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Modulation of Notch1 signaling regulates bone fracture healing

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Abstract

Fracture healing involves interactions of different cell types, driven by various growth factors and signaling cascades. Periosteal mesenchymal progenitor cells give rise to the majority of osteoblasts and chondrocytes in a fracture callus. Notch signaling has emerged as an important regulator of skeletal cell proliferation and differentiation. We investigated the effects of Notch signaling during the fracture healing process. Increased Notch signaling in osteochondroprogenitor cells driven by overexpression of Notch1 intracellular domain (NICD1) (α SMA^{CreERT2} mice crossed with Rosa-NICD) during fracture resulted in less cartilage, more mineralized callus tissue and stronger and stiffer bones after three weeks. Periosteal cells overexpressing NICD1 showed increased proliferation and migration *in vitro*. *In vivo* data confirmed that increased Notch1 signaling caused expansion of α SMA-derived cells including α SMA-derived osteoblasts in the callus without affecting osteoclast numbers. In contrast, anti-NRR1 antibody treatment to inhibit Notch1 signaling resulted in increased callus cartilage area, reduced callus bone mass, and reduced biomechanical strength. Our study shows a positive effect of induced Notch signaling on the fracture healing process, suggesting that stimulating the Notch pathway could be beneficial for fracture repair.

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Data availability and sharing:

The data that support the findings of this study are available from the corresponding author on request.

Keywords

Notch signaling; Notch1; bone fracture; periosteal progenitors; osteoblast differentiation; inducible Cre; alpha smooth muscle actin

Introduction

Bone fractures represent a frequent clinical problem with 5–10% of all fractures having delayed healing, which leads to increased medical expenses and lower quality of life.¹ Fracture healing is a complex process involving many different cell lineages, growth factors, cytokines, and mechanical stimuli. The periosteum is the main contributor of progenitor cells that participate in fracture healing.² Mesenchymal progenitor cells (MPCs) in the periosteum surrounding the fracture site rapidly expand followed by differentiation into distinct cell lineages in the callus including chondrocytes and osteoblasts. As healing progresses, soft callus is replaced with a bony callus that remodels until the original structure is restored.³ However, the mechanisms that regulate MPC recruitment, proliferation and differentiation during fracture healing are not well defined.

Recent studies have suggested that the Notch signaling pathway plays a role during bone healing.^{4–6} Notch signaling is activated when Notch ligands (Jagged 1 and 2, and Delta like ligand1, 3 and 4) bind to one of the Notch (1–4) receptors on a nearby cell. The extracellular Notch receptor domain is important for ligand binding, the transmembrane domain is the site of cleavage by the γ -secretase complex following ligand binding, and the intracellular domain regulates transcriptional activity.⁷ Upon ligand binding, NICD is cleaved by a γ -secretase complex, then translocates to nucleus where it binds to RBPj κ and mastermind-like (Mam1) and activates transcription of downstream target genes including the *Hes* and *Hey* family of transcriptional repressors.⁷

In the osteoblast lineage, the effects of Notch signaling are dependent on the differentiation stage.⁸ During osteolineage cell differentiation, Notch1 signaling increases proliferation and maintains the progenitor pool, while inhibiting differentiation.⁹ Furthermore, Notch signaling activation promotes differentiation of osteoblasts to osteocytes and induces bone formation.^{10–13} Disregulated Notch signaling, specifically due to mutations in Notch2, is responsible for Hajdu Cheney syndrome which causes osteoporosis in humans.¹⁴

Despite the accumulated knowledge on Notch signaling effects on bone homeostasis, only a few studies have addressed the role of Notch signaling in injury models.^{4–6; 15–17} Expression of Notch receptors and ligands have been detected during all phases of fracture healing indicating a potential role of Notch signaling during the bone repair process.⁴ Genetic inhibition of Notch signaling in most cell types affects the callus size and healing progression due to global inflammatory processes.¹⁵ However, inhibition of Notch signaling by PrxCre mediated deletion of RBPJk resulted in non-union due to depletion of progenitor populations.⁶ Treatment of a bone defect with the Notch ligand Jagged1 promotes osteoblast differentiation and healing without excess bone formation.¹⁷ Our previous work showed that periosteal cells derived from alpha smooth muscle actin (α SMA) expressing progenitors have decreased expression of Notch receptors, ligands and downstream genes (*Hes1* and

Hey1) in the early stages of fracture healing indicating regulation of Notch signaling during healing in mice.⁵

The majority of the previous studies utilized global or constitutive deletion or activation of Notch signaling, but understanding Notch signaling during the fracture healing requires tissue targeting and evaluation of developmentally unperturbed bones. In contrast to previous studies we used an inducible mouse model to overexpress the Notch1 intracellular domain (NICD1) in MPCs, and evaluated the effects on fracture healing. We have also examined the effect of pharmacological inhibition of Notch1 during fracture.

Materials and Methods

Animal models

All animal procedures were approved by an UConn Health Institutional Animal Care and Use Committee prior to performing the study. Mice were housed in ventilated cages maintained at $22 \pm 2^\circ\text{C}$, $55 \pm 5\%$ humidity, and 12-hour light/dark cycle. To generate mice overexpressing NICD1 we used $\alpha\text{SMACreERT2}$ mice¹⁸ and bred them with previously described $\text{Gt(ROSA)}^{26\text{Sortm1(Notch1)Dam/J}}$ (termed $\text{Rosa}^{\text{NICD1}}$) mice obtained from Jackson labs # 008159¹⁹ to generate $\alpha\text{SMACreNICD1}$. Cre^- littermates were used as controls. For the lineage tracing experiments we crossed $\alpha\text{SMACreNICD1}$ mice with the Ai9 reporter mice (Jackson labs, stock # 007909) to generate $\alpha\text{SMA9/NICD1}$. We determined genotype for Cre, NICD and DNA recombination by PCR (Supplemental table 1)⁸. To study the effect of Notch1 signaling inhibition αSMA9 mice were used.^{5; 20} We utilized males and female mice on a C57Bl/6J background.

Femoral fractures

Fractures were performed in 8–9-week-old animals. Mice were anesthetized with isoflurane and a closed transverse diaphyseal fracture on right femur was created using a drop-weight blunt guillotine device, after inserting a 24G needle into the intramedullary canal to stabilize the fracture.²¹ To trigger overexpression in $\alpha\text{SMACreNICD1}$ mice, tamoxifen (Sigma Aldrich, St. Louis, MO, USA) dissolved in corn oil (Acros Organics, Belgium) was injected intraperitoneally (*ip*) at a dose of 75 $\mu\text{g/g}$ of body weight on 0, 2 and 4 days post fracture (DPF) to experimental and control groups. Buprenorphine (0.1 mg/kg) was given subcutaneously during the first 2 days of healing. Fractures were confirmed immediately after the procedure and the healing process was monitored by X-ray (Faxitron LX 60) on 7, 14, 21- and 42 DPF.

Gene expression analysis

Total RNA from cultured periosteal cells was extracted using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) and RNA isolated by the manufacturers protocol. RNA concentration and purity were assessed on a NanoDrop one (Thermo Fisher Scientific, Waltham, MA, USA). Following DNase treatment, 1 μg of RNA was used for cDNA synthesis by ImProm II (Promega, USA). qRT-PCR was performed with TaqMan probes and Universal Mastermix (Thermo Fisher Scientific, UK) to assess *Hey1* (Mm00468845_m1), *BSP* (Mm00492555_m1), *OCN* (Mm03413826_mH) and *GAPDH* (Mm99999915_g1)

expression or SYBR Select Master Mix (Thermo Fisher Scientific, Austin, Tx, USA) and primers for *Hes1* from Canalis et al.⁸ and *GAPDH* from Lin et al (Supplemental table 1).²² Data are presented as relative gene expression to *GAPDH* housekeeping gene, calculated using 2^{-C_t} and presented as fold change compared to control group.

Histology

Bones were fixed in 4% paraformaldehyde for 3 days at 4°C, intramedullary pins removed, then incubated in 30% sucrose/PBS overnight and embedded in Cryomatrix (Thermo Scientific, Waltham, MA, USA). Tissue was sectioned using a tape transfer system (Section-lab, Japan). Callus area, mineralization and cartilage area within the callus was determined by von Kossa and Safranin O staining. In brief, for mineral tissue staining, 7 µm sections were stained with 4% silver nitrate, exposed to UV light, washed and coverslipped. To determine cartilage area within the periosteal callus, samples were stained with Weigert's hematoxylin for 5 min, washed, stained with 0.2% Fast green, washed with 1% acetic acid, then stained with 0.1% Safranin O for 30 sec, washed and coverslipped. To determine osteoclast numbers, TRAP staining was performed on 14-day fractures with Leukocyte acid phosphatase kit (Sigma Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol. Sections were evaluated using Osteomeasure software (Osteometrics Inc., Atlanta, GA, USA). For osteocalcin staining, sections were incubated in Epitope Retrieval Solution (IHC World) at 60°C for 6 hr, washed in 0.1% Tween 20/PBS, and blocked with 10% goat serum in 0.1% Tween 20/PBS for 1h at room temperature. Osteocalcin antibody (1:100, Cat. No. PA5-78870, Thermo Fisher Scientific, Waltham, MA, USA) incubated overnight at 4°C, followed by washing in 50 mM Tris-Cl, 150 mM NaCl and incubating with secondary antibody conjugated with Alexa 647 (1:300, Cat. No. A21244, Thermo Fisher, Waltham, MA, USA). A minimum of two sections from the middle of the fractured bone were stained, scanned using Axioscan microscope (Zeiss, Germany) and analyzed using automated cell counting within the callus area using ImageJ software (NIH, USA). Before analyzing scanned images, standardized thresholds were set for each colour, and watershed step applied for segmenting clustered nuclei.²³ This allowed calculation of the proportion of αSMA-derived cells (red nuclei/total nuclei), the proportion of OCN+ osteoblasts in the callus (green nuclei/total nuclei), and the proportion of OCN+ osteoblasts derived from αSMA progenitors (dual red-green/green nuclei).

Micro computed tomography (µCT) and torsion testing

Two, three and six weeks after fracture, femurs were dissected and scanned using µCT (µCT40, Scanco Medical AG, Bassersdorf, Switzerland) with a voxel size of 12 µm, 55 kV and intensity of 145 µA. Since the callus tissue is not trabecular, nor cortical, algorithms calculating bone parameters might not reflect reality (analysis would not accurately segment larger and/or more mineralized regions), so density, and callus volume was determined and bone mass within the callus calculated. One hundred slices above and below the fracture site were evaluated. Following µCT imaging, the samples were soaked in PBS and stored at -20°C until torsion testing. Samples were potted in methyl methacrylate bone cement (Orthodontic Resin, Dentsply Caulk Inc. Milford, DE, USA) in custom aluminum jigs. Biomechanical properties were measured using the TestBench™ Torsion Testing system (Bose Corporation ElectroForce Systems Group, Eden Prairie, MN, USA), with data

acquisition rate of 10 Hz, and 1 degree/s torsion. Data were plotted as a torque to rotation and bone strength, stiffness, twist to failure and toughness were determined.

Flow cytometry

Callus tissue was pooled from 2 fractured femurs 5 DPF and digested.²⁴ In brief, muscle tissue was dissected from the bone, bone marrow flushed and the thickened periosteum around the fracture site (“periosteal callus”) was scraped and digested in PBS containing 0.05% Collagenase P (Roche, IN, USA) and 0.2% hyaluronidase (Sigma Aldrich, St Louis, MO, USA) for 1 hour at 37°C. Cells were washed and stained with Zombie aqua (1:500, BioLegend, San Diego, CA, USA) to exclude dead cells. To exclude hematopoietic and endothelial cells staining for CD45 violet Fluor 450 (Clone: 30-F11, Tonbo biosciences), Ter119 eFluor 450 (Clone: TER-119, Invitrogen, Waltham, MA, USA) and CD31 eFluor 450 (Clone: 390, eBioscience) was performed. LSR II flow cytometry system was used (BD Bioscience, San Jose, CA, USA). Voltages and gates were set based on unstained samples and single stained controls. Diva8 software (BD Bioscience, San Jose, CA, USA) was used for analyzing the data.

Periosteal progenitor cell culture

Periosteal cells were isolated from both the tibia and femur. Muscle tissue was dissected away and thickened periosteum around the fracture site or periosteum from intact bone was scraped, and digested as described above. 2.5×10^5 cells were seeded in α MEM/10 % FBS in 6-well plates. Following 48 hr at 37°C with 5% O₂, half media was changed and cultures were transferred to ambient oxygen. Cells were treated with 1 μ M 4-hydroxytamoxifen (Sigma Aldrich, MO, USA), on day 2 and 4. At 70% confluent, osteogenic medium (50 μ g/ml ascorbic acid, 4 mM β -glycerophosphate) was added. After 7, 10 and 14 days of differentiation, cells were harvested for RNA isolation or stained for alkaline phosphatase, von Kossa and crystal violet. For assessing proliferation, after 24 h of serum starvation the cells were labeled with EdU for 4 h, and stained according to the manufacturer’s protocol (Click-iT® EdU Alexa Fluor® 647 Imaging Kit, Thermo Fisher) and EdU incorporation determined by flow cytometry in CD45 negative cells.

To measure migration, confluent cells were cultured for 24h in serum free medium. Next, scratch-wound was made on the monolayer and closure determined after 4, 8, 12, 24 and 30 hours.

Treatment with NRR1 antibody

Fractures were performed in 8–9 week old male C57Bl/6J animals, and on 0, 2 and 4 DPF, 2.5 mg/kg of anti-NRR1 antibody (aNRR1, Genentech, Inc. San Francisco, CA, USA) or isotype control (anti-gD, Genentech, Inc. San Francisco, CA, USA) was injected *ip*. To determine efficiency of Notch1 signaling inhibition, we evaluated thymocyte differentiation ability²⁵ by flow cytometry assessing frequency of CD4⁺CD8⁺ double positive cells using CD4-PE (Clone: GK1.5, eBioscience) and CD8-biotin (Clone: 53–6.7, Invitrogen, Waltham, MA, USA) and stained with secondary antibody conjugated with APC eFluor780 (eBioscience).

Statistical analysis

All data are expressed as mean $\pm$ standard deviation (SD) and assessed by two-tailed Student t-tests. $P < 0.05$ was set as statistically significant difference. Real time PCR data is presented as mean $\pm$ standard error of the mean (SEM). Statistical analyses were performed using Graph Prism 6 software (GraphPad software, CA, USA).

Results

Effect of Notch1 Signaling overexpression on fracture healing

We utilized an inducible model overexpressing NICD1 in α SMA osteoprogenitor cells during fracture healing using α SMACreERT2 mice¹⁸ and bred them with previously described Rosa^{NICD1} mice. We have evaluated effects 1, 2, 3- and 6-weeks following fracture using x-ray, histological analysis, μ CT and mechanical testing (Figure 1A). We confirmed that DNA recombination had occurred in fracture callus tissue at 7 days post fracture (DPF) (Figure 1A). Significantly increased expression of *NICD1*, as well as downstream Notch target gene *Hes1* in α SMACreNICD1 male animals compared to littermate controls was detected in primary cells derived from periosteal callus tissue 5 DPF. These cells were expanded 3 days to enrich the population of periosteal progenitor cells²⁶ (Figure 1B).

To visualize the cell population with targeted overexpression of NICD1, α SMACreNICD1 mice were crossed with Ai9 reporter mice and α SMA9/NICD1 expression traced at 5, 10 and 14 DPF (Figure 2B). Consistent with our previous studies^{5; 18}, α SMACre targeted various mesenchymal populations in the fracture callus including osteoblasts and chondrocytes. Compared to controls, mice overexpressing NICD1 showed an increased proportion of α SMA9⁺ cells in the callus indicated by histology and flow cytometry at 5 DPF (Figure 2C).

The *in vivo* effect of NICD1 overexpression on fracture healing was evaluated by histological analysis. Total callus area was unchanged between the groups at all time-points examined and no difference was observed in cartilage area and bone area on day 7. However, male mice with targeted NICD1 overexpression showed a significantly lower percentage of cartilaginous area 10 DPF, and at 14 DPF had increased mineralized area (Figure 3A). μ CT evaluation of fractured femurs 14 DPF did not reveal a significant difference in callus volume, callus bone mass, or bone density between mice overexpressing NICD1 and controls (data not shown). The 21 DPF α SMACre⁺NICD1 mice had a significant increase in callus bone mass ($p < 0.05$). Furthermore, torsion testing showed increased strength and stiffness ($p < 0.001$) of the fractured femurs 21 DPF in mice overexpressing NICD1 (Figure 3B, C). Six weeks after fracture, we did not detect a difference in the bone volume or bone mass of the remodeling callus (Figure S-2A, B). Furthermore, bone strength and stiffness were not statistically different between α SMACre⁺NICD1 and control groups (Figure S-2B). This data confirms that NICD1 overexpression in osteoprogenitor cells does not lead to bone tissue overgrowth or abnormal cortical structure and that normal function was established as confirmed by mechanical testing. In contrast to males, *NICD1* and *Hes1* expression in females did not differ from controls, and μ CT analysis 21 DPF did not show

difference in healing between α SMA^{Cre}+NICD1 and their Cre⁻ littermate controls (Figure S-1). Altogether, histological analysis, μ CT data, and torsion testing suggest accelerated fracture healing in male mice overexpressing NICD1 in α SMA osteoprogenitor cells. We also determined increase strength and stiffness 21 DPF in contralateral femurs in animals overexpressing NICD1 compared to their littermate controls (Figure S-3A).

We used the lineage tracing model to track the frequency of commitment of SMA9/NICD1⁺ cells into mature osteoblasts by assessing osteocalcin expression. Callus tissue overexpressing NICD1 in α SMA cells had increased frequency of OCN⁺ cells, and also increased frequency of OCN⁺ cells derived from α SMA osteoprogenitors (Figure 4). A small proportion of the cells at 10DPF were OCN⁺. In NICD overexpressing animals $56.5 \pm 13.7\%$ osteocalcin positive osteoblasts arise from the α SMA⁺ osteoprogenitor cells compared to $36.5 \pm 11.7\%$ in control animals (Figure 4C). Although osteocalcin was detected in hypertrophic chondrocytes in some studies²⁷, we stained same slides for Safranin O and did not detect osteocalcin positive cells within the cartilage (Figure S-4), consistent with other published findings.²⁸

Previous studies show that activating the Notch signaling pathway in osteocytes increases osteoprotegerin expression and suppresses bone resorption.¹² Calluses from α SMA^{Cre}+NICD1 mice did not show differences in osteoclast perimeter (Oc.Pm), osteoclast number per bone area, or osteoclast number per bone perimeter compared to control animals 14 DPF (Figure S-5).

In order to further elucidate the effects of NICD1 overexpression in periosteal osteoprogenitors, we performed *in vitro* assays (Figure 5A). Higher expression of Notch downstream target gene *Hey1* confirmed that Notch signaling was increased (Figure 5B). Osteogenic differentiation of cells overexpressing NICD1 was reduced as indicated by reduced alkaline phosphatase staining, and expression of bone sialoprotein and osteocalcin (Figure 5A, B). Periosteal cells with NICD1 overexpression in α SMA cells had significantly increased proliferation (Figure 5C), and significantly increased ability for wound closure indicative of enhanced cell migration 24 and 30 hours after the wound was made ($p < 0.01$, and $p < 0.001$) (Figure 5D). Collectively our data indicates that both *in vitro* and *in vivo*, NICD1 overexpression is responsible for expansion of progenitor cells.

Effect of Notch1 Signaling Inhibition on fracture healing

Using an aNRR1 antibody, we systemically inhibited the Notch1 signaling pathway in the early stages of fracture healing (aNRR1 antibody injected on 0, 2, 4 DPF). Since Notch1 signaling is needed for T cell differentiation, we evaluated thymocyte differentiation 5 DPF. The proportion of CD4⁺CD8⁺ thymocytes was 65% lower in the aNRR1 treated group ($p < 0.01$), a confirmation that the dosing regimen caused systemic inhibition of the Notch1 signaling pathway (Figure S-6). In contrast to overexpression of NICD1, we observed that inhibition of Notch1 signaling during the early phases of fracture healing leads to decreased callus area and increased cartilaginous area fraction compared to anti-gD control 10 DPF (Figure 6A). Mineralized area was unchanged. Furthermore, μ CT data three weeks after the fracture showed significantly decreased callus volume and bone mass (24.7% smaller callus volume and 24.2% less bone mass, $p < 0.05$, and $p < 0.005$, respectively) in aNRR1 treated

animals compared to controls (Figure 6B, C). Bone strength was also significantly decreased 21 DPF in fractures with aNRR1 treatment (Figure 6B), but no change in bone strength and stiffness was observed in unfractured contralateral femora (Figure S-3B).

To investigate the effect of Notch1 signaling inhibition on cell populations in the fracture callus, we used α SMA9 mice and evaluated proportion of α SMA cells 5 DPF (Figure 7A). Animals treated with aNRR1 had fewer α SMA9 labeled cells (Figure 7B).

Discussion

We have previously demonstrated that α SMA⁺ cells are osteoprogenitors that actively participate in bone healing.^{5, 18} Periosteal α SMA⁺ osteoprogenitor cells show down-regulation of Notch receptors, ligands and downstream targeting genes (*Hes1* and *Hey1*) after fracture (2 and 6 DPF), compared to cells from uninjured tissue.⁵ These changes were specific to the α SMA9-labeled population, and were not seen in callus tissue overall. However, Dishowitz et al. showed that expression of Notch signaling genes is upregulated during healing of both tibia fractures and calvarial defects, peaking around day 10 post injury.⁴ Immunostaining indicated expression of various pathway components in osteoblasts at all stages of differentiation. It is therefore evident that Notch signaling is active during fracture healing, but the level of signaling is likely dependent on the cell type in question as well as its stage of differentiation.

Despite an accumulation of knowledge on role of Notch signaling in development and bone homeostasis and pathology, the mechanisms by which Notch signaling regulates bone healing have been understudied. In part, this is due to the lack of the suitable models for targeting specific lineage of cells during fracture healing. For studying effects on fracture healing it is critical to have animal models that do not have preexisting musculoskeletal phenotypes. Previous studies targeting Notch signaling utilized inhibition using Mx1Cre;dnMAML mice, inhibiting Notch in most cell types during callus formation, resulted in impaired fracture healing, likely due to a prolonged inflammatory phase.¹⁵ Mice with constitutively targeted RBPj κ deletion in the skeletal lineage (Prx1-Cre;RBPj κ ^{fl/fl} mice) also have impaired fracture healing; callus formation is delayed, with more fibrous tissue within the fractured gap, and reduced BMSC numbers, suggesting that the exhausted osteoprogenitor pool is unable to properly heal fractured bone which leads to non-union.⁶ The disadvantage of the Prx1-Cre based model is that RBPj κ is knocked out in the limb mesenchyme from early in development, which could confound these results. These animals at the age of 8-weeks have a high-bone-mass phenotype with 730% increase in BV/TV, and significantly more active osteoblasts, but with aging their MPC pool diminishes, and they have age-dependent bone loss.²⁹

We used α SMACreERT2 to specifically target MPCs by induction at the time of fracture resulting in an increased proportion of α SMA9 cells when NICD1 is overexpressed. Our data indicate that forced Notch1 activity in osteoprogenitors resulted in improvement of fracture healing parameters, induction of osteogenesis and increased bone formation in the callus as assessed by μ CT and mechanical testing. Notch 1 overexpression in α SMA cells did not result in negative effects on healing 6 weeks after fracture such as delayed callus

remodeling. Our model uses induction of Notch 1 overexpression and fractures are done in biologically unperturbed adult bone confirming the benefits of using inducible models. In contrast to this, a mouse model reproducing the Hajdu-Cheney syndrome (HCS, mice with 6955C→T gain of function mutation in the Notch2 locus) have shorter femurs than WT and exhibited cancellous and cortical bone osteopenia.³⁰ This phenotypic difference HCS mice from NICD1 overexpression phenotype can be a result of constitutively active mutation in Notch2 receptor but also due to differences in expression and function of different Notch receptors in the skeletal biology. Inducible COIN by inversion model for HCS are available, and these will provide a better comparison to our inducible Notch1 findings.^{31; 32} In line with our data is recent work by Youngstrom et al. showing that local delivery of Notch ligand Jagged-1 at the time of injury promotes regeneration of femoral and calvarial defects in mice and rats, with more physiological tissue formation than what was achieved with BMP2.¹⁷ In contrast, fractures in mice treated systemically during the early phases of healing with the γ -secretase inhibitor DAPT also appeared to have enhanced healing.¹⁶ γ -secretase activity is necessary for cleavage of Notch receptors to release the NICD. While Wang and colleagues demonstrate that Notch targets are transiently downregulated following DAPT treatment, confirming the expected inhibition of Notch signaling by this compound, this inhibitor is not specific for Notch signaling.^{33; 34}

In our study, a specific antibody was used to inhibit Notch1 signaling by targeting the negatively regulated region (NRR) of Notch1 receptor using anti-NRR1 antibody.²⁵ When anti-NRR1 antibody binds the NRR1 region of the extracellular domain, it blocks conformational change of the NRR1 region, and protease cleavage is prevented, leading to specific inhibition of Notch1 signaling pathway.²⁵ We have demonstrated that systemic treatment of mice with aNRR1 antibody during days 0–4 of fracture healing leads to impaired fracture healing evidenced by smaller, less mineralized callus formation, and reduced bone strength. The treatment window corresponds to the inflammation and soft callus formation phases, and is very similar to the treatment regimen used for DAPT.¹⁶ As well as Notch-independent effects, DAPT would also target signaling by all the Notch receptors, which are also expressed in fracture, and could contribute to the differences between the two studies.

Periosteal cells from these mice make less cartilage, and differentiate to osteoblasts and incorporate more mineral earlier, so the fractured bones are initially stronger than controls. Although Notch signaling in osteoblasts can modulate osteoclastogenesis, this does not appear to be the mechanism in this case. The enhanced healing was unexpected based on our *in vitro* data in this and our previous study⁵, as following Cre activity, NICD1 would be permanently overexpressed in the cells which would be expected to impair terminal osteoblast differentiation. The reason for this discrepancy requires further investigation, as it likely relates to interaction of Notch and other signaling pathways present in the microenvironment specific to fracture healing.

While α SMA CreERT2 has advantages over constitutive Cre models for fracture studies, it does not specifically target the periosteum, so it is possible that non-periosteal NICD1 overexpression is influencing fracture healing. We have previously shown that in the muscle, α SMA9 labels perivascular and satellite cells.²⁰ The perivascular subset of α SMA-labeled

cells are capable of BMP2-stimulated osteogenesis. Muscle may exert supportive effect on fracture healing, with callus tissue mineralizing fastest at the edges of the callus. Muscle can also be a source of osteoprogenitors in cases where injury extends into the adjacent muscle tissue.^{35; 36} We did not observe any significant changes in other organ systems in which α SMA9 expression was detected up to 42 DPF.

The apparent discrepancy in the effects of NICD1 overexpression *in vitro* and *in vivo*, could have a few potential explanations. Our *in vivo* study suggest that the targeted cells make a greater contribution to osteoblasts following Notch overexpression. Mice overexpressing NICD1 using *Osx-Cre;Rosa(Notch)* at one month of age had osteopenia, but at 3 months of age had increased bone volume, and increased osteoblasts number.⁸ Next, Canalis' group showed that overexpressing NICD1 in DMP1 positive osteocytes lead to increased bone formation and decreased bone resorption increasing expression of OPG and activating Wnt signaling.¹² This data suggests that NICD1 inhibition of osteoblast differentiation is lineage stage specific and might be overcome with sufficient osteogenic signals provided within the cells or by the environment. Overexpression of NICD1 can trigger regulation of other signaling pathways that exert stronger induction of osteogenic differentiation, such as C3H10T1/2 cells that showed significantly improved osteoblastic differentiation after BMP-2 induction with NICD1 overexpression.³⁷ Besides the autocrine effects of Notch signaling overexpression, it is possible that NICD1 overexpressing cells have paracrine effects on other progenitors that promote healing. Complex signaling and cellular diversity present at the fracture site, is different from cultured cells and could be an explanation for persistence of NICD1 inhibitory effect on osteogenesis *in vitro*.

The majority of data presented are from male mice. As indicated in Figure S-1, females with targeted NICD1 overexpression did not demonstrate a bone healing phenotype. It is well-established that bone mass and microarchitecture are different between male and female C57BL/6 mice (females have lower trabecular number and spacing, BV/TV, and decreased ALP activity and differentiation markers in cultured BMSC)³⁸, although differences in healing are not well defined. A number of bone phenotypes related to dysregulation of Notch signaling are evident in both males and females, including in mice combining *Rosa-NICD1* with other Cre drivers.^{8; 12; 29; 39} However, it has been reported that females have decreased α SMA expression¹⁷, and in our studies, we did not see overexpression of Notch1 signaling in the females which would be consistent with lower recombination efficiency resulting in no significant changes in healing (Figure S-1). As most Cre inducible models are not 100% efficient, using *in vitro* experiments we were determined recombination efficiency of α SMACreERT2 to about 40–50%.⁴⁰ In conjunction with no increase in *NICD1* and nonsignificant increase of *Hes1* this might be a likely reason why we do not see effects on fracture healing in females. None of the other studies looking at modulating Notch signaling during fracture healing systematically compared males and females, in fact most either perform studies only in male mice, or do not report which sex was studied.^{6; 15–17}

In summary, enhancing Notch signaling in early fracture healing via forced NICD1 expression in α SMA⁺ progenitors accelerated fracture healing in male mice, with earlier loss of cartilage, increased OCN⁺ osteoblasts, increased mineralization, and stronger and stiffer healing bones. This is likely due to increased expansion of progenitor cells during the

early stages of healing. Notch1 inhibition via aNRR1 during the initial stages of fracture has the opposite effect, with smaller fracture calluses, larger cartilaginous area within the callus tissue, and weaker bones. We conclude that regulation of Notch1 signaling is crucial in the fracture healing process. All this data suggests that treatment increasing Notch1 signaling in fractured bone if applied at the appropriate time early in the healing process might have a therapeutic effect.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Validation of NICD1 overexpression.

A. Experimental design for Rosa-NICD1 fracture studies. Cre recombination was confirmed by DNA recombination in callus tissue 7 DPF, each lane represents a band from single animal callus tissue sample. B. Relative expression of Notch pathway target genes of male mice from primary periosteal cells derived from callus tissue digested 5 DPF and additionally expanded for 3 days in cell culture, determined by qRT-PCR. Results are presented as fold change normalized to Cre⁻ expression, n=4–6, *p<0.05.

Figure 2. Tracing the αSMA^+ cells in fracture healing using $\alpha\text{SMACreERT2/Rosa-NICD1}$ mice crossed with Ai9 reporter mice.

A. Experimental design for $\alpha\text{SMA9/NICD1}$ lineage tracing during the fracture healing. Tamoxifen was injected on the day of the fracture, 2 and 4 DPF, and fractured femurs collected for histological analysis 5, 10 and 14 FPF and flow cytometry 5 DPF. B. Representative images show presence of TdTomato reporter in αSMA cells in controls ($\alpha\text{SMA9/NICD1}^-$) and with NICD1 overexpression ($\alpha\text{SMA9/NICD1}^+$) 5, 10 and 14 DPF. Dotted lines are surrounding the periosteal callus. Scale bar = 500 μm . PC-periosteal callus, CB-cortical bone, BM-bone marrow, PT-pin track, M-muscle. C. Representative dot plots and graph present αSMA9 cell frequency in $\alpha\text{SMA9/NICD1}^-$ and $\alpha\text{SMA9/NICD1}^+$ from digested periosteal callus tissue 5 DPF, *p<0.05. The results are presented as mean $\pm$ SD, (n=5 for Cre- and n=6 for Cre+). **p<0.01.

Figure 3. NICD1 overexpression enhances fracture healing.

We produced a stabilized fracture on the right femur of male α SMACreNICD1 mice. A. Representative images of Safranin O (left) and von Kossa staining (right) are shown. a' - d' shows magnified cartilage area within the callus. Scale bar = 500 μ m. Graphs indicate quantification of cartilage and mineralized area from the histological sections. (D7, n=4-7; D10 n=6-7; D14, n=16-18 (Cre+) and D7, n=3-7; D10, n=8-9; D14, n=13-14 for (Cre-)). B. μ CT analysis at 21 DPF was used to calculate callus volume and bone mass. Torsion testing was then used to determine strength and stiffness (n=9). C. Reconstruction of representative calluses 21 DPF. Scale bar = 1 mm Results are presented as mean $\pm$ SD. * p<0.05, **p<0.01, ***p<0.001.

Figure 4. NICD1 overexpression in αSMA osteoprogenitor cells increases proportion of αSMA and osteocalcin expressing cells.

Stabilized femur fracture was produced in male $\alpha\text{SMACreERT2/Rosa-NICD1}$ mice crossed with Ai9 reporter mice and collected 10 DPF. A. Representative images of osteocalcin staining. Scale bar = 500 μm . B. Stained cells were quantified as a proportion of DAPI-stained nuclei using image analysis. C. Osteoblasts positive for osteocalcin derived from αSMA cells were determined by counting double positive cells (positive for αSMA^+ and OCN^+) out of OCN^+ cells. Arrows indicate tdTomato⁺ osteoblasts. n=6 for NICD⁻ and n=8 for NICD⁺. *p<0.05, **p<0.01.

Figure 5. NICD1 overexpression in αSMA cells inhibits differentiation but enhances migration of periosteal cells *in vitro*.

Primary periosteal cells were isolated from 8-week-old male mice, and cultured as indicated in the diagrams. A. Alkaline phosphatase staining. B. Expression of indicated genes during differentiation, mean $\pm$ SD (n=3 for Cre-, and n=5 for cre+). C. EdU incorporation over 4h, (n=5 for Cre-, and n=6 for Cre+). D. Wound closure following a scratch assay, n=5.. * p<0.05. *Hey1*, Hairy/Enhancer-Of-Split Related with YRPW Motif Protein 1; *BSP*, bone sialoprotein; DOM, days in osteogenic medium; *OCN*, osteocalcin. Blue bars = Cre- and Red bars = Cre+.

Figure 6. Notch1 inhibition impairs fracture healing.

A. Treatment scheme for aNRR1. B. Histology of femur fractures treated with aNRR1 or control antibody at 10 DPF. Cartilaginous and mineralized area within the callus were determined by Safranin O or von Kossa staining and images analyzed in ImageJ software. n=11 for control, and n=9 for aNRR1 treated group. C. Fractured femurs were analysed by μ CT to determine callus volume and bone mass, and tested for biomechanical properties by torsion testing at 21 DPF. D. Representative images show reconstructed calluses 21 DPF in control and aNRR1 treated mice. Scale bar = 1 mm, n=7–9 for control, and n=10–11 for aNRR1 treated group. The results are presented as mean $\pm$ SD. * $p < 0.05$, *** $p < 0.001$.

Figure 7. Proportion of α SMA cells in fracture healing during early Notch1 signaling inhibition. A. Fractures were performed in α SMA9 mice, and tamoxifen and aNRR1 antibody were injected on 0, 2, and 4 DPF, n=5. B. The results are presented as mean $\pm$ SD. Representative dot plots are showing gating of (CD45/Ter119/CD31)⁻ cells and histograms of α SMA9 that are (CD45/Ter119/CD31)⁻ in two experimental groups. **p<0.01.