FGF signaling in the osteoprogenitor lineage non-autonomously regulates postnatal chondrocyte proliferation and skeletal growth

Kannan Karuppaiah¹, Kai Yu¹,², Joohyun Lim³, Jianquan Chen³, #, Craig Smith¹, Fanxin Long³, and David M. Ornitz¹*

Departments of ¹Developmental Biology, ³Orthopaedic Surgery, Washington University School of Medicine, St. Louis, MO 63110 USA. ²Division of Craniofacial Medicine, Department of Pediatrics, University of Washington and Center for Developmental Biology and Regenerative Medicine, Seattle Children's Research Institute, Seattle, WA 98101 USA.

#Current address: Orthopedic Institute, Soochow University, China

Key words: FGF Signaling; PTHLH; IHH; Skeletal Development; Endochondral bone formation; Osteoblast; Chondrocyte

*Corresponding author

David M. Ornitz
Washington University School of Medicine,
Department of Developmental Biology
Campus Box 8103,
660 S. Euclid Avenue,
Saint Louis, MO 63110 USA

Tel: 314-362-3908
Fax: 314-362-7058
Email: dornitz@wustl.edu
Fibroblast Growth Factor (FGF) signaling is important for skeletal development; however, cell-specific functions, redundancy, and feedback mechanisms regulating bone growth are poorly understood. FGF receptors 1 and 2 (Fgfr1 and Fgfr2) are both expressed in the osteoprogenitor lineage. Double conditional knockout mice (DCKO) mice, in which both receptors were inactivated using an osteoprogenitor-specific Cre driver, appeared normal at birth; however, DCKO mice showed severe postnatal growth defects that include an ~50% reduction in body weight and bone mass, and impaired longitudinal bone growth. Histological analysis showed reduced cortical and trabecular bone in DCKO mice, suggesting cell autonomous functions of FGF signaling during postnatal bone formation. Surprisingly, DCKO mice also showed growth plate defects and an arrest in chondrocyte proliferation. We provide genetic evidence that revealed a non-cell autonomous feedback pathway regulating Fgf9, Fgf18, and Pthlh expression, which together led to increased expression and signaling of Fgfr3 in growth plate chondrocytes and suppression of chondrocyte proliferation. These observations show that FGF signaling in the osteoprogenitor lineage is obligately coupled to chondrocyte proliferation and the regulation of longitudinal bone growth.

**SUMMARY STATEMENT**

Conditional ablation of Fgfr1 and Fgfr2 in the osteoblast lineage reveals a feed-forward loop controlling FGFR3 signaling and chondrocyte proliferation.
INTRODUCTION

Human genetic disease and conditional gene inactivation experiments in mice have demonstrated essential roles for FGFRs 1 and 2 in development of the appendicular and axial skeleton (Ornitz and Marie, 2002; Ornitz and Marie, 2015; Su et al., 2014). Although both of these receptors are expressed in the osteoprogenitor lineage, redundant functions of these FGFRs and mechanisms that couple FGFR signaling in the osteoprogenitor lineage to chondrogenesis and longitudinal bone growth are not known.

In mice, Fgfr1 has been targeted with T(brachyury)-Cre, Ap2-Cre, Prx1-Cre, Col2a1-Cre, Coll-Cre, OC(osteocalcin)-Cre, and Dmp1-Cre (Jacob et al., 2006; Karolak et al., 2015; Li et al., 2005; Verheyden et al., 2005; Xiao et al., 2014; Yu and Ornitz, 2008; Zhang et al., 2014). With the exception of Col1-Cre, OC-Cre, and Dmp1-Cre, which target relatively late stages of development, inactivation of Fgfr1 was in multiple cell lineages that include condensing mesenchyme, chondrocytes, and osteoprogenitors. Observed phenotypes for Prxl-Cre and T-Cre include impaired limb bud development, increased cell death, and reduced size of mesenchymal condensations (Li et al., 2005; Verheyden et al., 2005; Yu and Ornitz, 2008). Col2a1-Cre targets chondrocytes and osteoblasts, and inactivation of Fgfr1 resulted in an expanded hypertrophic chondrocyte zone (Jacob et al., 2006; Karolak et al., 2015); however, whether this was a cell-autonomous function of FGFR1 in hypertrophic chondrocytes or a non-cell-autonomous effect of inactivation of Fgfr1 in the osteoblast lineage could not be determined from these experiments.

Use of Coll-Cre or OC-Cre to target Fgfr1 in mature osteoblasts resulted in increased bone mass and osteoblast number and no reported effect on bone length (Jacob et al., 2006; Zhang et al., 2014). Use of Dmp1-Cre to target Fgfr1 in osteocytes resulted in decreased osteocyte-specific gene expression but no overt skeletal phenotype (Xiao et al., 2014).
Mice in which the Fgfr2c splice variant has been inactivated (Fgfr2c<sup>−/−</sup>) were viable but showed reduced postnatal growth (Eswarakumar et al., 2002). Fgfr2 has also been conditionally targeted with Dermo1(Twist2)-Cre or suppressed using RNA interference in limb bud mesenchyme. Inactivation of Fgfr2 with Dermo1-Cre, which effectively targets the chondrocyte and osteoblast lineage, also showed that Fgfr2 is necessary for postnatal bone growth (Yu et al., 2003). Suppression of Fgfr2 expression in limb bud mesenchyme in the Ap2-Cre lineage showed that FGFR2 is important for digit and tarsal bone development and ossification (Coumoul et al., 2005). None of the Fgfr2 gene inactivation studies provided a mechanism to explain decreased bone growth.

Fgfr1 and Fgfr2 have considerable overlap in their expression patterns in developing limb bud and bone (Orr-Urtreger et al., 1991; Peters et al., 1992; Yu et al., 2003). Inactivation of Fgfr1 and Fgfr2 in limb mesenchyme with Prx1-Cre resulted in severe skeletal hypoplasia (Yu and Ornitz, 2008). Analysis of phenotypes in distal limb bud mesenchyme identified a role for FGFR signaling in regulating cell survival, but not proliferation (Yu and Ornitz, 2008). The severity of the phenotype in the limb bud precluded analysis of embryonic or postnatal skeletal development.

Fgfr3 is expressed in proliferating and prehypertrophic chondrocytes and functions to inhibit postnatal chondrogenesis (Chen et al., 2001; Havens et al., 2008; Naski et al., 1998; Ornitz and Marie, 2015; Su et al., 2014). Loss of function of FGFR3, either globally or specifically in chondrocytes leads to skeletal overgrowth in mice, sheep, and humans (Beever et al., 2006; Colvin et al., 1996; Deng et al., 1996; Makrythanasis et al., 2014; Ornitz and Marie, 2015; Toydemir et al., 2006; Zhou et al., 2015). The inhibitory activity of FGFR3 on growth plate chondrocytes explains the pathogenic consequences of gain of function mutations in
FGFR3 on suppressing pre-pubertal skeletal growth in Achondroplasia and related chondrodysplastic disorders (Laederich and Horton, 2012; Naski et al., 1998; Naski et al., 1996). The signaling mechanisms by which FGFR3 suppresses chondrogenesis involves activation of STAT1, ERK1/2, and p38, increased expression of Snail1 (Snai1), decreased expression of AKT, and activation of protein phosphatase 2a (PP2a), which dephosphorylates (activates) the retinoblastoma family members, p107 and p130. Activation of p107 (and p130) and increased expression of the cell cycle inhibitor, p21\textsuperscript{Waf1/Cip1} function to directly suppress chondrocyte proliferation (Aikawa et al., 2001; Cobrinik et al., 1996; Dailey et al., 2003; de Frutos et al., 2007; Kolupaeva et al., 2013; Kolupaeva et al., 2008; Kurimchak et al., 2013; Laplantine et al., 2002; Legeai-Mallet et al., 2004; Priore et al., 2006; Raucci et al., 2004; Su et al., 1997).

Although much is known about signals downstream of FGFR3 in chondrocytes, the mechanisms that regulate FGFR3 expression and activation and coordinate osteogenesis and chondrogenesis are poorly understood.

Here we investigate cell-autonomous FGFR1 and FGFR2 signaling in the osteoprogenitor lineage. We show that inactivation of FGFR1 and FGFR2 with Osx-Cre (Rodda and McMahon, 2006) results in decreased bone mass. Unexpectedly, we found that loss of FGFR1/2 in the osteoprogenitor lineage has a profound effect on chondrogenesis and postnatal longitudinal bone growth. The mechanism by which osteoprogenitor FGFR1/2 signaling regulates chondrogenesis involves activation of FGFR3 expression and signaling in chondrocytes through reduction in the expression of parathyroid hormone related peptide (Pthlh) and increased expression of Fgf9 and Fgf18, ligands that normally regulate postnatal endochondral bone growth.
RESULTS

Postnatal growth defects in mice lacking Fgfr1 and Fgfr2 in the osteoprogenitor lineage

Fgfr1 and Fgfr2 are expressed in the perichondrium and periosteum during skeletal development (Yu et al., 2003). Both FGFR1 and FGFR2 have similar in vitro signaling potency and ligand response profiles to FGF9 and FGF18 (Zhang et al., 2006), ligands that have key roles in regulating skeletal development (Hung et al., 2016; Hung et al., 2007; Liu et al., 2007; Liu et al., 2002; Ohbayashi et al., 2002). In several tissues, including the limb bud, palate, lung, kidney, liver, cerebellum, epidermis, and inner ear, Fgfr1 and Fgfr2 show significant functional redundancy (Bohm et al., 2010; Huh et al., 2015; Meyer et al., 2012; Ornitz and Itoh, 2015; Poladia et al., 2006; Sims-Lucas et al., 2011; Smith et al., 2012; White et al., 2006; Yang et al., 2010; Yu et al., 2015; Yu and Ornitz, 2008). To study the roles of FGFR signaling in the osteoprogenitor-lineage the Osx-GFP::Cre (Osx-Cre) allele was crossed to floxed alleles of Fgfr1 and Fgfr2 (Rodda and McMahon, 2006; Trokovic et al., 2003; Yu et al., 2003). Osx-Cre efficiently targets the osteoprogenitor lineage (trabecular bone and cortical bone), bone marrow stroma, a small percentage of chondrocytes, and some other non-skeletal cell types (Chen et al., 2014a; Rodda and McMahon, 2006).

Osx-Cre;Fgfr1f/f;Fgfr2f/f double conditional knockout (Osx-Cre;DCKO), Fgfr1f/f;Fgfr2f/f double floxed control (DFF), and Osx-Cre control mice appeared normal at birth. Body weight was not significantly different between Osx-Cre;DCKO, DFF, and Osx-Cre control mice before postnatal day 4 (P4) (Fig. 1A, supplementary material Fig. S1A). Inactivation of Fgfr1 and Fgfr2 in the Osx-Cre lineage was confirmed by quantitative PCR evaluation of mRNA isolated from cortical bone from P21 DFF and Osx-Cre;DCKO mice (supplementary material Fig. S2). Histological evaluation of embryonic day 18.5 (E18.5) Osx-Cre;DCKO proximal tibia showed an increased height of the hypertrophic chondrocyte zone and narrowing of the growth plate and
diaphysis, but no other changes in cortical, trabecular, or growth plate histology (Fig. 1B). Furthermore, bone architecture of Osx-Cre;DCKO mice, as determined by Alizarin-Red and Alcian Blue staining of P0 skeletons also showed slightly narrowed long bones, but normal mineralized regions and cartilaginous growth plates (Fig. 1C).

Osx-Cre;DCKO mice failed to gain normal body weight when compared to DFF or Osx-Cre control mice. This growth defect became statistically significant (p<0.05) after P4 (Fig. 1A). By three weeks of age, DCKO mice were approximately half normal size but otherwise healthy (Fig. 1A, D). Because Osx-Cre is active in some non-skeletal lineages, including stromal cells, adipocytes, perivascular cells in the bone marrow, olfactory glomerular cells, and a subset of gastric and intestinal epithelial cells (Chen et al., 2014a), we questioned whether inactivation of Fgfr1 and Fgfr2 with Osx-Cre could influence growth by affecting the nutritional or hormonal status of the mice. Analysis of bone density and total body fat content, using Dual Energy X-Ray Absorptiometry (DEXA), showed a 29±2% (n=4, p<0.01) decrease in bone mineral content in Osx-Cre;DCKO compared to DFF mice, but no significant change in body fat content (Fig. 1E). Additionally, litters were placed on a high fat, high calorie diet at birth until 5 weeks of age. On this diet, Osx-Cre;DCKO and DFF mice both showed an elevated (19±1%, n=4, p<0.05) body fat content but Osx-Cre;DCKO mice still showed a decrease in bone mineral content (30±1%, n=4, p<0.01). We conclude that the growth defect in Osx-Cre;DCKO mice is most likely a consequence of impaired FGF signaling in Osx-Cre targeted cell lineages within skeletal tissue and not a consequence of extrinsic hormonal or nutritional changes.

The Osx-Cre allele, by itself, has been reported to have variable effects on skeletal growth that could depend on genetic background (Huang and Olsen, 2015; Wang et al., 2015). To evaluate a potential contribution of the Osx-Cre allele in the mixed C57BL/6J; 129X1
background used in these studies, wild type hybrid mice were compared to littermate *Osx-Cre* mice by following growth and by endpoint skeletal micro CT and histological analysis. Growth curves for wild type and *Osx-Cre* mice revealed a slight delay in *Osx-Cre* mice at P30 that normalized after P36 (supplementary material Fig. S1A). Micro CT analysis of cortical and trabecular bone showed no significant difference in the bone volume to total volume (BV/TV) ratio or bone mineral density (BMD) between P21 wild type and *Osx-Cre* mice (supplementary material Fig. S1B, C). Growth plate histology and *Fgfr3* expression at P21 were also similar between wild type and *Osx-Cre* mice (supplementary material Fig. S1D, E). These studies show that the *Osx-Cre* allele has a minimal effect on bone growth on the genetic background used in these studies.

**Decreased bone formation in *Osx-Cre;DCKO* mice**

Radiographic analysis of intact skeletons of three-month-old mice revealed that *Osx-Cre;DCKO* mice had shorter bones and reduced bone density compared to control mice (Fig. 1F). The overall shape of the bones was normal. MicroCT analysis of intact long bones (femur, tibia) revealed that the *Osx-Cre;DCKO* mice had reduced trabecular and cortical bone (Fig. 1G). This was reflected in a significantly reduced trabecular and cortical BV/TV ratio and BMD (Fig. 1H). Consistent with the microCT analysis, von Kossa-stained histological sections of P21 tibia revealed a reduced area of mineralized cortical bone, trabecular bone (primary spongiosa), and secondary ossification centers in *Osx-Cre;DCKO* mice (Fig. 2A). Although *Osx-Cre;DCKO* mice clearly have less mineralized trabecular and cortical bone and thus decreased numbers of osteoblasts, histological analysis of the trabecular region revealed normal osteoblast density and similar intensity of type I collagen (*Col1*) expression in osteoblasts (Fig. 2B, C). Consistent with this, histomorphometric analysis revealed a normal number of osteoblasts (N.Ob) and osteoblast surface area (Ob.S), when normalized to bone surface area (Fig. 2D).
**Decreased growth plate size in Osx-Cre;DCKO mice**

Growth plate histology of P21 Osx-Cre;DCKO mice compared to DFF controls showed a significant decrease in the overall length of the growth plate (GP) and the length of the proliferating (columnar) chondrocyte zone (p) (24% and 36%, p< 0.02, respectively) (Fig. 3A,B). At this stage of postnatal development, the hypertrophic chondrocyte zone (h), which was expanded at E18.5, was not significantly different from controls. Normalization of the length of the hypertrophic zone could be due to compensatory changes in the number of available input cells (assessed by chondrocyte proliferation) and changes in distal loss of hypertrophic chondrocytes through apoptosis, degradation of the extracellular matrix, or differentiation into trabecular osteoblasts.

Chondrocyte proliferation in P21 mice was evaluated by BrdU labeling. Osx-Cre;DCKO mice showed a 58% reduction in chondrocyte proliferation (Fig. 3C,D). Cell death, evaluated by expression of activated caspase 3 immunostaining was decreased in distal hypertrophic chondrocytes in Osx-Cre;DCKO mice (supplementary material Fig. S3A,B), and matrix degradation potential, evaluated by measuring osteoclast number (N.Oc) and osteoclast surface (Oc.S) per bone surface area was not significantly different in DFF and Osx-Cre;DCKO mice (Fig. 3E, F). Collectively, these data suggest that normalization of the hypertrophic chondrocyte zone in P21 Osx-Cre;DCKO mice results from both decreased chondrocyte proliferation that is partially compensated for by decreased cell death in distal hypertrophic chondrocytes.

**Decreased chondrocyte proliferation is due to effects of non cell-autonomous loss of Fgfr1 and Fgfr2.**

Given that Osx-Cre targets a small percentage of prehypertrophic and hypertrophic chondrocytes (Chen et al., 2014a) and Fgfr1 is expressed in hypertrophic chondrocytes, it was necessary to determine if inactivation of Fgfr1 (and Fgfr2) in growth plate chondrocytes could
contribute to the observed decrease in chondrocyte proliferation. The aggrecan enhancer-driven, tetracycline-inducible Cre (ATC) transgene allele, which efficiently targets proliferating and hypertrophic chondrocytes during embryonic development (Dy et al., 2012), was used to inactivate floxed alleles of Fgfr1 and Fgfr2. Female mice carrying ATC;Fgfr1<sup>F/f</sup>;Fgfr2<sup>F/f</sup> (ATC;DCKO) embryos were placed on doxycycline throughout gestation and pups were maintained on doxycycline until P21. In situ hybridization shows Fgfr1 expression in hypertrophic chondrocytes in DFF control mice and decreased expression in ATC;DCKO mice (Fig. 3G). PCR analysis of isolated growth plates from P21 mice demonstrated inactivation of Fgfr1 (Fig. 3H). However, at P21, DFF control mice and ATC;DCKO mice were of similar weight, and showed no difference in growth plate histology (Fig. 3I), or chondrocyte proliferation (Fig. 3J,K). We conclude from these data that FGFR1 (and FGFR2, which is not expressed in chondrocytes) does not have a major cell-autonomous impact on embryonic or postnatal chondrogenesis.

**Increased expression of Fgf9 and Fgf18 in Osx-Cre;DCKO mice**

We hypothesized that inactivation of Fgfr1 and Fgfr2 in the Osx-Cre lineage could lead to a compensatory upregulation of Fgf9 or Fgf18, ligands that are each necessary for normal embryonic skeletal development (Hung et al., 2007; Liu et al., 2007; Liu et al., 2002; Ohbayashi et al., 2002) and together display marked redundancy in skeletal development (Hung et al., 2016). Because FGF9 and FGF18 are also thought to function as ligands that signal to FGFR3 during postnatal bone growth to negatively regulate chondrocyte proliferation, compensatory upregulation of Fgf9 or Fgf18 expression due to loss of FGFR1/2 signaling in the osteoprogenitor lineage could aberrantly activate FGFR3 in the growth plate and suppress chondrocyte proliferation. To test this hypothesis, we performed in situ hybridization analysis of paraffin fixed intact bone tissues and qRT-PCR on distal bone tissue from DFF and Osx-
Cre;DCKO mice. In situ analysis revealed that Fgf9 expression was induced in perichondrial tissue, adjacent connective tissue, reserve, proliferating, and prehypertrophic chondrocytes of Osx-Cre;DCKO mice (Fig. 4A). Consistent with in situ expression, qRT-PCR analysis of distal bone tissue showed a ~3.5 fold increased Fgf9 expression in tissue from Osx-Cre;DCKO compared to DFF mice (Fig. 4B). Analysis of the expression pattern of Fgf18 by in situ hybridization showed increased expression in reserve, proliferating, and prehypertrophic chondrocytes in Osx-Cre;DCKO compared to DFF mice (Fig. 4C). Consistent with these data, qRT-PCR showed a ~1.5 fold increase in Fgf18 expression in Osx-Cre;DCKO compared to DFF distal bone tissue (Fig. 4D).

Increased Fgfr3 expression and signaling Osx-Cre;DCKO growth plate

Evaluation of Fgfr3 expression by in situ hybridization revealed a striking increase in expression in Osx-Cre;DCKO compared to DFF mice in both proliferating and prehypertrophic chondrocytes (Fig. 5A). This increase was confirmed by qRT-PCR analysis of distal bone tissue from P21 distal femur and proximal tibia (Fig. 5B). The Snail1 transcription factor is induced by FGFR3 and is required for the activation of both the STAT1 and MAPK branches of the FGFR3 signaling pathway (de Frutos et al., 2007). Consistent with increased FGFR3 expression and signaling, Snail1 expression was highly increased in Osx-Cre;DCKO compared to DFF mice (Fig. 5C). Immunostaining for the chondrocyte-specific transcription factor, SOX9, showed mildly elevated levels of expression in Osx-Cre;DCKO compared to DFF mice (Fig. 5D).

Activation of FGF9 in the perichondrium suppresses chondrocyte proliferation.

The ability of FGF9 to signal from perichondrial tissue to growth plate chondrocytes has been inferred from phenotypes seen in Fgf9−/− embryos (Hung et al., 2007). Additionally, transgenic mice that overexpressed FGF9 in chondrocytes (Col2a1-Fgf9) showed short limbs and a smaller growth plate and died by 5 weeks of age (Garofalo et al., 1999). However, whether
FGF9 has the capacity to signal from periosteal and trabecular osteoblasts to growth plate chondrocytes during prepubertal growth was not known. To conditionally overexpress Fgf9 in periosteal and trabecular osteoblasts, Runx2-rtTA (Chen et al., 2014b) and TRE-Fgf9-ires-eGFP (White et al., 2006) transgenic mice were mated to generate biallelic Runx2-rtTA;TRE-Fgf9-ires-eGFP (RunxTFG) mice. In the presence of doxycycline, GFP fluorescence was observed in the perichondrium, periosteum, and trabecular bone of RunxTFG mice, but not in proliferating or hypertrophic chondrocytes (Fig. 6A). Compared to control (single transgenic mouse), RunxTFG transgenic mice showed a significantly (p<0.01) reduced body weight at P21 (Fig. 6B). Growth plate histology revealed that compared to control, RunxTFG transgenic mice had significantly (p<0.01) smaller proliferating and hypertrophic chondrocyte zones compared to controls (Fig. 6C, D). The height of the trabecular zone in RunxTFG transgenic mice was reduced, but otherwise histologically normal, and osteoclast numbers and morphology appeared normal (Fig. 6C, E). Most notably, chondrocyte proliferation was significantly (p<0.001) reduced in RunxTFG transgenic mice compared to control mice (Fig. 6F, G). Finally, in situ hybridization revealed that activation of Fgf9 in the perichondrium/periosteum and trabecular bone induced the expression of Fgfr3 in proliferating chondrocytes (Fig. 6H).

**PTHLH links Osx-Cre lineage FGFR1/2 signaling to Fgfr3 expression and chondrocyte proliferation in the postnatal growth plate.**

Indian hedgehog (IHH) and parathyroid hormone-related peptide (PTHLH) are critical regulators of endochondral bone growth (Kozhemyakina et al., 2015; Long and Ornitz, 2013). IHH stimulates chondrocyte proliferation and Pthlh expression, while PTHLH suppresses chondrocyte maturation and Ihh expression. Because we have observed apparent non-cell autonomous effects of loss of Osx-Cre lineage FGFR1 and FGFR2 on chondrocyte growth, it was important to examine the potential activity of other signaling pathways that regulate growth
plate function. Compared to controls, \textit{Ihh} was decreased in the P21 growth plate of \textit{Osx-Cre;DCKO} mice (Fig. 7A,B). Interestingly, we found that \textit{Pthlh} expression was also reduced in reserve chondrocytes in \textit{Osx-Cre;DCKO} mice (Fig. 7C). Quantitative RT-PCR analysis of distal bone tissue showed an overall reduction in \textit{Pthlh} mRNA (Fig. 7D). Consistent with FGFR3 signaling suppressing \textit{Ihh-Pthlh} expression (Chen et al., 2001; Li et al., 2010; Minina et al., 2002; Naski et al., 1998), in mice induced to overexpress \textit{Fgf9}, expression of \textit{Pthlh} was reduced in reserve zone chondrocytes (Fig. 7E).

Analysis of \textit{Fgfr3} promoter function \textit{in vitro} shows that \textit{Fgfr3} expression could be directly regulated (suppressed) by PTHLH activation of Protein Kinase A (PKA) (McEwen et al., 1999). To test whether PTH signaling could suppress \textit{Fgfr3} expression \textit{in vivo} in \textit{Osx-Cre;DCKO} mice that highly overexpress \textit{Fgfr3}, \textit{Osx-Cre;DCKO} mice were injected intermittently (daily) with PTH from P15 to P21, a treatment regimen known to stimulate the anabolic effects of PTH signaling on bone (Esen et al., 2015; Xie et al., 2012). Compared to control \textit{Osx-Cre;DCKO} mice that were only injected with PBS, PTH injected \textit{Osx-Cre;DCKO} mice showed an increase in the size of the growth plate, increased thickness of trabecular bone, decreased expression of \textit{Fgfr3}, and increased chondrocyte proliferation (Fig. 7F-J).
DISCUSSION

The growth plate is a transient component of developing endochondral bone that mediates longitudinal bone growth from late stages of embryonic development through puberty (Hunziker and Schenk, 1989; Noonan et al., 1998). FGFR3 is a well-established negative regulator of postnatal bone growth, functioning in the growth plate in proliferating and prehypertrophic chondrocytes. Activating mutations in FGFR3 are responsible for Achondroplasia, the most common form of dwarfism in humans (Horton et al., 2007; Ornitz and Marie, 2015). As signaling pathways that function downstream of FGFR3 are well established,(Ornitz and Itoh, 2015) the identification of non-cell autonomous mechanisms that regulate FGFR3 expression and signaling and postnatal growth plate function are essential for further elucidating complex regulatory networks that control endochondral bone formation.

Inactivation of Fgfr1 and Fgfr2 in the Osx-Cre lineage disrupted a non cell-autonomous feedback loop resulting in activation of FGFR3 signaling in growth plate chondrocytes and suppression of chondrocyte proliferation and longitudinal bone growth (Fig. 8). The precise cell-type(s) that maintains this feedback loop is not known; however, it is likely to be an immature osteoprogenitor, as similar phenotypes are not observed when Fgfr1 and Fgfr2 are inactivated in mature osteoblasts with the Osteocalcin-Cre allele (unpublished data). A likely early event eliciting this phenotype is increased expression of Fgf9 in osteoprogenitor cells in the perichondrium, resulting in increased signaling through FGFR3 in adjacent chondrocytes. Activation of FGFR3 inhibits Ihh expression and signaling in prehypertrophic chondrocytes (Naski et al., 1998), a factor that is required to maintain Pthlh expression in reserve and articular chondrocytes (Hilton et al., 2005; Koziel et al., 2005; St-Jacques et al., 1999; Vortkamp et al., 1996). Propagating events include increased Fgfr3 expression and signaling in the growth plate, which may further suppress Ihh and Pthlh and increase Fgf9 and Fgf18 expression. Such non
cell-autonomous cellular signaling pathways thus coordinate osteoprogenitor development and longitudinal bone growth.

**FGFR1/2 function in the osteoprogenitor lineage.**

Although FGFR1 and FGFR2 signaling have robust functions in limb bud mesenchyme, their function in the osteoprogenitor lineage during embryonic development is surprisingly mild. Osx-Cre;DCKO mice were born alive and showed no patterning defects in the appendicular skeleton. However, Osx-Cre;DCKO mice exhibited a calvarial ossification defect at birth (data not shown) and a postnatal reduction in cortical bone growth that indicates that osteoprogenitor lineage FGFR signaling is required for osteoblast growth and maturation that is independent of chondrogenesis. The precise role of FGFR signaling in osteoblasts will require further investigation.

**FGFR signaling in osteoprogenitor cells indirectly affects growth plate activity.**

The most striking feature of Osx-Cre;DCKO mice was the profound reduction in chondrocyte proliferation and longitudinal bone growth. We posited that this phenotype resulted from non-cell autonomous changes in chondrocytes that are secondary to loss of FGFR1 and FGFR2 signaling in osteoprogenitor cells. Because Osx-Cre targets a small percentage of chondrocytes (Chen et al., 2014a), there remained a possibility that the observed phenotype could result from inactivation of Fgfr1 and Fgfr2 in chondrocytes. However, this is unlikely because Fgfr1 expression is restricted to hypertrophic chondrocytes and Fgfr2 is not expressed in proliferating or hypertrophic chondrocytes. Nevertheless, to rule out cell autonomous effects of FGFR1 and FGFR2 in chondrocytes, these genes were inactivated specifically in chondrocytes using the ATC-Cre allele. The normal development of ATC-Cre;DCKO mice demonstrated that inactivation of Fgfr1 and Fgfr2 in proliferating and hypertrophic chondrocytes does not significantly affect chondrogenesis or prepubertal longitudinal bone growth.
A second feature of the Osx-Cre;DCKO phenotype was the prominent increase in Fgfr3 expression in proliferating and hypertrophic chondrocytes. In vitro analysis of the Fgfr3 promoter identified a regulatory sequence that results in decreased promoter activity in response to cAMP (McEwen et al., 1999). This in vitro data suggested that the observed decrease in Pthlh expression could contribute to increased Fgfr3 expression. In support of this model, intermittent injection of Osx-Cre;DCKO mice with PTH(1-34) peptide suppressed Fgfr3 expression in chondrocytes and increased chondrocyte proliferation (Fig. 8).

A third feature of the Osx-Cre;DCKO phenotype was reduced bone volume and density. This could result from cell-autonomous effects of FGFR signaling in osteoblasts, or to the reduced levels of Pthlh expression. Haploinsufficiency of Pthlh results in osteopenia in mice (Miao et al., 2005), with similar morphologies to that observed in Osx-Cre;DCKO mice.

**Regulation of embryonic vs. postnatal growth plate.**

The experiments presented here focus on the postnatal growth plate of 21 day old Osx-Cre;DCKO mice. Although, the Osx-Cre allele used to target Fgfr1 and Fgfr2 is active as early as embryonic day 12.5 (Ono et al., 2014; Rodda and McMahon, 2006), the extent of the embryonic phenotype appears to be limited to expansion of the hypertrophic chondrocyte zone, similar to the phenotype observed when the Col2-Cre allele was used to inactivate Fgfr1 (Jacob et al., 2006). Thus, FGFR1/2 signaling either does not have a major role in the osteoprogenitor lineage prior to establishment of a secondary ossification center and formation of a mature growth plate or the non cell-autonomous mechanism that we identified is not activated during embryonic development. Most studies investigating skeletal development focus on the embryonic growth plate. However, the postnatal growth plate is the developmental structure that accounts for the majority of organismal skeletal growth and, yet, gene expression patterns and
the molecular and cellular mechanisms that regulate the postnatal growth plate are poorly defined.

In the embryonic growth plate IHH is involved in a feedback loop that regulates Pthlh expression in the distal periarticular perichondrium (Kronenberg, 2003). However, in postnatal bone, there is a reorganization of the growth plate, and Pthlh expression shifts to reserve zone chondrocytes and IHH signaling (Gli1) and Pth1r expression remains prominent in reserve/proliferating and prehypertrophic chondrocytes, respectively (Chau et al., 2011; Chen et al., 2008; Koziel et al., 2004). Thus, in the postnatal growth plate, PTHLH and IHH responsive cells overlap with Fgfr3 expression patterns.

Loss of FGFR1/2 signaling in perichondrial and osteoprogenitor cells may disrupt growth plate homeostasis by initially triggering increased expression of FGF9 and FGF18. We posit that these events lead to increased FGFR3 expression and signaling (modeled by forced expression of Fgf9 in perichondrial cells and osteoblasts). Secondarily, increased FGFR3 signaling could suppress Ihh expression and signaling and lead to suppression of Pthlh in chondrocytes, leading to an aberrant feed forward signal that further increases the expression of Fgfr3 (Fig. 8). The ability to block this feed forward loop by administration of PTH(1-34) supports a model in which PTHLH regulates communication between osteoprogenitors, chondroprogenitors, and growth plate chondrocytes in a mature postnatal growth plate.

Termination of skeletal growth.

Osx-Cre;DCKO mice show increased expression of Fgf9 and Fgf18 in reserve, proliferating, and prehypertrophic chondrocytes and in cells at the periphery of the growth plate that may include chondroprogenitors in the groove of Ranvier. This may represent an amplification of a normal feed forward induction of Fgf9 and Fgf18 that could function to
permanently suppress growth plate chondrocyte proliferation at puberty and suppress articular chondrocyte proliferation and differentiation in adults. This model is consistent with continued expression of endogenous Fgf18 in the postnatal growth plate and perichondrium, and in adult articular chondrocytes (Ellsworth et al., 2002; Lazarus et al., 2007; Mori et al., 2014).
MATERIALS AND METHODS

Mice

Mice were housed in a pathogen-free facility and handled in accordance with standard use protocols, animal welfare regulations, and the NIH Guide for the Care and Use of Laboratory Animals. All protocols were approved by the Washington University Animal Studies Committee. Osx-GFP::Cre (Osx-Cre) (Rodda and McMahon, 2006), Fgfr1<sup>f/f</sup> (Trokovic et al., 2003), Fgfr2<sup>f/f</sup> (Yu et al., 2003), aggrecan enhancer-driven, tetracycline-inducible Cre (ATC) (Dy et al., 2012), Runx2-rtTA (Chen et al., 2014b), TRE-Fgf9-ires-eGFP (White et al., 2006) have been previously described.

Homozygous floxed alleles of Fgfr1 and Fgfr2 are maintained as double flox mice (DFF) and are outbred to hybrid C57BL/6J;129X1 mice every second generation and then backcrossed to homozygosity. Double conditional knockout (DCKO) breeding males (Osx-Cre;Fgfr1<sup>f/f</sup>;Fgfr2<sup>f/f</sup>) were generated by crossing Osx-Cre mice with DFF mice, backcrossing to DFF and suppressing the Cre activity of Osx-Cre with doxycycline. To inactivate Fgfr1/2 in the osteoprogenitor-lineage, DFF female mice were crossed with Osx-Cre;DCKO breeder male mice resulting in an 50% yield of Osx-Cre;DCKO mice and DFF controls. Osx-Cre control mice were generated by crossing Osx-Cre;DCKO mice or Osx-Cre mice to wild type hybrid mice. A similar breeding strategy was used to generate ATC;DCKO mice. To express Fgf9 in the osteoblasts lineage, Runx2-rtTA mice (Chen et al., 2014b) were crossed to TRE-Fgf9-ires-Gfp (White et al., 2006) to generate RunxTFG double transgenic mice. Females were induced with doxycycline chow (Bio-Serv Inc. 200 mg/kg green pellets, S3888)) from E0 to P21. High fat high calorie diet included breeder chow (PicoLab® Mouse. Diet 20) supplemented with Nutri-Cal® (Patterson Veterinary Supply) from birth to 5 wk of age.
Body weights were measured on multiple litters two to three times per week until animals were sacrificed for analysis. Growth curves represent cumulative pooled data from multiple litters and overlapping time points covering the entire time course.

**Histology, Immunohistochemistry, and Immunofluorescence**

For histological analysis of long bones, intact femur-tibia were isolated, fixed in 4% PFA/PBS overnight at 4°C or fixed in 10% buffered formalin overnight at room temperature. Bones were rinsed in water several times and decalcified in 14% EDTA/PBS for two weeks. Paraffin embedded tissue section (5 µm) were stained with hematoxylin and eosin (H&E), tartrate resistant acid phosphatase (TRAP), VonKossa, or Alizarin-Red.

For immunohistochemistry, paraffin sections or cryo-sections were rehydrated and treated with 0.3% hydrogen peroxide in methanol for 15 min to suppress endogenous peroxidase activity. Antigen retrieval was achieved by microwaving the sections in 10 mM citrate buffer for 10 min followed by gradual cooling to room temperature. Sections were incubated overnight at 4°C with the following primary antibodies: SOX9 (Millipore, AB5535, rabbit polyclonal, 1:100), anti-active Caspase -3 (BD Pharmingen, #559565, 1:100). Fluorescently labeled Alexa Fluor 488 donkey anti-rabbit (Life Technologies, A-21206, 1:1000). Colorimetric detection used the ABC kit (Invitrogen #95-9943). Immunofluorescence imaging was performed on a Zeiss Apotome Fluorescence Microscope. Data is representative of at least three independent experiments.

For in situ hybridization analysis, tissues were fixed and decalcified at 4°C. For frozen sections, the tissues were fixed as described above and decalcified for 3 days, transferred to 30% sucrose (S0389, SIGMA) for 24 hr, embedded in OCT compound, sectioned at 5 µm, and stored at -20 until analysis. Non-radioactive in situ hybridization was performed as previously described (Naski et al., 1998). In situ probes: Fgf9 (Colvin et al., 1999); Fgf18 (Liu et al., 2002);
Fgf3 (Peters et al., 1993); Snail1 (Vega et al., 2004); Pthlh (Lee et al., 1996; Long et al., 2001); Ihh (Bitgood and McMahon, 1995); Coll (Rossert et al., 1995). Data is representative of at least three independent experiments.

Cell proliferation was determined by injecting U (5-Bromo-2’ deoxyuridine, SIGMA, 9285) 0.1 mg/gram body weight 2 hr before tissues were harvested. Anti BrdU mouse monoclonal (BD Biosciences, #347580) was used at a 1:200 dilution. BrdU labeling was normalized to the total number of cells in the proliferating zone or to the area of the proliferating zone. Data were then normalized to that of DFF control mice. At least three mice and 2-3 sections per mouse were analyzed for each genotype.

**Histomorphometry**

H&E and TRAP stained sections were used for quantification of osteoblast and osteoclast number and surface, using BioQuant OSTEO 2010 software. Measurements of growth plate length in H&E stained sections were made using Canvas X software (ACD systems). All lengths were normalized to the total length of the DFF control growth plate. Statistical analysis (Student’s t test) was based on measurements of tissue samples from at least three control and three experimental mice.

**MicroCT and DEXA analysis**

For microCT, intact long bones were isolated and fixed in 70% ethanol overnight at 4°C and then stored at -20°C until analysis. Bones were embedded in 1.5% agarose and scanned (µCT40, Scanco Medical). MicroCT analysis of trabecular and cortical bone was performed as follows: for trabecular bone, 100 to 150 sections were selected below the growth plate for reconstruction and quantification. For cortical bone quantification, 50 to 100 sections were selected from the mid diaphysis of the femur or tibia. Quantification was performed using
SCANCO Medical microCT systems software. Dual-Energy X-Ray Absorptiometry (DEXA, GE/Lunar PIXImus) was used for measurements of whole body bone density and body fat content. Data is representative of at least three mice per genotype.

**Real time quantitative PCR**

Distal bone, containing the growth plate, perichondrium, and trabecular bone was dissected. Immediately after isolation, the tissues were individually frozen in liquid nitrogen and stored at -80°C until analysis. Frozen tissues were pulverized in a dry ice cooled stainless steel flask with a ball bearing in a Micro Dismembrator (Sartorius) at 2000 RPM for 20 seconds. RNA was stabilized with Trisol (TRIsol Reagent, Ambion/RNA) and total RNA isolation was prepared according to the manufacturer’s instructions. cDNA was synthesized using the iScript™Select cDNA synthesis Kit (#170-8841, BIO-RAD). mRNA expression was measured using TaqMan™ Fast Advanced Master Mix (#4444557, Life Technologies) and TaqMan™ assay probes for Ihh, Pthlh, Fgf9, Fgf18, and Fgfr3. Hprt was used a normalization control.

**PTH treatment**

For in vivo treatment of mice with PTH, 15 day-old Osx-Cre;DCKO mice were injected i.p. once per day (morning) with synthetic PTH-related peptide (I-34) (H-6630, Bachem) at a concentration of 80 µg/kg body weight or with PBS (control). Mice were injected for five days and then sacrificed at P21.

**Statistics**

The data are reported as the mean ± SD and changes with p values less than 0.05 were considered to be statistically significant. Data was analyzed using a two-tailed Student’s *t*-test. Numbers of mice used per group per experiment are stated in the figure legends.
Acknowledgements

This work was supported by NIH grant HD049808 (D.M.O.), AR055923 (F.L.), DE025077 (K.Y.), the Washington University Musculoskeletal Research Center NIH P30 AR057235 and by internal funds from the Craniofacial Center of Seattle Children’s Hospital and Center for Developmental Biology and Regenerative Medicine of Seattle Children’s Research Institute (K.Y.).

Author Contributions

References


Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. *Genes Dev.* **13**, 2072-2086.

Role of FGF/FGFR signaling in skeletal development and homeostasis: learning from mouse models. *Bone Research* **2**.


FGFR1 is independently required in both developing mid- and hindbrain for sustained response to ischemic signals. *Embo J* **22**, 1811-1823.


FGF9 and SHH signaling coordinate lung growth and development through regulation of distinct mesenchymal domains. *Development* **133**, 1507-1517.


Intermittent PTH (1-34) injection rescues the retarded skeletal development and postnatal lethality of mice mimicking human achondroplasia and thanatophoric dysplasia. *Hum Mol Genet*.


Figure 1. Postnatal growth defects in mice lacking Fgfr1 and Fgfr2 in the osteoprogenitor lineage.
A. Growth curve of control (DFF) and Osx-Cre;DCKO (DCKO) mice showing reduced growth of DCKO mice after P4 (data is pooled from 35 DFF mice and 26 DCKO mice). Not all mice were weighed at every time point. B. Proximal tibia histology (H&E) of DFF and DCKO mice at E18.5 showing a normal proliferating chondrocyte zone (p) and an expanded hypertrophic chondrocyte zone (h). C. Alizarin-Red and Alcian Blue staining of whole skeleton at P0 showing similar skeletal architecture of control and DCKO mice. D. DFF and DCKO mice at P21 showing smaller size of DCKO compared to control. E. Whole body DEXA analysis of DFF and DCKO mice (age 24-26 days, n=4) showing normal body fat content. F. Radiographic images of hindlimb (left) and forelimb (right) of three month-old DFF and DCKO mice showing reduced bone density of DCKO compared to control. G. Micro CT analysis of DFF and DCKO mice at P21 showing reduced trabecular and cortical bone in DCKO mice. H. Quantification of micro CT showing reduced cortical and trabecular bone volume to total volume ratio (BV/TV) and bone mineral density (BMD) in DCKO mice (n=3); Error bars, SD; *P<0.05; **P<0.01.
Figure 2. Decreased cortical and trabecular bone formation in Osx-Cre;DCKO mice

A. Histology of DFF and DCKO proximal tibia of P21 mice, showing reduced mineralized bone (von Kossa stain) in DCKO mice. B: Histology of DFF and DCKO mice (H&E stain) showing normal osteoblast morphology in the trabecular region adjacent to the chondro-osseous junction (asterisk). C. Type I collagen (Col1) expression detected by in situ hybridization in DFF and DCKO mice. D: Histomorphometry of DFF and DCKO mice (n=3) showing normal osteoblast number per bone surface area and normal osteoblast surface per bone surface. Arrows in B and C indicate osteoblasts. Scale bars: A, 500 µm; B, C, 50 µm. ns, non significant.
Figure 3. Decreased growth plate size and Decreased chondrocyte proliferation in Osx-Cre; DCKO mice
A. Histology (H&E) of the distal femur showing smaller growth plate and trabecular bone thickness in P21 Osx-Cre;DCKO compared to DFF mice. inset, 2X magnification. B. Growth plate measurements showing reduced total growth plate and proliferative zone length, and normal hypertrophic zone length in P21 Osx-Cre;DCKO mice compared to DFF mice. Data is normalized to the total growth plate height of DFF mice (n=3). C. BrdU immunohistochemistry showing reduced chondrocyte proliferation in Osx-Cre;DCKO compared to DFF mice. D. Quantification of BrdU labeled cells in the proliferating chondrocyte zone of DFF and Osx-Cre;DCKO growth plates (n=6, expressed as percent of control). E. TRAP staining (red) of DFF and Osx-Cre;DCKO proximal tibia. F. Histomorphometric analysis showing no difference in osteoclast number per bone surface, and normal osteoclast surface per bone surface of DFF and Osx-Cre;DCKO mice. G. Expression of Fgfr1 (in situ hybridization) in the proximal tibia showing reduced expression in hypertrophic chondrocytes in ATC;DCKO compared to DFF mice. inset, 2X magnification. H. Confirmation of deletion of Fgfr1 in the growth plate of ATC;DCKO mice: flox, unrecombined Fgfr1 flox allele; del, Fgfr1 deleted allele; Cre, Cre recombinase allele; M, markers (nt). I. Histology (H&E) of distal femur showing similar growth plate size in DFF and ATC;DCKO mice. J. BrdU immunohistochemistry showing no difference in chondrocyte proliferation in DFF and ATC;DCKO growth plate. K. Quantification of BrdU labeled cells in the proliferating chondrocyte zone on DFF and ATC;DCKO growth plates (n=3, expressed as percent of control). p, proliferating chondrocytes; h, hypertrophic chondrocytes; tb, trabecular bone. Scale bars: A, C, I, 200 µm; E, G, J, 100 µm; Error bars, SD; *P<0.02; **P<0.001; ns, not significant.
Figure 4. Increased expression of Fgf9 and Fgf18 in Osx-Cre;DCKO mice

A. Expression of Fgf9 (in situ hybridization) in DFF and Osx-Cre;DCKO proximal tibia of P21 mice showing increased expression in the perichondrium, reserve, proliferating, and prehypertrophic chondrocytes of Osx-Cre;DCKO mice. B. qRT-PCR analysis of Fgf9 expression in DFF and Osx-Cre;DCKO proximal tibia metaphysis (n=3). C. Expression of Fgf18 (in situ hybridization) in P21 DFF and Osx-Cre;DCKO proximal tibia showing increased expression in articular cartilage, proliferating, and prehypertrophic chondrocytes in the growth plate and in trabecular bone of Osx-Cre;DCKO mice. D. qRT-PCR analysis of Fgf18 expression in DFF and Osx-Cre;DCKO proximal tibia metaphysis (n=3). rc, reserve chondrocytes; p, proliferating chondrocytes; h, hypertrophic chondrocytes; ac, articular chondrocytes; tb, trabecular bone; pc, perichondrium. Scale bars: A, D, 100 µm; Error bars, SD; *P<0.05; **P<0.002.
Figure 5. Increased Fgfr3 expression and signaling in Osx-Cre;DCKO mice

A,C. Expression (in situ hybridization) of Fgfr3 (A) and Snail1 (C) in DFF and Osx-Cre;DCKO distal femur. B. qRT-PCR analysis of Fgfr3 expression in DFF and Osx-Cre;DCKO proximal tibia and distal femur metaphysis (n=3). D. Immunohistochemistry of DFF and Osx-Cre;DCKO proximal tibia showing mildly increased SOX9 in Osx-Cre;DCKO compared to DFF mice. p, proliferating chondrocytes; h, hypertrophic chondrocytes; tb, trabecular bone. Scale bars: A, C, D, 100 µm; Error bars, SD; *P<0.05.
Figure 6. Activation of *Fgf9* in the perichondrium suppresses chondrocyte proliferation.
A. Fluorescence imaging of induced GFP expression in trabecular bone (tb), cortical bone (e, endosteum; po, periosteum) and perichondrium (pc) of RunxTFG mice. GFP was not observed in hypertrophic chondrocytes (h). B. Decreased body weight of P21 RunxTFG mice (n=3) compared to Runx2-rtTA single transgenic control (n=4). C. Histology (H&E) of the proximal tibia showing a smaller growth plate in P21 RunxTFG compared to Runx2-rtTA single transgenic control. D. Growth plate measurements showing reduced total growth plate, proliferative zone, and hypertrophic zone size in P21 RunxTFG compared to Runx2-rtTA single transgenic control. E. TRAP staining of P21 control and RunxTFG mice showing normal osteoclast number. F. BrdU immunohistochemistry showing reduced chondrocyte proliferation in RunxTFG compared to control P21 mice. G. Quantification of BrdU labeled cells in the proliferating chondrocyte zone of P21 control and RunxTFG growth plates (n=3). H. Expression of Fgfr3 (in situ hybridization) in P21 control and RunxTFG distal femur. rc, reserve chondrocytes; p, proliferating chondrocytes; h, hypertrophic chondrocytes; tb, trabecular bone. Scale bars: A1,A3,C-H 100 µm; A2, 20 µm; Error bars, SD; *P<0.01; **P<0.001.
Figure 7. Rescue of the Osx-Cre;DCKO growth plate phenotype by administration of PTH(1-34).
A. Expression of \textit{Ihh (in situ hybridization)} in P21 \textit{DFF} and \textit{Osx-Cre;DCKO} distal femur showing decreased expression in the growth plate of \textit{Osx-Cre;DCKO} mice. B. qRT-PCR analysis of \textit{Ihh} expression in \textit{DFF} and \textit{Osx-Cre;DCKO} proximal tibia metaphysis (n=3). C. Expression of \textit{Pthlh (in situ hybridization)} in P21 \textit{DFF} and \textit{Osx-Cre;DCKO} distal femur showing decreased expression in the peripheral growth plate in \textit{Osx-Cre;DCKO} mice (inset: 2X magnification). D. qRT-PCR analysis of \textit{Pthlh} expression in \textit{DFF} and \textit{Osx-Cre;DCKO} proximal tibia metaphysis (n=3). E. Expression of \textit{Pthlh} in P21 control and \textit{RunxTFG} proximal tibia. F. Histology (H&E) of the proximal tibia showing a larger growth plate and increased trabecular bone in P21 PTH treated compared to PBS (control) treated \textit{Osx-Cre;DCKO} mice. G. Growth plate measurements showing increased total growth plate, proliferative, and hypertrophic zone size in PTH treated (n=3) compared to PBS treated (n=4) mice. H. Expression of \textit{Fgfr3 (in situ hybridization)} in the distal femur of P21 PTH treated compared to PBS (control) treated \textit{Osx-Cre;DCKO} mice. I. BrdU immunohistochemistry showing increased chondrocyte proliferation in P21 PTH treated compared to PBS (control) treated \textit{Osx-Cre;DCKO} mice. J. Quantification of BrdU labeled cells in the proliferating chondrocyte zone of PTH treated compared to PBS (control) treated \textit{Osx-Cre;DCKO} mice (n=3). rc, reserve chondrocytes; p, proliferating chondrocytes; ph, prehypertrophic chondrocytes; h, hypertrophic chondrocytes; tb, trabecular bone. Scale bars: A, C, 50\(\mu\)m; E, F, H, I, 100 \(\mu\)m; Error bars, SD; \(\ast P<0.05; \ast\ast P<0.005; \ast\ast\ast P<0.001\).
Figure 8. Model of FGF regulated interactions between osteoprogenitor lineages and growth plate chondrocytes in postnatal endochondral bone growth.

(A) FGFR1 and FGFR2 in the osteoprogenitor lineage are regulated by FGF9 expressed in osteoprogenitors and adjacent connective tissue and periosteum. Inactivation of FGFR1 and FGFR2 results in compensatory increased expression of Fgf9, which (B) aberrantly activates FGFR3 and downstream Snail1 to suppress chondrocyte proliferation and hypertrophy. Increased FGFR3 signaling also promotes Fgf9 and Fgf18 expression in chondrocytes and suppresses expression of Ihh and Pthlh. PTHLH functions to suppress Fgfr3 expression and reduced Pthlh contributes to increased Fgfr3 expression. The aberrant activation of FGFR3 (expression and signaling in chondrocytes) may initiate a feed-forward signaling loop in chondrocytes that functions to terminate chondrogenesis. HS, heparan sulfate.