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A Murine Model of Neurofibromatosis Type 1 Tibial Pseudarthrosis Featuring Proliferative Fibrous Tissue and Osteoclast-like Cells

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ABSTRACT

Neurofibromatosis type 1 (NF1) is a common genetic condition caused by mutations in the *NF1* gene. Patients often suffer from tissue-specific lesions associated with local double-inactivation of *NF1*. In this study, we generated a novel fracture model to investigate the mechanism underlying congenital pseudarthrosis of the tibia (CPT) associated with NF1. We used a Cre-expressing adenovirus (AdCre) to inactivate *Nf1* in vitro in cultured osteoprogenitors and osteoblasts, and in vivo in the fracture callus of $Nf1^{flox/flox}$ and $Nf1^{flox/-}$ mice. The effects of the presence of $Nf1^{null}$ cells were extensively examined. Cultured $Nf1^{null}$-committed osteoprogenitors from neonatal calvaria failed to differentiate and express mature osteoblastic markers, even with recombinant bone morphogenetic protein-2 (rhBMP-2) treatment. Similarly, $Nf1^{null}$-inducible osteoprogenitors obtained from $Nf1^{null}_{MyoD}$ mouse muscle were also unresponsive to rhBMP-2. In both closed and open fracture models in $Nf1^{flox/flox}$ and $Nf1^{flox/-}$ mice, local AdCre injection significantly impaired bone healing, with fracture union being <50% that of wild type controls. No significant difference was seen between $Nf1^{flox/flox}$ and $Nf1^{flox/-}$ mice. Histological analyses showed invasion of the $Nf1^{null}$ fractures by fibrous and highly proliferative tissue. Mean amounts of fibrous tissue were increased upward of 10-fold in $Nf1^{null}$ fractures and bromodeoxyuridine (BrdU) staining in closed fractures showed increased numbers of proliferating cells. In $Nf1^{null}$ fractures, tartrate-resistant acid phosphatase–positive (TRAP+) cells were frequently observed within the fibrous tissue, not lining a bone surface. In summary, we report that local *Nf1* deletion in a fracture callus is sufficient to impair bony union and recapitulate histological features of clinical CPT. Cell culture findings support the concept that *Nf1* double inactivation impairs early osteoblastic differentiation. This model provides valuable insight into the pathobiology of the disease, and will be helpful for trialing therapeutic compounds. © 2012 American Society for Bone and Mineral Research.

KEY WORDS: ADENOVIRUS; FRACTURE MODELS; ORTHOPEDICS; NEUROFIBROMATOSIS TYPE 1; LOCAL GENE KNOCKOUT; PSEUDARTHROSIS

Introduction

Neurofibromatosis type 1 (NF1) is a common genetic condition characterized by cutaneous and subcutaneous neurofibromas, café au lait macules, and musculoskeletal lesions.[1] Genetic linkage studies have identified the *NF1* gene as being responsible for this condition. *NF1* encodes for the neurofibromin protein, a key negative regulator of the small GTPase Ras.[2,3] Affected patients have been shown to be heterozygous for *NF1*; however, some disease phenotypes have been attributed to sporadic somatic mutations within defined cell types.[4,5] This not only includes neurofibromas and other NF1-associated tumors, but also more recently skeletal lesions associated with focal bone defects.[4,5] Nevertheless, the pathobiological importance of heterozygous versus homozygous *NF1* cells as well as crosstalk between these cell types remains unclear, particularly in the context of bone.[6–8]

The bone and orthopedic manifestations of NF1 can have a significant negative impact on patient quality of life. Generalized osteopenia and/or osteoporosis, scoliosis, compromised bone repair and pseudarthrosis,[9] and other bony manifestations can affect up to 50% of individuals with NF1. In particular, the orthopedic complications have a poor prognosis and can be recalcitrant to current medical interventions. Genetic mouse models have been quintessential to the improved understanding of the role of NF1 in bone. $Nf1^{+/-}$ primary osteoblasts

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showed a decreased osteogenic potential in culture.[10] Heterozygous $Nf1^{+/-}$ deficient mice exhibited an impaired response in BMP-7 in an ectopic bone formation assay[11] as well as reduced bone healing in a distal tibial fracture model.[12] More advanced models have been subsequently generated in an attempt to model the double inactivation of NF1 seen in some human bone defects. $Nf1^{-/-}_{Prx1}$ mice lacking Nf1 in the calvaria and limb mesenchyme showed runting, congenitally bowed tibias, and impaired bone healing in a drill hole defect.[13,14] Osteoblast-specific knockout mice ($Nf1^{-/-}_{Ob}$) showed an increased bone phenotype and impaired bone healing, albeit with increased callus size.[15] Whereas these models indicate double inactivation of *Nf1* can have profound and negative effects on bone, these models do not optimally model the local focal defects hypothesized to be causal of congenital tibial dysplasia. In this study we report a new genetic and surgical model that utilizes a Cre-expressing adenovirus (Ad-Cre) to locally knock out *Nf1* in healing fractures. Midshaft fractures were performed and bony union was assessed after 3 weeks. The effects of double inactivation of *Nf1* in committed and inducible osteoblasts were also examined in vitro. In cell culture we investigated the effects of early and late double inactivation of *Nf1* in cultured calvarial osteoblasts to explore the hypothesis that NF1 is important for early osteoblast differentiation. This was further explored in an alternative model for examining recombinant bone morphogenetic protein-2 (rhBMP-2)-induced osteogenesis in heterozygous or homozygous muscle-derived cells that can function as inducible osteoprogenitors.[16,17]

In addition to an osteoblastic deficiency, $Nf1^{+/-}$ osteoclasts have been implicated in the NF1 bone phenotype.[18] Interactions between $Nf1^{null}$ osteoblasts and $Nf1^{+/-}$ preosteoclasts may also be significant. To explore this concept, fracture models were performed in both $Nf1^{flox/flox}$ and $Nf1^{flox/-}$ mice. The former genotype has wild type osteoclast precursors whereas the latter has functionally $Nf1^{+/-}$ osteoclast precursors. It was speculated that the presence of $Nf1^{+/-}$ osteoclasts precursors may lead to a more severely affected repair phenotype.

Materials and Methods

Animal Models

$Nf1^{flox/flox}$ mice originally generated by Prof L. Parada were sourced from the National Cancer Institute (NCI) mouse repository (Bethesda, MD, USA).[19] $Nf1^{+/-}$ mice were a generous gift from Prof L. Parada (University of Texas Southwestern Medical Center, Dallas, TX, USA).[20] We maintained both colonies on a C57/Bl6 background. We bred $Nf1^{flox/flox}$ mice with $Nf1^{+/-}$ mice for two generations to generate experimental mice of $Nf1^{flox/flox}$ and $Nf1^{flox/-}$ genotypes. *MyoD-cre* transgenic mice were a gift from Prof David Goldhamer (University of Connecticut, Storrs, CT, USA)[21]; we crossed them in house with the $Nf1^{flox/flox}$ line to generate $Nf1^{-/-}_{MyoD}$ mice. *Z/AP* transgenic reporter mice were a gift from Prof Patrick Tam (Children's Medical Research Institute, The University of Sydney, Sydney, Australia) with the permission of Prof Andreas Nagy,[22] we backcrossed this strain onto a C57Bl/6 background in house. We used female mice for fracture experiments. Mice were housed in autoclaved polypropylene solid boxes with stainless steel lids and polycarbonate water bottles with food and mouse chow supplied *ad libitum*. Animal experiments were performed with approval from the South Western Area Health Service (SWAHS) Animal Ethics Committee.

Adenovirus production and purification

HEK293 cells were grown to 80% confluence in T175 culture flasks and transduced with Cre-expressing adenovirus (AdCre), a gift from Dr Thomas Gajewski (University of Chicago, Chicago, IL, USA),[23] or adenovirus containing green fluorescent protein (AdGFP) (AdEasy System, Microbix, Mississauga, Ontario, Canada). We harvested cells within 72 hours after transduction, then pelleted them and subjected them to cell lysis and DNase treatment. We ultracentrifuged the resulting supernatant for 1 hour in multiple tubes; viral fractions were then pooled and re-spun for 20 hours. Centrifugation was performed in a standard CsCl gradient at 35,000 rpm in a Beckman SW-41Ti rotor. We dialyzed purified virus in a Slide-A-Lyzer Dialysis Cassette, 10- kDa cutoff (Thermo Scientific, USA) for up to 3 days in a 10 mM Tris (pH 8.0) solution. Following dialysis, virus was titered using the Adeno-X titration kit (Clontech, USA) and used or frozen at -80°C in 10% glycerol.

Cell culture

We generated calvarial cultures from $Nf1^{flox/flox}$ neonatal mice harvested within 1 week of birth using published culture methods.[24] In summary, mice were decapitated and the calvaria were washed in Dulbecco's PBS while stripping any remaining fibrous tissue. Calvaria were minced and digested three times in Collagenase D at 1 mg/mL (Roche) in α modified essential medium (α-MEM) (Invitrogen) at 37°C for 15 minutes per digestion. The resulting calvarial fragments were plated in 10% fetal bovine serum in α-MEM with penicillin/streptomycin and cells were allowed 3 to 5 days to grow onto the plate before splitting for experiments. $Nf1^{flox/flox}$ calvarial osteoblasts were treated with either AdCre[23] or AdGFP via transduction with the Geneporter reagent (Stratagene, USA).[25] Cre expression induces recombination and excision of exons 31 and 32 of the *Nf1-flox* allele, creating a nonfunctional protein product.[19]

We derived primary muscle cell cultures from $Nf1^{-/-}_{MyoD}$ conditional knockout mice. Neonatal hind limb muscles were harvested from pups within a week of birth, washed in PBS, and then minced with surgical scissors. Tissue fragments were plated in 10% fetal bovine serum (FBS) in DMEM with penicillin/streptomycin, on a collagen coated dish for 3 to 5 days until cells were ready to be split for experiments.

Cultured cells underwent osteoblastic differentiation in α-MEM containing 10% FBS and penicillin/streptomycin, supplemented with 10 mM β-glycerophosphate, 50 μg/mL ascorbic acid, and 100 to 200 ng/mL of rhBMP-2 (Medronic Australasia).

Surgical methods and adenovirus delivery

We used female mice aged to 8 to 12 weeks in closed and open fracture surgery experiments. All fractures were generated at the

tibial mid-shaft, near the fibular junction, by experienced staff. This mouse midshaft fracture model has been previously described.[12] In summary, anesthesia was induced with ketamine (35 mg/kg) and xylazine (4.5 mg/kg) intraperitoneally (i.p.) and maintained using inhaled isoflurane. A small incision was made slightly below the knee and an entry point made using a 27G needle. An intramedullary rod (0.3 mm-diameter stainless steel insect pin) was surgically inserted inside the medullary canal of the tibia, and a second pin was inserted for stability. Fractures were generated manually by three-point bending using a modified set of surgical staple removers. For open fractures only, an incision was then made in mid-diaphyseal region and the periosteum was damaged, but not extensively stripped. Fractures were confirmed to be transverse, above the fibular junction, and without comminution via radiography using a digital X-ray machine (Faxitron X-ray Corp, USA).

We injected approximately 1×10^8 infectious units of purified AdCre adenovirus at the fracture site and surrounding soft tissue in an isotonic solution (10 mM Tris, pH 8.0). All experimental mice received AdCre treatment, regardless of genotype. Pain relief was managed using buprenorphine (0.05 mg/kg subcutaneously postoperatively, then every 12 hours as required). Fracture repair was monitored by weekly X-ray and in the event that internal fixation had failed (due to pin slippage, bending, or breakage) the affected mouse was euthanized and excluded from subsequent analysis. Select animals received bromodeoxyuridine (BrdU) labeling reagent 2 hours prior to euthanasia at 21 days (Invitrogen). At the experimental endpoint, animals were euthanized using carbon dioxide and specimens collected postmortem for radiography and histology.

Histological analysis

We removed fractured tibias with surrounding soft tissue, fixed them overnight in 4% paraformaldehyde, and then stored them in 70% alcohol at 4°C until ready for decalcification. Bone samples were decalcified in 0.34 M EDTA (pH 8.0) for 21 days at 4°C on shaker with changes every 2 to 3 days. Samples were embedded in paraffin and 5-μm-thick sections were stained with Picro Sirius Red/Alcian Blue to differentiate bone and cartilage. Adjacent sections were stained for tartrate-resistant acid phosphatase (TRAP) expression to highlight osteoclasts, and counterstained with 0.4% Light Green.[11] Sections were also stained for BrdU using a commercial kit (Invitrogen). Stained sections were quantified and analyzed by a blinded assessor using the Bioquant Analysis System. Statistical analyses and graphing were done using GraphPad Prism, and a two-tailed *t* test or Fisher's exact test were used to determine statistical significance.

For cell tracking experiments to validate AdCre infection and recombination, we fixed Z/AP specimens and cut cryosections. Samples were heat treated for 40 minutes at 70°C to inactivate any endogenous alkaline phosphatases and then stained for the heat-resistant human placental alkaline phosphatase (hAP) using a nitro-blue tetrazolium and 5-bromo-4-chloro-3'-indolyphosphate (NBT/BCIP) solution (Roche Diagnostics; Cat #1161451001) diluted 1:50 in a staining buffer (0.1 M NaCl, 0.1 M Tris-HCl pH 9.4, 0.1 M $MgCl_2$, 0.1% Tween-20).

PCR and quantitative PCR

We harvested DNA and RNA from cells using Trizol Reagent (Invitrogen), and cDNA using Superscript III (Invitrogen). qPCR was performed using a SYBR green master mix (Stratagene, USA) using the following primer sequences:

ALP-F 5′-GGGACTGGTACTCGGATAACGA-3′
ALP-R 5′-CTGATATGCGATGTCCTTGCA-3′
OCN-F 5′-CGGCCCTGAGTCTGACAAA-3′
OCN-R 5′-GCCGGAGTCTGTTCACTACCTT-3′
Runx2-F 5′-AGCCTCTTCAGCGCAGTGAC-3′
Runx2-R 5′-CTGGTGCTGCGATCCCAA-3′.

Radiographic analysis

We examined bone repair using microcomputed tomography (μCT) using a SkyScan 1174 compact μCT scanner (SkyScan, Kontich, Belgium). Samples were scanned in 70% ethanol at 9.4-μm pixel resolution, 0.5-mm aluminum filter, 50-kV X-ray tube voltage, and 800-μA tube electric current. The images were reconstructed using NRecon, version 1.5.1.5 (SkyScan). A global threshold was used representing bone tissue. Representative three-dimensional fractures were reconstructed with sagittal slices using CTVol Realistic Visualization software version 2.1.0.0 (SkyScan).

We defined union based on 3-week X-ray radiographs of each fracture. X-rays from 1 week and 2 weeks were initially reviewed and any specimens showing a premature loss of fixation were excluded from subsequent analysis. Fractures were defined as not united when a complete lack of bridging of the outer neocortex was observed by X-ray. This was previously used to show reduced fracture healing in $Nf1^{+/-}$ mice.[12] A lack of union at 3 weeks does not necessarily imply that fractures would proceed to non-union, only that normal healing was delayed. In this study, fractures were assessed by up to two blinded assessors, and statistical significance was determined using a Fisher's exact test.

Results

Nf1 deletion impairs the differentiation of committed osteoprogenitors but not more differentiated osteoblasts

To validate the capacity of AdCre to induce deletion of the *Nf1-flox* allele in vitro, we transduced cultured osteoblasts and extracted genomic DNA for further analysis. PCR experiments using primers specific for the excised *Nf1* allele confirmed recombination in AdCre-treated test samples but not in AdGFP-treated controls (Fig. 1*A*).

To functionally evaluate the role of Nf1 in osteoblast differentiation, we generated $Nf1^{null}$ cells by treating $Nf1^{flox/flox}$ calvarial osteoblasts with AdCre and rhBMP-2 to promote osteogenic differentiation. Early treatment with AdCre of committed osteoprogenitors prior to rhBMP-2 treatment led to delayed mineralization at days 5 and 7 compared to AdGFP-treated cells (Fig. 1*B*). In contrast, late AdCre exposure of calvarial

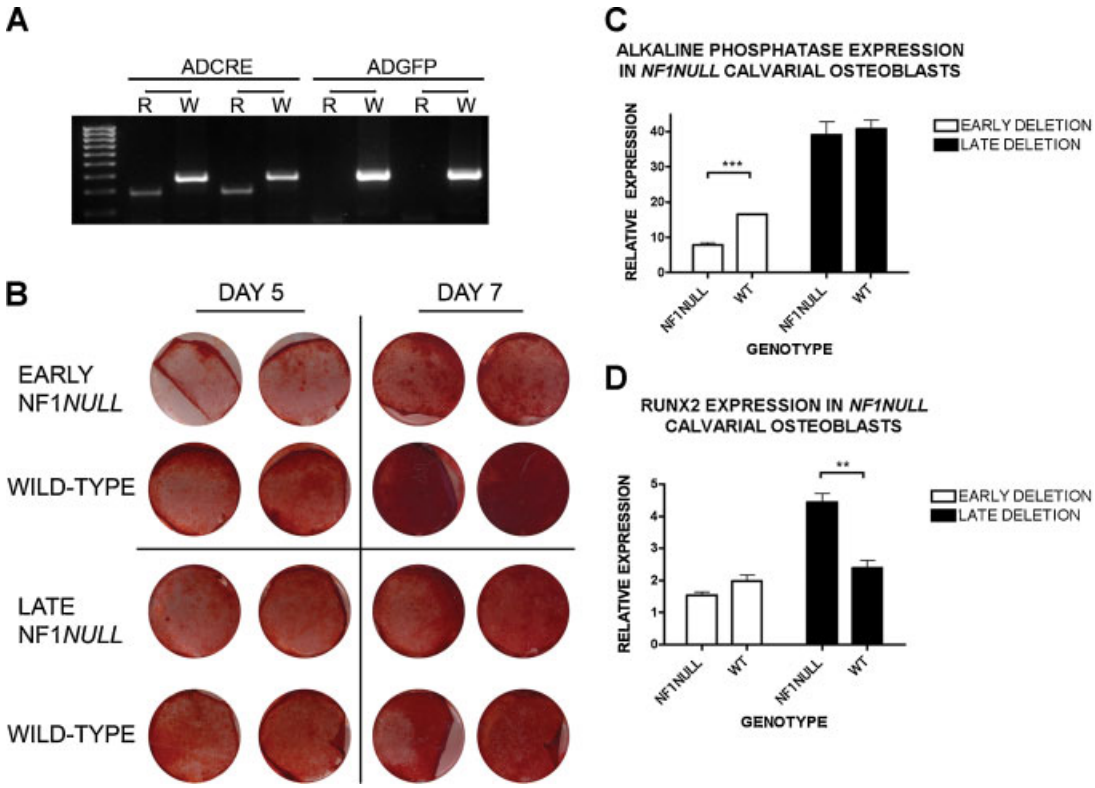

Fig. 1. Early knockout of *Nf1* leads to decreased osteoblastic mineralization potential. $Nf1^{flox/flox}$ calvarial cells were transduced with AdCre adenovirus leading to recombination of *Nf1*. (*A*) Recombination specific primers in duplicate samples confirm that *Nf1* has been recombined (R) in AdCre-treated cells compared to AdGFP-treated cells that have that maintain the wild-type allele (W). (*B*) *Nf1* knockout before rhBMP-2 treatment leads to delayed osteoblast differentiation as shown by Alizarin Red S stain. (*C*) *Alkaline phosphatase (Alp)* gene expression confirms impaired osteogenic differentiation of $Nf1^{null}$ osteoprogenitors after 5 days in culture. (*D*) *Runx2* was decreased but not significantly reduced in $Nf1^{null}$ osteoprogenitors; however, it was significantly upregulated in $Nf1^{null}$ osteoblasts pretreated with rhBMP-2 prior to AdCre transduction ($n = 4$, *t* test).

cells following 48 hours of rhBMP-2 treatment to induce osteoblastogenesis did not affect further $Nf1^{null}$ osteoblast differentiation or mineralization (Fig. 1*C*). These findings were further confirmed by assays for gene expression for *alkaline phosphatase* (*Alp*) and *Runx2* after 5 days in culture (Fig. 1*D*). These data are consistent with NF1 influencing the differentiation of osteoprogenitors but not later stages of osteoblastic differentiation.

rhBMP-2–induced osteogenesis is impaired in $Nf1^{null}$-inducible osteoprogenitors from muscle

We cultured neonatal myoblasts as an alternate type of inducible osteoprogenitor from $Nf1^{+/-}_{MyoD}$ and $Nf1^{-/-}_{MyoD}$ conditional knockout mice. Control cells were sourced from *MyoD-Cre* negative littermates. *Nf1* excision was confirmed by PCR from explant muscle tissue (Fig. 2*A*). When treated for 4 days with rhBMP-2 to induce osteogenesis in vitro, control cells underwent complete matrix mineralization as seen by Alizarin Red S staining. In contrast, functionally *Nf1* heterozygous and homozygous cells show a graded insensitivity to rhBMP-2 (Fig. 2*B*). Gene expression analysis confirmed that *alkaline phosphatase* expression was significantly decreased, and *osteocalcin* and *Runx2* showed a downward trend in expression that did not reach significance. Myoblasts that were not treated with rhBMP-2 did not express any osteoblastic markers (Fig. 2*C*).

Validation of AdCre-induced gene deletion in fracture repair

Whereas it has been previously reported that $Nf1^{+/-}$ mice show delayed healing in the distal tibia,[12] we generated a novel model of AdCre-mediated deletion of both *Nf1* alleles. To confirm local recombination in mice, AdCre was injected directly into the fracture site at the time of fracture. PCR assays were performed on genomic DNA isolated from tissue isolated from the entire fracture callus at 5 days postsurgery. Positive bands indicating recombination of an $Nf1^{flox}$ allele were seen in AdCre-treated but not AdGFP-treated specimens (Fig. 3*A*).

As an alternate method of demonstrating AdCre-induced recombination, we injected *Z/AP* reporter mice with AdCre following fracture. The *Z/AP* reporter mouse responds to Cre expression by expressing a heat resistant human placental alkaline phosphatase (hAP) as described by Lobe and colleagues.[22] In the *Z/AP* mice, *Nf1* recombination was not induced. Specific hAP staining showed recombined cells persisting within the callus 3 weeks postfracture (Fig. 3*B*, arrows). No staining was seen in other osteogenic sites (such as the growth plate), confirming heat inactivation of endogenous AP.

Local double-inactivation of *Nf1* impairs fracture repair

To identify the role of *Nf1* in fracture healing, we used the AdCre virus to generate $Nf1^{null}$ fractures in $Nf1^{flox/flox}$ and $Nf1^{flox/-}$ mice.

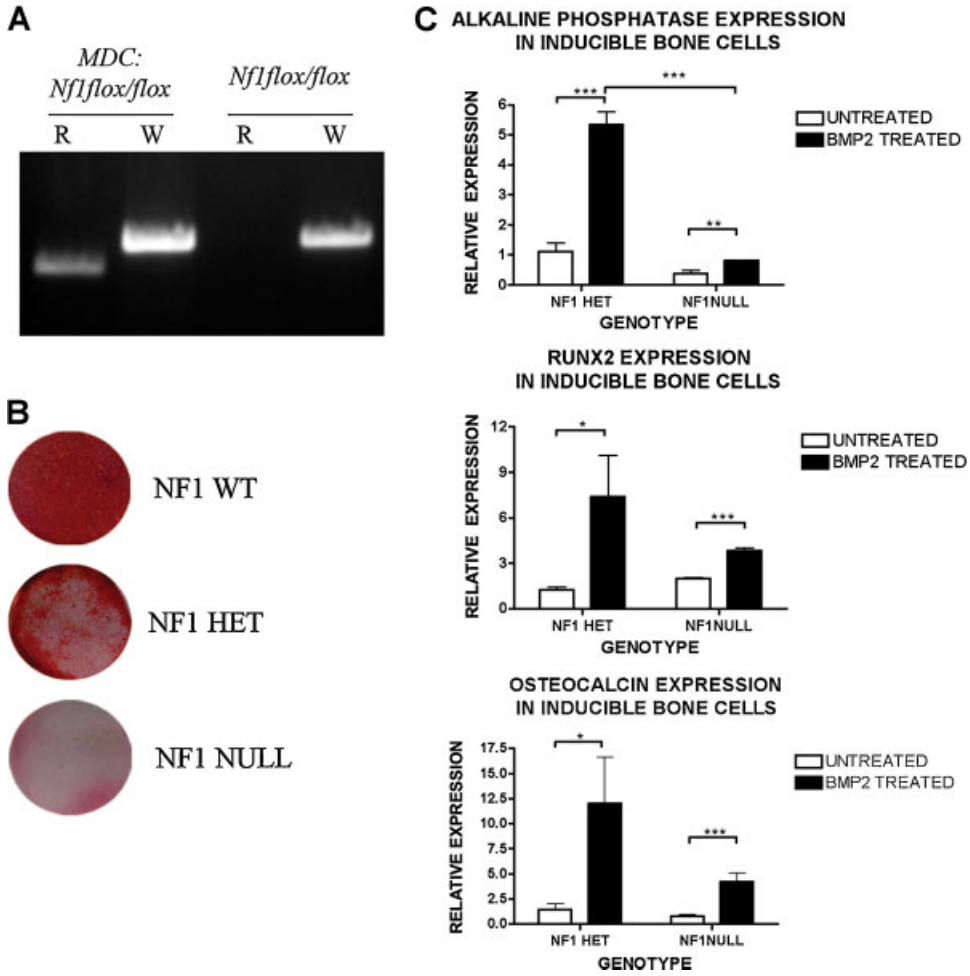

Fig. 2. $Nf1^{-/-}_{MyoD}$ inducible osteoprogenitors from muscle were treated with rhBMP-2 to induce osteogenic differentiation. (*A*) Genomic DNA from explant cultures confirmed that the *Nf1-flox* allele is recombined (R) in $Nf1^{-/-}_{MyoD}$ (Nf1 null) specimens but not control specimens that only show a wild-type allele (W). (*B*) With 200 ng/mL rhBMP-2, wild-type cells stain positive for Alizarin Red S within 4 days in culture, while $Nf1^{-/-}_{MyoD}$ (Nf1 null) cells remained undifferentiated, and $Nf1^{+/-}_{MyoD}$ (Nf1 het) cells have a graded response. (*C*) Gene expression data in rhBMP-2 treated $Nf1^{-/-}_{MyoD}$ cells compared to $Nf1^{+/-}_{MyoD}$ cells confirmed that *Alp* is significantly downregulated, and that *Runx2* and *osteocalcin* had a downward trend that did not reach significance ($n = 4$, *t* test).

AdCre was also given to C57Bl/6 mice to control for any effects of virus dosing alone. Both closed fractures (intact skin and periosteum) and open fractures (open injury with periosteal stripping) were examined.

At 3 weeks postfracture, $Nf1^{null}$ fractures displayed poor union rates based on radiographic analyses. In the closed fracture model all control (wild type) fractures healed within 3 weeks. However, $Nf1^{null}$ fractures showed a significant impairment with union rates of 38% and 50% in the $Nf1^{flox/flox}$ and $Nf1^{flox/-}$ mouse lines, respectively ($p < 0.05$) (Fig. 4*A*). In the open fracture model, 77% of control fractures healed within 3 weeks. Again, less $Nf1^{null}$ fractures were bridged at this time, with 31% and 23% union in $Nf1^{flox/flox}$ and $Nf1^{flox/-}$ mice, respectively ($p < 0.05$) (Fig. 4*B*).

We processed specimens for histological analysis to investigate the potential cellular mechanism underlying healing delay. Picosirius Red/Alcian Blue staining showed that $Nf1^{null}$ fractures were characterized by a large amount of mesenchymal and fibrotic tissue in the fracture site. In both closed and open $Nf1^{null}$ fractures this nonosseous tissue represented an average of 15% to 20% of the callus area whereas only 1% was observed in control fractures (Fig. 5*A*,*B*). These elements were primarily fibrotic, with approximately 75% of the nonosseous tissue being mesenchymal or fibrotic. The predominance of fibrous tissue over cartilage normally seen in endochondral bone repair indicates a potential pathologically process leading to non-union.

Increased cellular proliferation is seen in $Nf1^{null}$ closed fractures

To examine proliferation within the $Nf1^{null}$ fracture calluses, we labeled mice with BrdU. Following staining, quantification was performed on cells lining the bone surface or within the callus area (including any nonosseous tissue but excluding hematopoietic cells within the marrow cavity). In $Nf1^{null}$ closed fractures a significant increase was seen in the number of proliferating cells at 3 weeks, compared to wild type control fractures that had largely healed (Fig. 6*A*). Proliferating cells were present in the fibrous tissue, as shown in the representative section, but were also generalized in the callus, suggesting an overall proliferative callus in $Nf1^{null}$ closed fractures.

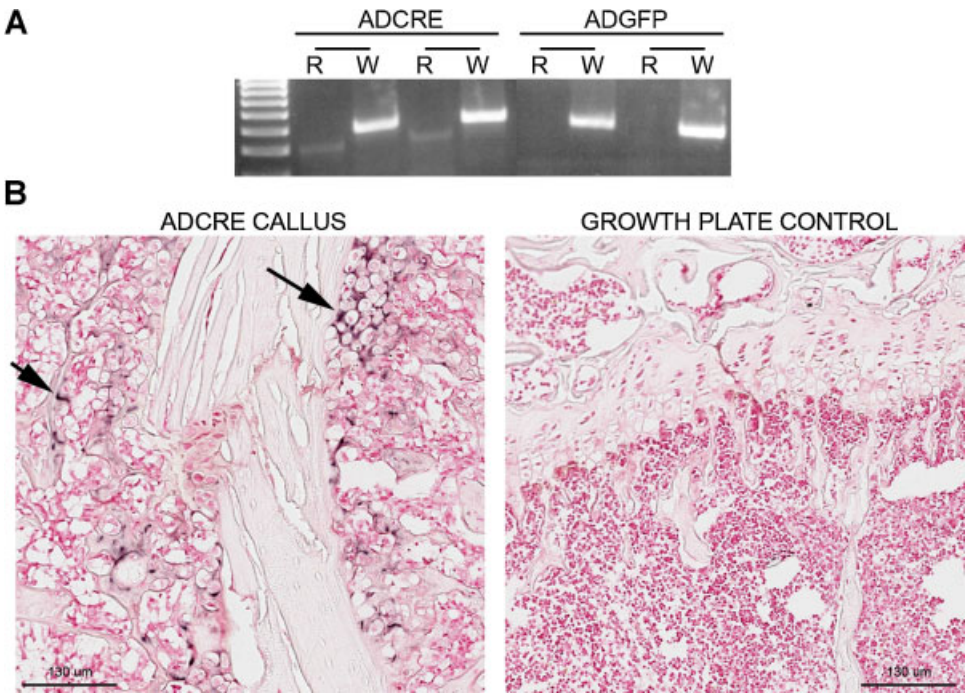

Fig. 3. AdCre adenovirus recombination efficiency was examined in vivo by local injection into *Nf1*$^{flox/flox}$ and *Z/AP* reporter mice. (*A*) Five days after surgery, genomic DNA was harvested from two fracture calluses and specific primers detect the recombined allele at the fracture site of AdCre-treated animals (R). In AdGFP-treated animals, the recombined allele was not present, only the wild-type allele is amplified (W). (*B*) Upon Cre-mediated recombination, *Z/AP* mice express a heat-resistant human alkaline phosphatase (hAP), distinct from the heat-labile endogenous alkaline phosphatase seen in bone cells. AdCre-treated *Z/AP* mice showed hAP+ cells at the fracture site 3 weeks postfracture (eg, black arrows). Heat inactivation completely ablated endogenous phosphatases; the growth plate was used as an internal control and no background staining was observed.

In contrast, open fractures were not as advanced in healing and thus showed high levels of proliferating cells in both *Nf1*^{null} and control fractures such that no significant difference was seen.

TRAP+ cells are present in *Nf1*^{null} fibrous tissue not adjacent to bone surfaces

To examine whether bone resorption was increased in *Nf1*^{null} fractures, we performed TRAP staining on fracture sections. Osteoclast number and surface relative to bone surface were not significantly increased in *Nf1*^{null} fractures compared to controls (data not shown), but a large number of TRAP-positive osteoclast-like cells were observed within the fibrous tissue, and not adjacent to a bone surface (Fig. 7). Quantification revealed that these cells were more prevalent in open fractures, where they were observed in up to 80% of *Nf1*^{null} animals (Fig. 7*B*). A comparable phenomenon was recently reported in NF1 patient samples.[26]

Discussion

The findings in this study support the concept that *Nf1*^{null} cells in the fracture site can significantly impair bone healing. In this mouse model, the vast majority of *Nf1*^{null} fractures not only failed to heal, but the histological appearance was that of a fibrous non-union. For midshaft fractures, no impairment was seen with Nf1 deficiency in the previous *Nf1*$^{+/-}$ mouse model.[12] In contrast, double inactivation of *Nf1* in a midshaft fracture was able to induce a fibrous non-union. The presence of fibrous tissue is a key complication in congenital pseudarthrosis of the tibia; cultured cells from this tissue is poorly responsive to osteogenic signals and also secrete high levels of receptor activator of NF-κB ligand (RANKL) and low levels of osteoprotegerin (OPG), inducing osteoclastogenesis.[27] Fibrous tissue invasion prevents ingrowth of functional bone tissue and provides inadequate biomechanical support. The generalized clinical approach to management requires the excision of this fibrous tissue. Fibrosis has been previously reported in an alternative model of *Nf1* double inactivation in mice. In the *Nf1*$^{-/-}_{Prx1}$ calvarial and limb specific knockout mouse, fibrosis and impaired healing were seen in a drill hole defect model.[14] The precise source of fibrous tissue seen in our fracture model has yet to be fully characterized; however, we hypothesize that it results primarily from *Nf1*^{null} osteoprogenitors that fail to commit to an osteogenic lineage and proliferate at the fracture site. Consistent with this concept, BrdU labeling showed high cell proliferation in the callus and in closed fractures at 3 weeks; it remained high in *Nf1*^{null} fractures but was significantly lower in wild type controls. No difference was seen in open fractures with all samples showing high proliferation. This suggests that cell proliferation itself may not be the sole factor responsible for instigating a fibrous non-union.

We examined impairment of osteogenic differentiation in cell culture models. Studies with the *Col2.3Cre:Nf1*$^{flox/flox}$ mouse (also known as the *Nf1*$^{-/-}_{Ob}$ mouse) reported increased bone formation in the spinal processes.[28] This is again contrary to the clinical presentation of local osteopenia in congenital pseudarthrosis of the tibia (CPT) as well as systemic osteopenia in up to 30% of NF1

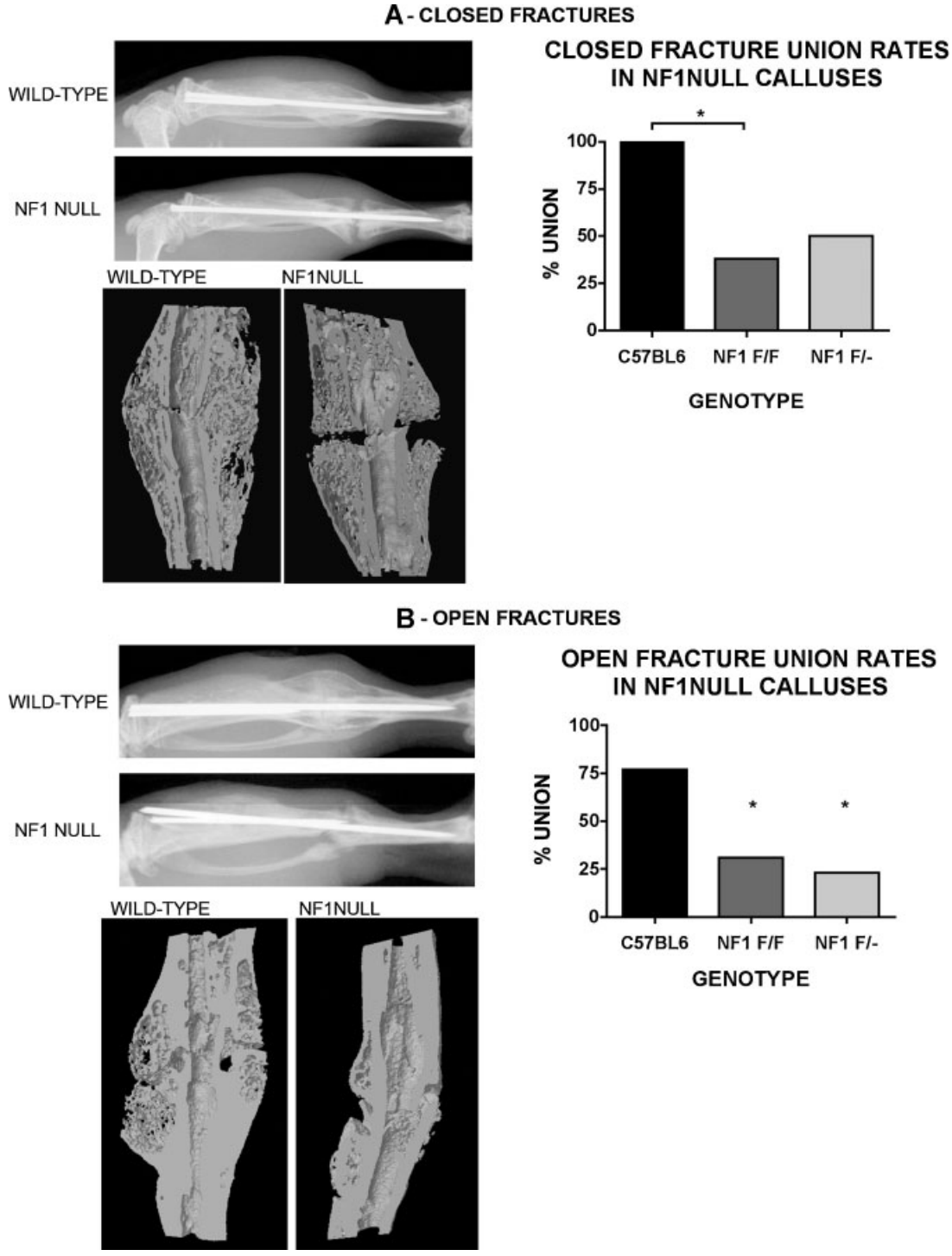

Fig. 4. *Nf1*^{null} fracture calluses showed impaired healing in closed and open fracture models. (*A*) Analysis of *Nf1*^{null} fractures using radiographic and μCT analysis revealed that these fractures are characterized by poor healing and high rates of non-unions compared to C57Bl/6 mice treated with AdCre ($n = 6$–16, Fisher's exact test). (*B*) Analysis of *Nf1*^{null} open fractures via radiographic and μCT analysis shows that these fractures show poor bone formation at the fracture gap ($n = 13$, Fisher's exact test).

patients.[29,30] However, in this mouse strain the *Nf1* gene is specifically inactivated in mature osteoblasts only, leading us to hypothesize differential roles for NF1 in osteoprogenitor differentiation versus mature osteoblast function. Consistent with this, we found that *Nf1*^{null} osteoprogenitors were impaired in their ability to differentiate into mature bone cells in vitro. However, when *Nf1*$^{flox/flox}$ cells were pretreated with rhBMP-2 to induce osteogenic differentiation and then subsequently treated with AdCre to induce recombination, *Nf1*^{null} mature osteoblasts were able to continue an osteogenic program. From a translational standpoint, these data may be interpreted to imply that targeted pathway-based therapies would only be required to convert *Nf1* progenitors into osteoblasts and would not be required for maintenance of osteoblast function.

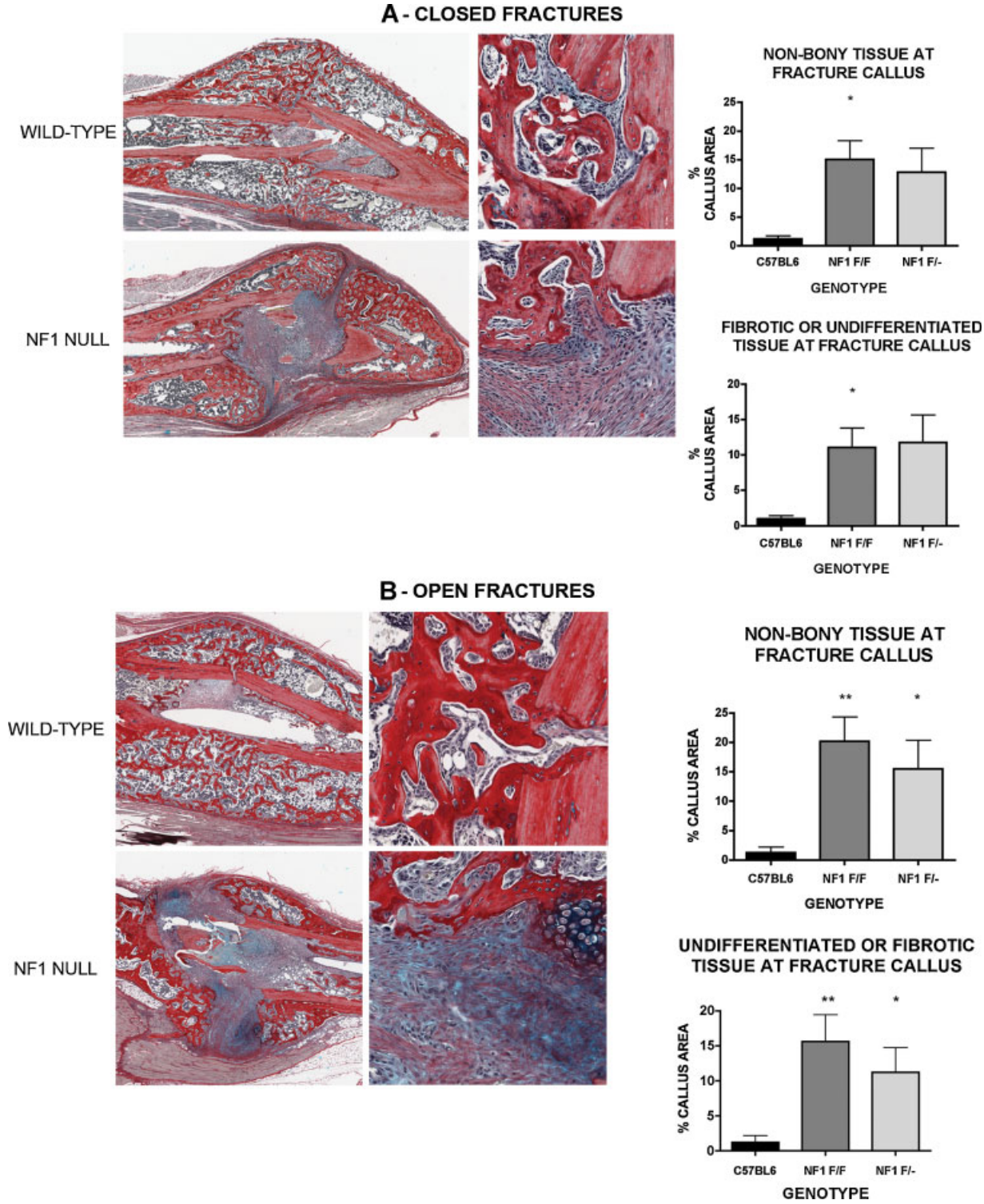

Fig. 5. *Nf1*^{null} fractures showed an abundance of undifferentiated fibrotic tissue. Picosirius Red/Alcian Blue staining of (*A*) closed and (*B*) open fractures displayed similar accumulation of non-bony elements in the fracture callus. Notably, 15% of the callus area was filled with undifferentiated fibrotic tissue in *Nf1*^{null} calluses compared to 1% in WT calluses. ($n = 4$–16, two-tailed t test).

In this study we utilized both *Nf1*$^{flox/flox}$ mice and *Nf1*$^{flox/-}$ mice and both genotypes yielded similar impairments of repair with no significant worsening in the *Nf1*$^{flox/-}$ mice. This is inconsistent with previous studies suggesting a principal role for *Nf1*$^{+/-}$ osteoclasts in deficient bone healing.[18] Rather, these data indicates that the major deficiency in CPT may involve poor *Nf1*^{null} bone anabolism rather than increased responsiveness of *Nf1*$^{+/-}$ osteoclast precursors. The fact that increased TRAP+ cells were seen in fibrous tissue in *Nf1*$^{flox/flox}$ mice and *Nf1*$^{flox/-}$ mice indicates that increased bone catabolism may be present, but it is likely secondary and due to signaling from *Nf1*^{null} cells, more so than a specific *Nf1*$^{+/-}$ sensitivity to these signals.[27] TRAP+ cells

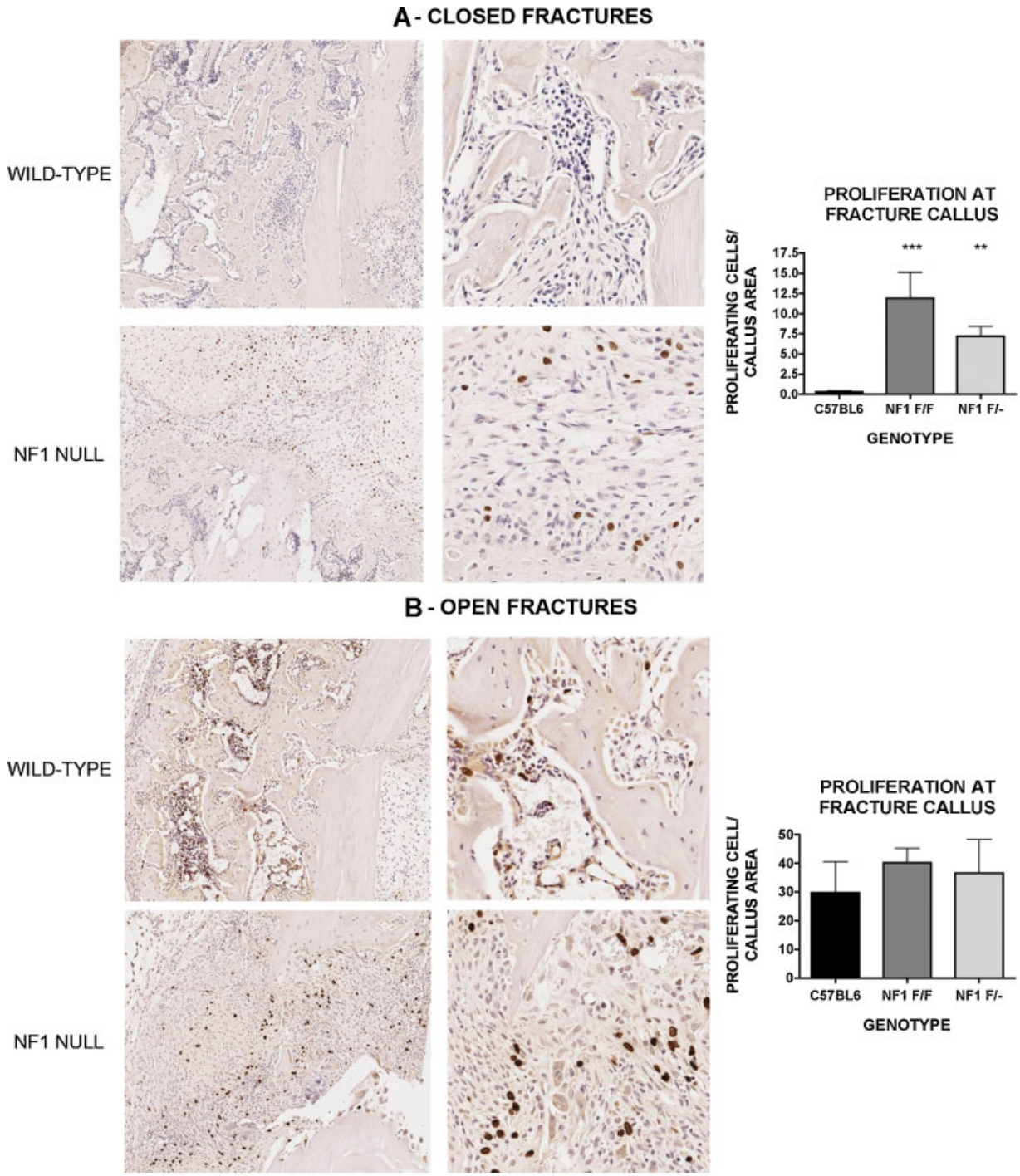

Fig. 6. *Nf1* impacts proliferation during fracture healing. Mice underwent BrdU staining to analyze the number of proliferating cells in bone, cartilage, and fibrous tissue. (*A*) Closed fracture calluses that are deficient in *Nf1* displayed a significant increase in the number of proliferating cells in the affected regions. (*B*) In open fractures, all genotypes showed a high number of proliferating cells ($n = 4–7$, two-tailed t test).

are rarely present away from bone surfaces; however, such cells have been previously reported in human fibrotic pseudarthrosis tissue, yet their origin or function is unknown.[26]

The AdCre system is functionally effective in impairing bone healing, but does not appear to lead to widespread ablation of *Nf1* at the fracture site. Based on PCR results showing some retention of the *Nf1-flox* allele (functionally wild-type) and *Z/AP* reporter mouse studies showing limited hAP staining with AdCre injection, it would appear that impairment can be brought about by a mix of $Nf1^{null}$ and normally functioning cells. Mechanistically, future studies will be required to address whether this is due to increased proliferation and physical impairment (fibrosis) by $Nf1^{null}$ cells or due to aberrant signaling from $Nf1^{null}$ cells to other cells in the fracture callus. Although this model adequately represents the attenuated response to fracture seen in congenital pseudarthrosis, further work is required to elucidate the cause of the tibial dysplasia and bowing that usually precedes fracture and pseudarthrosis.

In summary, we have generated a novel fracture model that accurately recapitulates critical characteristics of a human

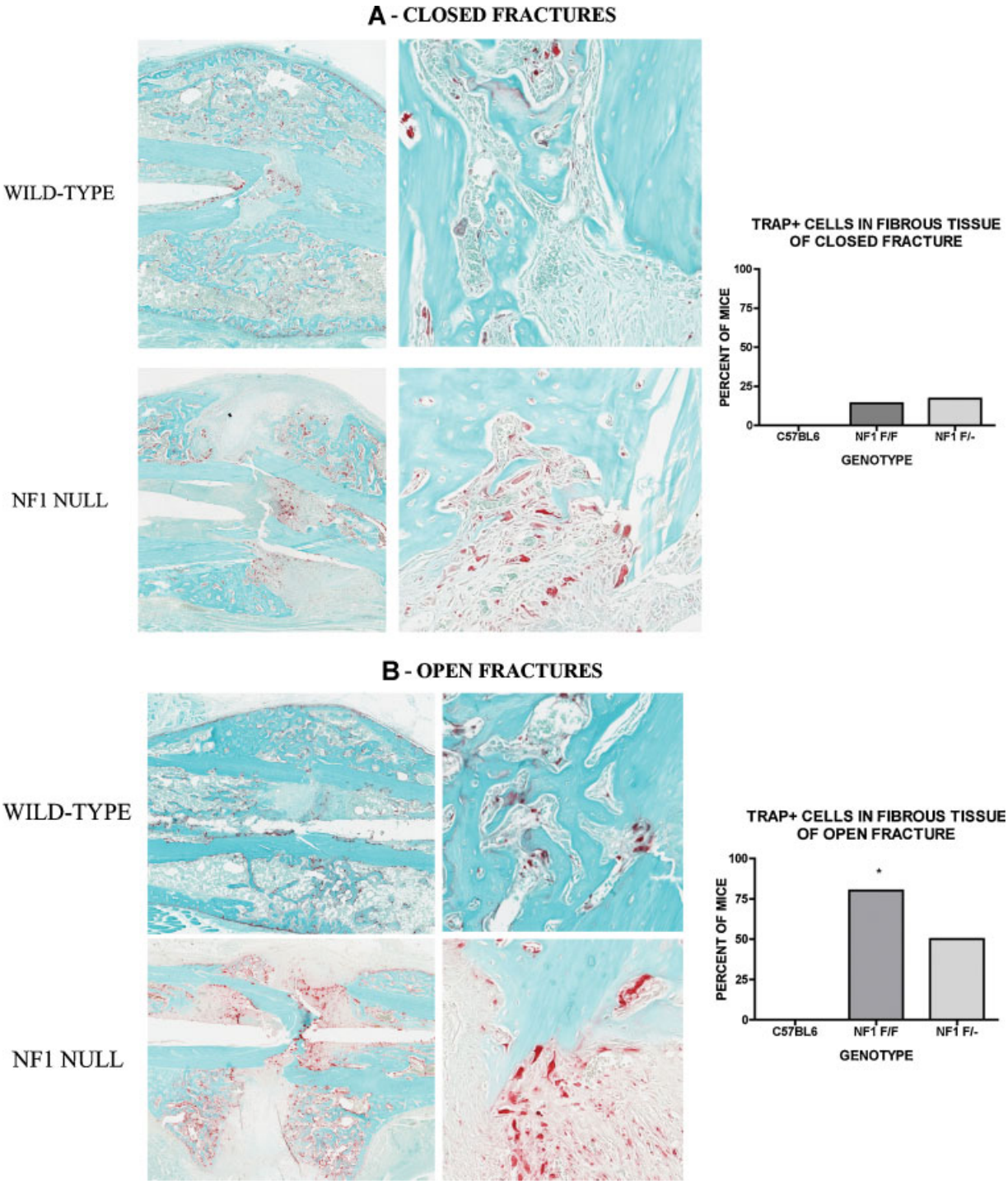

Fig. 7. TRAP-positive osteoclast-like cells were present within the fibrous tissue of *Nf1*^{null} fractures. These cells were observed in both (*A*) closed and (*B*) open fracture models, but they were more pronounced in the open fracture model ($n = 4–7$, Fisher's exact test).

pseudarthrosis. Local *Nf1* deletion in a subset of cells early in the fracture healing process is sufficient to induce a large fibrotic callus that is highly proliferative and contains large TRAP+ like cells. Complete inactivation of *Nf1* in all cells within the fracture callus is not required, with a limited number of cells being sufficient to significantly impair healing. This model provides new insights into the pathobiology of congenital pseudarthrosis, and represents a key advancement as a resource for the screening of new pathway-based adjunctive drug therapies.

Disclosures

All authors state that they have no conflicts of interest.

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