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IFT80 is required for fracture healing through controlling the regulation of TGFβ signaling in chondrocyte differentiation and function

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Abstract

Primary cilia are essential cellular organelles that are anchored at the cell surface membrane to sense and transduce signaling. Intraflagellar transport (IFT) proteins are indispensable for cilia formation and function. Although major advances in understanding the roles of these proteins in bone development have been made, the mechanisms by which IFT proteins regulate bone repair have not been identified. We investigated the role of the IFT80 protein in chondrocytes during fracture healing by creating femoral fractures in mice with conditional deletion of IFT80 in chondrocytes utilizing tamoxifen inducible Col2α1-CreER mice. Col2α1^{cre}IFT80$^{f/f}$ mice had smaller fracture calluses than IFT80$^{f/f}$ (control) mice. The max-width and max-callus area were 31% and 48% smaller than those of the control mice. Col2α1^{cre}IFT80$^{f/f}$ mice formed low-density/porous woven bony tissue with significantly lower ratio of bone volume, Trabecular (Tb) number, Tb thickness and greater Tb spacing compared to control mice. IFT80 deletion significantly down-regulated the expression of angiogenesis markers-VEGF, PDGF and angiopoietin and inhibited fracture callus vascularization. Mechanistically, loss of IFT80 in chondrocytes resulted in a decrease in cilia formation and chondrocyte proliferation rate in fracture callus compared to the control mice. Meanwhile, IFT80 deletion down-regulated the TGF-β signaling pathway by inhibiting the expression of TGF-βI, TGF-βR, and phosphorylation of Smad2/3 in the fracture callus. In primary chondrocyte cultures in vitro, IFT80 deletion dramatically reduced chondrocyte proliferation, cilia assembly and chondrogenic gene expression and differentiation. Collectively, our findings demonstrate that IFT80 and primary cilia play an essential role in the fracture healing, likely through controlling chondrocyte proliferation, differentiation and TGF-β signaling pathway.

Keywords

primary cilia; IFT80; fracture healing; TGF-β; chondrocytes; conditional knockout; type II collagen; cre

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Introduction

It is estimated that about 7.9 million fractures occurs annually in the United States with 5–10% having either delayed or nonunion fracture healing [(1)]. Treatment of fractures costs $21 billion dollars every year [(2)]. Therefore, the efficacious treatment of fractures is important to patient well-being and the healthcare system. Fracture healing is a complex biological process mediated by both intramembranous and endochondral bone formation. It is initiated by inflammation that is followed by recruitment of mesenchymal stem cells, formation of chondrocytes and production of a cartilaginous callus. The soft callus is replaced by hard callus through revascularization and calcification, and finally remodeled by resorption of cartilage and replacement by bone to restore the osseous structure and function. Recent studies demonstrated that chondrocytes play a critical role in this process [(3,4)].

Primary cilia are essential cellular organelles that are anchored at the cell surface membrane to sense environmental cues and transduce extracellular signals. They have complicated structures containing a microtubule-based cytoskeleton called the axoneme and a mother centriole formed basal body. In humans, defects in ciliary structure and/or function cause a broader set of diseases with severe skeletal abnormalities referred to as ciliopathies [(5)]. Primary cilia are essential for hedgehog (Hh) signaling transduction and regulate platelet-derived growth factor and Wnt signaling [(6–8)]. In addition to the chemical-sensing role, primary cilia play an important role in mechanotransduction and function as a calcium signaling nexus [(9–12)].The presence of cilia on chondrocytes was first reported a half century ago [(13)]. Later studies showed that primary cilia modulate chondrocyte differentiation, signal transduction, endocytosis, and apoptosis [(14)]. Intraflagellar transport (IFT) mediated by IFT proteins and motors are indispensable for cilia formation and function. Mutations in IFT proteins cause various human diseases including bone disorders [(5,15)]. Two IFT protein complexes have been identified, complex A and complex B, containing 20 IFT proteins. IFT complex A facilities retrograde transport from the cilia tip to cytosol, while IFT complex B facilities anterograde transport from the cytosol to cilia tip. Mutations of some IFT proteins have been reported to cause cilia loss [(16,17)]. Work from our laboratories identified an IFT complex B protein, IFT80, which regulates chondrocyte and osteoblast differentiation [(7)]. Conditional deletion of IFT80 in chondrocytes disrupts cilia formation and impairs epiphyseal and articular cartilage formation [(18)]. These findings strongly suggest that IFT80 plays an indispensable role in chondrocyte behavior and function.

Cilia regulate bone formation by modulating expression of genes that participate in growth factor signaling pathways [(19)]. TGF-β upregulation is observed early in fracture healing [(20)] and stimulates repair by binding to cell surface receptors that activate Smad protein, particularly phosphorylation of Smad2/3. Activation of Smad2/3 promotes mesenchymal stem cells proliferation and differentiation, stimulates chondrogenesis, increases the production of matrix proteins and new blood vessel formation in the fracture callus [(21)]. TGF-β receptors localize to primary cilia of human embryonic stem cells [(22)] and TGF-β signaling cascade has been found to occur within the microdomain of the primary cilium and mediate cell migration in human mesenchymal stem cells [(23)]. TGF- β signaling is enhanced and balanced by internalization of activated receptors through clathrin-mediated endocytosis in human foreskin fibroblasts [(24)]. Since the ciliary pocket region is a site for extensive

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clathrin-mediated endocytosis, primary cilia are considered to play a role in regulating TGF-β signaling.

Major advances have been made in understanding the roles of IFT proteins in bone development. Deletion of Kif3a, an anterograde motor in primary cilia in osteoblasts/osteocytes impairs responses to load-induced bone formation, fluid flow shear stress, and strain fields caused by implant displacement, suggesting that primary cilia play an essential role in bone remodeling [25–27]. However, the role of IFT proteins in regulating fracture healing has not been investigated. The purpose of this study is to investigate the role of IFT80 in the fracture healing focusing on how it affects chondrocytes. Col2α1-CreER mice were used to delete IFT80 in the chondrocyte lineage cells by administration of tamoxifen, allowing us to study the role of IFT80 in postnatal fracture repair. Indeed, we found that conditional ablation of IFT80 in chondrocytes significantly impaired fracture healing. IFT80 deletion dramatically reduced cilia number and cilia length, which interfered with chondrocyte differentiation and proliferation. The changes induced were linked to IFT80 deletion down-regulated TGF-βI and TGF-βR expression, and significantly reduced Smad2/3 activation in the fracture callus. Thus, our findings demonstrate for the first time that ciliary IFT80 plays an essential role in fracture healing by controlling chondrocyte proliferation and differentiation via the TGF-β signaling pathway. A better understanding of the mechanisms of fracture healing provided by these results may provide insight into new treatment modalities for bone fracture and other defects.

Materials and methods

Experimental animals

All animal procedures were approved by and conducted in accordance to the University of Pennsylvania Institutional Animal Care and Use Committee. $IFT80^{f/f}$ mice were generated as we previously described [18] by inserting two LoxP sites flanking exon 6 in the IFT80 gene. Col2α1-CreER mice was purchased from the Jackson Laboratory (Stock # 6774; FVB; Bar Habor, ME, USA), which has tamoxifen-regulated Cre deletion restricted to chondrogenic lineage cells [28]. $IFT80^{f/f}$ mice were bred with Col2α1-CreER mice to produce $Col2\alpha1^{cre}$; $IFT80^{f/+}$ mice. $Col2\alpha1^{cre}$; $IFT80^{f/+}$ mice were mated with each other to generate $Col2\alpha1^{cre}IFT80^{f/f}$ mice. Without tamoxifen injection, $Col2\alpha1^{cre}IFT80^{f/f}$ mice were indistinguishable from wild type, Col2α1-CreER, and $IFT80^{f/f}$ mice. Therefore, $IFT80^{f/f}$ mice were then crossed with $Col2\alpha1^{cre}IFT80^{f/f}$ mice to produce $IFT80^{f/f}$ (used as control) and Col2 $a1^{cre}IFT80^{f/f}$ mice.

Murine femur fracture and tamoxifen injection

Closed femoral fractures with intramedullary nail fixation were created in 12-week (wk)-old experimental and control mice described above Col2α1(N=6-8 per group per time point) as described [29]. Briefly, closed fractures were generated by a three-point blunt guillotine driven by a dropped weight, which creates a uniform transverse fracture of the femur. In this model MSCs differentiate to chondrocytes prior to Day (D) 6 post fracture, while Col2α1 mRNA expression peaks around D9 [30]. We deleted IFT80 by intraperitoneally (i.p.)

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injection of 180 mg/kg tamoxifen (Sigma, T5648) at D3, D5 and D7 post -fracture. The fractured femurs were harvested at D9 and D21 for analysis.

Micro-computed tomography (microCT) imaging and radiography

Mice were anesthetized by isoflurane and the fracture sites were radiographed using a Faxitron MX-20 (Faxitron X-Ray) at D0, D7, D14 and D21 to monitor the progress of fracture healing. Bone microarchitecture of the fractured femur (D21) was tested using Scanco Medical μCT 35 at the Imaging Core, Penn Center for Musculoskeletal Disorders. Scans were performed at 55keV and 3D images were reconstructed at the fracture line extending at least 3 mm to the proximal and distal. The ratio of the bone volume to the total volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th, mm), and trabecular spacing (Tb.Sp, mm) were determined in the fracture callus with density thresholds set at 300 mg/cm3 HA.

Histological and histomorphometric analyses

Fracture calluses were excised, fixed with 4% paraformaldehyde and decalcified in 10% ethylenediaminetetraacetic acid (EDTA) for a minimum three weeks at 4°C. The samples were then embedded in OCT. Longitudinal sections (8 μm thick) were cut from the mid-portion of the callus and mounted serially on gelatin-coated glass slides. Safranin O staining was performed to visualize cartilage and asses the proteoglycan. Callus sections were stained with Weigert's iron hematoxylin and fast green, and then stained with 0.1% safranin O solution. Quantification of maximal cartilage width and cartilage area was done with Image J (NIH, Bethesda, MD, USA).

Cells and cell culture

Primary chondrocytes were isolated using previously published methods [18]. Briefly, control mice were euthanized at postnatal D7. The articular cartilage from the femoral heads, femoral condyles, and tibia plateaus was isolated by first removing the soft tissue and bone thoroughly followed by incubation with collagenase type IV (Worthington, Lakewood, NJ) (3 mg/mL) for 45 min at 37°C. Cartilage pieces were obtained and incubated in 0.5 mg/mL collagenase type IV solutions overnight at 37°C. Cells were then filtered through a 40 micrometer cell strainer, collected, and seeded at a density of 8×10^3 cells per cm^2 and then cultured in DMEM culture media with 10% FBS [31].

Primary chondrocytes from $IFT80^{f/f}$ mice were infected with adenoviruses, which overexpress either Cre (Ad-CMV-Cre, #1405, Vector Biolabs) or GFP (Ad–GFP, #1060, Vector Biolabs) as described previously [7,32]. Ad-CMV-Cre infection yielded 85% deletion of IFT80 in primary chondrocytes and Ad–GFP treated cells were used as a control.

Differentiation of primary chondrocytes

To induce cell differentiation, primary chondrocytes were cultured with media supplemented with ITS (insulin-10 μg/mL, transferrin-5.5 μg/mL, and sodium selenite-5 ng/mL, Sigma-13146) for 2 weeks [33]. The medium was changed every three days for 14 days and alcian blue staining was performed as described [18]. Briefly, cells were fixed with 4% paraformaldehyde for 15 min, washed, and stained with 1% Alcian blue in 3% acetic acid

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(pH = 2.5) overnight. The cells were washed repeatedly with distilled water before imaging. For quantitative analysis, dye was extracted with 150 μL of 6M guanidine-HCl for 2h at room temperature. The extracted dye was transferred to 96-well plates and the optical density measured at 620 nm [7]. This experiment was run in triplicate. For micromass cultures, the protocol was described by De Bari et al [34] and modified by Greco et al [35]. Briefly, micromasses were obtained by pipetting 20 μL of primary chondrocytes into individual wells of 24-well plates. Following a 3-h attachment period without medium, the growth medium was gently added. Differentiation was promoted by ITS supplementation. On D1, D3 and D5, micromasses were harvested for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) gene expression analysis of marker genes for chondrocytes.

Immunofluorescence (IF) staining

IF staining was performed to visualize cilia, cell proliferation, collagen II and blood vessels. Fixed chondrocytes or fracture callus sections were incubated with primary acetylated α-tubulin antibody (specific for cilia, 1:500, Sigma, St. Louis, MO), Ki67 antibody (1:300, ab16667, Abcam, Cambridge, MA), collagen II antibody (1:300, ab34712, Abcam) or von Willebrand factor antibody, which identifies mature endothelial cells (1:500, sc8068, Santa Cruz Biotechnology, Dallas, TX) overnight at 4°C and then localized with Alexa Fluor 647 or 488 conjugated anti-mouse IgG (Invitrogen) antibodies. Slides were washed and mounted with VectaShield containing DAPI. All images were captured and processed on a fluorescence microscope equipped with a digital camera system (Leica, Allendale, NJ).

Western blot analysis

Snap-frozen fracture callus samples in liquid nitrogen were finely crushed and homogenized in RIPA buffer (Thermo Scientific) supplemented with protease and phosphatase inhibitors (Thermo Scientific). The lysates were centrifuged at 12000 g for 10 min at 4 °C. Protein concentration of the resulting supernatant was determined using a Bio-Rad Protein Assay (Bio-Rad; Hercules, CA). Equal amounts of protein from each group was solubilized in sodium dodecyl sulfate (SDS) buffer, separated on 8–10% polyacrylamide-SDS gel and approximate molecular weight was established by comparison to protein molecular weight standards. Proteins were transferred to polyvinylidene difluoride (PVDF) membrane in 25 mM Tris, 192 mM glycine, and 20% methanol. Membranes were blocked in 5% milk-TBST and incubated with rabbit anti-IFT80 antibody (1:400, PAB15842, Abnova), phospho-SMAD2/3 antibody (Ser423/425; 1:500; sc11769-R; Santa cruz) or SMAD2/3 antibody (1:500; #5678s; Cell Signaling) overnight at 4°C. Following several washes in tris buffer saline Tween20 (TBST:20 mM Tris–HCl [pH 7.5]; 137 mM NaCl and 0.1% Tween20), membranes were incubated in the appropriate horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature, washed, incubated for 5 minutes in ECL reagent (Thermo Scientific, Waltham, MA), and then imaged using the ChemiDoc Touch (Bio-Rad; Hercules, CA) imaging system. Protein band intensities were measured using ImageJ software, normalized to beta actin.

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Quantitative real-time polymerase chain reaction (qPCR):

Total RNA was isolated from callus specimens that were carefully trimmed of soft tissue, snap frozen and finely crushed. RNA from callus samples and primary chondrocytes was isolated with Trizol Reagent (Life Technologies), treated with DNAse (Promega; Madison, WI) and cDNA synthesized from 1 μg total RNA using the SuperScript III reverse transcriptase kit (Invitrogen) in a final volume of 20 μL. Resulting cDNA was subjected to real-time PCR using Fast SYBR Green Master Mix (appliedbiolgystems) in a CFX96 real-time system (Bio-Rad; Hercules, CA) with the mouse-specific primers (Table).

Results

IFT80 expression increases after fracture.

To detect the expression pattern of IFT80, we harvested fracture calluses on days 6 and 9 after fracture, which is chondroctye rich stage. As shown in Fig 1A and 1B, IFT80 expression was dramatically upregulated when compared to the intact (non-facture) femur shaft. IFT80 mRNA expression increased and reached its peak on D9 post fracture, which resulted in a 7-fold increase ($P<0.05$, Fig. 1A). IFT80 upregulation in fracture callus was further confirmed by western blot (Fig. 1B). In primary chondrocytes, we also noted a significant elevation of IFT80 at D1, D3 and D5 compared to that at D0 during cell differentiation in micromass culture (Fig. 1C). These results indicate that IFT80 is highly up-regulated in fracture calluses and is expressed throughout the healing period assessed.

Since IFT80 expression peaks when cartilage predominates [30], we conditionally deleted IFT80 in chondrocytes in experimental mice by tamoxifen injections on D3, D5 and D7 post fracture (Fig. 1D). To evaluate the deletion efficiency and specificity, we assessed the IFT80 expression in fracture callus in both Col2α1^{cre}IFT80$^{f/f}$ and IFT80$^{f/f}$ control groups at D9. The levels of IFT80 mRNA decreased by 56% ($P<0.05$) and protein decreased by 45% ($P<0.05$) in the Col2α1^{cre}IFT80$^{f/f}$ group compard with that of the IFT80$^{f/f}$ control group (Fig. 1E and 1F).

Loss of IFT80 in chondrocyte lineage cells reduces bone formation in fracture calluses.

In view of newly formed bone of the fracture calluses, serial X-ray images were performed at D0, 7, 14, and 21 post-fracture. Images of fractured femurs in both Col2α1^{cre}IFT80$^{f/f}$ and IFT80$^{f/f}$ control groups showed remarkable differences during the period of the fracutre callus formation. Col2α1^{cre}IFT80$^{f/f}$ mice had a clear decrease in callus size with less bone formation (Fig. 2A). Micro-CT images, which covered around 3mm proximal and 3mm distal to the fracture site, were acquired at 21 days post-fracture. Similarly, 3D reconstrution of microCT images confirmed the significant reduction of bone formation in Col2α1^{cre}IFT80$^{f/f}$ mice compared to IFT80$^{f/f}$ mice. Col2α1^{cre}IFT80$^{f/f}$ mice had much less bone mass with low-bone density and porous woven bone compared to IFT80$^{f/f}$ (Fig. 2B). Accordingly, axial microCT images showed alterations in composition and stucture. The ratio of BV/TV was decreased by 48%, Tb.N by 26% and Tb.Th by 54%, while Tb.Sp was increased by 69% in Col2α1^{cre}IFT80$^{f/f}$ mice compared to those in the IFT80$^{f/f}$ mice ($P<0.05$, Fig. 2C).

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Loss of IFT80 in chondrocytes lineage cells inhibits the formation of fracture calluses.

To evaluate the effect of IFT80 deletion in chondrocytes on the formation of cartilageious callus, we examine the fracture calluses size in Col2α1^{cre}IFT80$^{f/f}$ mice and IFT80$^{f/f}$ mice. The max-diameter of soft calluses were measured at D9 post-fracture. The result showed that the average diameter of the soft calluses in Col2α1^{cre}IFT80$^{f/f}$ mice was significantly smaller than that in IFT80$^{f/f}$ mice by 19.05% (P<0.05, Fig 3A). Moreover, the newly-formed cartilage area were quantitatively assessed from Safranin O staining using histomorphometric measurements . As shown in Fig. 3B, Col2α1^{cre}IFT80$^{f/f}$ mice had smaller callus area, and the max callus area and newly formed cartilage are significantly lower than control mice by 48% and 32%, respectively (P<0.05). Thus, these findings demonstrated that deletion of IFT80 in chondrocytes impaired the formation of fracture calluses and cartilage tissue, suggesting that IFT80 is essential for endochondral bone formation during fracture healing.

Conditional deletion of IFT80 in chondrocytes impairs cilia formation and chondrocyte prolferation.

To investigate whether IFT80 deletion influences cilia formation, the cilia were visualized by IF staining for acetylated tubulin (pseudo-colored red) and gamma tubulin (green label the centrosome) at D9 post fracture. About 61% cells in IFT80$^{f/f}$ in the sections showed apparent cilia, while in Col2α1^{cre}IFT80$^{f/f}$ group, only 18% cells had cilia (P<0.05, Fig. 4A). To examine if loss of IFT80 affects chondrocytes proliferation, Ki67 IF staining was performed. The results showed a significant reduction of Ki67^{+} cells by 65% in Col2α1^{cre}IFT80$^{f/f}$ group compared to those in IFT80$^{f/f}$ group (P<0.05, Fig. 4B).

To further investigate the role of IFT80 in chondrocyte proliferation and cilia formation in vitro, chondrocytes were isolated from the articular cartilage of IFT80$^{f/f}$ mice and infected with Ad-CMV-Cre to delete IFT80 as described in Methods. Ad-GFP-infected IFT80$^{f/f}$ cells were used as control. qPCR showed that Ad-CMV-Cre transduction yielded ~85% reduction of IFT80 mRNA expression in chondrocytes in vitro (P<0.05, Fig. 4C). Chondrocytes infected by Ad-CMV-Cre showed reduced ciliated population by 68% and shorten cilia length by 23% compared to Ad-GFP infected cells after 24 hours of serum starving, confirming the critical role of IFT80 in cilia formation (P<0.05, Fig. 4D). Meanwhile, IFT80 deletion significantly decreased the abundance of proliferating cells by 27%, as measured by BrdU assay (P<0.05, Fig. 4E).

Loss of IFT80 inhibits chondrogenic differentiation in fracture callus.

To study whether IFT80 deletion influenced chondrogenic differentiation in fracture calluses, we assessed the mRNA expression of chondrogenic makers: Sox9, collagen 2, aggrecan and collagen 10. We first performed collagen II IF staining to estimate the chondrocytes population. We found that collagen II positive cells were significantly reduced by ~41-52% in the Col2α1^{cre}IFT80$^{f/f}$ mice (P<0.05, Fig. 5A). qPCR results showed that IFT80 deletion significantly down-regulated the expression of collagen II, aggrecan and collagen X by 74%, 66% and 76%, respectively (P<0.05, Fig. 5B).

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To investigate the role of IFT80 in chondrogenic differentiation in vitro, chondrocytes from IFT80$^{f/f}$ were infected with Ad-GFP or Ad-Cre, and subsequently induced with chondrogenic medium for 14 days. Alcian blue staining was used to detect sulfated proteoglycan deposits. The results showed that Ad-Cre infected cells displayed significantly less sulfated proteoglycan deposits by 71% compared to Ad-GFP infected chondrocytes ($P<0.05$, Fig. 5C). Consistent with the defects in chondrogenic differentiation, loss of IFT80 in chondrocytes resulted in the reduced expression of the chondrocyte marker genes: Sox-9, type II collagen, Aggrecan, and type X collagen by 48%, 41%, 31%, and 58% respectively, ($P<0.05$, Fig. 5D). These results demonstrate that deletion of IFT80 impaired chondrocyte differentiation and its marker expressions.

Loss of IFT80 inhibits angiogenesis in fracture callus.

Given that new blood vessels are required for vascularizing the fracture callus and bone formation [36], to get insight whether vascularization in the fracture callus was affected, we therefore performed the IF staining for an endothelial cell marker, von Willebrand Factor (VWF), to assess the blood vessel formation in the callus and found a clear reduction in VWF+ area by 69% in the Col2α1^{cre}IFT80$^{f/f}$ mice in the cartilage zone compared to that in control mice ($P<0.05$, Fig. 6A). Consistently, qPCR results showed that IFT80 deletion significantly down-regulated the expression of angiogenesis markers: vascular endothelial growth factor (VEGF); Platelet Derived Growth Factors (PDGF) and angiopoietin 1 (Ang1) by 36%, 41% and 38% respectively ($P<0.05$, Fig. 6B). In primary chondrocytes harvested from IFT80f/f mice, IFT80 deletion reduced the mRNA expression of angiogenesis markers expression as well during cell differentiation in micromass culture (Fig. 6C).

Loss of IFT80 impairs chondrocyte differentiation through the decrease of TGF-β signaling activity.

Since TGF-β plays an essential role in fracture healing, to gain further insight into the molecular mechanism of primary cilia and IFT80, we characterize the activation of TGF-β signaling pathway. Data show that TGF-βI expression is dramatically up-regulated in fracture calluses until D16, (Fig. 7A) consistent with previous reports [21]. To further investigate downstream events we examined activation of TGF-β signaling in fracture calluses of mice with chondrocyte-specific IFT80 deletion. IFT80 ablation significantly down-regulated TGF-βI and TGF-βRI mRNA expression by 71% and 37% in fracture callus ($P<0.05$, Fig. 7B). Immunostaining and western blot results show a marked decrease in P-Smad2/3 expression, indicating the critical role of IFT80 on the TGF-β signaling activity (Fig. 7 C and D). In primary chondrocytes harvested from IFT80f/f mice, IFT80 deletion reduced the TGF-βI and TGF-βRI mRNA expression during cell differentiation in micromass culture (Fig. 7E). In addition, IFT80 deletion significantly inhibited TGF-βI-induced SMAD2/3 phosphorylation (Fig. 7F). These results suggest that activation of TGF-β-Smad signaling is dependent on primary cilia function.

Discussion

Fracture repair is a complex physiological process, which involves characteristic phases of inflammation, bony callus formation and bone remodeling [37]. Although major signaling

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pathways have been identified, the gene regulatory networks that control bone regeneration during fracture healing remain to be delineated. In this study, we demonstrated for the first time that IFT80 plays an essential role in chondrocytes during fracture healing. Moreover, the results provide mechanistic insight into the role of IFT80 in chondrocyte proliferation, facilitating TGFβ signaling and promoting the expression of angiogenic factors that are critical for normal repair. Moreover, the impact of IFT80 specifically on chondrocytes was propagated to the formation of a hard callus which was smaller and had lower bone quality.

A number of studies have reported that IFT proteins are required in primary cilia assembly and maintenance of cilia function [(38–42)]. Protein synthesis is not carried out in the primary cilia, all ciliary proteins are presumably synthesized in the rough endoplasmic reticulum and trafficked to ciliary pocket via vesicular trafficking and transported into cilium by IFT. IFT proteins function as adaptors to assemble cargo proteins and operate the bidirectional transport movement of proteinaceous material sandwiched between the microtubule-based axoneme and the ciliary membrane [(43)]. Defects in some IFT proteins results in impaired cilia formation, contributing to ciliopathies in mouse models [(6,44)]. IFT80 is a major component of IFT complex B. Loss of IFT80 in zebrafish and Tetrahymena thermophila results in a shortened and reduced number of cilia [(45,46)]. Our previous studies showed that silence of IFT80 caused the shortening of cilia or cilia loss in mesenchymal progenitor cells line C3H10T1/2 and bone marrow derived stromal cells (BMSCs) [(7)]. By crossing IFT80$^{f/f}$ mice with inducible Col2α1-CreER mice, we recently reported that IFT80 deletion in chondrocytes resulted in 50–60% loss of cilia in chondrocytes in the growth plate area [(18)]. In the current study, we noted the significant elevation of IFT80 expression in fracture cartilaginous callus and in primary chondrocytes during cell differentiation in vitro, suggesting a critical role for IFT proteins in chondrocytes. We also found that loss of IFT80 in chondrocyte lineage led to a significant cilia loss in both fracture callus and primary chondrocytes culture. These results provide support that IFT80 is essential for the cilia assembly in postnatal endochondral bone formation. Recently, Taschner et al present a crystal structure of IFT80 to show an unusual N-terminal double β-propeller followed by a-solenoid structure. Using gene-editing to delete IFT80 in IMCD3 mouse cells, they reported that IFT80 is essential for initiation of the ciliary axoneme and absolutely required for ciliogenesis [(47)].

We found that deletion of IFT80 in chondrocyte lineage during fracture healing impairs chondrocyte proliferation and differentiation and cartilaginous callus formation. The impact of IFT80 ablation in chondrocyte lineage cells resulted in a smaller fracture calluses that had lower-density bony tissue compared to IFT80$^{f/f}$ control mice. Additionally, the expression of the chondrocyte marker genes Sox-9, aggrecan, and collagen X significantly decreased in IFT80-deficient chondrocytes, demonstrating IFT80 is essential for cartilaginous callus formation during fracture healing.

Generally, cartilage forms by the first week after the fracture and invasion of cartilage by blind-ending vascular loops started to occur by the second week [(48)]. The vascularization process is regulated by a VEGF-dependent pathway [(49)]. Both osteoblasts and hypertrophic chondrocytes express high levels of VEGF, which promotes both the aggregation and proliferation of endothelial mesenchymal stem cells into a vascular plexus, and growth of

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new vessels from already existing ones [50]. It has recently been shown that the transcription factor FOXO1 mediates expression of VEGF by chondrocytes, which plays a significant role in angiogenesis during the transition from cartilage to bone in postnatal fracture healing [4]. In the current study, we found that IFT80 in chondrocytes was necessary for blood vessel formation in fracture callus and was needed for expression of VEGF, PDGF and angiopoietin 1. These data point to the important function of IFT80 in chondrocytes by contributing to the cellular events that occur in the formation of a hard fracture callus and provide mechanistic insight into the regulation of IFT80 in chondrocytes to promote angiogenesis.

TGF-β is one of the most important factors stimulating and controlling the bone healing process (refer to the review article [21]). TGF- β attaches to its receptors at the time between the inflammatory and repair phases during fracture healing, enabling recruitment of MSC, chondroprogenitor cells, osteoprogenitor cells, fibroblasts, and immune cells [51,52]. TGF-β is reported to present in the forming hematoma within 24 h post fracture. It's level will then gradually increase and most abundant TGF-β level within the callus is found between the 7th and 14th day of the fracture [53]. In this study, we found that deletion of IFT80 significantly reduced the expression of TGF-βI and TGF-βRI. How cilia regulate the TGF-β generation is not clearly understood. Xiao et al found that TGF-β can be regulated at transcriptional level through P38 MAPK, ERK, and JNK and translational levels by activation of Rho GTPase, PI3K, Akt, and mammalian target of rapamycin [54]. Primary cilium has been reported to plays a role in PDGFRα signaling to activate the MEK1/2-ERK1/2 and Akt pathways [55].

Most notably, our data showed a significant decrease in the phosphorylation of Smad2/3, implying the critical role of cilia and IFT80 on the TGF-β signaling transduction activity. These findings are supported by recent evidence implicating that primary cilia may serve as an intracellular site for internalization of TGF-β receptors via clathrin-dependent endocytosis. The ciliary pocket region, which locates at the proximal part of the primary cilium, is known as a unique site of clathrin-dependent endocytosis [56,57]. TGF-β receptors are found to localize at the ciliary tip and, on activation, are transported to the ciliary base in fibroblasts and TGFβ stimulation increases receptor localization and activation of SMAD2/3 [24]. Primary cilium has been reported to mediate TGFβ1-induced recruitment of human bone mesenchymal stem cells in a SMAD3-dependent manner [23]. A recent study revealed that mutation of the cilia-targeting signal abolished the trafficking of TGFβRI to the primary cilia [58]. In fibroblasts from $Tg737^{orpk}$ mutant mouse, where IFT88 is mutated, clathrin-dependent endocytosis is disrupted at stunted primary cilia and accumulation of TGFβ receptors and activation of SMAD2/3 are reduced [24]. Both IFT88 and IFT80 are the core protein in the IFT complex B, and deletion IFT88 or IFT80 resulted in cilia loss. Our results suggest that cilia loss and ciliary dysfunction caused by IFT protein mutations will interrupt the TGF-β signaling pathway to impair fracture healing. In chondrocytes, TGF-β treatment has been reported to suppress the levels of IFT88 mRNA and reduce the number of cilia positive cells and cilia length, suggesting that TGF-β signaling could play multiple roles in regulating cilia assembly [59]. Our study showed that cilia loss induced by IFT80 deletion reduces TGF- β expression and downstream signal transduction. However, we did not find that TGF- β reduced cilia number or length in ATDC5 chondrocytes under serum free

conditions (data not shown). It is possible that specific cell conditions such as cell density or the specific microenvironment affects cellular response to TGF-β treatment. Whether TGF-β regulates cilia formation and length in physiological and pathological condition needs further investigation.

Collectively, our findings demonstrate that ciliary IFT80 plays an essential role in the fracture healing, controlling chondrocyte proliferation and differentiation through TGF-β signaling pathways. Loss of IFT80 in chondrocytes interrupts soft callus formation, reduces the vascularization and impairs the activation TGF-β signaling pathway. Previous studies have demonstrated that cilia function as mechanical sensors. It is possible that primary cilia act as mechanosensors in chondrocytes to affect postnatal bone healing. Future studies are needed to establish the specific role of IFT proteins in mesenchymal lineage cells during fracture healing, their role in transducing mechanical signals during the healing process and to further investigate mechanisms by which the TGF-β signaling is regulated by primary cilia. Moreover, modulation of cilia and cilia-associated proteins may have translational value to prevent the negative impact on fracture healing and other bone disorders, which are affected by ciliogenesis.

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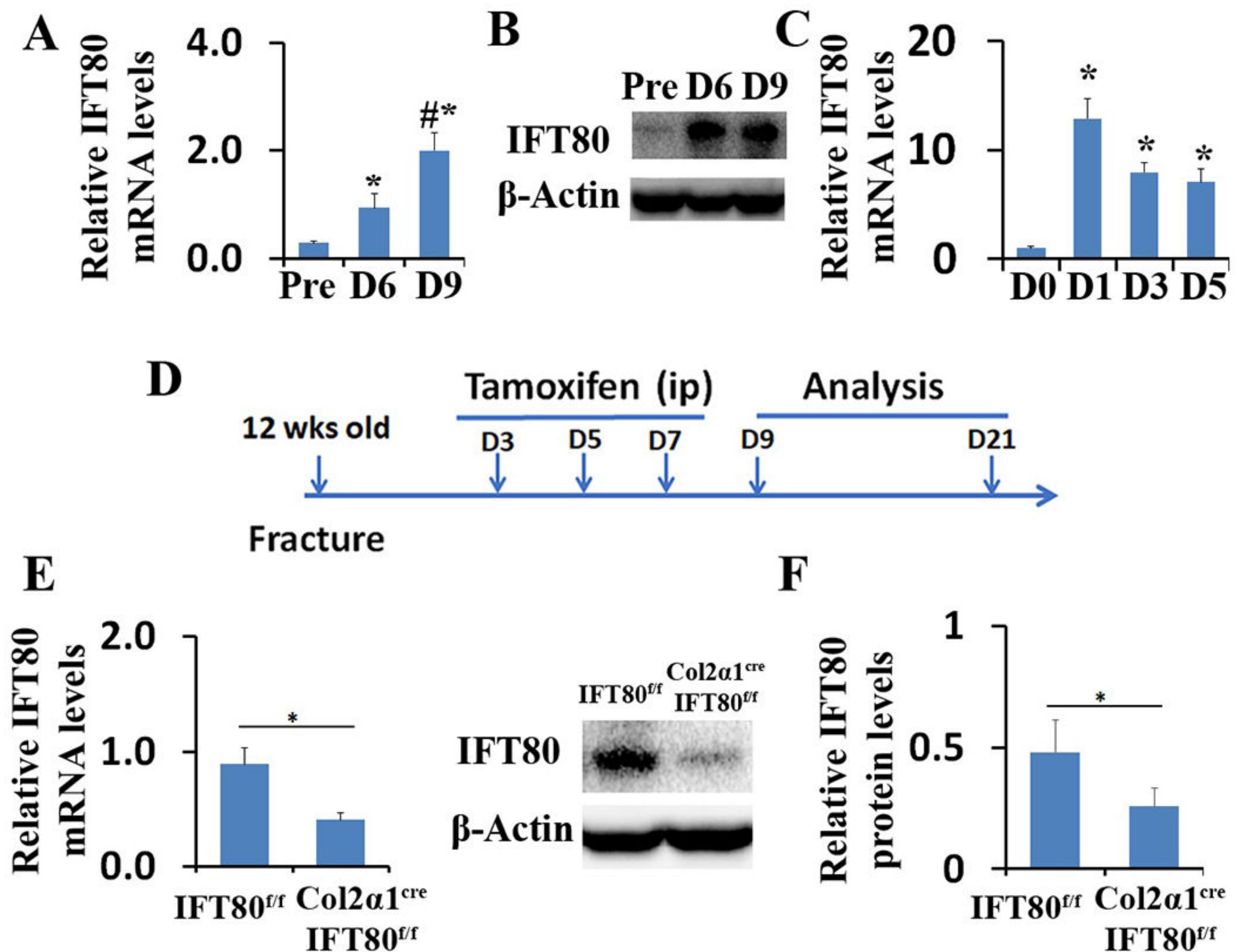

Fig 1:

IFT80 expression at the site of fracture. (A) qPCR analysis of IFT80 expression in femur collected at pre-fracture, D6 and D9 post fracture in IFT80$^{f/f}$ mice. IFT80 expression was normalized to GAPDH. (B) Western blot validation of IFT80 expression in femur collected at pre-fracture, D6 and D9 post fracture in IFT80$^{f/f}$ mice. *: P<0.05 compared to Pre; #: P<0.05 compared to D6. (C) qPCR analysis of IFT80 expression in micromass cultured primary chondrocytes at D1,3 and 5 after switching to differentiation medium. (D) Line drawing shows the time courses of tamoxifen administration and tissue harvest for data analysis. Femoral fractures were performed in 12-week (wk)-old IFT80f/f control mice control and experimental Col2α1^{cre}IFT80$^{f/f}$ mice, which were then given intraperitoneal (IP) injections of 180 mg/kg tamoxifen at Day(D) 3, 5 and 7 post fracture to induce IFT80 deletion, and fractured femur were harvested at D9 and D21 post fracture (N=6 per group). (E) qPCR analysis of IFT80 expression in fracture callus of IFT80$^{f/f}$ mice and Col2α1^{cre}IFT80$^{f/f}$ mice collected at D9 post fracture. IFT80 expression was normalized to GAPDH. (F) Western blot analysis of IFT80 expression in fracture callus of IFT80$^{f/f}$ mice and Col2α1^{cre}IFT80$^{f/f}$ mice collected at D9 post fracture.

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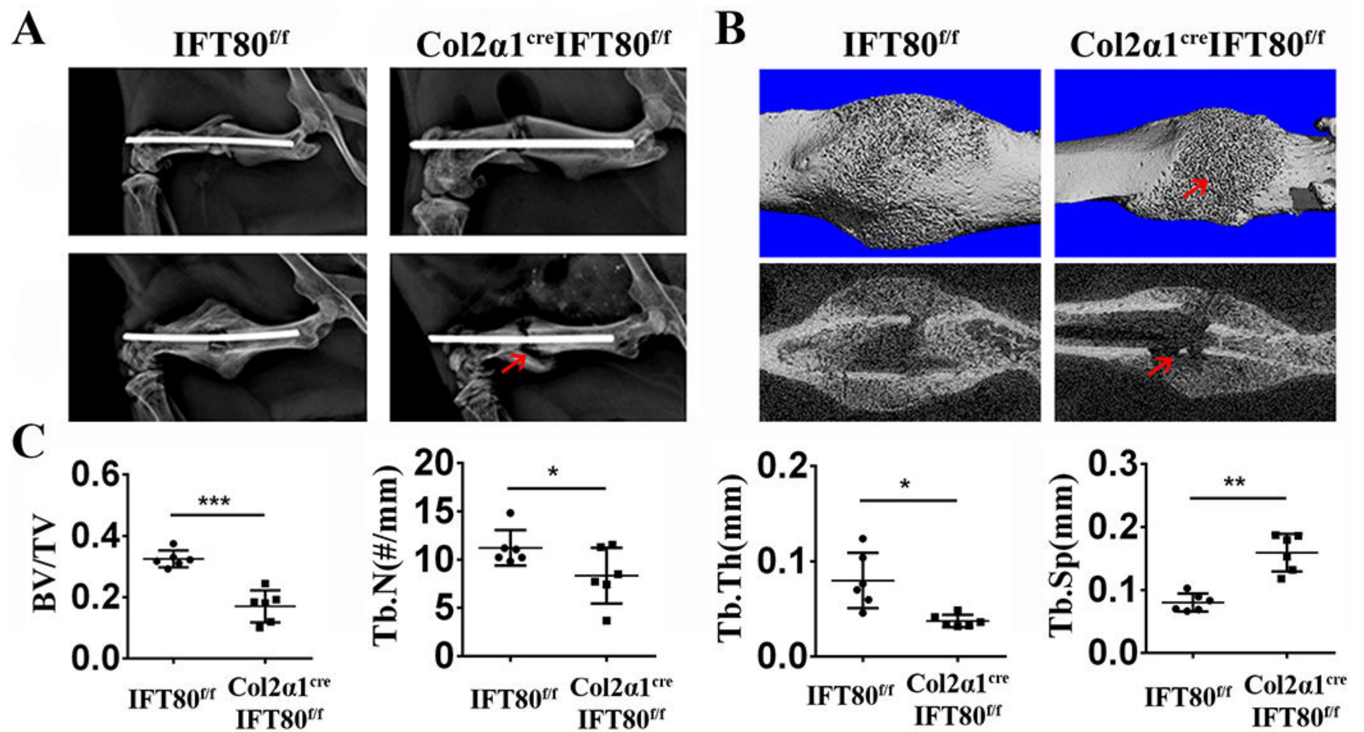

Fig 2:

Radiological assessment of fracture callus demonstrates IFT80 deletion impairs fracture healing. (A) Serial X-ray images were conducted at D0 and D21 post fracture. (B) Representative images of 3-D reconstruct and section of micro CT scan of the fracture site at D21 post fracture. (C) Quantitative measurements of the newly formed bone at the fracture gap. * P<0.05; ** P<0.01; *** P<0.001. Red arrows point out the marked less bones mass with low-bone density and porous woven bone in experimental in Col2α1^{cre}IFT80$^{f/f}$ mice. These data demonstrate that deletion of IFT80 in chondrocytes impairs fracture healing.

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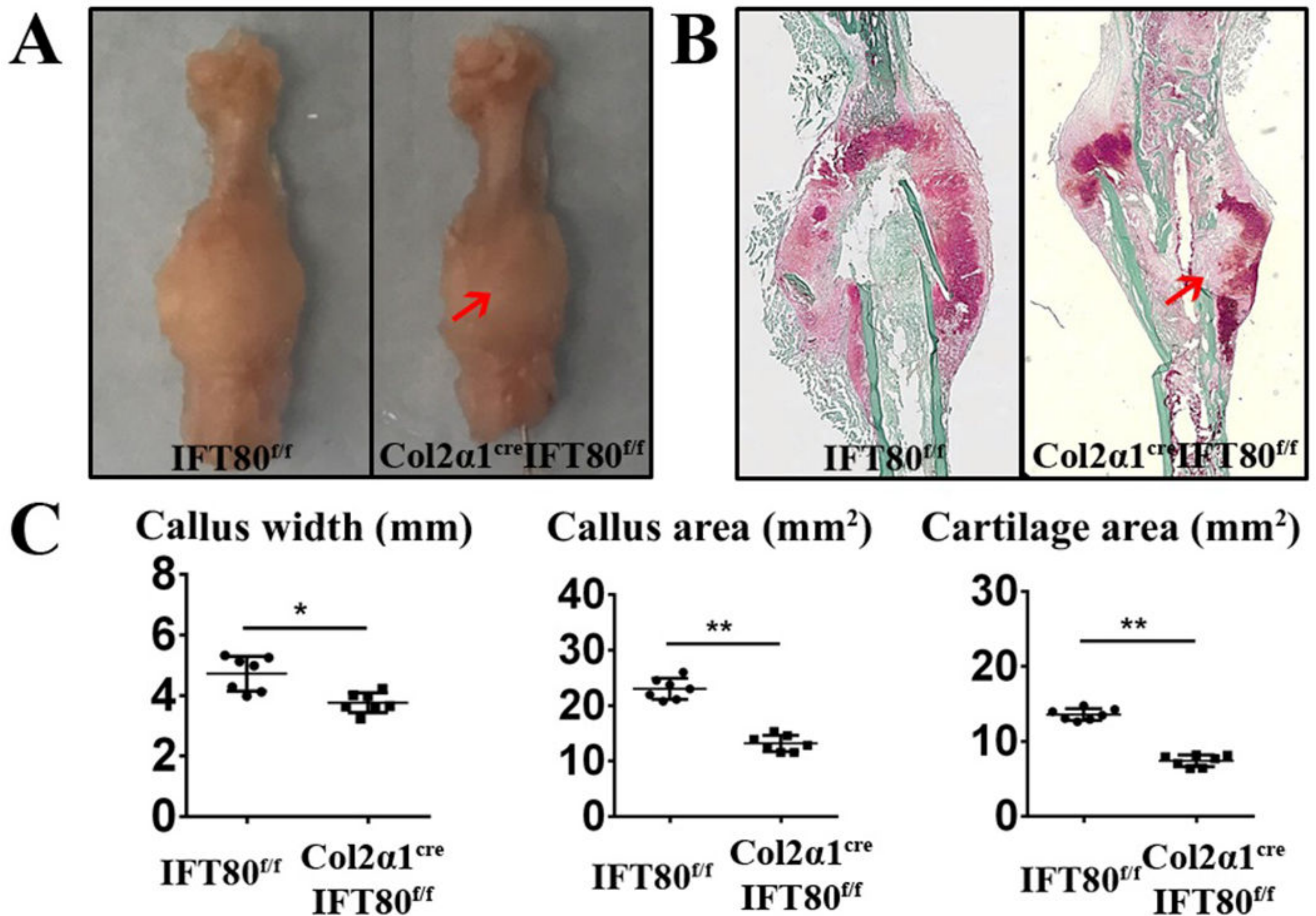

Fig 3:

Histological analysis of fracture callus. (A) Representative images of callus collected at D9 post femur fracture. (B) Safranin O staining was used to visualize cartilage and access the content of proteoglycan. (C) The quantitative assessment of fracture callus, cartilage area. * P<0.05; ** P<0.01. Red arrows point out the marked decrease in fracture callus and cartilage area in Col2α1^{cre}IFT80$^{f/f}$ mice.

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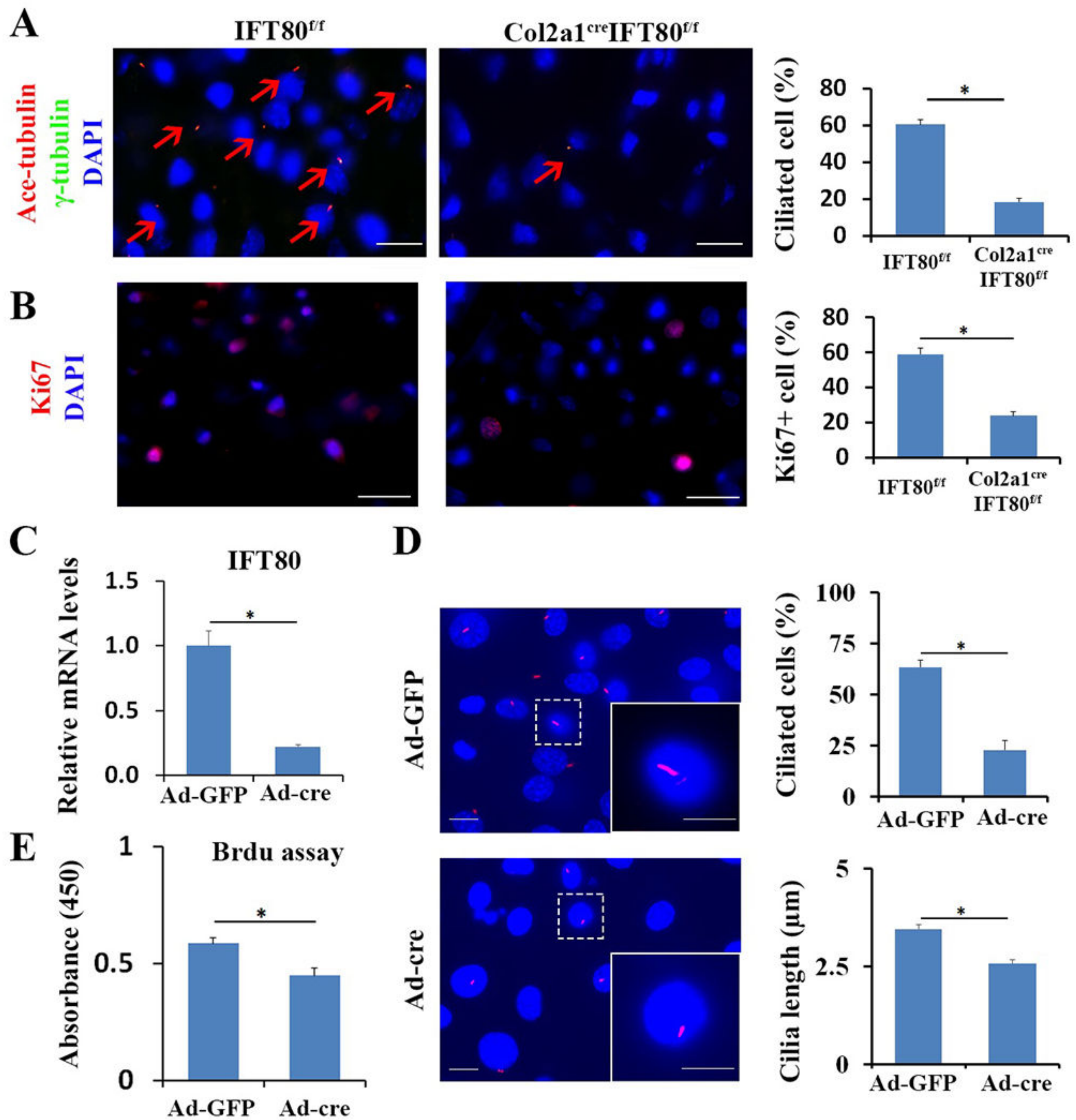

Fig 4:

IFT80 deletion in fracture callus reduced the cilia formation and impaired chondrocytes proliferation in fracture callus (A-B) and primary chondrocytes culture (C-E). To delete the IFT80 in primary chondrocytes, cells were infected with adenovirus, which overexpress either Cre or GFP (as control). (A) Representative immunofluorescence staining images and analysis of aceylated tubulin (ace-tubulin) and γ tubulin in the sections of fracture callus of IFT80$^{f/f}$ mice and Col2α1^{cre}IFT80$^{f/f}$ mice collected at D9 post fracture. (B) Representative immunofluorescence staining images and analysis of Ki67 in the sections of fracture callus

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of $IFT80^{f/f}$ mice and $Col2\alpha1^{cre}IFT80^{f/f}$ mice collected at D9 post fracture. The nuclei were labeled with DAPI (blue). (C) qPCR analysis of IFT80 expression in primary chondrocyte culture. (D) Immunofluorescence of chondrocyte stained with anti-acetylated α-tubulin (red) to visualize cilia and quantitative analysis of percentage of ciliated cells and cilia length. (E) Brdu assay was performed to assess the cell proliferation in primary chondrocytes cultures. * $P<0.05$. White bar: 10μm.

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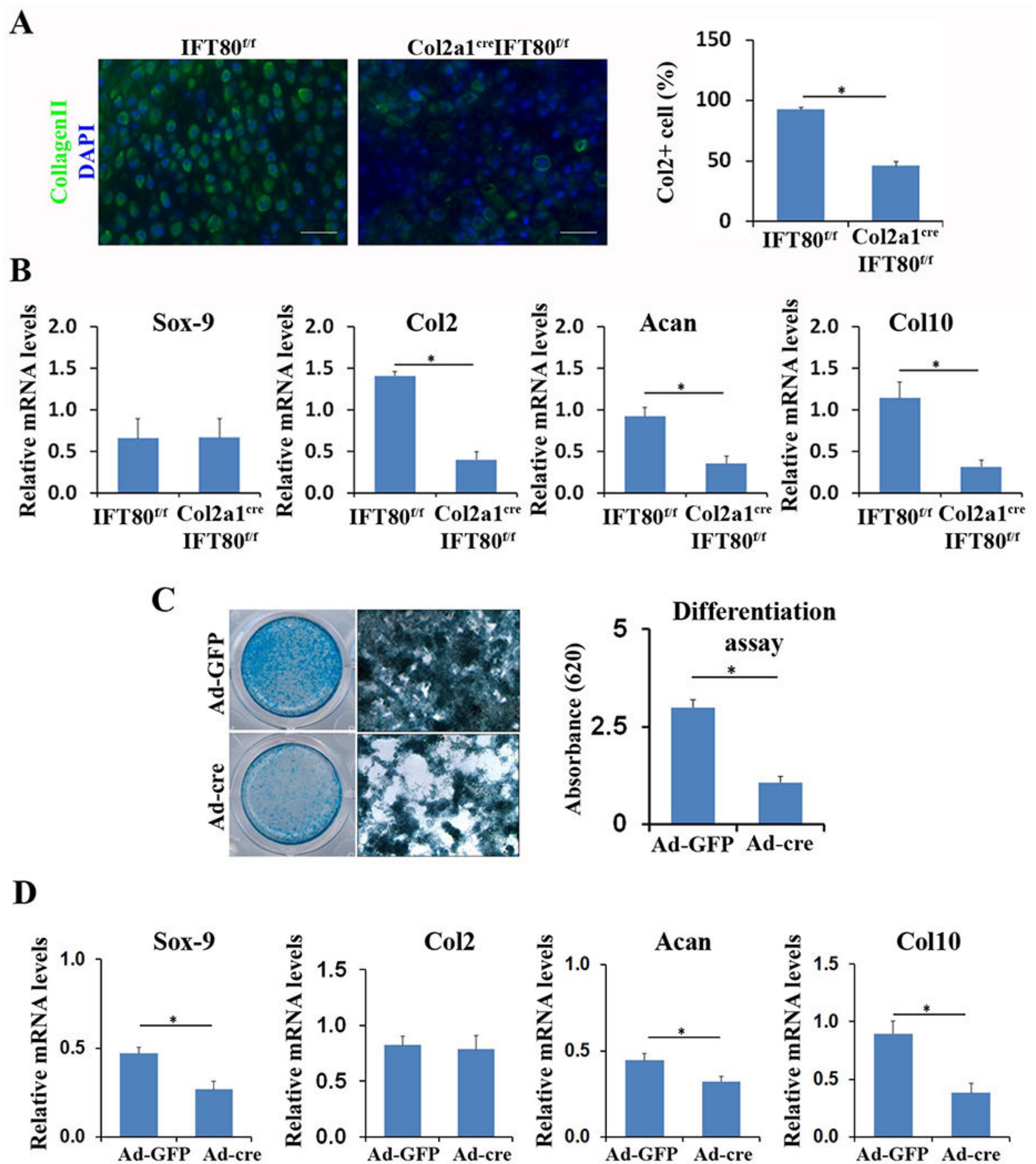

Fig 5:

IFT80 deletion in fracture callus impaired chondrocytes differentiation in fracture callus (A-B) and primary chondrocytes culture (C-E). (A) Representative immunofluorescence staining images and analysis of collagen II in the sections of fracture callus of IFT80$^{f/f}$ mice and Col2α1^{cre}IFT80$^{f/f}$ mice collected at D9 post fracture. The nuclei were labeled with DAPI (blue). (B) qPCR analysis of chondrocytes differentiation markers: Sox-9; Collagen II (Col2); aggrecan (Acan) and Collagen X (Col10) in fracture callus. (C) Proteoglycan production was assessed by Alcian blue staining at day 21 after chondrogenic induction and

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quantitative assessment of Alcian blue staining by measuring the optical density of the dyes extracted by 6 M guanidine-HCl from both groups. (D) qPCR analysis of chondrocytes differentiation markers: Sox-9; Collagen II (Col2); aggrecan (Acan) and Collagen X (Col10) in primary chondrocytes culture. *$p<0.05$ between groups. White bar: 20μm.

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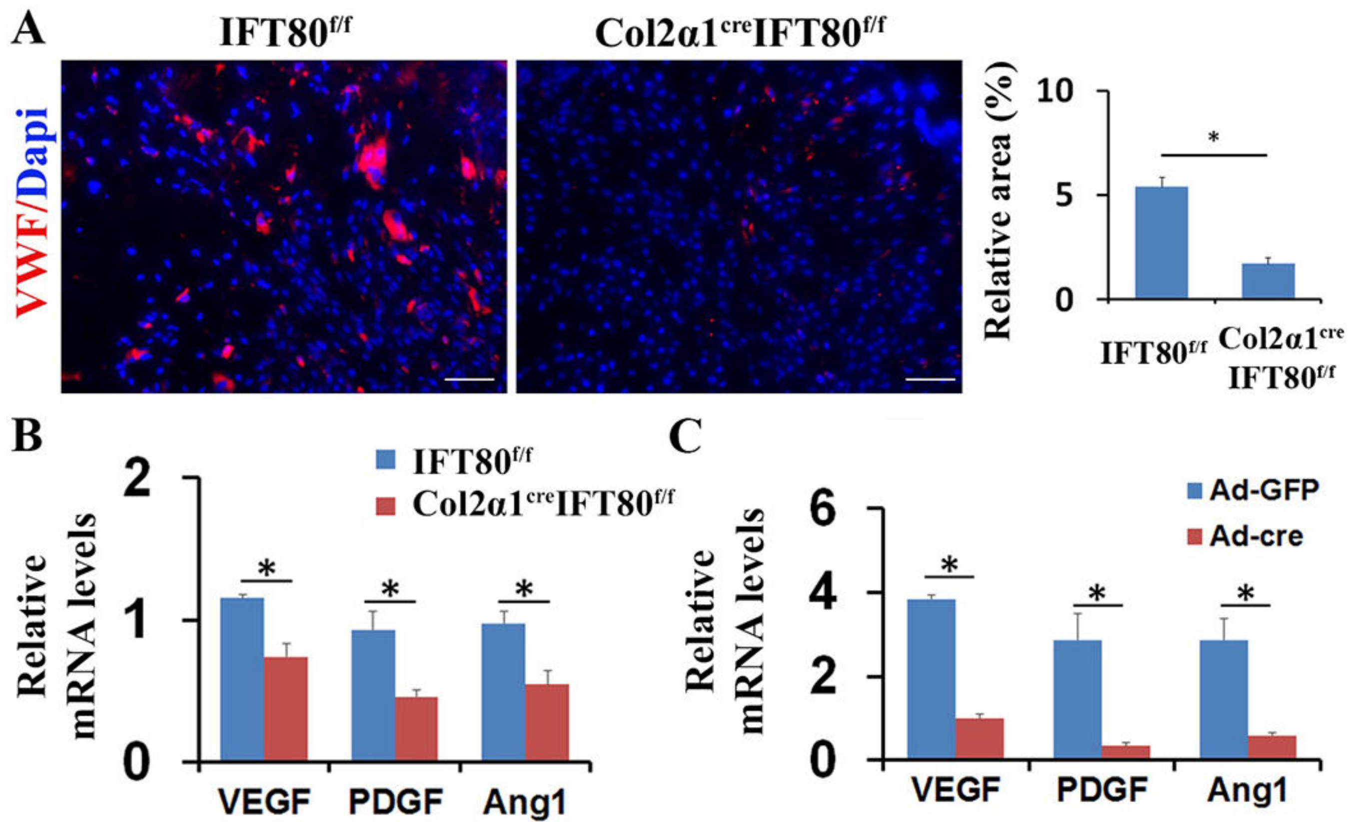

Fig 6:

Loss of IFT80 inhibits angiogenesis in fracture callus. (A) Representative immunofluorescence staining images of von Willebrand Factor (VWF), an endothelial cell marker. (B) qPCR analysis of angiogenesis markers: vascular endothelial growth factor (VEGF); Platelet Derived Growth Factors (PDGF) and angiopoietin 1 (Ang1) in fracture callus. (c) qPCR analysis of angiogenesis markers in micromass cultured primary chondrocytes harvested from IFT80f/f mice at Day3 after switching to differentiation medium treated with Ad-cre (to delete IFT80) or Ad-GFP (control). * $p<0.05$ between groups. White bar: 50μm.

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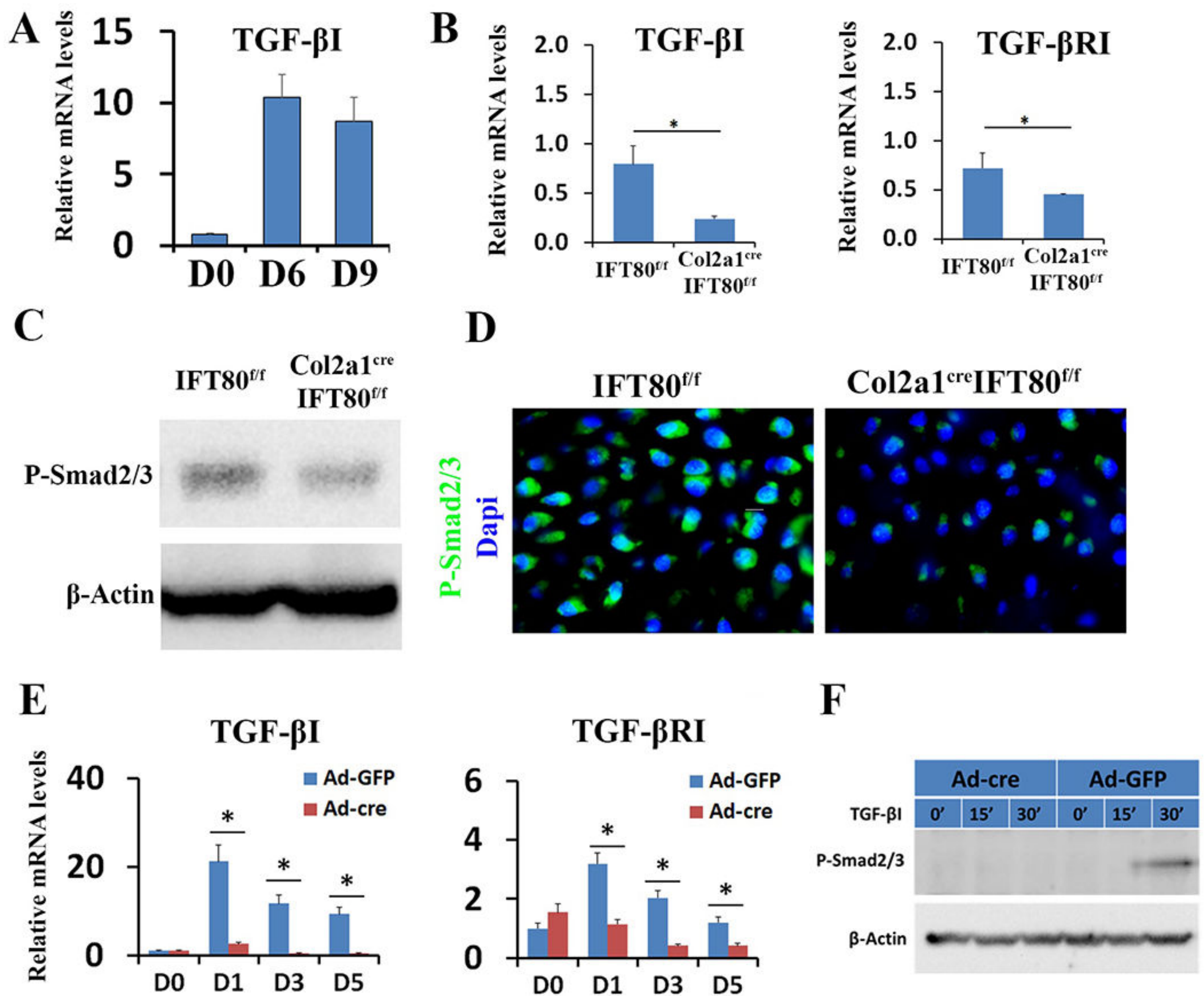

Fig 7:

IFT80 deletion down-regulates TGF-βI /TGF-βR expression and inhibits Smad 2/3 activation in primary chondrocytes and fracture callus. (A) TGFβI gene expression was upregulated after fracture. (B) mRNA expression in fracture callus collected at D9 post femur fracture in both IFT80$^{f/f}$ and Col2α1^{cre}IFT80$^{f/f}$ mice. (C) Western blots for P-Smad2/3 protein levels in fracture callus. (D) IF staining of P-Smad2/3 in fracture callus in both IFT80$^{f/f}$ and Col2α1^{cre}IFT80$^{f/f}$ mice. (E) qPCR analysis of TGF-βI and TGF-βR1 expression in micromass cultured primary chondrocytes harvested from IFT80f/f mice at D1,3 and 5 after switching to differentiation medium treated with Ad-cre (to delete IFT80) or Ad-GFP (control). (F) Western blots of P-samd2/3 protein levels in primary chondrocytes at 15min and 30min after TGF-βI stimulation.

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Table 1:

Primers

Name	Forward	Reverse
IFT80	AAGGAACCAAAGCATCAAGAATTAG	AGATGTCATCAGGCAGCTTGAC
Sox-9	CCTGGACTGTATGTGGATGTG	TAAGGTCTGTCCGATGTCTCT
Collagen II	GCAGAATGGGCAGAGGTATAA	AGTCTGGGTCTTCACAGATAATG
Aggrecan	GTGGAGAGTCTTCTGGCATTAC	CACTGAGTTCCACAGATCCTAAC
Collagen X	CCCTGGTTCATGGGATGTTT	TGGCGTATGGGATGAAGTATTG
Tgfb1	GGTGGTATACTGAGACACCTTG	CCCAAGGAAAGGTAGGTGATAG
Tgfbr1	CCTTGAGTCACTGGGTGTTATG	CCACTTAGCTGTCACCCTAATC