ABSTRACT
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 (FGF1 and FGF2) are both expressed in the osteoprogenitor lineage. Double conditional knockout mice, in which both receptors were inactivated using an osteoprogenitor-specific Cre driver, appeared normal at birth; however, these 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, suggesting cell-autonomous functions of FGF signaling during postnatal bone formation. Surprisingly, the double conditional knockout mice also showed growth plate defects and an arrest in chondrocyte proliferation. We provide genetic evidence of a non-cell-autonomous feedback pathway regulating Fgf9, Fgf18 and Pthlh expression, which led to increased expression and signaling of Fgfr1 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.

KEY WORDS: FGF signaling, PTHLH, IIH, Skeletal development, Endochondral bone formation, Osteoblast, Chondrocyte, Mouse

INTRODUCTION
Human genetic disease and conditional gene inactivation experiments in mice have demonstrated essential roles for FGFR1 and FGFR2 in development of the appendicular and axial skeleton (Ornitz and Marie, 2002, 2015; Su et al., 2014). Although both receptors are expressed in the osteoprogenitor lineage, redundant functions of these FGFRs and mechanisms that couple FGF signaling in the osteoprogenitor lineage to chondrogenesis and longitudinal bone growth are not known.

In mice, Fgfr1 has been targeted with a range of Cre drivers including brachyury (T), Ap2 (Tfap2a), Prxl (Prrx1), Col2a1, Col1, osteocalcin (OC, Bglap) and Dmp1 (Jacob et al., 2006; Karolak et al., 2015; Li et al., 2005; Verheyden et al., 2005; Xia et al., 2014; Yu and Ornitz, 2008). With the exception of Coll1-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 Prx1-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 Coll1-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 Fgfr2b splice variant has been inactivated (Fgfr2b<sup>−/−</sup>/−) were viable but showed reduced postnatal growth (Eswarakumar et al., 2002). Fgfr2 has also been conditionally targeted with a Dermol (Twist2) Cre driver or has been suppressed using RNA interference in limb bud mesenchyme. Inactivation of Fgfr2 with Dermol-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 (Counoul et al., 2005). None of the Fgfr2 gene inactivation studies provided a mechanism to explain the 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;
The inhibitory activity of FGFR3 on growth plate chondrocytes explains the pathogenic consequences of gain-of-function mutations in FGFR3 in suppressing pre-pubertal skeletal growth in achondroplasia and related chondrodysplastic disorders (Laederich and Horton, 2012; Naski et al., 1998, 1996). The signaling mechanisms by which FGFR3 suppresses chondrogenesis involve activation of STAT1, ERK1/2 (MAPK3/1) and p38 (MAPK14), increased expression of Snail1 (Snai1), decreased expression of AKT, and activation of protein phosphatase 2a (PP2a), which dephosphorylates (activates) the retinoblastoma family members p107 (RBL1) and p130 (RBL2). Activation of p107 (and p130) and increased expression of the cell cycle inhibitor p21^Cip1 (CDKN1A) function to directly suppress chondrocyte proliferation (Akaiwa et al., 2001; Cobrinik et al., 1996; Dailey et al., 2003; de Frutos et al., 2007; Kolupaeva et al., 2013, 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 signaling in the osteoprogenitor lineage 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) (Osx is also known as Sp7) 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 Pthlh and increased expression of Pgf9 and Pgf18, which encode ligands that normally regulate 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 peristemum during skeletal development (Yu et al., 2003). FGFR1 and FGFR2 have similar in vitro signaling potency and ligand response profiles to FGFR9 and FGFR18 (Zhang et al., 2006), ligands that have key roles in regulating skeletal development (Hung et al., 2016, 2007; Liu et al., 2007, 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 (Böhm 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;Fgfr1<sup>fl/fl</sup>;Fgfr2<sup>fl/fl</sup> double conditional knockout (abbreviated here as Osx-Cre;DCKO), Fgfr1<sup>fl/fl</sup>;Fgfr2<sup>fl/fl</sup> double floxed control (abbreviated here as 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 (P) 4 (Fig. 1A, Fig. S1A). Inactivation of Fgfr1 and Fgfr2 in the Osx-Cre lineage was confirmed by qRT-PCR evaluation of mRNA isolated from cortical bone from P21 DFF and Osx-Cre;DCKO mice (Fig. S2). Histological evaluation of embryonic day (E) 18.5 Osx-Cre;DCKO proximal tibia showed an increase in 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 compared with DFF or Osx-Cre control mice. This growth defect became statistically significant (P<0.05) after P4 (Fig. 1A). By 3 weeks of age, Osx-Cre;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 with 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 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 the 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 with 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 (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 in bone mineral density (BMD) between P21 wild-type and Osx-Cre mice (Fig. S1B,C). Growth plate histology and Fgfr3 expression at P21 were also similar between wild-type and Osx-Cre mice (Fig. S1D,E). These studies show that the Osx-Cre allele has a minimal effect on bone growth in the genetic background used in these studies.

Decreased bone formation in Osx-Cre;DCKO mice

Radiographic analysis of intact skeletons of 3-month-old mice revealed that Osx-Cre;DCKO mice had shorter bones and reduced bone density compared with control mice (Fig. 1F). The overall shape of the bones was normal. Micro-CT 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 micro-CT 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 a 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 with DFF controls showed a significant decrease in the overall length of the growth plate and the length of the proliferating (columnar) chondrocyte zone (24% and 36%, respectively; *P*<0.02) (Fig. 2A,B). At this stage of postnatal development, the hypertrophic chondrocyte zone, which was expanded at E18.5, was not significantly different from that of 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 the 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, as evaluated by activated caspase 3 immunostaining, was decreased in distal hypertrophic chondrocytes in Osx-Cre;DCKO mice (Fig. S3A,B), and matrix degradation potential, as evaluated by measuring osteoclast number (N.Oc) and osteoclast surface (Oc.S) per bone surface area, did not significantly differ between 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 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 that Fgfr1 is expressed in hypertrophic chondrocytes, it was necessary to determine whether inactivation of Fgfr1 (and Fgfr2) in growth plate
chondrocytes could contribute to the observed decrease in chondrocyte proliferation. The aggregan 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>fl/fl</sup>;Fgfr2<sup>fl/fl</sup> (ATC;DCKO) embryos were placed on doxycycline throughout gestation and pups were maintained on doxycycline until P21. In <i>in situ</i> 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, which encode ligands that are each necessary for normal embryonic skeletal development (Hung et al., 2007; Liu et al., 2007, 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 FGF3 during postnatal bone growth to negatively regulate chondrocyte proliferation, compensatory upregulation of Fgf9 or Fgf18 expression due to loss of FGF3/2 signaling in the osteoprogenitor lineage could aberrantly activate FGF3 in the growth plate and suppress chondrocyte proliferation. To test this hypothesis, we performed <i>in situ</i> hybridization analysis of paraffin-fixed intact bone tissues and qRT-PCR on distal bone tissue from DFF and Osx-Cre;DCKO mice. In <i>in situ</i> 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 the <i>in situ</i> expression data, qRT-PCR analysis of distal bone tissue showed a ~3.5-fold increase in Fgf9 expression in tissue from Osx-Cre;DCKO compared with DFF mice (Fig. 4B). Analysis of Fgf18 by <i>in situ</i> hybridization showed increased expression in reserve, proliferating and prehypertrophic chondrocytes in Osx-Cre;DCKO compared with DFF mice (Fig. 4C). Consistent with these data, qRT-PCR showed a ~1.5-fold increase in Fgf18 expression in Osx-Cre;DCKO compared with DFF distal bone tissue (Fig. 4D).

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

<i>In situ</i> hybridization revealed a striking increase in Fgfr3 expression in Osx-Cre;DCKO compared with 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 FGFFR3 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 strongly increased in Osx-Cre;DCKO compared with DFF mice (Fig. 5C). Immunostaining for the chondrocyte-specific transcription factor SOX9 showed mildly elevated levels of expression in Osx-Cre;DCKO compared with 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<sup>−/−</sup> 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-rTA (Chen et al., 2014b) and TRE-Fgf9-ires-eGFP (White et al., 2006) transgenic mice were mated to generate biallelic Runx2-rTA;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 with 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 with the control, RunxTFG transgenic mice had significantly (P<0.01) smaller proliferating and hypertrophic chondrocyte zones (Fig. 6C,D). The height of the trabecular zone in RunxTFG transgenic mice was
with metaphysis (expression in proximal tibia showing mildly increased SOX9 in reduced in (Fig. 6F,G). Finally, perichondrium. Error bars, s.d.; *chondrocytes; ac, articular chondrocytes; tb, trabecular bone; pc, reserve chondrocytes; p, proliferating chondrocytes; h, hypertrophic expression in and in trabecular bone of cartilage, proliferating and prehypertrophic chondrocytes in the growth plate

Fig. 5. Increased expression of Fgfr1 and Fgfr2 signaling to Fgfr3 expression and chondrocyte proliferation in the postnatal growth plate

Indian hedgehog (IHH) and parathyroid hormone-like peptide (PTHLH) are crucial regulators of endochondral bone growth (Kozhemya 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 with controls, Ihh was decreased in the P21 growth plate of Osx-Cre; DCKO mice (Fig. 7A,B). Interestingly, we found that Pthlh expression was also reduced in reserve chondrocytes in Osx-Cre; DCKO mice (Fig. 7C). qRT-PCR analysis of distal bone tissue showed an overall reduction in Pthlh mRNA (Fig. 7D). Consistent with Fgfr3 signaling suppressing Ihh-Pthlh expression (Chen et al., 2001; Li et al., 2010; Minina et al., 2002; Naski et al., 1998), in mice induced to overexpress Fgfr3, expression of Pthlh was reduced in reserve zone chondrocytes (Fig. 7E).

Analysis of Fgfr3 promoter function in vitro shows that Fgfr3 expression could be directly regulated (suppressed) by PTHLH activation of protein kinase A (PKA) (McEwen et al., 1999). To test whether parathyroid hormone (PTH) signaling could suppress Fgfr3 expression in vivo in Osx-Cre;DCKO mice that highly overexpress Fgfr3, Osx-Cre;DCKO mice were injected intermittently (daily) with PTH [PTH(1-34) peptide] 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 with control Osx-Cre;DCKO mice that were only injected with PBS, PTH-injected Osx-Cre;DCKO mice showed an increase in the size of the growth plate, increased thickness of trabecular bone, decreased expression of 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 Schenk, 1989; Noonan et al., 1998). FGFR3 is a well-known inhibitory factor that function downstream of FGFR3 are well established (Ornitz and Schenk, 1989; Noonan et al., 1998). FGFR3 is a well-known inhibitor of chondrocyte proliferation and IHH-Pthlh expression in the postnatal growth plate (Fig. 8).

The precise cell type(s) that maintain 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 OC-Cre allele (our unpublished data). A likely early event eliciting this phenotype is increased expression of Fgfr3 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. This non-cell-autonomous signaling pathway thus coordinates 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, the effect of disrupting 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, which 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 is 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), the possibility remained 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 allele. The normal development of ATC;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 is 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). These 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. 7).

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

Regulation of embryonic versus 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 E12.5 (Ono et al., 2014; Rodda and McMahon, 2006), 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 the 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 in the distal periarticular perichondrium (Kronenberg, 2003). However, in postnatal bone there is a reorganization of the growth plate. Pthlh expression shifts to reserve chondrocytes, and Ihh signaling (GLI1) and Pthlhr expression remain 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.

Fig. 7. Rescue of the Osx-Cre;DCKO growth plate phenotype by administration of PTH(1-34). (A) Expression of Ihh, assessed by in situ hybridization, in P21 distal femur showing decreased expression in the growth plate of Osx-Cre;DCKO mice. (B) qRT-PCR analysis of Ihh expression in DFF and Osx-Cre;DCKO proximal tibia metaphysis (n=3). (C) Expression of Pthlh, assessed by in situ hybridization, in P21 distal femur showing decreased expression in the peripheral growth plate in Osx-Cre;DCKO mice. Inset, 2× magnification. (D) qRT-PCR analysis of Pthlh expression in DFF and Osx-Cre;DCKO proximal tibia metaphysis (n=3). (E) Expression of Pthlh in P21 control and RunxTFG proximal tibia. (F) Histology (H&E staining) of the proximal tibia showing a larger growth plate and increased trabecular bone in P21 PTH-treated compared with PBS-treated (control) Osx-Cre;DCKO mice. (G) Growth plate measurements showing increased total growth plate, proliferative and hypertrophic zone size in PTH-treated (n=3) compared with PBS-treated (n=4) mice. (H) Expression of Fgfr3, assessed by in situ hybridization, in the distal femur of P21 PTH-treated compared with PBS-treated Osx-Cre;DCKO mice. (I) BrdU immunohistochemistry showing increased chondrocyte proliferation in P21 PTH-treated compared with PBS-treated Osx-Cre;DCKO mice. (J) Quantification of BrdU-labeled cells in the proliferating chondrocyte zone of PTH-treated compared with PBS-treated Osx-Cre;DCKO mice. *P<0.05, **P<0.005, ***P<0.001. Scale bars: A,C, 50 µm; E,F,H,I, 100 µm.

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 might 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 the 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>fl/fl</sup> (Trokovic et al., 2003), Fgfr2<sup>fl/fl</sup> (Yu et al., 2003), aggrecan enhancer-driven, tetracycline-inducible Cre (ATC) (Dy et al., 2012), Runx2-TTA (Chen et al., 2014b) and TRE-Fgf9-ires-eGFP (White et al., 2006) have been described previously.

Homozygous floxed alleles of Fgfr1 and Fgfr2 were maintained as double floxed mice (DFF) and outbred to hybrid C57BL/6J;129X1 mice every second generation and then backcrossed to homozygosity. Double conditional knockout breeding males (Osx-Cre::Fgfr1<sup>fl/fl</sup>;Fgfr2<sup>fl/fl</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 a 50% yield of Osx-Cre;DCKO mice and DFF controls. Osx-Cre control mice were generated by crossing Osx-Cre mice to wild-type hybrid mice. A similar breeding strategy was used to generate ATC;DCKO mice. To express Fgf9 in the osteoblast lineage, Runx2-rTA mice (Chen et al., 2014b) were crossed to TRE-Fgf9-ires-eGFP (White et al., 2006) to generate Runx2TPG double-transgenic mice. Females were induced with doxycycline chow (Bio-Serv, S3888; 200 mg/kg green pellets) 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 weeks of age.

Body weights were measured for 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 timecourse.

**Histology, immunohistochemistry and immunofluorescence**

For histological analysis of long bones, intact femur and tibia were isolated, fixed in 4% PFA/PBS overnight at 4°C or fixed in 10% buffered formalin overnight at room temperature. Bone samples were then decalcified in 14% EDTA/PBS for 2 weeks. Paraffin-embedded tissue sections (5 µm) were stained with Hematoxylin and Eosin (H&E), tartrate-resistant acid phosphatase (TRAP), von Kossa or Alizarin Red.

For immunohistochemistry, paraffin sections or cryosections were dehydrated 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 (pH 6.0) for 10 min followed by gradual cooling to room temperature. Sections were incubated overnight at 4°C with the following primary antibodies: anti-SOX9 (Millipore, AB5535, rabbit polyclonal; 1:100), anti-active caspase 3 (Cell Signaling, 9662S, rabbit polyclonal; 1:1000), and anti- Col1a1 (Millipore, AB1826, rabbit polyclonal; 1:1000). Detection was achieved by peroxidase staining using the ABC Kit (Vector Labs, PK-6100) and 3,3′-diaminobenzidine (DAB) (Vector Labs, SK-4100) for 5 min. Hematoxylin (H&E) counterstaining was achieved by submerging the sections (5 µm) into a solution of 0.1% hematoxylin and 0.02% sodium acetate-acetic acid (50:50) for 30 sec.

For immunofluorescence, paraffin sections or cryosections were incubated overnight at 4°C with the following primary antibodies: 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) and Col1 (Rossert et al., 1995). Data are representative of at least three independent experiments. Where necessary, image adjustments (to brightness/contrast) were made equally to allow clearer visualization of cellular expression in both control and knockout images.

Cell proliferation was determined by injecting BrdU (5-bromo-2′-deoxyuridine; Sigma, 9285) at 0.1 mg/g body weight 2 h before tissues were harvested. Anti-BrdU mouse monoclonal (BD Biosciences, 347580) was used at 1:200. 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 two or three 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.

**Micro-CT and DEXA analysis**

For micro-CT, 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). Micro-CT analysis of trabecular and cortical bone was performed as follows. For trabecular bone, 100 to 150 sections were selected below the growth plate. For cortical bone, 50 to 100 sections were selected from the mid-diaphysis of the femur or tibia. Quantification was performed using SCANCO Medical micro-CT systems software. DEXA (GE/Lunar PIXImus) was used for measurements of whole-body bone density and body fat content. Data are representative of at least three mice per genotype.

**Real-time quantitative PCR (RT-qPCR)**

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 Disembrator (Sartorius) at 2000 rpm for 20 s. RNA was stabilized with TRIzol (Ambion) and total RNA isolation was performed 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 Fgf3. Hprt was used as a normalization control.

**PTH treatment**

For *in vivo* treatment of mice with PTH, 15-day-old Oss-Cre;DCKO mice were injected intraperitoneally once per day (morning) with synthetic PTH-related peptide (1-34) (H6630, Bachem) at 80 μg/kg body weight or with PBS (control). Mice were injected for 5 days and then sacrificed at P21.

**Statistics**

Data are reported as mean±s.d. Data were analyzed using a two-tailed *t*-test) was based on measurements of tissue samples from at least three mice and two or three sections per mouse were analyzed for each genotype.

**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

Conceptualization and methodology, K.K., K.Y., F.L. and D.M.O.; investigation, K.K., K.Y., J.L., J.C., C.S. and D.M.O.; writing of original draft, K.K. and D.M.O.; manuscript review and editing, K.K., K.Y., F.L. and D.M.O.; funding acquisition, resources and supervision, F.L. and D.M.O.

**Funding**

This work was supported by National Institutes of Health (NIH) grants [HD049808 to D.M.O., AR055923 to F.L., DE02577 to 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.). Deposited in PMC for release after 12 months.

**Supplementary information**

Supplementary information available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.131722/-/DC1

References


