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REVIEW

The role of Nkx3.2 in chondrogenesis

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Abstract **Transcription factor, Nkx3.2, is a member of the NK family of developmental genes and is expressed during embryogenesis in a variety of mammalian model organisms, including chicken and mouse. It was first identified in *Drosophila* as the Bagpipe (bap) gene, where it has been demonstrated to be essential during formation of the midgut musculature. However, mammalian homolog Nkx3.2 has been shown to play a significant role in axial and limb skeletogenesis; in particular, the human skeletal disease, spondylo-megaepiphyseal-metaphyseal dysplasia (SMMD), is associated with mutations of the Nkx3.2 gene. In this review, we highlight the role of Nkx3.2 during musculoskeletal development, with an emphasis on the factor's role in determining chondrogenic cell fate and its subsequent role in endochondral ossification and chondrocyte survival.**

Keywords Nkx3.2, musculoskeletal development, chondrogenesis, chondrocyte hypertrophy

Introduction: Expression of Nkx3.2

Nkx3.2, also referred to as Bapx1, is a transcription factor that belongs to the NK family of homeobox genes (Kim and Nirenberg, 1989). This transcription factor was initially identified as the Bagpipe (bap) gene in *Drosophila*, which is expressed in dorsal mesoderm along with Tinman, another homeobox NK gene. Bagpipe is essential for the specification of the visceral mesoderm, the progenitor tissue of the visceral muscle (Azpiazu and Frasch, 1993). In the absence of Bagpipe, visceral mesodermal cells do not form the columnar shape and are significantly reduced in number (Tribioli et al., 1997; Lettice et al., 2001). Vertebrate Bagpipe homologs have been found in *Xenopus* (Newman and Krieg, 1999), mouse (Yoshiura and Murray, 1997), and human (Tribioli and Lufkin, 1997). Two homologs of Bagpipe, Nkx3.1 and Nkx3.2, were first identified in mouse and named based on the names of the investigators who identified the NK family genes (Kim and Nirenberg, 1989). While both Nkx3.1 and Nkx3.2 are expressed in the somite, Nkx3.1 is more specifically expressed in the prostate (Bieberich et al., 1996). It has been shown in a Nkx3.1 null mouse model that this factor plays a role in the epithelial branching and proliferation in the prostate and palatine glands (Tanaka et al., 2000). Subsequently, Nkx3.1 has been identified as a tumor suppressor of prostate cancer (Lei et al., 2006).

Nkx3.2 is first expressed in the somite of the chick embryo at stage 9 and the mouse embryo at E8.0 (Tribioli et al., 1997). Then starting from chick stage 10 and mouse E8.5, it is also expressed in the lateral plate mesoderm and exhibits left-right asymmetry in expression. Interestingly, it is expressed at a higher level on the left side than the right side of the chick embryo while at a higher level on the right side than the left side of the mouse embryo (Schneider et al., 1999). At a later stage, this asymmetry is lost, and Nkx3.2 is expressed in the mesoderm around the midgut epithelium, including the mesenchyme dorsal to the pancreatic bud associated with spleen development (Schneider et al., 1999; Lettice et al., 2001). At stage 24 in chick development and in E10.5 mouse embryos, Nkx3.2 also starts to be expressed in the developing limb. The Nkx3.2 knockout mouse exhibited skeletal malformation as well as a hypoplastic spleen and stomach abnormalities, which are phenotypes consistent with Nkx3.2's expression pattern (Tribioli Akazawa et al., 2000; Tribioli and Lufkin, 1999; Asayesh et al., 2006; Verzi et al.,

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2009). However, there was no evidence of left-right asymmetry defects and thus the function of its sided expression during early embryogenesis is still unclear. In this review, we focus on the role of Nkx3.2 in the context of musculoskeletal development during chondrogenesis.

Role of Nkx3.2 in chondrocyte cell fate determination

Regulation in the somite

During vertebrae embryogenesis, mesodermal spherical structures called somites emerge along either side of the neural tube from the unsegmented paraxial mesoderm (Brent and Tabin, 2002). At first, the somite appears as an epithelial structure. As it matures, the dorsal domain of the somite gives rise to the dermomyotome, which subsequently becomes the dermatome and which contributes to the formation of the dermis and the myotome, the precursor to the skeletal muscle of the body wall and the limb. The ventral portion of the somite goes on to form the mesenchymal sclerotome that ultimately proceeds to form the vertebral cartilage and bone, as well as tendon and the intervertebral disc. Nkx3.2 is expressed in the ventral somite before the formation of the sclerotome and maintains its expression in the sclerotome (Tribioli et al., 1997).

The important role of Nkx3.2 in chondrogenic differentiation from the sclerotome has been demonstrated by the timing of its expression in relationship to Sox9, the master transcriptional regulator of chondrogenesis (Lefebvre and Smits, 2005), as well as in gain- and loss-of-function analyses. Importantly, Nkx3.2 is expressed in the sclerotome before Sox9 is expressed in the same region, and ectopic expression of Nkx3.2 in the dermomyotome leads to inhibition of dermomyotomal genes and induction of Sox9 (Cairns et al., 2008). The critical role of Nkx3.2 during chondrogenesis has been demonstrated through the targeted ablation of this gene in mouse (Tribioli and Lufkin, 1999; Akazawa et al., 2000; Lettice et al., 2001). Here, such loss gave rise to defective axial skeleton formation, with decreased expression of Sox9 and chondrogenic marker type II collagen (Col II) (Tribioli and Lufkin, 1999; Akazawa et al., 2000; Lettice et al., 2001). However, knocking out Nkx3.2 does not completely inhibit sclerotome patterning (Lettice et al., 2001). This could be due to the partial functional redundancy of the two Nkx genes, as the double homozygous mouse mutant for Nkx3.2 and Nkx3.1 showed much more impaired sclerotomal development and malformation of the vertebral column (Herbrand et al., 2002; Shen and Abate-Shen, 2003).

In further investigation of the chick somite, it has been suggested that Nkx3.2 and Sox9 cooperate to promote chondrogenic differentiation and serve as mediators of Sonic Hedgehog (Shh)-induced chondrogenesis (Murtaugh et al., 2001; Zeng et al., 2002). Both Shh and bone morphogenetic proteins (BMPs) are important factors for chondrogenesis (Yoon and Lyons, 2004). Nkx3.2 is induced by Shh and maintained by BMPs, and acts to block transcription of Sox9 repressors, while Sox9 can maintain expression of both itself and Nkx3.2 (Zeng et al., 2002). One such repressor of chondrogenesis could be Pax3, an inducer of dorsal somitic cell fate, as ectopic expression of Pax3 in the somite inhibits Shh-induced cartilage gene expression(Cairns et al., 2008). In fact, Cairns et al have demonstrated that a gradient of Shh in the somite mutually represses the cartilage and muscle cell fates induced by Nkx3.2 and Pax3, respectively (Cairns et al., 2008). Low levels of Shh maintain the Pax3 expression induced by exogenous Wnt signals and subsequently, myogenesis. In contrast, high levels of Shh induce Nkx3.2 and Sox9 expression, thereby promoting chondrogenesis. Furthermore, forced expression of Nkx3.2 can repress Pax3 expression in the somite. Interestingly, BMP signaling is required for Nkx3.2 to serve as a transcriptional repressor in somitic chondrogenesis (Murtaugh et al., 2001; Zeng et al., 2002) and that its activity requires BMP-dependent association with Smad1 (Kim and Lassar, 2003).

The relationship between Nkx3.2 and Pax genes has been further demonstrated in the Pax1:Pax9 double mutant mouse, where Nkx3.2 was found to be absent, suggesting that Pax1 and Pax9 are required for Nkx3.2 expression in the sclerotome (Rodrigo et al., 2003). Furthermore, Pax1 could substitute Shh to activate Nkx3.2 by binding to its promoter to induce chondrogenesis in the sclerotome. However, after the establishment of the chondrogenic lineage, Pax1 expression is excluded from the chondrocytes, and forced expression of Pax1 actually inhibits Nkx3.2 expression and accumulation of proteoglycan (Takimoto et al., 2013) . Therefore, the regulation of Nkx3.2 can be quite dynamic depending on the stages of chondrocyte differentiation.

Regulation in satellite cells

In addition to promoting the chondrogenic fate in chondrocyte precursors, such as those in the somite, Nkx3.2 can also promote chondrogenesis in the stem cells that are normally fated to become muscle (Cairns et al., 2012). Here, it has demonstrated that muscle satellite cells, the myogenic progenitor stem cell population within muscle (Collins et al., 2005), can undergo chondrogenesis in the presence of BMP-2 or TGF-β3, which is reminiscent of the fracture healing process *in vivo*, and that during this process, Nkx3.2 and Sox9 are both induced and inhibit Pax3 promoter activity and Pax3 protein expression. The Nkx3.2 DNA binding domain fused with a generic transcriptional activation domain blocked the ability of Sox9 to promote chondrogenesis, thereby suggesting that Nkx3.2 acts as a transcriptional repressor and is required for Sox9 to promote chondrogenesis

in muscle satellite cells. These results demonstrate the similar molecular mechanisms that can be used to promote chondrogenesis in the muscle stem cells as in the somite.

Regulation in chondrogenic cell lines

In addition to primary cells, Nkx3.2 is known to promote chondrogenic differentiation in mesenchymal cell lines, such as the murine C3H10T1/2 chondrogenic cell line. In such cells, Nkx3.2 has been shown to promote chondrogenesis through upregulation of Sox9 and independently from Sox9, through activating collagen II (Col II) promoter (Kawato et al., 2012). As with the somite, it has also been shown in C3H10T1/2 cells that Sox9 induces Nkx3.2 expression. Another mechanism by which Nkx3.2 promotes chondrogenesis in these cells could be through the inhibition of runt-related transcription factor 2 (Runx2; also called Cbfa1). Runx2 is a master regulator of the osteoblast cell fate, as little bone formation was observed in the Runx2 knockout mice (Ducy et al., 2000). At the onset of chondrogenesis, Nkx3.2 was found to inhibit Runx2 promoter activity . Yamashita et al, has further shown that Nkx3.2 is a direct target for Sox9 for repression of Runx2; Sox9 was shown to be physically bound to the Nkx3.2 promoter. Sox9 ectopic expression decreased Runx2 expression and transient knockdown of either Nkx3.2 or Sox9 increased the expression of Runx2 (Yamashita et al., 2009). Interestingly in this work, a knockdown of Nkx3.2 expression with an overexpression of Sox9 resulted in diminished effect of Sox9 with respect to Runx2 expression, thereby suggesting that Nkx3.2 is required for Sox9's chondrogenic activity, a finding which is consistent with the study on muscle satellite cells (Cairns et al., 2012). The balance between Nkx3.2 and Runx2 was found not only under normal culture conditions, but also in hypoxic conditions, a key microenvironmental feature essential for chondrogenic differentiation and survival *in vivo* (Kawato et al., 2011). Under hypoxic conditions, Nkx3.2 overexpression suppresses Runx2 expression while down-regulation of Nkx3.2 expression restores Runx2 expression.

Role of Nkx3.2 on chondrocyte hypertrophy during endochondral ossification

Cartilage serves as an intermediate template for bone formation. After adopting the cartilage cell fate, chondrocytes undergo endochondral ossification. During long bone development, chondrocytes of different differentiation stages are positioned into various zones (resting, proliferative, pre-hypertrophic, and hypertrophic) that represent the cells' functional states (Mackie et al., 2008). As the cartilage template elongates, resting and proliferating chondrocytes within the cartilage template mature from a proliferative state to a hypertrophic state, where they exit the cell cycle and enlarge their cell sizes. A parathyroid hormone related peptide-Indian hedgehog (PTHrP-Ihh) regulatory loop that involves a number of other secreted factors, such as transforming growth factors (TGFs) and Wnts, and transcription factors govern the pace of the maturation process of chondrocytes during endochondral ossification (Baffi et al., 2004; Mackie et al., 2008; Guo et al., 2009). All newly committed chondrocytes express Nkx3.2; this expression becomes restricted to the proliferative, especially columnar chondrocytes in the growth plate, but decreases as chondrocytes mature and become more hypertrophic during long bone development (Church et al., 2005; Provot et al., 2006). However, Nkx3.2 is not expressed in cells at the very surface of the developing bone (Church et al., 2005), which likely contains cells that would form articular chondrocytes, a population distinct in origin and function from the transient chondrocytes that make up the cartilage intermediate during endochondral ossification (Pacifici et al., 2005).

The role of Nkx3.2 in controlling the pace of chondrocyte proliferation to maturation has been demonstrated in several gain- and loss-of-function experiments. In the chick wing bud, Nkx3.2 misexpression generated a significant delay in chondrocyte maturation (Provot et al., 2006). Consistent with this, in chick limb bud micromass cultures, Nkx3.2 overexpression promoted chondrogenesis as demonstrated by increased alcian blue staining (Church et al., 2005). Finally, in the mouse, Nkx3.2 overexpression through a transgene resulted the formation of prechondrogenic condensations, as well as polydactyly and tibial hypoplasia (Tribioli and Lufkin, 2006). All these results suggest Nkx3.2 is sufficient to promote a proliferative chondrocyte phenotype, and suggest that the maturation process requires a downregulation of Nkx3.2. Interestingly, Nkx3.2 knockout mouse does not show any phenotype in chondrocyte hypertrophic differentiation (Tribioli and Lufkin, 1999; Akazawa et al., 2000; Lettice et al., 2001; Provot et al., 2006;). Yet, in a study using the chick embryo, when an Nkx3.2 reverse function mutant, where Nkx3.2 was converted into a transcriptional activator, was ectopically expressed in the limb, accelerated maturation of the chondrocytes occurred. Together, these studies demonstrate that while Nkx3.2 is sufficient to repress chondrocyte maturation, there is redundancy with Nkx3.2 in suppressing chondrocyte maturation *in vivo*.

One of the mechanisms by which Nkx3.2 inhibits chondrocyte hypertrophy could be through the inhibition of transcription factor Runx2. In addition to its key role in determining osteogenic cell fate, Runx2 also plays an important role in promoting chondrocyte hypertrophy (Enomoto et al., 2000; Guo et al., 2006). As in the case of chondrogenic-osteogenic cell fate determination in C3H 10T1/2 cells, a mutually exclusive relationship between the two factors also plays a role in chondrocyte maturation. Nkx3.2 and Runx2 are inversely expressed in the different zones of the growth plate, such that hypertrophic chondrocytes express higher levels of Runx2 and lower levels of Nkx3.2 as compared to proliferating chondrocytes (Provot et

al., 2006). Here, it has been demonstrated that Nkx3.2 overexpression caused reduced Runx2 expression in chondrocytes in the hypertrophic zone in the developing chick limb; accordingly, overexpression of Runx2 accelerated chondrocyte maturation. Interestingly, for Runx2 to promote chondrocyte hypertrophy such as collagen X expression, these cells have to be expressing Sox9 and Nkx3.2 first, so that to ensure a chondrogenic state before the chondrocyte maturation program can take place (Kempf et al., 2007).

To explore the mechanism that regulates Nkx3.2 expression during chondrocyte proliferation and maturation, Provot et al. (2006) examined the relationship between Nkx3.2 and parathyroid hormone-related protein (PTHrP). The secreted factor PTHrP is expressed at the end of the developing bone, and promotes chondrocytes proliferation; chondrocytes located further away from the epiphysis receive less PTHrP signals and transition into the hypertrophic phase (Kronenberg, 2003). PTHrP overexpression in chick limb buds led to enlarged Nkx3.2 expression domain; similarly, mouse embryos lacking PTHrP or its receptor had undetectable levels of Nkx3.2 within the proliferative chondrocytes of the growth plate. Consistently, overexpression of PTHrP repressed Runx2 expression in the hypertrophic zone (Guo et al., 2006). BMP proteins can also regulate Nkx3.2 expression. It was shown that BMP2 induces hypertrophy, while BMP7 suppresses hypertrophy. As the knockdown of Nkx3.2 prevented BMP7 induced hypertrophic gene expression in the chondrocyte cell line ATDC5 cells, it suggests that Nkx3.2 mediates BMP7's activity in maintaining a chondrogenic potential (Caron et al., 2013).

Role of Nkx3.2 in chondrocyte survival

Nkx3.2 not only plays a critical role in chondrocyte cell fate determination and the pace of chondrocyte differentiation and maturation, but also is important for chondrocyte survival in the somites as well as in the chondrogenic cell lines (Zeng et al., 2002; Park et al., 2007). Extensive studies from the Kim laboratory showed that Nkx3.2 binds NF-κB, which leads to NF-κB nuclear localization, thereby repressing apoptosis and promoting cell survival. In growth arrest hypertrophic chondrocytes, where Nkx3.2 is not expressed, there is no Nkx3.2 binding to NF-κB, and thus no repression of this signaling pathway. As a result, hypertrophic chondrocytes become apoptotic, and eventually allowing bone formation to take place where cartilage has originally occupied. Subsequently, the study in the ATDC5 chondrocyte cell line showed that Nkx3.2 leads to NF-κB nuclear localization by promoting the disassociation of IkB from the NF-κB/IkB complex (Park et al., 2007; Yong et al., 2011). These results suggest that there must be mechanisms to tightly control Nkx3.2 protein expression in order for chondrocytes to undergo cell death, which would allow bone formation to take place in a timely manner. In addition to transcriptional regulation by secreted factors such as PTHrP, Nkx3.2 protein stability is also regulated. It was recently found that Ihh, a factor expressed in the prehypertrophic zone, triggers Nkx3.2 protein degradation in ATDC5 cells (Choi et al., 2012).

Role of Nkx3.2 in human skeletogenesis

In humans, the recessive mutation of Nkx3.2 results in spondylo-megaepiphyseal-metaphyseal dysplasia (SMMD) (Hellemans et al., 2009; Simon et al., 2012). This rare condition manifests with cervical spine deformation, spinal cord injury, and shorter stature; individuals with SMMD possess short, stiff necks and trunks, along with disproportionate limbs, fingers, and toes. While many of these symptoms resemble Nkx3.2 mouse mutants when compared with Nkx3.2 knockout mouse, a number of differences are present (Hellemans et al., 2009). Unlike patients with SMMD, dysplasia of the appendicular skeleton is not observed in the Nkx3.2 knockout mouse. This may be due to the lethality of the mutation in mice, which prevents studies on the role of Nkx3.2 in postnatal appendicular skeleton development. This may also be due to the functional redundancy of Nkx3.2 with other unidentified genes in the mouse in controlling limb morphogenesis. Unlike the mouse model, SMMD patients are not asplenic, suggesting that additional differences in the regulatory mechanisms between human and mouse in splenogenesis. It is unclear whether Nkx3.2 is the only gene affected in SMMD, and further studies on this front may help explain the differences in phenotype in the mouse Nkx3.2 mutant and the human disease. Nevertheless, the embryonic and molecular analyses on Nkx3.2 not only shed light on the understanding of the etiology of this Nkx3.2-related human disease, but also provide insights into the fundamental mechanisms of bone formation, which is relevant to many other skeletal-associated diseases.

Conclusions

In this review, we have highlighted the role of transcription factor Nkx3.2 in axial and limb skeletogenesis, with emphasis on its importance in regulating chondrocyte cell fate and subsequent role in endochondral ossification and chondrocyte survival. Nkx3.2 is expressed in chondrogenic progenitor cells and proliferating chondrocytes. In the process of cell fate determination, Nkx3.2 maintains an intricate balance with Pax and Runx2 transcription factors to promote the expression of Sox9 in specifying cartilage cell fate. After establishing chondrocyte cell fate, Nkx3.2 expression is restricted to the proliferating chondrocytes and prevents chondrocyte hypertrophy, which is a natural maturation process during endochondral ossification in preparation for bone formation. Finally, Nkx3.2 has been shown to play a critical role in

chondrocyte survival through the binding to NF-κB, which subsequently causes a repression of apoptosis. Therefore, the overall function of Nkx3.2 is to promote and maintain the chondrogenic phenotype in various ways. The clinical relevance of Nkx3.2's role in skeletogenesis is revealed by the discovery of Nkx3.2 mutations in spondylo-megaepiphyseal-metaphyseal dysplasia (SMMD), a genetic human disease that is characterized by short statures and malformation of the spine and limbs. While the role of Nkx3.2 in axial and appendicular skeletogenesis has been explored, little is known regarding its potential function in craniofacial bone formation or in postnatal bone development and cartilage maintenance. These may be areas that warrant further investigations in the future. The mechanistic analysis on Nkx3.2-mediated chondrogenesis will certainly shed light on the understanding of human diseases such as SMMD, and inspire the development of potential treatment options of these skeletal diseases.

Compliance with ethics guidelins

Roshni S. Rainbow, Heenam Kwon and Li Zeng declare that they have no conflict of interest. This manuscript is a review article and does not involve a research protocol requring approval by the relevant institutional review board or ethics committee.

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