NOTCH signaling in skeletal progenitors is critical for fracture repair

Cuicui Wang, …, Hani A. Awad, Matthew J. Hilton


Fracture nonunions develop in 10%–20% of patients with fractures, resulting in prolonged disability. Current data suggest that bone union during fracture repair is achieved via proliferation and differentiation of skeletal progenitors within periosteal and soft tissues surrounding bone, while bone marrow stromal/stem cells (BMSCs) and other skeletal progenitors may also contribute. The NOTCH signaling pathway is a critical maintenance factor for BMSCs during skeletal development, although the precise role for NOTCH and the requisite nature of BMSCs following fracture is unknown. Here, we evaluated whether NOTCH and/or BMSCs are required for fracture repair by performing nonstabilized and stabilized fractures on NOTCH-deficient mice with targeted deletion of _RBPjk_ in skeletal progenitors, maturing osteoblasts, and committed chondrocytes. We determined that removal of NOTCH signaling in BMSCs and subsequent depletion of this population result in fracture nonunion, as the fracture repair process was normal in animals harboring either osteoblast- or chondrocyte-specific deletion of _RBPjk_. Together, this work provides a genetic model of a fracture nonunion and demonstrates the requirement for NOTCH and BMSCs in fracture repair, irrespective of fracture stability and vascularity.
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We determined that removal of NOTCH signaling in BMSCs and subsequent depletion of this population result in fracture nonunion, as the fracture repair process was normal in animals harboring either osteoblast- or chondrocyte-specific deletion of RBPjk. Together, this work provides a genetic model of a fracture nonunion and demonstrates the requirement for NOTCH and BMSCs in fracture repair, irrespective of fracture stability and vascularity.

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 can include malnutrition, infection, metabolic disease, poor vascularization 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 recombination binding protein kappa J region (RBPjk) (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 RBPjκfl/fl mice (herein referred to as RBPjκ−/−) 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 Prxl-Cre and contribute to callus formation during fracture repair, we analyzed tibia fractures on Prxl-Cre R26LacZ 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 Prxl-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 RBPjκ−/− mutant mice at 0, 14, 28, and 42 dpf. Consistent with radiographic data, μCT analyses on mineralized calluses of WT fractures showed a nearly complete bridging of bony calluses by 14 dpf, followed by complete bridging at 28 dpf and beyond. RBPjκ−/− fractures presented with a large radiolucent space between broken cortices at 14 dpf that remained evident up to and beyond 42 dpf (Figure 1B). Reconstruction of μCT data revealed that mineralized callus volumes in WT fractures peaked at 14 dpf and rapidly decreased from 14 dpf to 42 dpf, suggesting continuous external callus remodeling. Quantitatively, substantial new external callus bone formation was observed in RBPjκ−/− mutant fractures, reflecting relatively normal and robust periosteal and soft tissue responses in these mutants. In contrast, bone remodeling was likely delayed in RBPjκ−/− mutant fractures, since callus volumes did not show a decline until beyond 28 dpf (Figure 1C).

A fibrous hypertrophic nonunion develops in RBPjκ−/− mutant fractures. Histological assessments of fracture repair were performed using alcian blue/hematoxylin/orange-g (ABH/OG) staining of RBPjκ−/− mutant and WT fracture calluses at 7, 14, 28, and 42 dpf (Figure 2A). At 7 dpf, WT and RBPjκ−/− 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 tibia, where the transition from cartilaginous to bony callus was nearly complete. By 28 dpf, the cortices in WT fractures were unidentified 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, RBPjκ−/− 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-
Translation was largely unaffected in Prx1RBPjκ 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 fractures mostly appeared within the internal or intramedullary callus area and ultimately caused the lack of any bone unification (Figure 3A). To determine the structural impact of the altered fracture-healing processes, we analyzed fractured WT and RBPjκPrx1 mutant tibiae at 42 dpf using biomechanical torsion testing. As expected, all biomechanical parameters, including bone strength, bone stiffness, and bone toughness, were markedly lower in RBPjκPrx1 mutant tibiae (~7-fold lower, P ≤ 0.002) (Figure 3B). No failure moment could be specifically detected in RBPjκPrx1 mutant tibiae, indicating the complete absence of any rigid unifying structure (Figure 3B). In addition, the minimal torsional stiffness possessed by RBPjκPrx1 mutant fractured tibiae suggested soft tissue bridging of bone fragments, resulting in a slow tearing rather than abrupt breaking during tests (Figure 3B). The fully restored mechanical competence in the WT group, compared with nearly undetectable parameters in the mutant group, demonstrated not just delayed fracture repair, but rather an actual and complete nonunion.

The fracture nonunions observed in RBPjκPrx1 mutants are likely due to the depleted and/or defective BMSC pool, rather than altered vascularization or osteoclast numbers. To determine whether alterations in vascularization contribute to fracture nonunions in RBPjκPrx1 mutants, we performed PECAM immunofluorescence (IF) on callus sections at 14 dpf. In regions of the external callus, we observed similar patterns of vascularization between WT and mutant fractures. The cartilaginous tissue was avascular, and new blood vessels invaded areas of new bone formation, indicating normal endochondral bone healing within external calluses from both groups (Supplemental Figure 3, B and E). Within areas of internal calluses of WT fractures, we also observed that bone fragments were bridged by avascular cartilaginous tissue flanked by vascular tissue (Supplemental Figure 3, A and C). Surprisingly, abundant PECAM expression was observed across the fracture gap containing mesenchymal-like fibrous tissue without evidence of bone formation in RBPjκPrx1 mutants (Supplemental Figure 3, D and F). Thus, it was unlikely that the fracture nonunions observed in RBPjκPrx1 mutants could be attributed to a disrupted vascular network, since the tissues within the fracture gap were highly vascularized. Vascularization at all stages of fracture repair was further evidenced by the presence of red blood cells and vascular tissues within the intramedullary and fibrous tissue domains observed within the ABH/OG-stained histology sections from the RBPjκPrx1 mutant fractures (Figure 2A). Additionally, tartrate resistant acid phosphatase (TRAP) staining for osteoclasts was also assessed to determine whether dysregulated osteoclastogenesis could be
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./BS) 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 fracture nonunion observed in Prx1mutant fractures (Figure 4B), suggesting that remaining cells were a more committed osteogenic cell population (Figure 4A), the percentage of bone surface covered by osteoclasts, we isolated cells from WT and Prx1mutant fractures. Since skeletal progenitors contribute significantly to the nonunion phenotype observed in Prx1mutant 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 ColIa1-Cre(2.3 kb)–expressing osteoblastic cells during fracture repair by analyzing the fracture callus of ColIa1-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). Gene-expression analyses from these cultures demonstrated direct NOTCH inhibition in BMSCs via the downregulation of the NOTCH target gene hairy and enhancer of split 1 (Hes1) (Figure 4E). DAPT treatments subsequently resulted in a dramatic downregulation of leptin receptor (Lepr) expression (Figure 4E), which marks a critical subset of clonogenic BMSCs specifically derived from the intramembranous and endochondral BM and serves as an important skeletal progenitor source that contributes to normal fracture repair (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 AIP 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 mutations 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 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 ColIa1-Cre(2.3 kb)–expressing osteoblastic cells during fracture repair by analyzing the fracture callus of ColIa1-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 bone toughness, respectively, were markedly lower in RBPjκmutant mutant repaired tibia than those in the WT controls. n = 7 mice per genotype. * P < 0.05 compared with WT by 2-way ANOVA followed by Dunnett’s post hoc 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 Colla1(2.3 kb)-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 Colla1-Cre(2.3 kb) transgenic line (Colla1-Cre; RBPjκ/κ; Col1a1-osteoblasts) specifically from within chondrocytes and not adjacent mesenchymal/osteogenic tissue (Figure 6B). Histological analyses confirmed that RBPjκ was efficiently deleted in osteoblasts of RBPjκ/κ mutant mice (Supplemental Figure 6). We then performed nonstabilized fracture calluses at 10 dpf during the endochondral phase confirmed an extremely efficient removal of RBPjκ specifically from within chondrocytes and not adjacent mesenchymal/osteogenic tissue (Figure 6B). These data demonstrate that NOTCH signaling is dispensable in the differentiated cell lineages of osteoblasts or chondrocytes during fracture repair, while NOTCH signaling in the earliest skeletal progenitors is absolutely required for normal fracture repair processes with timely bony union (Figure 7). These data demonstrate that NOTCH signaling is dispensable in the differentiated cell lineages of osteoblasts or chondrocytes during fracture repair, while NOTCH signaling in the earliest skeletal progenitors is absolutely required for normal bone healing and unification.

Insufficient fracture stabilization is not required for fracture nonunion observed in RBPjκ/κ mutants. To exclude the possibility that fracture nonunion observed in RBPjκ/κ 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 RBPjk<sup>cre</sup> 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 RBPjk<sup>cre</sup> mutant femurs (Figure 7A). Three-dimensional reconstructed μCT images also demonstrate impaired healing and nonunion in RBPjk<sup>cre</sup> 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 RBPjk<sup>cre</sup> mutants, indicating a lack of bone union and a high propensity of failure (Figure 7, C and D). Histologically, at 42 dpf, the 1.2-mm WT osteotomies exhibited complete continuity of the cortex with mature lamellar structure and normal BM, whereas no healing was evident in RBPjk<sup>cre</sup> mutants. In place of normal healing, RBPjk<sup>cre</sup> mutant osteotomies developed a cap-like structure sealing the medullary canal. Often, one segment would have some mineralization and 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 further 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 RBPjk<sup>cre</sup> mutants still exhibited a radiolucent area at the level of the osteotomy (Supplemental Figure 8A). μCT 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 RBPjk<sup>cre</sup> 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 osteotomy resulted in incomplete and inappropriate bridging. Therefore, osteotomies in RBPjk<sup>cre</sup> 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 specifi-
and BMSCs as possible causes of the failed or inappropriate intra-
leavement (34, 35). Our use of multiple fracture modalities and
chondrocytes leads to no impairment in fracture healing and bone
nonunion, while NOTCH removal in maturing osteoblasts and
progenitors results in clonogenic BMSC depletion and fracture
departure that NOTCH signaling removal specifically within skeletal
Our findings here provide what we believe is the first genetic evi-
dence that NOTCH signaling just prior to fracture prolongs the inflammatory
phase and alters fracture healing in mice (33). While these studies
have implicated NOTCH in the fracture-repair process, the precise
role of 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.
Collectively, 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 callus
development is known to occur at 3 specific loci: the medul-
lar 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 transplantations have demonstrated that BMSCs localize
to the fracture gap and may contribute to intramedullary or in-
canal formation (13, 14). Based on these and other findings,
we speculate that skeletal progenitors derived from the perios-
teon 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−/− mutant fractures show a relatively nor-
mal periosteal response with robust external callus formation, but
fail to produce an internal callus and bone union, likely due to
the depletion of local BMSC populations.

Since our study utilized a constitutively expressed Prx1-Cre
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
these 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-deficient fracture nonunions
can be repaired via the transplantation of appropriate BMSC popu-
lations at appropriate cell concentrations with appropriate cell car-
riers 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.](image-url)
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 RBPjκmutants 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 RBPjκprx1mice, 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.

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.

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.
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 RBPjκ/κ, Prxl-Cre, and Col1a1-Cre(2.3 kb), have been described previously (52–54). Prxl-Cre RBPjκ/κ, Prxl-Cre R26RLacZ/+, Colla1-Cre(2.3 kb) RBPjκ/κ, Colla1-Cre(2.3 kb) R26RLacZ/+, and Acan-CreERT2 RBPjκ/κ mice were viable and produced in Mendelian ratios. Mice were fractured at 8 to 10 weeks of age. Acan-CreERT2 RBPjκ/κ 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 μg/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 with a 0.66-mm wire Gigli saw and a cutting guide (RISystem). For the 0.66-mm defect, a transverse osteotomy was cut through the femoral middiaphysis 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 fracture 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 was 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 nonunified may have possessed non-zero PMOI values.

After μCT imaging of the fracture calluses, 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 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 at 4°C for 10 to 14 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 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 Prxl-Cre RBPjκ/κ, Colla1-Cre; RBPjκ/κ, 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).
Statistics. All results are presented as the mean ± SD. Comparisons between 2 groups were analyzed using 2-tailed, unpaired Student's t test. Two-way ANOVA followed by Dunnett's post hoc test was used for comparison of 2 groups at multiple time points. P < 0.05 was considered statistically significant.

Study approval. All animal studies were approved by the University of Rochester Committee on Animal Resources and the Duke University Institutional Animal Use and Care Committee.

Author contributions
CW conducted experiments, acquired data, analyzed data, and wrote the manuscript. JAI, AJM, YR, JS, and ZL conducted experiments, acquired data, and analyzed data. RJO and HAA assisted in experimental design, analyzed data, and edited the manuscript. MJH designed research studies, analyzed data, and wrote and edited the manuscript.

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 AOTrauma 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.

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