Introduction

Although most fractures progress to union, 10% to 20% result in nonunions and are often associated with morbidity, prolonged hospitalization, and increased expenses (1–3). Risk factors for fracture nonunion include malnutrition, infection, metabolic disease, poor vascularity or vascular disease, fracture comminution, and most commonly, inappropriate fixation or stabilization at the fracture site (4). However, there remain questions as to whether systemic factors or impaired cellular function may play a role in the pathogenesis of fracture nonunion, particularly in cases that do not heal after appropriate surgical intervention. A reduced pool of human bone marrow stromal/stem cells (BMSCs) correlates with altered bone repair in patients with fracture nonunions (5, 6). The administration of BMSCs has shown promise in treating patients with fracture nonunions in some settings (7–12). Interestingly, recent studies using reporter-tagged BMSCs have demonstrated that transplanted BMSCs specifically localize within the fracture gap and intramedullary or internal calluses rather than within the external callus tissues mostly derived from the periosteum and surrounding soft tissue, suggesting a localized and specific role for BMSCs in fracture repair (13, 14). While the importance of BMSC-associated osteogenesis during development has been established, the functional role and importance of BMSCs in fracture healing remain to be determined.

Through the use of mouse genetic studies, we and others have demonstrated that loss of NOTCH signaling in skeletal progenitors leads to an early increase in bone mass, depletion of the BMSC pool, and subsequent age-related bone loss (15, 16). The NOTCH signaling pathway is a known regulator of various stem cell populations that signals via single-pass transmembrane ligands (JAG1/2 and DLL1/3/4) and receptors (NOTCH1/4), culminating in the activation of a transcriptional complex composed of the NOTCH intracellular domain (NICD), the mastermind-like transcriptional coactivator (MAML), and the central regulator known as recombinant bone binding protein for immunoglobulin kappa J region (RBPjκ) (17). Although NOTCH signaling is critical for maintaining BMSCs during skeletal development (15, 16), little evidence exists for a particular role for NOTCH signaling during fracture repair. Here, we set out to test the hypothesis that NOTCH signaling in skeletal progenitors serves to preserve the numbers and maintain the progenitor status of BMSC populations that are critical for normal fracture repair and unification, while NOTCH signaling within more committed skeletal lineages may be largely dispensable.
Results

Loss of NOTCH signaling in skeletal progenitors results in fracture nonunion. To examine the role of NOTCH signaling during fracture repair, we generated a loss-of-function (LOF) mouse model in which floxed alleles for the transcriptional NOTCH effector RBPjκ were conditionally deleted in skeletal progenitors. Western blot analyses confirmed that RBPjκ was efficiently deleted in the skeletal lineages of Prx1-Cre RBPjκ fl/fl mice at 2 months of age (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI80672DS1). To identify the cell lineages targeted by Prx1-Cre and contribute to callus formation during fracture repair, we analyzed tibia fractures on Prx1-Cre R26S LacZ mice at 14 days post fracture (dpf). X-gal staining on fracture calluses revealed LacZ-positive cells in the periosteum, the BM of the diaphysis, and both the external and internal cartilaginous and bony callus (Supplemental Figure 2). These data demonstrate that all skeletal-related cells in the callus are derived from Prx1-expressing progenitors, which largely reside in both the periosteum and BM.

To determine whether normal fracture repair requires a NOTCH-maintained population of BMSCs, we performed nonstabilized tibia fractures on RBPjκvpr1 mutant mice at 0, 14, 28, and 42 dpf revealed persistent fracture lines (yellow arrows) at 42 dpf, suggesting an established fracture nonunion in RBPjκvpr1 mutants. n = 12 mice per genotype per time point. (B) μCT analyses of 14-, 28-, and 42-day-old WT and RBPjκvpr1 mutant fractures revealed substantial periosteal external callus formation by 14 dpf and beyond, but apparent radiolucent space (yellow arrows) between broken cortices at 42 dpf in RBPjκvpr1 mutants. n = 7 mice per genotype per time point. (C) Reconstruction of μCT data confirmed the normal and robust periosteal response in RBPjκvpr1 mutants; however, the new bone remodeling was delayed in these animals. n = 7 mice per genotype per time point. *P < 0.05 compared with WT by 2-way ANOVA followed by Dunnett’s post hoc test. Results are expressed as mean ± SD.

A fibrous hypertrophic nonunion develops in RBPjκvpr1 mutant fractures. Histological assessments of fracture repair were performed using alcian blue/hematoxylin/orange-g (ABH/OG) staining of RBPjκvpr1 mutant and WT fracture calluses at 7, 14, 28, and 42 dpf (Figure 2A). At 7 dpf, WT and RBPjκvpr1 mutant fracture calluses were comparable, with early signs of mesenchymal cell recruitment, cartilage formation in the external calluses, and the appearance of vascular tissue noted by the presence of red blood cells (Figure 2A). By 14 dpf, further callus formation in WT fractures was observed in 3 areas, including (a) the endosteal surface close to the fracture rim and within the intramedullary BM space, (b) the fracture gap directly adjacent to the fractured cortices, and (c) the periosteal surface of both proximal and distal sides of the tibiae, where the transition from cartilaginous to bony callus was nearly complete. By 28 dpf, the cortices in WT fractures were unified by bridging calluses in both the periosteal and intramedullary areas. Due to bony union and new bone remodeling, WT fractures were completely repaired, as illustrated by the restoration of the original lamellar structure of the cortical bone by 42 dpf (Figure 2A). Alternatively at 14 dpf, RBPjκvpr1 mutants exhibited no internal callus formation, but rather displayed a persistence of undifferentiated mesenchymal tissue within both the intramedullary space and the fracture gap. Interestingly, relatively normal external callus formation could be visualized along the periosteal surfaces, although the replacement of cartilage by bone was delayed, suggesting the periosteal-derived stem/progenitor cell (PSC) popu-
lation was largely unaffected in RBPjκPrx1 mutant mice. By 28 dpf and beyond, regions of the intramedullary space and fracture gap mostly were filled with mesenchymal fibrous tissue (Figure 2A). To determine the fibrotic nature of the mesenchymal tissue that developed and persisted at the fracture site, we performed immunohistochemistry (IHC) for COL3A1 (Figure 2B). COL3A1 expression, which was minimally visualized in WT fracture sections at 14 dpf, dissipated over time, as it does during normal fracture repair. Alternatively, COL3A1 appeared to be more pronounced over the course of healing in RBPjκPrx1 mutant fracture sections, especially within the fracture gap and the intramedullary mesenchymal area, confirming that a fibrous nonunion developed in these mutant mice (Figure 2B).

We next performed quantitative assessments of tissue composition of fracture calluses to further understand fracture healing in these mice. Consistent with histological observations, histomorphometric results revealed that the cartilage and bone areas were significantly increased in RBPjκPrx1 mutant fractures throughout much of the fracture repair process, which was attributed to the robust endochondral bone formation that occurred along the periosteal surfaces as well as the delayed bone remodeling (Figure 3A). The significantly greater amount of mesenchymal tissue observed throughout the healing process in RBPjκPrx1 mutant frac-
an underlying contributor to fracture nonunion. While the total number of TRAP-positive cells observed throughout mutant fracture sections was greater than that in WT controls (Supplemental Figure 4A), the percentage of bone surface covered by osteoclasts (OC/S./B.S.) was comparable or lower (Supplemental Figure 4B). Therefore, these data indicate that the fracture nonunions in RBPjκmutant mutants are not caused by enhanced osteoclastogenesis.

Previous studies have demonstrated that loss of NOTCH signaling in skeletal progenitors significantly enhanced trabecular bone mass in adolescent mice at the expense of depleting the BMSC or skeletal progenitor pool (15, 16). We reasoned that the effect on BMSC populations, such that they lose their progenitor status with time and that, following fracture, NOTCH-defective osteoblasts or chondrocytes do not result in fracture nonunion (18). Consistent with this reduction of skeletal progenitors or early osteogenic cells following 2 weeks of DAPT treatments, we observed a decrease in collagen type I alpha 1 (Col1a1) expression and a subsequent increase in Alp and osteocalcin (Oc) expression, suggesting that remaining cells were a more committed osteogenic cell population (Figure 4E). Collectively, these data argue that NOTCH signaling inhibition directly within skeletal progenitors results in a significant effect on BMSC populations, such that they lose their progenitor status with time and that, following fracture, RBPjκmutant mutants likely form fibrous nonunions due to the depletion of BMSC populations with altered differentiation potential.

Loss of NOTCH signaling in osteoblasts or chondrocytes does not result in fracture nonunion. Thus far, we could not rule out the possibility that NOTCH-defective osteoblasts or chondrocytes contribute significantly to the nonunion phenotype observed in RBPjκmutant mutant fractures, since the Prxl-Cre lineage traces to more committed osteoblast and chondrocyte populations during fracture repair (Supplemental Figure 2). Therefore, to determine whether loss of NOTCH signaling in osteoblasts could also lead to fracture nonunion, we first traced the fate of Col1a1(2.3 kb)–expressing osteoblastic cells during fracture repair by analyzing the fracture callus of Col1a1-Cre(2.3 kb) R26RLacZ mice at 14 dpf. X-gal staining revealed LacZ-positive cells in the cambium layer of the periosteum, but not in the BM (Supplemental Figure 5, D and F). Furthermore, we observed a large number of LacZ-positive cells in the external callus, in particular, in regions of hard

Figure 3. RBPjκmutant mutant fractures have altered callus composition with remarkably inferior biomechanical properties. (A) Histomorphometric quantifications of the cartilage, bone, and mesenchymal areas (Ar) from ABH/OG-stained sections show robust cartilage and bone formation in the external callus regions and the progression of fracture nonunion in RBPjκmutant mutant fractures. n = 5 mice per genotype per time point. *P < 0.05 compared with WT by 2-way ANOVA followed by Dunnett’s post hoc test. Results are expressed as mean ± SD. (B) Biomechanical torsion testing of WT and RBPjκmutant mutant fractures at 42 dpf. All biomechanical parameters, including the maximum torque, torsional rigidity, and energy to maximum, which represent the bone strength, bone stiffness, and bone toughness, respectively, were markedly lower in RBPjκmutant mutant repairedibia than those in the WT controls. n = 7 mice per genotype. *P < 0.05 compared with WT by 2-tailed, unpaired Student’s t test. Results are expressed as mean ± SD.
calluses. In contrast, no LacZ-positive cells were detected within the developing internal callus (Supplemental Figure 5, H and J). These results indicate that Col1α1-expressing osteoblasts in the periosteum only contribute to the formation of the external callus, but not the internal callus. We next analyzed fractures from mice with NOTCH signaling selectively removed from osteoblasts using the Col1α1-Cre (2.3 kb) transgenic line (Supplemental Figure 6). We then performed nonstabilized tibia fractures on tamoxifen-inducible (TM-inducible) AcanCreERT2 RBPjkfl/fl (hereafter RBPjkAcamTM) and WT control mice at 2 months of age. RBPjkAcamTM and WT mice received TM (1 mg/10 g body weight) via intraperitoneal injections at 3, 5, 7, and 9 dpf to induce recombination of RBPjk floxed alleles following initiation of fracture repair during the chondrogenic or endochondral phase. Radiographs showed the initiation of mineralized callus at 14 dpf and complete bridging and bony union in both WT and RBPjkAcamTM mutant mice by 28 dpf (Figure 6A). IHC analyses of DMSO- and DAPT-treated BMSC cultures show significantly reduced type 1 colonies (CFU-Fs) and ALP-positive colonies (CFU-OBs), but an increased ratio of CFU-OB to CFU-F. n = 6 mice per genotype. *P < 0.05 compared with WT by 2-tailed, unpaired Student’s t test. Results are expressed as mean ± SD of 4 independent experiments. (E) Relative gene expression for Hes1, Lepr, Col1a1, Alp, and Oc in DAPT-treated BMSCs as compared with DMSO-treated control. *P < 0.05, compared with DMSO control by 2-tailed, unpaired Student’s t test. Results are expressed as mean ± SD of 3 independent experiments.

To determine whether loss of NOTCH signaling in fracture callus chondrocytes could also lead to fracture nonunion similar to that observed in RBPjkAcamTM mutants, we performed nonstabilized tibia fractures on tamoxifen-inducible (TM-inducible) AcanCreERT2 RBPjkAcamTM and WT control mice at 2 months of age. RBPjkAcamTM and WT mice received TM (1 mg/10 g body weight) via intraperitoneal injections at 3, 5, 7, and 9 dpf to induce recombination of RBPjk floxed alleles following initiation of fracture repair during the chondrogenic or endochondral phase. Radiographs showed the initiation of mineralized callus at 14 dpf and complete bridging and bony union in both WT and RBPjkAcamTM mutant mice by 28 dpf (Figure 6A). IHC analyses of DMSO- and DAPT-treated BMSC cultures show significantly reduced type 1 colonies (CFU-Fs) and ALP-positive colonies (CFU-OBs), but an increased ratio of CFU-OB to CFU-F. n = 6 mice per genotype. *P < 0.05 compared with WT by 2-tailed, unpaired Student’s t test. Results are expressed as mean ± SD of 4 independent experiments. (E) Relative gene expression for Hes1, Lepr, Col1a1, Alp, and Oc in DAPT-treated BMSCs as compared with DMSO-treated control. *P < 0.05, compared with DMSO control by 2-tailed, unpaired Student’s t test. Results are expressed as mean ± SD of 3 independent experiments.

Insufficient fracture stabilization is not required for fracture nonunion observed in RBPjkAcamTM mutants. To exclude the possibility that fracture nonunion observed in RBPjkAcamTM mutants was promoted by insufficient stabilization, we employed a rigidly stabilized femur osteotomy model. Two different gap sizes (1.2 mm and 0.66 mm)
of osteotomy were created in femurs of \( \text{RBPj}^{\kappa} \) mutants and WT controls and stabilized with a rigid internal fixator. By 14 dpf, radiographs revealed complete periosteal bridging in the control mice with a 1.2-mm osteotomy. In contrast, although the osteotomy gap narrowed and obscured on radiographs, radiolucent space could still be observed at 42 dpf in \( \text{RBPj}^{\kappa} \) mutant femurs (Figure 7A). Three-dimensional reconstructed \( \mu \)CT images also demonstrate impaired healing and nonunion in \( \text{RBPj}^{\kappa} \) mutant femurs (Figure 7B). Quantitatively, the bony callus within the 1.2-mm defect, representing the volume of the internal callus, was significantly lower in the \( \text{RBPj}^{\kappa} \) mutants (Figure 5B). The opposing cortical bone did not show signs of healing or bone formation near the osteotomy site, while the other was occupied by loose, fibrous tissue (Figure 7E). IHC for COL3A1 on 42 dpf mutant osteotomy sections confirmed the fibrotic nature of the tissue within the osteotomy gap (Figure 7E).

For the 0.66-mm osteotomies, autoradiographs indicated bony bridging in the control group at 14 dpf; however, by 21 dpf, the \( \text{RBPj}^{\kappa} \) mutants still exhibited a radiolucent area at the level of the osteotomy (Supplemental Figure 8A). \( \mu \)CT scanning showed the same pattern of defective repair as observed in the autoradiographs (Supplemental Figure 8B). Histological analysis at 21 dpf demonstrated that new mineral deposition bridged the osteotomy gap, and islands of disorganized bone formed in the marrow space of WT controls. Alternatively, by 21 dpf, only one side of the bone fragments displayed bridging in \( \text{RBPj}^{\kappa} \) mutants, with small islands of mineralized trabecular bone in the intramedullary cavity. The opposing cortical bone did not show signs of healing or callus formation (Supplemental Figure 8C), indicating that the mutant osteotomy resulted in incomplete and inappropriate bridging. Therefore, osteotomies in \( \text{RBPj}^{\kappa} \) mutants developed either incomplete unions or nonunions, suggesting that insufficient stabilization is not required for fracture nonunion to occur, although defect size may contribute to the healing outcome.

**Discussion**

NOTCH signaling is a recently established pathway critical to skeletal development and disease in both mice (15, 16, 19–25) and humans (26–28). Fracture-repair mechanisms are believed to recapitulate a series of spatiotemporal cellular and signaling events that occur during skeletal development (29, 30), suggesting a potential involvement of NOTCH signaling. Evidence that further implicates NOTCH in the general processes of fracture repair has recently emerged, including: (a) an upregulation of some NOTCH components in murine callus tissues during fracture healing (31), (b) a downregulation of NOTCH signaling specifici-
and BMSCs as possible causes of the failed or inappropriate intra-

caly within certain mouse skeletal progenitors during early frac-
ture repair (32), and (c) evidence that systemic downregulation of
NOTCH signaling just prior to fracture prolongs the inflammatory
phase and alters fracture healing in mice (33). While these studies
have implicated NOTCH within specific cell lineages remained unknown.
Our findings here provide what we believe is the first genetic evi-
dence that NOTCH signaling removal specifically within skeletal
progenitors results in clonogenic BMSC depletion and fracture
nonunion, while NOTCH removal in maturing osteoblasts and
chondrocytes leads to no impairment in fracture healing and bone
unification (34, 35). Our use of multiple fracture modalities and
multiple gene targeting approaches has proven the requisite role
for BMSCs and NOTCH signaling within BMSCs during fracture
repair, irrespective of fracture stability and vascularization. Col-
lectively, our work implicates both defective NOTCH signaling
and BMSCs as possible causes of the failed or inappropriate intra-
medullary callus formation leading to fracture nonunions.

Current dogma suggests that various skeletal progenitors are
recruited to the fracture site during bone repair, with at least 2
likely participants being BMSCs and PSCs (36, 37). Fracture cal-
lus development is known to occur at 3 specific loci: the medul-
mary canal, the area between fractured cortices, and the extramed-
ullary space, including the subperiosteal layer and surrounding
soft tissues (13, 14). However, the precise identity of cells con-
tributing to callus development and their relative contribution are not well defined. It is difficult to distinguish the precise role
of various skeletal progenitors during fracture repair, partly due to
the complex nature of the fracture-healing process, but also due
to the lack of specific progenitor markers. Thus far, studies
assessing the contribution of progenitor sources to bone healing
have largely relied on transplantation approaches (13, 14, 38–41).
In vivo lineage analyses using transplanted live bone grafts have
demonstrated that the periosteum supports endochondral ossi-
fication, while BM/endosteum supports intramembranous ossi-
fication during bone repair (40). Periosteal progenitors give rise
solely to skeletal cells specifically localized within the develop-
ing external callus (39, 40). Alternatively, reporter-tagged BM/
BMSC transplantsations have demonstrated that BMSCs localize
to the fracture gap and may contribute to intramedullary or in-
ternal callus formation (13, 14). Based on these and other findings,
we speculate that skeletal progenitors derived from the perios-
teum and BM/endosteum contribute differently to bone healing.
While PSCs establish the external callus to provide rapid stability
to the fracture via endochondral ossification, BMSCs might act
to form the internal callus via intramembranous ossification and
contribute markedly to fracture unification. Consistent with this
hypothesis, the RBPjk<sup>Alcam</sup> transgene for the removal of NOTCH signaling in skeletal progeni-
tors throughout skeletal development, it remains a question as to
precisely when NOTCH signaling is required within skeletal pro-
genitors during fracture repair. It may be that NOTCH signaling is
required throughout development to maintain a functional pool of
BMSCs or skeletal progenitors that are ultimately needed follow-
ing skeletal injury for appropriate and complete fracture repair, or
alternatively, NOTCH signaling may be required within BMSCs or
skeletal progenitors strictly at the time of fracture repair in order
to provide the appropriate cues directing bone unification. Parsing
differences will require the identification of precisely which
skeletal progenitors or BMSC populations exhibit functionally
relevant NOTCH signaling and will also require the development
or use of appropriate inducible Cre-expressing transgenic mouse
lines. As we develop these tools, future studies will also examine
whether these particular NOTCH-expressing fracture nonunions
can be repaired via the transplantation of appropriate BMSC popu-
lations at appropriate cell concentrations with appropriate cell car-
rriers or scaffolds. Further development of these tools and data will
aid in establishing the critical nature of NOTCH signaling within
specific BMSC populations that are required for normal fracture

Figure 6. Loss of NOTCH signaling in fracture callus chondrocytes does not result in fracture nonunion. (A) A real-time radiographic comparison of 2 representative nonstabilized tibia fractures from WT and RBPjk<sup>Alcam</sup> mutant mice at 0, 14, and 28 dpf, revealed normal fracture repair in RBPjk<sup>Alcam</sup> mutants. n = 5 mice per genotype per time point. (B) IHC- and ABH/OG-
stained callus sections from RBPjk<sup>Alcam</sup> mutants and controls at 10 and 28
dpf. IHC analyses for RBPjk shows an extremely efficient removal of RBPjk protein in RBPjk<sup>Alcam</sup> mutant cartilage calluses. ABH/OG-stained callus sec-
tions indicate no identifiable tissue or cellular alterations in fracture repair between WT and RBPjk<sup>Alcam</sup> mutant fractures. n = 5 mice per genotype per
time point. Original magnification, ×20 (IHC); ×5 (ABH/OG).
Fracture nonunions remain a challenging problem in orthopedic surgery. Traditionally, fracture nonunions have been classified as hypertrophic and atrophic. Hypertrophic nonunions are often associated with inadequate mechanical stabilization; therefore, immobilization alone may be sufficient for treatment. However, for atrophic nonunions and a distressing number of cases of hypertrophic nonunions that do not heal after appropriate surgical intervention, the causes have not been explicitly defined, and treatment options are limited. Many nonunion animal models are currently available; however, most of these models rely on creating critical-sized segmental defects (42, 43) or removing periosteum and BM (44, 45). These models infrequently simulate the clinical human scenario and rarely reflect the mechanisms for nonunions occurring in patients. In this study, we identified a genetic mouse model for both hypertrophic and atrophic nonunions. Specifically, we have demonstrated that nonstabilized fractures in RBPjκ\textsuperscript{cre}\textsuperscript{mut} mutants display (a) a persistent fracture line, (b) no bridging callus formation between cortices, (c) fibrosis within the fracture gap, and (d) poor biomechanical performance, all of which are consistent with the clinical assessment of human hypertrophic nonunions. Furthermore, we observed persistence of the osteotomy gap and osseous capping of the intramedullary canal in rigidly stabilized fractures of RBPjκ\textsuperscript{prx1} mice, which represent endpoint characteristics of atrophic nonunions (2, 46). Therefore, these results support the concept that BMSCs and potentially NOTCH signaling are key cellular and signaling participants in the pathogenesis of both hypertrophic and atrophic nonunion.

Figure 7. Insufficient fracture stabilization is not absolutely required for the fracture nonunion observed in RBPjκ\textsuperscript{cre}\textsuperscript{mut} mutants. (A) A real-time radiographic comparison of 1.2-mm osteotomies in WT and RBPjκ\textsuperscript{cre}\textsuperscript{mut} mutants. n = 6 mice per genotype. (B) Representative \(\mu\)CT images of 1.2-mm osteotomies in WT and RBPjκ\textsuperscript{cre}\textsuperscript{mut} mutants at 42 dpf. n = 6 mice per genotype. (C and D) Amira analyses of \(\mu\)CT data revealed significantly lower bone volume and minimum PMOI in defect zone. n = 6 mice per genotype. \(^*\)P < 0.05 compared with WT by 2-tailed, unpaired Student’s t test. Results are expressed as mean ± SD. (E) ABH/OG staining and IHC for COL3A1 staining on femur fracture sections (1.2-mm osteotomy) from WT and RBPjκ\textsuperscript{cre}\textsuperscript{mut} mutants at 42 dpf revealed the formation of mesenchymal-like fibrous tissue (red arrows) in the 1.2-mm gap. n = 6 mice per genotype. Original magnification, ×5.

Fracture nonunions are sometimes considered to be avascular, although recent data suggest that no statistically significant change in the median vessel counts of biopsies from the fracture gap of patients with healing fractures, hypertrophic nonunions, or atrophic nonunions can be identified (47). These findings are supported by preclinical models, which have demonstrated the highly vascular nature of many nonunions, including atrophic nonunions (48, 49). Our study further demonstrates that internal callus formation can fail and fracture nonunion can occur, even in the presence of a well-vascularized fracture. Collectively, these data have extended our understanding of the pathophysiology of fracture nonunions and suggest that the fracture nonunions characterized here are likely due to the biological impairment of local skeletal progenitors at the fracture site or their depletion and that NOTCH signaling in particular is a key regulator.

It is of note that children with Alagille syndrome caused by JAG1 or NOTCH2 mutations have an especially high risk of lower extremity fractures, originally thought to be due to altered bone development and metabolism brought on by improper calcium, vitamin, and mineral regulation and/or altered osteoblast differentiation or function (50). Management of these pathological lower extremity fractures in Alagille syndrome patients can sometimes be challenging, with reports of recurrent fractures in some patients and poor healing outcomes and/or postfracture deformities in others (50, 51). Therefore, it may be important to assess BMSC status and CFU-F frequency in the BM of Alagille patients with complicated fracture repair scenarios and to consider BMSC or BM aspirate treatments to repair and unification and may also provide the basis for developing cell- and/or molecular-based therapeutics aimed at challenging skeletal repair and nonunion scenarios.
enhance fracture repair. Furthermore, based on our findings, it may be relevant to establish the NOTCH signaling status within BMSCs and CFU-F frequency in all cases of prolonged fracture nonunion, especially when fractures do not heal even after appropriate surgical intervention, as it may be that these individuals have deficient or defective NOTCH signaling within their clonogenic populations of BMSCs.

Methods

**Experimental animals.** All mouse strains, including RBPjκfl/fl, Prx1-Cre, and Coll1a1-Cre(2.3 kb), have been described previously (52–54). Prx1-Cre RBPjκfl/fl, Prx1-Cre R26RLacZfl/fl, Coll1a1-Cre(2.3 kb) RBPjκfl/fl, Coll1a1-Cre(2.3 kb) R26RLacZfl/fl, and Acan-CreR222 RBPjκfl/fl mice were viable and produced in Mendelian ratios. Mice were fractured at 8 to 10 weeks of age. Acan-CreR222 RBPjκfl/fl mice received TM (1 mg/10 g body weight) via intraperitoneal injections on 3, 5, 7, and 9 dpf.

**Fracture model.** Prior to surgery, mice were anesthetized with 2.5% avertin (15 μl/g body weight) injected intraperitoneally. In the nonstabilized tibia fracture model, after the mice were anesthetized, an incision along the anterior side of the tibia was made. A transverse osteotomy was unilaterally performed at the mid-shaft of the tibia with a rotary bone saw. Fractured bones were repositioned without fixation, and the incision was closed. In the rigidly stabilized femur-fracture model, the right femur was exposed by a direct lateral approach, and a 4-hole titanium plate was installed across the anterolateral surface using 4 titanium screws (55) (RISystem). For the 0.66-mm defect, a transverse osteotomy was cut through the femoral mididiaphysis using a 0.66-mm wire Gigli saw and a cutting guide (RISystem). The 1.2-mm osteotomy was created by making 2 transverse cuts with a 0.22-mm wire Gigli saw and a cutting guide. The wound was closed, and the bone was allowed to heal for up to 6 weeks. Following surgery, mice were kept in cages after recovery from anesthesia, allowing free unrestricted weight bearing, and buprenorphine was administered subcutaneously (0.1 mg/kg) to manage pain every 6 to 12 hours, beginning at the time of sedation, for up to 3 days following surgery. Fractures were confirmed immediately after surgery, and healing of the fractures was monitored weekly after surgery under anesthesia using a Faxitron Cabinet X-Ray System (Faxitron X-Ray Corp.).

**μCT assessment of the mineralized callus and biomechanical torsion testing.** After careful dissection, repaired tibiae and femurs from days 14, 28, and 42 were imaged using a μCT system (VivaCT 40, Scanco Medical), with an integration time of 300 ms, a current of 145 mA, and an energy setting of 55 kV. The threshold was chosen using 2D evaluation of several slices in the transverse anatomic plane so that mineralized callus was identified, but surrounding soft tissue was excluded. Quantification for the volumes of the bony calluses was determined as previously described using Scanco analysis software (56).

**μCT image processing and analysis.** μCT images were also performed using Amira software (Amira 5.4.5, FEI Visualization Sciences Group). The volume of newly formed bone was measured from the 1.2-mm region (114 slices) that corresponded with the initial osteotomy after applying a bone mineral density threshold of 435 mg HA/cm³ to binarize the image. Additionally, the PMOI, which is correlated with the bone’s resistance to twisting, was calculated for each slice within the 1.2-mm defect region. The mean PMOI of the minimum 10% of slices was reported, as this is more indicative of the bone’s lack of union or propensity to fail. Specimens with no bone growth for 11 consecutive slices possessed PMOI values of zero, but other specimens that were visually nonunited may have possessed non-zero PMOI values.

After μCT imaging of the fracture callus, the specimens were moistened with PBS and frozen at −20°C until thawed for biomechanical testing as previously described (57). Briefly, specimens were potted in polymethyl methacrylate (PMMA) bone cement (DePuyOrthopaedics Inc.) in a square aluminum tube holders and allowed to rehydrate in PBS at room temperature for 1 to 2 hours. Specimens were tested in torsion using an EnduraTec TestBench system (200 N.mm torque cell; Bose Corp.) at 1/s until failure. The torque data were plotted against the rotational deformation to determine the maximum torque, torsional rigidity, and energy to maximum.

**Analysis of mouse tissue sections.** From 5 to 7 specimens (tibia or femur) in each group obtained at all time points were harvested, fixed in 10% neutral buffered formalin for 3 days, decalcified in 14% EDTA (pH 7.2) for 10 to 14 days, paraffin processed, embedded, and sectioned at a thickness of 3 μm. Sections were stained using ABH/OG staining and TRAP in order to analyze the cartilage composition and osteoclast formation in the fracture callus tissues. IHC stainings for COL3A1 (Abcam, ab7778) and RBPjκ (Cell Signaling) were performed on paraffin sections following the traditional antigen retrieval and colorimetric development methodologies. Tissues prepared for frozen sections were fixed in 4% PFA for 2 hours at 4°C, decalcified with 14% EDTA at 4°C for 10 days, infiltrated with gradient sucrose for 3 days, embedded with Tissue-Tek OCT medium, and sectioned at a thickness of 10 μm. LacZ staining and IF for PECAM (BD Biosciences, 550274) were performed on frozen sections. Cartilage area, bone area, mesenchyme area, and OC.S. S/B. were quantified on ABH/OG-stained and TRAP-stained sections using the Visiopharm Integrator System (Visiopharm).

**CFU and molecular assays.** BMSCs were isolated from fractured mice at 42 dpf or from WT mice at 2 months of age. Femurs and tibiae were removed and BM cells were flushed from the marrow cavity. Cells were plated at a density of 1 × 10⁶ cells/well in 6-well tissue culture plates for 14 days without change of mouse MSC medium (Stem Cell Technologies). DAPT/DMSO-treated cultures were grown in standard mouse MSC medium for 3 days and then supplemented with DAPT (10 μM) or DMSO vehicle control for an additional 14 days. On either day 14 or 17 after plating, cells were fixed for crystal violet and/or ALP staining. Type 1 colonies (CFU-F), as previously described (15), and ALP-positive colonies (CFU-OB) were scored. RNA was isolated using the RNeasy Mini Kit (QIAGEN) according to the manufacturer’s instructions. Real-time quantitative PCR (qPCR) was performed to analyze relative gene expression using the Bio-Rad CFX Connect Real-Time qPCR System. Gene expression was normalized to β-actin prior to being normalized to control samples. Mouse primers for Hes1, LepR, Colla1, Alp, Oc, and β-actin are listed in Supplemental Table 1.

**Western blot.** Bone proteins were extracted from femora and tibiae of Prx1-Cre RBPjκfl/fl, Colla1-Cre; RBPjκfl/fl, and control mice with RIPA buffer supplemented with protease inhibitors and phosphatase inhibitors (Thermo Scientific, 78440) after BM cells were flushed away. Proteins were fractionated in an SDS-PAGE gel, transferred to a nitrocellulose membrane, and detected with the RBPjκ antibody (Cell Signaling, 5313).
Acknowledgments

This work was supported in part by the following NIH grants: R01 grants (AR057022 and AR063071), an R21 grant (AR059733 to M.J. Hilton), a P50 Center of Research Translation grant (AR054041 to R.J. O’Keefe), and a P30 Core Center grant (AR061307 to M.J. Hilton and H.A. Awad). This work was also supported by a grant from the AO Trauma Research Clinical Priority Program on Bone Infection (to H.A. Awad). J.A. Inzana was supported by a National Science Foundation grant (NSF Award DGE-1419118). We would like to gratefully acknowledge the technical expertise and assistance of Sarah Mack, Kathy Maltby, Ashish Thomas, and Michael Thullen within the Histology, Biochemistry, and Molecular Imaging Core and the Bio-mechanics and Multimodal Tissue Imaging Core in the Center for Musculoskeletal Research at the University of Rochester Medical Center.

Address correspondence to: Matthew J. Hilton, Duke Orthopaedic Cellular, Developmental, and Genome Laboratories, Departments of Orthopaedic Surgery and Cell Biology, Duke University School of Medicine, 450 Research Drive, LSRC B321C, Durham, North Carolina 27710, USA. Phone: 919.613.9761; E-mail: matthew.hilton@dm.duke.edu.

The Journal of Clinical Investigation


