasts. (A) Transcript levels of the hedgehog target genes, Ptch1 and Gli1, were analyzed using rt-PCR and compared to undifferentiated controls. Bone marrow stromal cells from Col1-Cre;SmoStab, Col1-Cre;SmoNull, and control mice were cultured under osteogenic conditions. Bone-marrow stromal cell aspirates were plated onto tissue culture plastic and differentiated to osteoblasts. (B) Alkaline phosphatase (empty) and Von Kossa (solid) stains were used to visualize the cell-surface marker and matrix mineralization, respectively and to determine the number of (C) colony forming units (CFU). Osteoblastogenesis was further investigated by analyzing amounts of (D) Col1 transcript and (E) Alp transcript after 12 days in osteogenic media.
enhances osteoblastic differentiation and osteoblastic activity. This work confirms activation of hedgehog signaling in osteoblasts positively affects bone fracture repair and indicates the potential use of hedgehog modulators as therapeutics in bone repair and to aid in osseous integration of implants.

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G.B. designed the study, conducted the experiments, analyzed the results, and drafted and edited the manuscript. D.S. designed and conducted experiments. P.N. designed and analyzed the results. B.A. designed the study, analyzed the results, and edited the manuscript.

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