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Research Article

A combined series of *Fgf9* and *Fgf18* mutant alleles identifies unique and redundant roles in skeletal development

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ABSTRACT

Fibroblast growth factor (FGF) signaling is a critical regulator of skeletal development. *Fgf9* and *Fgf18* are the only FGF ligands with identified functions in embryonic bone growth. Mice lacking *Fgf9* or *Fgf18* have distinct skeletal phenotypes; however, the extent of overlapping or redundant functions for these ligands and the stage-specific contributions of FGF signaling to chondrogenesis and osteogenesis are not known. To identify separate versus shared roles for FGF9 and FGF18, we generated a combined series of *Fgf9* and *Fgf18* null alleles. Analysis of embryos lacking alleles of *Fgf9* and *Fgf18* shows that both encoded ligands function redundantly to control all stages of skeletogenesis; however, they have variable potencies along the proximodistal limb axis, suggesting gradients of activity during formation of the appendicular skeleton. Congenital absence of both *Fgf9* and *Fgf18* results in a striking osteochondrodysplasia and revealed functions for FGF signaling in early proximal limb chondrogenesis. Additional defects were also noted in craniofacial bones, vertebrae, and ribs. Loss of alleles of *Fgf9* and *Fgf18* also affect the expression of genes encoding other key intrinsic skeletal regulators, including IHH, PTHLH (PTHrP), and RUNX2, revealing potential direct, indirect, and compensatory mechanisms to coordinate chondrogenesis and osteogenesis.
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1. Introduction

Skeletal development in vertebrate organisms occurs via intramembranous ossification with mesenchymal cells differentiating directly to osteogenic cells, and endochondral ossification, with an intermediary cartilaginous template prefiguring the future bone (Erlebacher et al., 1995; Karsenty and Wagner, 2002; Long and Ornitz, 2013). Following mesenchymal cell condensation, chondrogenic differentiation produces immature, type II collagen-expressing chondrocytes, while cells at the periphery differentiate into the surrounding perichondrium, expressing type I collagen. Immature chondrocytes initially separate into a population of round, reserve chondrocytes with low mitotic indices and a population of ovoid, columnar, CyclinD1-positive proliferating chondrocytes with high mitotic indices that will subsequently mature to hypertrophic chondrocytes. Centrally located cells are the first to undergo proliferative and hypertrophic differentiation. During hypertrophic maturation, chondrocytes expand dramatically to drive elongation of the cartilage anlagen and secrete a type X collagen-rich matrix. Trabecular bone and bone marrow replace distal hypertrophic chondrocytes to form the primary ossification center in the bone shaft (Gibson, 1998; Gerber and Ferrara, 2000). Cortical bone is formed separately by osteoblasts derived from osteoprogenitor cells in the perichondrium (Caplan and Pechak, 1987). Chondrogenesis and osteogenesis are tightly regulated by multiple signaling pathways and molecules.

The involvement of FGF pathways in skeletogenesis was realized with the discovery that activating mutations in FGF receptors (FGFRs) caused human skeletal dysplasia and craniosynostosis syndromes, including the most common form of genetic dwarfism, achondroplasia (Rousseau et al., 1994; Shiang et al., 1994; Bellus et al., 1995; Tavormina et al., 1995; Wilkie, 1997; Cohen, 2000; Wilkie, 2000; Britto et al., 2001; Morriss-Kay and Wilkie, 2005; Johnson and Wilkie, 2011). A distinctive feature in achondroplasia is rhizomelia, where the proximal limb is more severely affected than distal elements. FGF-related craniosynostotic patients also exhibit limb abnormalities of variable severity. Normal FGF signaling requires binding of FGF ligands and heparan sulfate chains to FGFRs at the cell surface, inducing receptor dimerization and

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activation of downstream signaling (Eswarakumar et al., 2005; Ornitz and Itoh, 2015). Thus far, physiologic roles in endochondral bone growth have been demonstrated for only three FGF ligands, FGFs 2, 9 and 18, although others, such as *Fgfs 7, 8,* and *17,* are also expressed in some skeletal elements (Mason et al., 1994; Finch et al., 1995; Xu et al., 1999). The difficulty in identifying additional FGFs has been attributed to potential redundancy between ligands; however, direct evidence of this phenomenon has been lacking in skeletogenesis.

Fgf2 is expressed in differentiated chondrocytes, periosteum, and osteoblasts (Sullivan and Klagsbrun, 1985; Hurley et al., 1994, 1999; Sabbieti et al., 1999, 2005). Though a function for *Fgf2* has not been identified in chondrogenesis, *Fgf2* null mice have reduced trabecular bone mass, indicating a role in osteogenesis (Montero et al., 2000). *Fgf9* and *Fgf18* share partially overlapping expression patterns in limb bud mesenchyme, in mesenchyme surrounding condensations, and in the perichondrium (Maruoka et al., 1998; Colvin et al., 1999; Garofalo et al., 1999; Ohuchi et al., 2000; Liu et al., 2002; Hung et al., 2007). Previous studies characterizing *Fgf9* and *Fgf18* single knockout skeletons identified similar roles for these genes (Liu et al., 2002, 2007; Ohbayashi et al., 2002; Hung et al., 2007). Specifically, the encoded ligands individually promote increased rates of cell division in proliferating chondrocytes and positively regulate initiation of chondrocyte hypertrophy at midgestational stages. Each of these ligands possesses unique roles as well. FGF9 activity was restricted to stylopod elements (the most proximal limb compartments such as hindlimb femur) while FGF18 activity was present throughout the developing limb. Additionally, FGF18 functions were biphasic in skeletogenesis: FGF18 negatively regulates chondrocyte proliferation and hypertrophic differentiation at later gestational stages (embryonic day 16.5; E16.5), but positively regulates these processes at earlier time points (E14.5). Opposing functions were not observed for FGF9 at different developmental stages. Biphasic activity of FGFR3 has also been observed *in vivo*: at E14.5–E15.5, FGFR3 promotes chondrocyte proliferation, while at late embryonic and postnatal stages FGFR3 inhibits chondrocyte proliferation and differentiation (Iwata et al., 2000).

Besides FGF signaling, additional pathways serve as important regulators of endochondral ossification, including Indian hedgehog (IHH), Parathyroid hormone-like peptide (PTHLH), and Runt-related transcription factor 2 (RUNX2) (de Crombrugghe et al., 2001; Karsenty and Wagner, 2002). IHH induces *CyclinD1* expression and chondrocyte proliferation, and promotes differentiation of immature chondrocytes to proliferating chondrocytes (Karp et al., 2000; Long et al., 2001). $Ihh^{-/-}$ mice have shortened limbs due partly to a severe chondrocyte proliferation defect and delayed initiation of chondrocyte maturation (St-Jacques et al., 1999). IHH also induces *Pthlh* expression, which, with its receptor, Parathyroid hormone 1 receptor (PTH1R), maintains chondrocytes in a proliferative state and inhibits chondrocyte maturation. Disruption of the PTHLH pathway in mice results in short-limbed dwarfism and extensive premature chondrocyte hypertrophy (Karaplis et al., 1994; Lanske et al., 1996). RUNX2 is an essential transcription factor that is required for osteogenesis (Komori et al., 1997; Otto et al., 1997) and functions to promote hypertrophic chondrocyte differentiation (Enomoto et al., 2000; Takeda et al., 2001; Ueta et al., 2001; Stricker et al., 2002). $Runx2^{-/-}$ embryos exhibit delayed chondrocyte maturation throughout the developing skeleton, and proximal limb elements lacked hypertrophic chondrocytes (Inada et al., 1999; Kim et al., 1999). RUNX2 also influences chondrocyte proliferation and differentiation through direct transcriptional activation of *Ihh* (Yoshida et al., 2004).

Our past studies showed similar expression sites and suggested overlapping roles for *Fgf9* and *Fgf18* in skeletal development. Here, we demonstrate profound functional redundancy of FGF9 and FGF18. By generating a series of *Fgf9* and *Fgf18* mutant alleles, we further characterize their roles in different phases of skeletogenesis and identify differential limb compartment-specific activity levels. In the developing stylopod, FGF9 and FGF18 are required for initial differentiation of chondrocytes from an immature state to CyclinD1-positive, columnar proliferating cells. Together, they promote *Ihh* and *Runx2* expression in E12.5 cartilaginous condensations and are required to maintain expression of these key signaling molecules during midgestation (E14.5). These findings demonstrate important regulatory functions for FGF9 and FGF18 in developing bone.

2. Results

2.1. Loss of Fgf alleles causes a severe osteochondrodysplasia

Viable compound heterozygotes ($Fgf9^{+/-}$; $Fgf18^{+/-}$) were generated by breeding mice harboring one functional allele of *Fgf9* ($Fgf9^{+/-}$) (Colvin et al., 2001) with mice harboring one functional allele of *Fgf18* ($Fgf18^{+/-}$) (Liu et al., 2002). The $Fgf9^{+/-}$; $Fgf18^{+/-}$ mice were subsequently intercrossed to obtain a series of *Fgf9* and *Fgf18* mutant alleles. All possible genotypes were recovered in Mendelian ratios; however, mice homozygous for *Fgf9* and/or *Fgf18* null alleles exhibited perinatal lethality, as reported previously (Colvin et al., 2001; Liu et al., 2002; Ohbayashi et al., 2002).

Skeletal preparations at E18.5 were stained with Alcian blue and Alizarin red for gross assessment of the combined roles of *Fgf9* and *Fgf18* (Figs. 1 and 2). As previously reported, *Fgf18*-deficient skeletons had delayed mineralization of endochondral and intramembranous bones. Axial skeletal abnormalities included wavy ribs and increased spinal curvatures, which we observed in ~30% of $Fgf18^{-/-}$ mice (Fig. 1B). Limb skeletal defects seen in all individuals included an angulated radius, a curved ulna, and a bowed tibia. A small unossified fibula was present in ~30% of $Fgf18^{-/-}$ mice (Fig. 2B). Cleft palate was present in >95% of these animals (not shown). *Fgf9*-deficient skeletons (Fig. 2D) had 100% penetrant proximal limb anomalies including an enlarged deltoid tuberosity in the humerus and disproportionate shortening of the femur with delayed mineralization (Liu et al., 2002, 2007; Ohbayashi et al., 2002; Hung et al., 2007).

Skeletons of compound heterozygous mice ($Fgf9^{+/-}$; $Fgf18^{+/-}$) were similar to controls, although a mild developmental delay in chondrocyte maturation was observed at E14.5 (Fig. S1). However, when one allele of *Fgf9* was removed in the context of *Fgf18* nullizygosity ($Fgf9^{+/-}$; $Fgf18^{-/-}$), $Fgf18^{-/-}$ skeletal phenotypes became 100% penetrant with regard to long bone, rib, and vertebral column abnormalities (Figs. 1B, C and 2B, C), and Alizarin red staining was absent in fibular remnants and autopod phalanges. In the reciprocal experiment, removal of one copy of *Fgf18* from *Fgf9*-deficient mice ($Fgf9^{-/-}$; $Fgf18^{+/-}$) worsened the rhizomelic hindlimb phenotype and reduced the area of Alizarin red staining (Figs. 1D, E and 2D, E).

Congenital absence of *Fgf9* and *Fgf18* ($Fgf9^{-/-}$; $Fgf18^{-/-}$) caused an extreme osteochondrodysplasia that was 100% penetrant (Fig. 1F). Multiple craniofacial defects were apparent, including striking calvarial agenesis with only small, ossified, frontal bones present, maxillary and mandibular hypoplasia, and a wide cleft palate (not shown). This phenotype is consistent with both *Fgf9* and *Fgf18* expression in cranial mesenchyme (Kim et al., 1998; Ohbayashi et al., 2002; Reinhold and Naski, 2007), and supports a previously unidentified role for *Fgf9* in intramembranous as well as endochondral ossification (Fig. 1F) (Fakhry et al., 2005). Patterning and growth of the axial skeleton was also abnormal, including rib, sternal, and vertebral anomalies. Rhizomelic shortening was observed throughout the appendicular skeleton, and

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