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Exogenous Hedgehog Antagonist Delays but does not Prevent Fracture Healing in Young Mice

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Abstract

Fracture healing recapitulates many aspects of developmental osteogenesis. The hedgehog (Hh) signaling pathway, essential to skeletal development, is upregulated during fracture healing, although its importance is unclear. Our goal was to assess the functional importance of Hh signaling in endochondral fracture healing. We created closed, transverse diaphyseal femur fractures in mice, stabilized with an intramedullary pin, and administered a systemic Hh inhibitor or vehicle. Because Hh pathway activation is mediated by the receptor Smoothened (Smo), we used the Smo antagonist GDC-0449 (GDC, 50 mg/kg, twice daily) to target the pathway. First, in vehicle-treated 10-wk female C57BL/6 mice we confirmed that Hh signaling was increased in fracture callus compared to intact bone, with >5-fold upregulation of target genes *Ptch1* and *Gli1*. Additionally, using 10-wk male and female Gli1 reporter mice, we saw a strong activation of the reporter in the osseous regions of the fracture callus 7–10 days after fracture. GDC treatment significantly blunted these responses, indicating effective inhibition of fracture-induced Hh signaling in bone. Moreover, microCT analysis revealed that GDC treatment significantly reduced cancellous and cortical bone volume at non-fracture sites (tibial metaphysis and diaphysis), suggesting that the drug inhibited normal bone formation. GDC treatment had a modest effect on fracture healing, with evidence of delayed callus mineralization radiographically (significantly lower Goldberg score at day 14) and by microCT (reduced callus vBMD at 14 days), and a delay in the recovery of torsional rotation to normal (elevated rotation-at-peak torque at 21 days). On the other hand, GDC treatment did not inhibit qPCR or morphological measures of chondrogenesis or angiogenesis, and did not impair the recovery of failure torque (at day 14 or 21), a measure of biomechanical competence. In summary, GDC treatment inhibited Hh signaling, which delayed but did not prevent fracture healing in young mice. We conclude that Hh signaling is strongly induced after fracture and may play a role in early callus mineralization, although it does not appear to be required for eventual healing.

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Keywords

Fracture healing; mouse femur fracture; hedgehog antagonist; Gli1 reporter; callus bone mineral density

Introduction

Fractures are one of the most prevalent medical conditions, accounting for 16% of all musculoskeletal injuries. Annually, 6.8 million cases occur, incurring approximately \$100 billion in medical costs and productivity loss in the United States [1–3]. While most fractures heal without difficulty, up to 10% of fractures result in healing deficiencies [3–5]. Improved understanding of the mechanisms of fracture healing will aid development of novel therapies to reduce healing deficiencies and minimize patient morbidity.

Long bone fractures typically heal by secondary fracture healing through a cartilage template intermediate. This process recapitulates events that take place during embryonic development [6–9]. One of the signaling pathways instrumental in the growth and differentiation of bone during development is the hedgehog (Hh) pathway. Of the three Hh ligands identified in vertebrates, Indian hedgehog (Ihh) is particularly critical in skeletogenesis. Ihh regulates proliferation and differentiation of chondrocytes, development of perichondrial osteoblasts, and vascularization of endochondral bone [10–12]. Apart from its role in skeletal development, Hh signaling is critical to vascular development in general, acting in part through BMP and VEGF pathways [13–15]. Thus, Hh is a key regulator of several developmental processes that are also critical in fracture healing – chondrogenesis, osteogenesis and vasculo/angiogenesis [15].

Several important mediators in the Hh pathway include Patched1 (Ptch1), a 12-transmembrane receptor that inherently inhibits Hh signaling, Smoothened (Smo), a 7-transmembrane receptor, and Glioma-associated oncogene (Gli1), a terminal effector of the pathway. The binding of Hh ligand to Ptch1 on the cell surface releases the baseline suppression of Smo by Ptch1, resulting in downstream Gli1 transcription, which leads to the activation of a signal cascade [16, 17]. Ptch1 negatively regulates Hh signaling via this feedback loop, making its expression a marker of increased Hh pathway activity [18, 19]. Recent advances in targeted anticancer therapies provided an effective competitive antagonist of Smo, GDC-0449 (Vismodegib) [20–23], which was FDA-approved for clinical use for basal cell carcinoma in 2012.

Despite the known importance of Hh signaling in skeletogenesis, little is known about the role of Hh signaling in post-natal fracture healing. Studies have shown that Ptch1 expression is upregulated during non-endochondral woven bone formation in response to stress fracture [24]. Also, endochondral ossification of unstabilized fractures in mice was associated with increased Ihh expression [25], and a similar study in rat femurs demonstrated expression of Ihh in chondrocytes and osteoblasts during fracture healing [26]. Two recent studies showed a functional role for Hh in bone healing. First, genetic inactivation of Hh in osteoblasts blunted bone matrix deposition during endochondral healing in mice [27]. Second, the use of the pharmacological Smo antagonist, GDC-0449, reduced osteogenesis and angiogenesis

during stress fracture healing in rats [28]. Our goal here was to extend these previous studies and assess the functional importance of Hh signaling in endochondral fracture healing. We utilized the mouse femur fracture model to determine whether pharmacological inhibition of the Hh pathway could modulate endochondral healing. We hypothesized that Hh pathway inhibition using GDC-0449 reduces Hh signaling and delays fracture healing in young mice.

Methods

Wild-type Mice

A total of 136 female, C57BL/6 wild-type mice (Charles River Lab, MA) were obtained at 8–9 weeks of age and housed until 10 weeks old in standard conditions (12hr light/dark cycles). The mice were randomly but equally divided into two groups of 68. The treatment group received GDC-0449 (ChemieTek, Indianapolis, IN; 50mg/kg; 20mg/mL dissolved in DMSO), hereafter referred to as GDC, twice daily by oral gavage (#20 gavage needle) until designated euthanasia time points. This regimen was selected based on our previous work [28] and pilot studies demonstrating suppression of *Ptch1* and *Gli1* expression in bone (confirmed in Results below). The control group received equivalent volume (2.5 ml/kg) of DMSO vehicle, hereafter referred to as Veh, twice daily by oral gavage. Treatment and vehicle animals were housed in separate cages. Access to food and water was *ad libitum* with unrestricted cage activity. Body weight was recorded daily. Euthanasia was performed by CO₂ asphyxiation. All protocols were approved by Animal Studies Committee at Washington University in St. Louis School of Medicine.

Cre Reporter Mice

A total of 19 Gli1Cre reporter mice were bred in the animal barrier and allowed to mature to 10 weeks. Mice were a cross between an Ai14 tdTomato reporter (B6.Cg-Gt(ROSA)26Sortm14(CAG-tdTomato)Hze/J, stock 007914, from Jackson Labs, Bar Harbor, ME) and a tamoxifen-inducible Gli1Cre originally reported by Ahn and Joyner [29]. Mice were either homozygous or heterozygous for Ai14 and had a single copy of Cre (Ai14; Gli1CreERT2). In pilot studies there were no differences in tdTomato expression with between homozygous or heterozygous Ai14 mice. Activation of Gli1Cre expression was induced using tamoxifen (Tmx) chow (TD.130859, Envigo, Madison, WI) started a week prior to surgery and given until euthanasia. The mice were randomly divided into Veh with normal chow (Veh/Ctrl), Veh with Tmx (Veh/Tmx), and GDC with Tmx (GDC/Tmx) groups. The GDC and Veh were administered orally in the same manner as stated in the previous section. Mice were sacrificed 7 or 10 days after surgery and processed for calcified frozen histology. Frozen sections were counterstained with DAPI and imaged (Hamamatsu NanoZoomer HT model). The tdTomato expression was cumulative throughout the experiment and labeled any cell or progeny expressing Gli1Cre.

Fracture surgery

The morning doses of Veh and GDC were given to the 10-wk old mice prior to surgery. Mice were sedated (1–3% isoflurane) in preparation for surgery. The right hindlimb was shaved and sterilely prepared. An incision was made over the distal femur and patella subluxated to expose the femoral condyles and release the quadriceps. With the mid-

diaphysis of the femur exposed, a small notch on the medial surface was made with a #12 blade in order to pre-dispose this site to fracture. A #70 drill bit was used to open the intercondylar notch. A series of 27G, 25G, and 23G needles were used to ream the intramedullary canal. A fracture of the right femur was created using three-point bending loading, with the load applied using a materials testing machine (Instron) as described [30]. The incision was lavaged with sterile saline for better visualization of the fracture site and proximal fracture fragment. A 23G pin with flattened ends was inserted until fully seated in the intramedullary space. The pin was cut to the appropriate length and the incision was closed with 3-O nylon sutures (McKesson Medical-Surgical, Mn). Buprenorphine was injected for postoperative pain control (0.1mL/20g). Mice were returned to cage for recovery and allowed unrestricted cage activity. Day of surgery was defined as post-operative day zero (POD0). For all experiments the right femur (RF) was injured and the left femur (LF) was used as an uninjured internal control.

Radiographs

After the surgery, the lower extremities were radiographed at 3x magnification with the mouse placed prone and the hips abducted (Faxitron UltraFocus 100; Tucson, AZ). Radiographs were taken again in the same manner at the time of sacrifice (either POD 7, 10, 14, or 21), to ensure fracture stability and alignment during the course of treatment and to note the stage of mineralization. The radiographs (POD7-21) were independently graded in a blinded manner by two investigators using a subset of the Goldberg Scale (0=no union; 1=partial union; 2=complete union) [31]. Any discrepancies were adjudicated by the senior investigator (MJG). For any mice where the radiograph displayed an absent or unstable pin (partial pullout) the femur was excluded from all analysis.

Ex vivo micro-CT of fractured femurs and intact tibias

On POD14 or 21, mice were euthanized and bilateral femurs dissected free of adjacent tissues. The femurs were placed in 10% neutral buffered formalin (NBF) overnight, washed twice with phosphate buffered saline (PBS), and placed into 70% ethanol. The proximal 2–3 mm of the fractured right femurs were potted in 2% agarose in a 21 mm diameter tube and the entire femur scanned in air using a microCT (VivaCT, Scanco Medical AG, Switzerland). Scan settings were 21 μm voxel size, 55 kVp, 145 μA , and 300 ms integration time. Images were filtered (gauss sigma 1.2) and the fracture midpoint was identified. The region of interest (ROI) was defined as 300 slices (6.3mm, encompassing the entire callus) centered at the fracture midpoint. Contours marking the outer margin of the callus were drawn. An upper threshold of 460/1000 (16 bit grey scale normalized to 1000; equal to 840 mgHA/cm³) was used to segment only cortical bone, while a lower threshold of 150/1000 (16 bit grey scale normalized to 1000 157.4 mgHA/cm³) was used to segment any mineralized tissue. Total tissue volume (TV, mm³), bone volume (BV, mm³), bone volume fraction (BV/TV), tissue mineral density (including cortical bone) (TMD, mg HA/cm³), volumetric bone mineral density of callus (excluding cortical bone) (vBMD, mg HA/cm³), and callus volume (= TV - cortical bone, mm³) were analyzed according to previously published methods [30]. Following microCT, femurs were decalcified and processed for histology.

To assess the effect of GDC on cancellous and cortical bone structure at a non-fracture site, intact tibias from POD21 mice were scanned using similar parameters as above, but with a 10.5 μm voxel size. For cortical bones, the ROI was defined as 1.05 mm (100 slices) of bone starting 5 mm distal to the tibia fibular junction. Contours were drawn on the periosteal surface. For cancellous bone, the ROI was at the proximal tibia metaphysis region just distal to the growth plate and extended 0.735 mm (70 slices) toward the midshaft of the bone. Analysis sigma was set at 0.4 and support at 1. A threshold of 160/1000 (179.3 mgHA/cm³) was used to segment bone for the cancellous analysis, from which trabecular (Tb) tissue volume (TV), bone volume (BV), BV/TV, thickness (Tb.Th), number (Tb.N), separation (Tb.Sp), vBMD, and connectivity density (Conn.D) were determined [32]. A threshold of 250/1000 (377.5 mgHA/cm³) was used for the cortical analysis. Total area (TA), bone area (BA), medullary area (MA), cortical thickness (Ct.Th), polar moment of inertia (pMOI), and TMD were determined.

Gene Expression

On POD7, 10 or 14, cohorts of mice were euthanized and bilateral femurs dissected free of adjacent tissues and flash frozen in liquid nitrogen. A 6–8mm length segment of mid-diaphysis (corresponding to the fracture callus) was resected and the marrow removed by centrifugation. Segments were pulverized with Mikro-Dismembrator U (B. Braun Biotech International, Germany) and stabilized with 1mL of Trizol (Ambion, CA). The stabilized samples were stored in –80C freezer if RNA was not extracted immediately following pulverization. Samples were thawed and mRNAs were extracted using RNeasy Mini Kit with RNase free DNase step (Qiagen). RNA concentration was quantified (Nanodrop ONE, Thermal Scientific) and RNA integrity was evaluated (Bioanalyzer 2100, Agilent Technologies). RNA quality was ensured prior to proceeding with 260/280nm absorbance ratio of 1.8–2.1 and electropherograms consistent with RNA integrity numbers (RIN) ≥ 7 . First strand cDNA was produced (iScript, Biorad) from 500ng of total RNA, and used for qRT-PCR, which was performed using TAQMAN probes on 96 $\times$ 96 high-throughput chips on the BioMark HD System at the Genome Technology Access Center (GTAC, Washington University in St. Louis). A total of 22 target genes were evaluated in triplicate, two reference genes—TATA box binding protein (*Tbp*) and importin 8 (*Ipo8*); four Hh pathway mediators—*Gli1*, *Ptch1*, *Ihh*, and desert hedgehog (*Dhh*); four osteogenic genes—integrin binding sialoprotein (*Ibsp*), bone gamma-carboxyglutamate protein (*Bglap*, a.k.a., osteocalcin), osterix (*Sp7*), and collagen type 1- $\alpha 1$ (*Col1a1*); three chondrogenic genes—aggrecan (*Acan*), sex determining region Y-box 9 (*Sox9*), and collagen type 2- $\alpha 1$ (*Col2a1*); and nine vascular/angiogenic genes—fibroblast growth factor 2 (*Fgf2*), endothelial tyrosine kinase (*Tek*), vascular endothelial growth factor-a (*Vegfa*), heme oxygenase 1 (*Hmox1*), nitric oxide synthase 2 (*Nos2*), angiogenin ribonuclease (*Ang* and *Ang2*), tyrosine kinase with Ig-like and EGF-like domains 1 (*Tie1*), and pleiotrophin (*Ptn*) (Supplemental Table 1). Expression was determined normalizing to both reference genes (2^{-CT}) and then fold change of gene expression was determined comparing fractured right femurs versus the intact left femurs (2^{-CT}).

Immunohistochemistry

Histological analysis was performed at POD7, 14, and 21. POD7 femurs were dissected with intact adjacent tissues and POD14 and 21 femurs were dissected free of adjacent tissues. All femurs were fixed with 10% formalin overnight. Both POD14 and 21 femurs were first scanned with microCT. The femurs were decalcified in 14% EDTA for two weeks with solution change every fourth day. The decalcified femurs were stored in 70% ethanol, embedded in paraffin, longitudinally sectioned (5 μ m), and stained with hematoxylin and eosin, and picrosirius red/alcan blue. The stained slides were imaged and quantified with NanoZoomer at 20X magnification (Hamamatsu NanoZoomer HT model). Average callus area and average cartilage area were measured on picrosirius red/alcan blue stained samples by three repeated measures of a section at the midpoint region of the callus.

Fracture callus osteoclast activity was quantified on tartrate-resistant acid phosphatase (TRAP) staining of POD14 and 21 samples. Four images were taken of each callus at 20x magnification section, one from each quadrant. TRAP positive cell numbers (OcN), TRAP positive cell contact (with woven bone) surface (OcS), and total callus surface (BS) were measured and averaged using Bioquant software (v16, Nashville, TN).

Vascular Perfusion

To assess vascularity at the fracture site, POD7 and 14 mice were perfused with radiopaque compound followed by microCT (CT-angiography). In preparation for perfusion, three 10 mL syringes were connected to the independently valved inlets of a 6 in Y-connector (Smiths Medical, OH) filled with PBS/heparin, formalin, and perfusion solution, respectively. A 23G butterfly needle was connected to the outlet of the Y-connector. Perfusion solution consisted of MicroFil (MV-120, Flow Tech, Inc.) diluted 3:1 with diluent. 2 mL of curing agent was mixed with the perfusion solution immediately prior to the start of the procedure. After ensuring adequate anesthesia and sedation with ketamine/xylazine cocktail (87 mg/kg ketamine; 13 mg/kg xylazine), the abdominal cavity and rib cage of the mouse was opened. The right atrium was nicked and the 23G butterfly needle was used for intracardial cannulation of the left ventricle. The mouse circulation was first perfused with 10 mL of 0.1% heparinized solution in phosphate-buffered saline (heparin, Sigma, St. Louis). To fix the tissues, 10mL of 10% formalin was used followed by perfusion with 10 mL of the prepared MicroFil solution. The procedure took approximately 15 min during which the animals died under anesthesia. Obvious pigmentation of the eyes, tongues, and tails served as reliable markers for systemic vascular perfusion. The MicroFil solution was allowed to polymerize at 4°C overnight, after which the right and left femurs were resected and kept in 70% ethanol for microCT scanning.

MicroCT 40 (Scanco Medical) was used to scan the perfused femurs to assess vascularity at 45 kV, 177 μ A, 300 ms, and 6 μ m resolution. Because the microCT attenuation of bone mineral and Microfil are similar, it is difficult to assess the vasculature on a mineralized bone scan. Therefore, each sample was scanned before and after decalcification with 14% EDTA; the pre-decalcification scan was used to visualize the callus morphology, while the after-decalcification scan was used for the actual vascular assessment. The scanning region was the same 6.3 mm length (1050-slice) as for the bone scans described above. From

POD14 femurs, the mineralized CT scans were used to measure the callus diameter at 0, 25, 50, 75, and 100% of the callus length; these diameters were then used to define the callus ROI ("tissue volume") on the decalcified scans. Due to the lack of mineralized callus at POD7, the ROI of POD7 femurs was determined using the average of POD14 callus diameters. Three-dimensional reconstruction of the blood vessels (Gauss Sigma=1.2, Gauss Support=2, Lower Threshold=200, Upper Threshold=1400) in the ROI was performed using Scanco software (uCT Ray V4.0). The vascular network parameters and tissue volume were extracted from the images. Vascular density (BVV/TVV) was defined as the ratio of blood vessel volume (BVV) to total tissue volume (TTV).

Mechanical Testing

Bilateral femora from mice at POD14 and 21 were subjected to torsion testing. To facilitate gripping, the ends of each femur were potted in 6 mm diameter x 12 mm length acrylic tubes. The femurs were centered with a fixture that ensured the alignment of the proximal and distal potting tubes. Approximately 2 mm of the proximal and distal ends of the femurs were embedded into the potting tubes using polymethylmethacrylate (Ortho-Jet, Lang Dental), and cured overnight. The resulting samples had fully exposed calluses or approximately 7 mm of intact diaphyseal bone. Prior to testing, each sample was wrapped with PBS-soaked gauze for 15 min to ensure hydration. Samples were placed in a custom-built torsion tester equipped with a 250 in-oz load cell; LabVIEW was used for control and data acquisition (LabVIEW 2014, National Instruments, TX). With one end held fixed, the opposite end was rotated at 1 deg/sec from 0–40 deg. The upper limit of 40 degs was more than twice the angle at fracture of intact femurs tested in pilot tests (12–18 deg); nevertheless, not all of the right (*in vivo* fracture) femurs exhibited a clear failure point during the torsion test. From torque-rotation plots, peak torque and angle at peak torque were determined.

Sample Size

Group sample sizes (n) were chosen based on a priori sample size calculations (alpha [significance] = 0.05, and beta [type II error] = 0.2). Historical data were used to estimate variance, and effect sizes were based on judgement of biological importance. Target sample sizes were as follows: qPCR, n=6; microCT, n=10; torsion, n=10; vascular perfusion, n=6. Actual sample sizes are stated in the Results.

Statistical Analysis

The radiographic union assessment based on the Goldberg scale was compared between treatment groups at each time point using Chi-square analysis. Left femur versus right femur gene expression data (2^{-CT}) were compared using paired t-tests for each timepoint and treatment separately. A two-factor ANOVA with Bonferroni multiple comparisons test was performed for fold change gene expression (2^{-CT}), histological cartilage and callus measurements, TRAP analysis, vascular perfusion outcomes and torsion outcomes; time and treatment were independent factors. Tibia cancellous and cortical bone measures were evaluated with unpaired t-tests between treatment groups. Any outcome that did not adhere to the assumption for parametric statistical analysis (normality [D'Agostino & Pearson or Shapiro-Wilk test] and variance [F test]) was assessed using a non-parametric analysis

(Mann-Whitney test, <10% of all outcomes). Statistical significance was considered at $p < 0.05$. All statistical analyses were performed using commercial software (GraphPad Prism 7, CA).

Results

The Hh antagonist GDC-0449 diminishes Hh signaling after femur fracture

Gene expression of fracture callus of the right femora and the uninjured diaphyseal region of the left femora were evaluated by qPCR ($n = 6-10$ /treatment/timepoint). In Vehicle-treated mice, expression of *Ptch1* and *Gli1* were both significantly increased in fractured samples versus intact at POD7 and POD10 (ANOVA, $p < 0.05$) (Figure 1A, B, Supplemental Table 2), with upregulation greatest at POD10. This is consistent with activation of the Hh pathway early during fracture healing. Importantly, in the GDC-treated mice, this early upregulation was significantly blunted ($p < 0.001$). *Ihh* was also strongly upregulated in fractured limbs with a peak >50-fold on POD10, but this upregulation was not affected by GDC ($p = 0.62$) (Figure 1C, Supplemental Table 2). These results confirm that GDC inhibition of the Hh pathway occurred downstream of transcriptional regulation of this key Hh ligand.

Frozen histology of Ai14; Gli1CreERT2 reporter mice showed strong TdTomato expression in Vehicle-treated mice on Tmx chow at POD10, with abundant Gli1+ cells noted in regions where woven bone predominated, and to a lesser extent in the cartilaginous regions of the callus (Figure 1D, $n = 2-3$ /treatment/timepoint). Similar results were seen at POD7. Gli1+ cells were also observed in the articular cartilage and in the growth plates (not shown). TdTomato expression was markedly reduced by GDC treatment (Figure 1E). There was little TdTomato expression in non-Tmx controls, indicating the reporter was functioning correctly (not shown). Together with the qPCR results, these findings confirmed that GDC treatment inhibited Hh signaling in bone.

GDC delays radiographic signs of fracture healing

Healing progression was assessed qualitatively by inspection of radiographic images (Figure 2A). There were no visible differences on POD7 between GDC and Veh groups, with negligible mineralized callus at this early timepoint. At POD10, the GDC-treated samples still showed negligible bony callus, whereas the Veh group had visible mineralization at the callus periphery. By POD14, femora from both groups had well developed bony calluses, but the GDC group had less evidence of central bony bridging. By POD21, the outer calluses in both groups were fully bridged, although the interior callus appeared denser in the GDC group, while this region was more radiolucent in samples from the Veh group.

Semi-quantitative assessment of healing was assessed based on blinded scoring using the Goldberg scale (0: no union; 1: partial union; 2: full union). The GDC-treated group had a lower proportion of samples with full union, and a higher proportion with no or partial union, as compared to the Veh-treated group ($p < 0.05$; Figure 2B).

GDC causes transient reduction in callus BMD but negligible effect on osteogenic gene expression or osteoclast number

Representative 3D reconstructions of GDC and Veh samples suggested time-dependent differences in callus mineralization, with GDC samples appearing less mineralized at POD14 but more mineralized at POD21 (Figure 3A, n=13–15/treatment/timepoint). Quantitative evaluation confirmed these observations: callus vBMD was significantly less in the GDC group vs. the Veh group on POD14 ($p<0.05$), whereas this difference was reversed at POD21 ($p<0.01$) (Figure 3B). The GDC effect was not observed in the size of the callus, as there were no differences in total callus volume between groups at either timepoint (Figure 3C, Supplemental Table 3).

qPCR analysis revealed a significant upregulation of the osteogenic genes *Sp7*, *Ibsp* and *Col1a1* in fractured limbs compared to intact femora on POD7–14, which is consistent with the radiographic appearance of mineralized callus starting on POD10 and increasing thru POD21. GDC treatment had little effect on expression of these genes. The one notable effect was reduced expression of *Sp7* on POD10 in GDC vs. Veh group ($p < 0.05$), although this effect was not observed on POD7 or 21 (Figure 3D), which indicates only a transient suppression of osteoblast differentiation by GDC. Bone matrix genes *Ibsp*, *Bglap* and *Col1a1* were not significantly affected by GDC treatment (Figures 3E–G). (Complete gene expression results are presented in Supplemental Table 2).

TRAP stained sections were analyzed to assess osteoclasts in the fracture callus. Osteoclast number (Oc.N/BS) and surface (Oc.S/BS) per bone surface were increased from POD14 to 21 (ANOVA, $p<0.05$, n=3–5/treatment/timepoint), consistent with increased remodeling of the mineralized callus at the later timepoint. Neither of these measures were significantly affected by GDC treatment (Supplemental Table 4).

Chondrogenesis was not significantly affected by GDC treatment

Histological and gene expression analyses of fractured samples revealed no significant effect of GDC treatment on chondrogenesis (n=3–6/treatment/timepoint). For both GDC- and vehicle-treated groups, Alcian blue staining revealed immature chondral tissue on POD7, with more mature cartilage in the center of the callus region on POD14, and finally a near absence of cartilage on POD21 (Figure 4A). The cartilage and callus areas were quantified, and neither the absolute cartilage area nor the ratio of cartilage/callus area was significantly different between groups (Figure 4B, Supplemental Table 5). qPCR analysis revealed that chondrogenic genes *Acan*, *Sox9* and *Col2a1* were 10- to 100-fold more highly expressed in fracture callus compared to intact bone on POD 7 and 14 (Figure 4C–E; Supplemental Table 2). This upregulation persisted on POD14, although to a lesser degree (5- to 10-fold). Importantly, chondrogenic gene expression was not significantly affected by the GDC treatment, consistent with the histological findings.

GDC did not affect callus vascularity or strongly alter expression of angiogenic genes

Callus vascularity was assessed by microCT angiography on POD7 and 14 (n=6–7/treatment/timepoint). Three-dimensional reconstructions illustrate a network of vessels on the margins of the callus, with a relatively avascular zone in the callus center corresponding

to the cartilage region (Figure 5A). The pattern of vascularity was highly variable between samples in both experimental groups. Quantitation of callus vascular density (BVV/TTV) revealed a significant increase with time from POD7 to 14 (ANOVA, $p < 0.05$), but no significant effect of GDC treatment (Figure 5B).

qPCR revealed that the expression of several vascular- and angiogenic-related genes (*Hmox1*, *Ang2*, *Nos2*, *Ptn*) was increased in fractured limbs compared to control (Figures 5C–F), with *Hmox1*, *Nos2* and *Ptn* showing highest upregulation on POD7 (2- to 7-fold). Of these, *Hmox1*, *Nos2*, and *Ang2* were significantly affected by GDC treatment ($p < 0.05$, ANOVA). Post-hoc testing revealed that *Hmox1* and *Ang2* were significantly increased by GDC treatment on POD7 (Figure 5C–D). Interestingly, there were no angiogenic-related genes downregulated by GDC treatment.

Torsion tests revealed delayed callus maturation in GDC-treated mice

Callus samples were tested in torsion to assess biomechanical competence ($n=8-10$ /treatment/timepoint). Regardless of treatment, average peak torque (a measure of whole-bone strength) increased significantly from POD14 to POD21 ($p < 0.01$; Figure 6A). GDC treatment did not significantly alter peak torque. Rotation at peak torque decreased from POD14 to POD21, consistent with increased callus maturation (Figure 6B). Importantly, the GDC-treated bones had significantly greater rotation at peak torque on POD21 compared to Veh, indicating delayed functional maturation.

GDC treatment reduced cancellous and cortical bone volume at non-fracture sites

Intact tibiae of POD21 animals were scanned by microCT to assess the effects of Hh inhibition on cancellous and cortical bone structure ($n=10$ /treatment). 3D reconstructions illustrated no gross differences in bone shape or structure between GDC and Vehicle groups (Figure 7A). Nonetheless, quantitative analysis at the tibial metaphysis indicated that GDC treatment decreased cancellous BV and BV/TV by 20% ($p < 0.05$, Figure 7C), via its tendency to increase trabecular separation ($p < 0.05$, Figure 7E) with no effect on thickness (Supplemental Table 6). At the tibial diaphysis, GDC had similar effects, namely a 10% reduction in cortical area ($p < 0.05$, Figure 7D) due mainly to a reduction in periosteal size (-10% Tt.Ar, $p < 0.01$, Figure 7F). There was also a small but significant reduction in cortical TMD (-1% TMD, $p < 0.05$, Supplemental Table 6). These findings indicate that 3 weeks of GDC treatment had a negative effect on bone mass at non-fracture sites.

Discussion

The Hedgehog signaling pathway is critical to developmental osteogenesis [11, 33–35], a process that is recapitulated in part during fracture healing [7, 9]. Our goal was to assess the functional importance of Hh signaling in endochondral fracture healing. Using a mouse model of endochondral fracture healing, we confirmed that Hh signaling was increased in fracture callus compared to intact bone, and that this signaling was significantly blunted by treatment with the Smo antagonist GDC-0449 (“GDC”). Moreover, GDC treatment reduced cancellous and cortical bone volume at non-fracture sites, suggesting that the drug inhibited normal bone formation. However, GDC treatment had only a modest effect on fracture

healing, with evidence of delayed callus mineralization radiographically and by microCT, and a delay in the recovery of torsional rotation to normal. GDC treatment did not inhibit chondrogenesis or angiogenesis, and did not impair the recovery of failure torque, a measure of biomechanical strength. Thus, GDC treatment inhibited Hh signaling, which delayed but did not prevent fracture healing in young mice.

Previous studies have demonstrated that the Hh signaling pathway is activated during fracture healing, and our findings corroborate those reports [25–27]. We observed marked increases in mRNA expression of *Ihh* in fracture callus compared to intact bone, consistent with reports that *Ihh* is expressed in chondrocytes in the early stages of endochondral fracture healing [25, 26] and later in osteoblasts [26]. Importantly, we and others [27] observed increases in expression of *Gli1* and *Ptch1*, which are indicators of pathway activity. We extended these findings with the use of the Gli1-ERT2; Ai14 reporter mouse, wherein we show abundant Gli1-positive cells in the mineralized regions of the callus, indicating Hh activity in osteoblasts.

Taken together, these prior studies clearly indicate that Hh activation is a feature of fracture healing, although the importance of the pathway in this process is less clear. To address this question, Baht et al. [27] used a Cre-loxP strategy to selectively delete *Smo*, the co-receptor that transduces Hh signaling. Deletion using a ubiquitous, tamoxifen inducible Cre (CAGGCre-ERTM) did not cause a significant impairment in healing assessed at day 28, suggesting that the pathway is dispensable. Moreover, deletion in chondrocytes (using Col2Cre) did not affect fracture healing, indicating that Hh signaling in chondrocytes is not essential. On the other hand, deletion in the osteoblast lineage (using Col1Cre) significantly reduced the callus bone volume at day 28, indicating a role for osteoblastic Hh signaling in bone formation during fracture healing. Consistent with this latter result, we previously found that the Smo antagonist GDC-0449 diminished intramembranous bone formation after stress fracture [28]. Taken together, the previous studies indicate the Hh may play a role in osteogenesis during bone healing.

The results of the current study support a role for the Hh pathway in bone formation during fracture healing, although it does not appear to be absolutely required. We found that while many aspects of fracture healing are unaffected by GDC inhibition of Hh signaling, bone formation was only modestly affected. GDC treatment caused a significant reduction in *Sp7* expression on day 10, a delay in radiographic healing based on subjective scoring, and a significant reduction in callus mineral density at day 14. On the other hand, other genes related to bone formation were not affected by GDC treatment, and by day 21 the callus mineral density was increased in the GDC group. The effects of GDC on biomechanical properties were likewise mixed, with no effect on peak torque (a measure of whole-bone strength) but an effect on rotation-at-peak-torque on day 21, indicating more compliant, less mature calluses. Collectively, these results indicate that GDC treatment caused a delay in the initial phases of bone formation (reduced callus bone mass at day 14) that appears to delay callus remodeling (increased bone mass at day 21) and delay mineralization (reduced TMD of callus and non-loaded tibia at day 21), but with minimal eventual functional consequence.

During embryonic development, Hh signaling is essential for chondrogenesis [11, 33, 34] and is a key regulator of angiogenesis in the perichondrial bone collar [10]. In contrast, we found that Hh inhibition had no significant effect on chondrogenesis or callus vascularity during fracture healing. In *Gli1* reporter mice, we observed relatively low reporter expression in the cartilage regions of the fracture callus but much stronger expression in the bone regions (Figure 1C). Moreover, we found no effect of Hh inhibition on the post-fracture upregulation of chondrogenic genes (Figure 4C–E) or the histological appearance of cartilage in the central region of the callus (Figure 4A). These findings indicate that Hh activity is less important to chondrogenesis in the setting of post-natal fracture healing than it is in developmental skeletogenesis. A similar conclusion regarding angiogenesis is supported by the findings that GDC inhibition did not affect callus vascularity and did not inhibit vascular- and angiogenic-related gene expression (Figure 5).

Apart from the modest effects of Hh inhibition on the osteogenic aspects of fracture healing, we also observed a negative effect on cancellous and cortical bone mass at a non-fracture site. This result is consistent with a previous study, wherein we noted that GDC treatment significantly reduced expression of osteogenic genes and periosteal bone formation in normal rat ulnae (i.e., not subjected to mechanical injury) [28]. These findings are also consistent with the findings of Ohba et al. [36], who reported that treatment of mice with a Hh inhibitor (cyclopamine, a Smo antagonist) decreased bone formation and bone mass. These findings suggest that Hh signaling is a positive regulator of bone anabolism in the young-adult skeleton.

The modest effects of GDC treatment in our study raise the possibility that the drug may not have been effective in antagonizing the Hh pathway. The potency of GDC has been reported in several clinical and pre-clinical studies, albeit mainly in the setting of cancer [20–22]. The dose (50 mg/kg) and frequency of administration (twice daily) were selected based on preliminary studies that showed effective downregulation of two genes (*Ptch1* and *Gli1*) that are indicators of Hh pathway activity. Our final qPCR results show 75–85% inhibition based on mRNA expression of *Ptch1* and *Gli1* in fracture callus on days 7 and 10. Moreover, at these same timepoints *Gli1* reporter mice treated with GDC had markedly fewer Gli1-positive cells than Vehicle-treated mice. We did observe that by day 14, the effectiveness of inhibition was diminished but still > 50%. These findings indicate that GDC treatment was effective in downregulating the Hh pathway in the setting of fracture healing, especially in the first 10 days, when the pathway is strongly activated.

Because GDC strongly inhibited the Hh pathway in the fracture callus yet did not prevent fracture healing, our results indicate that the pathway is not required for fracture healing. This conclusion is somewhat at odds with Baht et al. [27], who reported that disruption of Hh signaling in osteoblasts led to reduced callus bone volume in transgenic mice. Their results were based on histological analysis at a single timepoint (day 28); additional timepoints and assessment methods were not reported. It is possible that their findings were influenced by use of a Cre (*Col1Cre*) that deletes Smo in the osteoblast lineage starting embryonically, while our approach used real-time pharmacological inhibition in wild type mice.

Because GDC-treated mice in our study were able to heal their fractures, it suggests that one or more compensatory pathways may have overcome any change in the normal process of fracture healing caused by Hh inhibition. For example, BMP and FGF pathways interact with Hh during osteoblast differentiation and developmental bone formation [37, 38], and one of these pathways may have compensated for diminished Hh activity. Identifying any such compensation was beyond the scope of our study.

There are several translational implications of this study. Hh antagonists are currently being assessed to treat a variety of human cancers [20, 22, 23]. We have shown here that 3 weeks of treatment using the Hh antagonist GDC had a negative effect on bone mass. This suggests that patients being treated with Hh antagonists may be at risk for developing osteopenia or osteoporosis, a possibility that deserves further study. Moreover, our finding that callus mineralization was delayed by GDC treatment suggests that fracture healing may be negatively affected in patients being treated with a Hh inhibitor. Conversely, these findings also suggest that the use of an Hh agonist to activate the pathway might increase bone mass and enhance callus mineralization during fracture healing. Using transgenic mice, Baht et al. [27] found that activation of Hh signaling can increase matrix deposition at the fracture site. Future studies are required to determine if activation of Hh using pharmacological approaches are beneficial to bone homeostasis and fracture healing.

In summary, our study in young mice demonstrated a strong increase in Hh activity in the bony regions of the callus during fracture healing. Systemic administration of a Hh antagonist, GDC-0449, significantly attenuated Hh signaling in the callus, and modestly affected bone formation and some mechanical properties. Hh inhibition did not inhibit chondrogenesis or callus vascularity, nor did it prevent fracture healing or retard the accrual of bone strength. We conclude that Hh signaling is strongly induced after fracture and may play a role in early callus mineralization, although it does not appear to be required for eventual healing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- In a mouse fracture Hh signaling was blunted by treatment with the Smo antagonist GDC-0449 (GDC)
- GDC reduced bone volume at non-fracture sites, suggesting inhibition of normal bone formation
- GDC modestly effected fracture healing, with delays in callus mineralization
- GDC delayed the recovery of torsional rotation to normal

Figure 1.

Markers of Hh pathway signaling in femora of GDC and Veh-treated mice. A,B) The upregulation of *Ptch1* and *Gli1* expression in fractured limbs from Vehicle-treated mice was significantly reduced in the GDC group on POD7 and POD10 ($p < 0.01$), but not at POD14 (*Ptch1*, $p = 0.051$; *Gli1*, $p = 0.22$). C) *Ihh* was strongly upregulated in fractured limbs and not affected by downstream inhibitor GDC ($p = 0.62$). D,E) Representative frozen histology sections from Ai14; Gli1CreERT2 mice showed prominent TdTomato expression on POD10 in Veh/Tmx-treated mice, indicative of Hh signaling; this was noticeably less in GDC/Tmx-treated mice. Similar results were seen at POD7 (not shown). D1,E1) At the midpoint of the callus there was sparse TdTomato signal. D2,E2) In cartilaginous areas the TdTomato signal was visible in some, but not all cells, and diminished with GDC treatment. D3,E3) In areas of woven bone formation the TdTomato signal was strongly expressed in Veh and absent with GDC treatment. ^a $p < 0.05$ fractured (RF) vs uninjured (LF) paired t-test, ** $p < 0.01$ GDC fold change vs Veh fold change 2-way ANOVA. Scale bar: D,E 2.5 mm; D1-3,E1-3 100 μ m.

Figure 2.

A) Representative radiographic images of GDC and Veh treated samples on POD7, 10, 14, and 21. There was no visible callus on POD7 in either group. By POD10, the Veh group had visible bony callus formation at the periphery, which was less noticeable on the GDC group. By POD14, both Veh and GDC groups had well developed bony calluses but GDC group had less central bony bridging. By POD21, radiolucency of the bony calluses was noted in the Veh group; samples in both Veh and GDC groups were fully healed. B) Chi-squared analysis of the Goldberg scale evaluation of the radiographs showed significant differences between the GDC and Veh groups at POD14 ($p < 0.05$).

Figure 3.

A) Representative 3D reconstructions of GDC and Veh samples on POD14 and 21. B) Callus density (vBMD) in Veh group did not change with time. Callus density was reduced in GDC group on POD14. The callus density was significantly higher in GDC group on POD21. C) Callus volumes were not different between the GDC and Veh groups. D) *Sp7* expression was significantly reduced in GDC group on POD10. E–G) *Ibsp*, *Bglap* and *Col1a1* expression were not significantly affected by GDC. ^a p<0.05 fractured (RF) vs uninjured (LF) paired t-test, *p<0.05, ** p<0.01 GDC fold change vs Veh fold change 2-way ANOVA.

Figure 4.

A) Representative picrosirius red/Alcian blue-stained sections showing collagen/bone (red) and glycosaminoglycans/cartilage (blue). At POD7 the cartilage tissue from both GDC and Veh groups appears diffuse and lightly stained, while at POD14 it is more defined with darker staining; by POD21 there is little cartilage. B) Quantification of histological images found that Cartilage/Callus area ratio was not different between the treatment groups. C–E) Chondrogenic gene expression was upregulated in fractured femurs compared to the contralateral uninjured femurs, but was not significantly affected by GDC treatment. ^a $p < 0.05$ fractured (RF) vs uninjured (LF) paired t-test.

Figure 5.

A) Representative 3D reconstruction of the nascent callus vasculature. B) Vascular volume (BVV/TTV) on POD7 and 14 was not significantly affected by GDC treatment. C) On POD7, *Hmox1* expression was significantly upregulated in the GDC group and, while not significant, its expression was also increased at POD10 and 14. D) *Ang2* expression on POD7 GDC group was also significantly upregulated. The upregulation was apparent in POD7 and 10 but the effect disappeared by POD14. E) *Nos2* expression showed increasing trend in GDC group, but the effect vanished by POD10 and POD14. F) *Ptn* expression was not significantly different between the GDC and Veh group. ^a p<0.05 fractured (RF) vs uninjured (LF) paired t-test; * p<0.05, ** p<0.01 GDC fold change vs Veh fold change 2-way ANOVA.

Figure 6.

A) Average peak torque on POD21 was significantly higher than on POD14 (ANOVA, $p < 0.05$), but was unaffected by treatment. Neither timepoint was as strong as intact left femurs (dashed line). B) Rotation at peak torque significantly decreased from POD14 to POD21 (ANOVA, $p < 0.05$) toward intact femur averages (dashed line). Treatment with GDC kept rotation significantly elevated at POD21 compared to Veh. * Veh vs. GDC, $p < 0.05$ unpaired t-test.

Figure 7.

A,B) Representative 3D reconstructions of cancellous and cortical regions of tibias after 21 days of treatment. GDC significantly reduced cancellous BV/TV (C), consistent with increased trabecular separation (E). GDC significantly reduced cortical bone area (D), as well as total area (F). * Veh vs. GDC, $p < 0.05$; ** Veh vs. GDC, $p < 0.01$.