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Snail genes and embryonic bone development

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In the past two decades, Snail family genes have been extensively studied in mouse development and cellular function, including the regulation of epithelial-mesenchymal transitions (EMTs) during both embryonic development and metastasis of epithelial tumors, cell survival and cell death, the determination of cell fate, and the establishment of left-right asymmetry. However, their role in bone development is not as widely reported. The purpose of this review is to briefly overview embryonic long bone development and summarize recent studies that provide evidence for Snail signaling in long bone development

Embryonic bone development mice

Bone formation, also known as osteogenesis or ossification, is a process by which bone tissue replaces embryonic connective tissue to form the skeleton from childhood to early adulthood. Ossification lengthens and thickens bone, and also provides mechanical strength. In addition, the process of ossification is used throughout life to remodel bones. During embryogenesis, the cells that form the vertebrate skeleton are derived from three distinct embryonic lineages. The cranial neural crest cells form much of the craniofacial skeleton, the paraxial mesoderm (somites) generates axial skeleton, and the lateral plate mesoderm becomes limb skeleton. At the beginning of bone development, the distinct embryonic lineages migrate to the specific sites in the embryo where skeletal elements will develop. These cells then form highly condensed aggregates of mesenchymal cells, and differentiate into osteoblasts (bone-forming cells) or chondrocytes (cartilage-forming cells). There are two distinct ways that bones can develop in mammals: intramembranous ossification and endochondral ossification. During intramembranous ossification, mesenchymal precursor cells proliferate and subsequently differentiate directly into osteoblasts which mineralize into bone. In contrast, endochondral ossification is a multistep process in which mesenchymal cells condense to form a cartilage template that subsequently becomes replaced by mineralized bone. Both of these types of ossification are critical for embryonic development and adult life. Flat bones, like cranial skull bones and clavicles, are produced by intramembranous ossification; long, short, and irregular bones, like limb bones, are generated by endochondral ossification. In the following section, I will mainly focus on describing endochondral ossification, as this review concerns long bone development, which occurs by endochondral ossification.

Endochondral ossification, the replacement of a cartilage template by bone and bone marrow, occurs in more than 95% of the skeleton in most vertebrates (Epstein et al., 2004). This process has several steps. For the long bones of the limbs, the process begins when lateral plate mesoderm-derived mesenchymal progenitor cells migrate into the limb bud. These cells form mesenchymal condensations that produce a cartilage template, the shape of the future bone. Normally, mesenchymal condensation in mouse limb buds starts at around embryonic day (E)11.5 (Bi et al., 2001), and is a prerequisite of chondrogenesis during embryonic development (Thorogood and Hinchliffe, 1975).

After mesenchymal condensation is complete, cells in the condensation keep proliferating. Cells in the center of the condensation differentiate into flattened, lens-shaped

chondrocytes. The chondrocytes then align into vertical columns and produce an extracellular matrix (ECM) largely composed of type II collagen and specific proteoglycans such as aggrecan. In the meantime, the peripheral mesenchymal cells differentiate into the perichondrial cells to form perichondrium that separates developing skeletal elements from the surrounding mesenchyme. At this point, the cartilage primordia form.

Shortly after the formation of the cartilage primordia (E13.5), rapidly proliferating chondrocytes near the center of each growing element then stop dividing to enter into a transition stage called prehypertrophic chondrocytes, which quickly undergo further maturation to become enlarged hypertrophic chondrocytes. As hypertrophy proceeds, the hypertrophic chondrocytes continue to enlarge and synthesize type X collagen as well as several growth factors to generate a mineralized matrix. Collagen X has been extensively used as a marker for hypertrophic chondrocytes, as it is a unique component of this population (Iyama et al., 1991). The growth factors produced by hypertrophic chondrocytes send critical signals to the surrounding perichondrial cells to induce these cells to become osteoblasts. In parallel, hypertrophic chondrocytes secrete angiogenic factors, such as vascular endothelial growth factor A (VEGF-A), to initiate sprouting of blood vessels from the perichondrium, which allow the osteoblasts to travel into the cartilage mold secreting bone matrix to form true bone (E15.5). Hypertrophic chondrocytes eventually undergo apoptosis. Subsequently, growth plates, the zones of cartilage at each end of the long bones, sustain themselves and elongate developing bones. In brief, growth plate development is a step by step process in which chondrocytes keep proliferating and give rise, layer by layer, to maturing chondrocytes. Therefore, chondrocytes play a central role in this process. During development, the growth plate stratifies into zones of resting, proliferating, prehypertrophic, and hypertrophic chondrocytes, forming a highly organized and interactive structure.

Various genetic regulators and signaling pathways contributing to the regulation of the proliferation, differentiation, and maturation of growth plate chondrocytes at all stages of endochondral ossification have been extensively described in the last two decades, including SOX (Wagner et al., 1994; Lefebvre and de Crombrugghe, 1998; Lefebvre et al., 1998; Bi et al., 1999; Smits et al., 2001; Akiyama et al., 2002; Kist et al., 2002; Han and Lefebvre, 2008; Hattori et al., 2010) and RUNX (Stricker et al., 2002; Yoshida and Komori, 2005; Sato et al., 2008) transcription factors, bone morphogenetic proteins (BMPs) (Yi et al., 2000; Zhang et al., 2005; Yoon et al., 2006; Retting et al., 2009; Karamboulas et al., 2010), fibroblast growth factors (FGFs) [reviewed in (Degnin et al., 2010)], the IHH-PTHrP feedback loop (Kobayashi et al., 2002; Kronenberg, 2006; van Donkelaar and Huiskes, 2007), WNT signaling (Rudnicki and Brown, 1997; Stott et al., 1999; Tufan and Tuan, 2001), NOTCH signaling (Karlsson and Lindahl, 2009; Mead and Yutzey, 2009), and so on. Some of these factors and pathways accelerate chondrocyte development, and some of them inhibit this process [reviewed in (Long and Ornitz, 2013)]. All these molecules and signaling pathways highly interact with each other, forming a complex network to maintain bone growth and homeostasis (Fig. 1).

Overview of the Snail family genes

The Snail gene was first described in Drosophila melanogaster (Grau et al., 1984), where it is essential for formation of the mesoderm (Alberga et al., 1991). The Snail gene superfamily contains the Snail and Scratch families (Barrallo-Gimeno and Nieto, 2009). In the past 20 years, three members of the Snail gene family have been described in vertebrates: *Snai1* (also known as Snail), *Snai2* (Slug) and *Snai3* (Smuc). Snail family proteins are composed of a highly conserved C-terminus, containing four or five Cys2-His2 (C2H2)-type

zinc finger regions, and a more divergent N-terminus that contains the evolutionarily-conserved SNAG domain. The zinc finger regions are sequence-specific DNA-binding domains which are capable of recognizing E2-box sequences (CAGGTG and CACCTG). Both SNAI1 and SNAI2 proteins can recruit and bind other proteins, such as histone deacetylase-1 (HDAC-1), to the E2 boxes of target genes to form a transcriptional repression complex (Tripathi et al., 2005; Peinado et al., 2007) to suppress the transcription of target genes. Therefore, Snail transcriptional factors are currently considered as transcriptional repressors (Peinado et al., 2007).

Snai1 and *Snai2* have been widely studied due to their ability to trigger the epithelial to mesenchymal transition (EMT) during multiple processes of developmental and cancer biology. In addition, the SNAI1 and SNAI2 proteins also have demonstrated roles in other important developmental and cellular processes, such as the protection of cells from programmed cell death, the establishment of left-right asymmetry and the regulation of cell motility (Barrallo-Gimeno and Nieto, 2005; Haraguchi, 2009; Wu and Zhou, 2010). *Snai3* was first identified in adult skeletal muscle (Kataoka et al., 2000), and was subsequently found to be highly expressed in thymus, and at much lower levels in heart, lung and spleen (Zhuge et al., 2005). The role of *Snai3* during development in mouse has been described in detail in a recent paper (Bradley et al., 2013). All evidence indicates that Snail family factors are transcriptional repressors that can suppress the activation of downstream target genes (Nieto, 2002; De Craene et al., 2005). To date, numerous direct target genes of the SNAI1 and SNAI2 proteins have been characterized, including gene encoding the endothelial adherens junction component, VE-cadherin (Peinado et al., 2004; Peinado et al., 2007), and genes encoding tight junction components such as occludin and Claudins (Ikenouchi et al., 2003), the vitamin D receptor (Palmer et al., 2004), Puma (a mediator of p53-induced apoptosis) (Wu et al., 2005), cyclin D1 (Vega et al., 2004), Runx2, and type II collagen (Seki et al., 2003). Most interestingly, the *Snai1* gene itself is a target for repression by the SNAI1 protein (Peiro et al., 2006).

Snail family genes and long bone development

In addition to their role in epithelial cells, the *Snai1* and *Snai2* genes also function in some non-epithelial cells, such as chondrocytes and osteoblasts. *Snai3* is not expressed during embryonic bone development (my unpublished data).

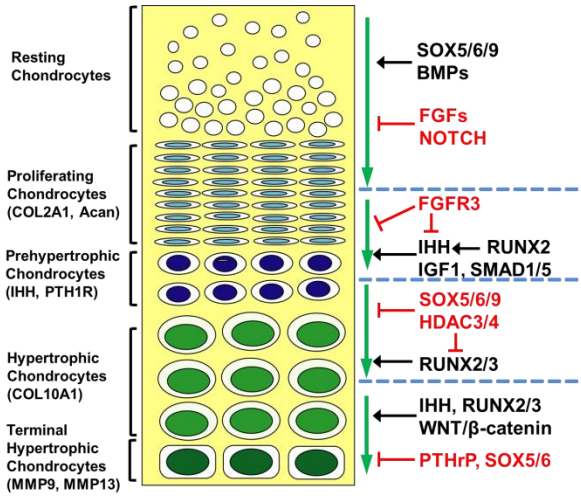

Figure 1. Schematic diagram of the molecular control of growth plate chondrocytes. Black arrows indicate stimulatory pathways, and red crossed lines indicate inhibitory pathways. Not all the molecules involved in this process are included.

Snai1 is first expressed in condensing pre-cartilage cells at the early stage of limb development (Nieto et al., 1992; Smith et al., 1992). During embryonic bone development, the *Snai1* and *Snai2* genes are highly expressed in chondrocytes and osteoblasts (Nieto et al., 1992; Oram et al., 2003), and have been implicated in cartilage (chondrocytes) and bone (osteoblasts) development. SNAI1 works as a necessary regulator by activating the early differentiation marker, Collagen I and

Osteopontin, and repressing Runx2 expression during osteogenic differentiation from mesenchymal stem cells (Park et al., 2010). Sustained *Snai1* activation in adult mouse osteoblasts facilitates the formation of unmineralized ECM but inhibits terminal differentiation of osteoblast (bone forming cells) and osteoclast (bone resorbing cells), leading to a defective mineral deposition (de Frutos et al., 2009). *SNAI2* is expressed in normal human osteoblasts. However, in vitro knockdown of *SNAI2* in human osteoblasts revealed that *SNAI2* was positively correlated with osteoblast markers, including Runx2 (Lambertini et al., 2009). These data suggest that SNAI1 and SNAI2 may act as positive or negative transcriptional regulators of Runx2 during different stages of osteogenesis.

Nieto's group showed that, in a gain of function mouse model, overexpression of *Snai1* in the developing limb bone inhibited chondrocyte proliferation and differentiation, and led to defects in both chondrocytes and osteoblasts, and consequently caused achondroplasias, the most common genetic forms of dwarfism, in mice (de Frutos et al., 2007). In their mouse model, *Snai1* was demonstrated as a critical downstream gene of fibroblast growth factor receptor 3 (FGFR3) and to be required for the transduction of FGFR3 signaling during bone development. Loss-of-function studies will help to better understand whether Snail family genes have an essential, physiological role during normal bone development.

Recently, our lab generated a loss-of-function mouse model in which we conditionally deleted the *Snai1* gene in limb bud mesenchymal progenitor cells by *Prrx1*-Cre on a *Snai2* null background. Using this mouse model, we demonstrated that *Snai1* and *Snai2* genes are required for chondrogenesis in mouse limbs by controlling chondrocyte proliferation and differentiation (Chen and Gridley, 2013a). Simultaneous deletion of the *Snai1* and *Snai2* genes led to shortened long bones, defects in chondrocyte morphology and organization, inhibited trabecular bone formation and delayed ossification. Most interestingly, we observed that when one of these two genes was mutated, the expression domain of the remaining gene (both mRNA and protein) expanded into the expression domain of the deleted gene, which means these two genes compensate for the lack of function in the other quantitatively, spatially, and temporally during this process. In addition to our in vivo data, our in vitro results support the model that expression of the *Snai1* and *Snai2* genes is negatively regulated by their protein products occupying each other's promoter during chondrogenesis, which helps provide an explanation for the genetic redundancy observed in the mouse loss of function models (Chen and Gridley, 2013b). The observations that either activation or deletion of Snail genes caused bone defects would suggest that normal bone development requires carefully-regulated expression of the *Snai1* and *Snai2* genes.

Prospective

A physiological role of the *Snai1* and *Snai2* genes during normal chondrogenesis has been explored using the *Prrx1*-Cre line in a loss-of-function mouse model (Chen and Gridley, 2013a). Using *the Prrx1*-Cre driver line results in the conditional knockout of the *Snai1* gene on a *Snai2* null background early in skeletal system development, in both cartilage and mineralized bone. It will be very interesting to see whether Snail genes behave in the same way during osteogenesis. In addition, more molecular targets of Snail family genes during bone development needed to be determined.

It has been reported that SNAI1 expression was highly increased in a human stillborn bearing the most severe and lethal achondroplastic condition (thanatophoric dysplasia type II; a K650E FGFR3 mutant) (de Frutos et al., 2007). In addition, the attenuation of one of *Snai1*'s upstream genes, FGFR3, has been long thought to be an effective therapy for achondroplasias. Because *Snai1* functions downstream of FGFR3 (de Frutos et al., 2007), modulation of Snail

genes activity could be a possible therapeutic avenue for treatment of achondroplasias.

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