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Making no bones about it: Transcription factors in vertebrate skeletogenesis and disease

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Abstract

Skeletogenesis is a complex multi-step process, which involves many genes and pathways. The tightly regulated interplay between these genes in these pathways ensures a correct and timely organogenesis and it is imperative that we have a fair understanding of the major genes and gene families involved in the process. This review aims to give a deeper insight into the roles of 3 major transcription factor families involved in skeleton formation: *Sox, Runx* and *Pax* and to look at the human skeleotogenic phenotypes associated with mutations in these genes.

Keywords

skeletogenesis; *Sox*; *Runx*; *Pax*; microRNAs

INTRODUCTION

The vertebrate skeleton is a highly complex organ, which helps in maintaining balance and giving mechanical support and protection to vital internal organs. The incidence of skeletogenic disorders in humans is about in 1 in 4000 with many being lethal at an early age [1]. To have a better understanding of the process it is imperative that we understand the genes and the gene families involved and the molecular processes behind them. This will lead to a better understanding and management of the various diseases associated with the skeletal system.

Chondrocytes are the first skeleton-specific cell type to appear during development and defects in chondrogenesis lead to chondrodysplasias and osteoarthritis [2, 3]. Early chondrocyte differentiation and subsequent maturation are controlled by *Sox9* and its family members, *Sox5* and *Sox6* [4, 5] and these continue to play an important role all the way through to late skeletogenesis [6].

Runx2 is important for the initial commitment of perichondrial cells to the osteoblast lineage [7, 8], whereas *Runx3* has no independent role in skeletogenesis, double knockout mice of *Runx2* and *Runx3* show complete lack of hypertrophic chondrocytes [9, 10].

Pax1 and *Pax9* are two other important transcription factors (TFs) involved in skeletogenesis. In *Pax1* null mice the entire axial skeleton is defective with reduced or loss of ossification centers [11], whereas the *Pax9* null mice display no vertebral column defects

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but show preaxial polydactyly, cleft secondary palate and lack the derivatives of pharyngeal pouches [12].

This review will attempt to look in a greater detail at these genes and how they affect skeletogeneis and known human diseases associated with them.

Sox5, *Sox6* and *Sox9* during skeletogenesis

Sox stands for *Sry*-related high mobility group (HMG) box as *Sry* was the first member in the family to be discovered. *Sox9* belongs to the *SoxE* subgroup whereas *Sox5* and *Sox6* are part of the *SoxD* subgroup. These proteins have the most important role to play in the initiation and progression of chondrogeneis and skeletogeneis. The first skeletal specific cell type to appear during chondrogenesis is the chondrocyte. Precartilaginous condensation marks the first step of chondrogenesis. At this stage, these skeletal precursor cells stop expressing type I collagen and hyaluronan. Instead, they start expressing adhesion proteins like N-cadherin and tenascin-C which allow the cells to aggregate tightly [13]. The transcription factor *Sox9* has been shown to be required for these precartilaginous cell condensations and their survival but the mechanism behind it remains elusive [14, 15]. These skeletal precursor cells which are bipotential at this stage have the ability to become chondrocytes or osteoblasts. They express both *Sox9* as well as *Runx2* which is a master regulator in osteoblastogenesis. The commitment to chondrogenesis is largely determined by the key chondrogenic transcription factor, *Sox9*, whose expression is absolutely necessary [16]. It inhibits *Runx2* expression through another transcription factor, *Nkx3.2* (also known as *Bapx1*), and interacts directly with *Runx2* to repress its activity pivoting it towards the chondrogenic fate [17, 18]. Other transcription factors have been implicated in establishing chondrocyte commitment but the *in vivo* relevance has only been shown for *Pax1*, *Pax9*, *Nkx3.1* and *Nkx3.2* [17, 19–21].

During the next stage of chondrogenesis, the prechondrocyte cells in the centre of the precartilaginous condensation undergo differentiation to form the early chondrocytes. Studies have shown that *Sox9* has the ability to directly activate a 48-bp enhancer sequence in the intron of *Col2a1* which is highly expressed during this phase and this activity was potentiated by two other proteins, *Sox5* and *Sox6* [22, 23]. *Sox9* was found to bind as a homodimer to a pair of consensus sequence in cartilage genes like *Col2a1* and *Matn1* [24]. Early chondrocyte differentiation and subsequent maturation are governed by *Sox9*, *Sox5* and *Sox6*, also known as the Sox chondrogenic trio which activate cartilage-specific genes [4]. Overexpression of the Sox trio in cell culture was shown to be sufficient to induce chondrocyte differentiation of mesenchymal cells and nonchondrocyte cell lines, establishing the importance of the Sox trio in directing chondrogenesis [25].

Early chondrocytes further enlarge to form prehypertrophic chondrocytes. These cells eventually stop proliferating and become hypertrophic chondrocytes. This process requires the down-regulation of the Sox trio which negatively regulates hypertrophy to prevent the premature differentiation of prehypertrophic chondrocytes [26]. At the same time, the transcription factors *Runx2* and *Runx3* through the interactions with other factors positively regulate hypertrophy [27]. Other factors like *Msx2*, *Mef2c*, *Mef2d* and *Fra2* have also been associated with the positive regulation of hypertrophy though their mode of action is not well understood [28]. The Wnt/β-catenin pathway plays an important role in supporting osteoblastogenesis. It is hypothesized to down-regulate *Sox9* expression and upregulate *Runx2* expression, hence favoring the osteoblast differentiation [29–31]. The transactivation domain of *Sox9* was also shown to physically interact with β-catenin to enhance chondrocyte differentiation and with parts of the transcriptional machinery such as transcriptional co-activators CBP/p300 [24].

The control of the expression of the Sox trio themselves in chondrocytes remains unclear. Through *in vitro* studies, *Sox9* was proposed to self-regulate its expression via a positive feedback loop [32]. miR-145 was reported to negatively regulate chondrocyte differentiation by targeting *Sox9* in mesenchymal stem cells (MSCs) [33]. A recent study performed using rat chondrosarcoma cells found *Sox9* binding sites located within its introns which may support the proposition [34]. From the same study, *Sox9* binding sites were found in the *Sox5* promoter and its introns suggesting that *Sox9* may regulate *Sox5* expression directly. In another recent study, miR-194 was found to regulate chondrogenesis by targeting *Sox5* in human adipose-derived stem cells [35].

Relevance to human diseases and phenotype of knockout mice

Heterozygous mutations in and around *Sox9* were shown to cause a semi-lethal syndrome known as campomelic dysplasia, characterized by a severe form of human chondrodysplasia often accompanied by male sex reversal and defects in other non-skeletal organs, thus identifying *Sox9* as a critical player in chondrogenesis [35, 36]. Heterozygous *Sox9* mutant mice showed the same skeletal malformations as humans with campomelic dysplasia and die soon after birth [15]. A delay in chondrogenic mesenchymal condensation and premature mineralization was observed indicating that *Sox9* is needed for initiating condensation and the inhibition of hypertrophy in proliferating chondrocytes. This was supported by another observation that *Sox9*-null cells were excluded from wildtype cells during mesenchymal condensation and that these mutant cells do not express chondrogenic genes like *Col2a1*, suggesting that *Sox9* is required for a chondrogenic cell fate [16]. When *Sox9* was inactivated prior to the onset of mesenchymal condensation, mesenchymal condensations were completely absent, and *Runx2* expression was not detected, indicating that *Sox9* expression is required for the formation of osteochondroprogenitor cells [14]. Conditional *Sox9* inactivation after mesenchymal condensation using *Col2a1-Cre* was observed to cause condensed mesenchymal cells to stop differentiation and impaired those differentiated chondrocytes in their proliferation and maturation process.

The ectopic expression of *Sox9* in limb buds of transgenic mice showed ectopic formation of cartilage with the induction of *Sox5* and *Sox6* expression, while lack of *Sox9* abolishes *Sox5* and *Sox6* expression in chondrocytes, indicating that *Sox9* is required for the downstream expression of *Sox5* and *Sox6* [14, 36]. *Sox5* and *Sox6* are coexpressed with *Sox9* from the prechondrocyte stage onwards during chondrogenesis and are required for the overt chondrocyte differentiation [23, 37]. *Sox5*-null mice die at birth from respiratory distress and were observed to have a smaller ribcage and a cleft secondary palate whereas *Sox6*-null mice die at birth or soon after with a short sternum as compared to the wildtype [38]. In general, single gene knockouts for *Sox5* and *Sox6* demonstrated mild skeletal defects. However, when both genes are inactivated, the mice die three days before birth with severe defects in cartilage formation, demonstrating the functional redundancy between *Sox5* and *Sox6* in chondrogenesis. This severe chondrodysplasia phenotype observed is comparable to the phenotype of the conditional knockout of *Sox9* after mesenchymal condensation in the mice.

Runx2 and *Runx3* in skeletogenesis

The *Runx* family of genes encode for transcription factors that contain the characteristic DNA-binding runt domain which derived its name from the *Drosophila* pair-rule gene, *runt,* owing to the high degree of homology between the two sequences [39]. This highly conserved 128-amino-acid runt motif found proximal to the N-terminus has functions in (1) DNA binding, recognizing a canonical DNA motif TGPyGGTPy (where Py refers to pyrimidine) [40], (2) proteinprotein interactions [41] and (3) nuclear import that is in addition to the conserved nuclear matrix-targeting signal (NMTS) in the C-terminus [42].

Runx2 is a crucial factor for the initial commitment of perichondrial cells and condensed mesenchymal anlagen of the intramembranous bones to osteoblast lineage cells [7, 8]. In endochondral ossification, the link between chondrocyte maturation and osteoblast differentiation hinges on Ihh signalling. While *Runx2* regulates Ihh in the prehypertrophic chondrocytes, Ihh induces *Runx2* expression in the adjacent perichondrium [43]. *Runx2* expression, however, is not sufficient for osteoblast differentiation as reflected by the ectopic maturation of chondrocytes without any defects in osteoblast differentiation in transgenic mice constitutively expressing *Runx2* [44]. Further commitment of the *Runx2*-expressing osteoblast progenitor cells to fully committed osteoblasts in both endochondral and intramembranous bones requires a Krüppel-like zinc finger domain-containing transcription factor *Sp7* (*Osterix*) [45]. The activity of Osterix is enhanced through interaction with nuclear factor of activated T cells (Nfatc1) transcription factor [46].

The $Runx2^{+/-}$ mice appeared normal but on closer examination revealed a defect in intramembranous ossification characterized by hypoplastic clavicles and delayed fusion of the cranial fontanelles. These abnormalities reflected some of the symptoms in the human skeletal disorder, cleidocranial dysplasia [8, 47, 48]. $Runx2^{-/-}$ mice died from respiratory failure shortly after birth owing to the inability to respire due to a non-ossified rib cage. The mutant mice were clearly smaller with shorter limbs and snout and were devoid of an ossified skeleton. Analysis of all bones showed the absence of osteoblasts while chondrocytes were still present. This demonstrated that *Runx2* is essential for osteoblast differentiation and has no positive regulatory functions in chondrocyte differentiation and proliferation. Although the deletion of the *Runx2* gene has an impact on both intramembranous and endochondral ossification, the former appears more sensitive to *Runx2* deficiency.

Runx3, on the other hand, has no apparent role in skeletogenesis as the $Runx3^{-/-}$ mice either present a severe limb ataxia phenotype [49, 50] or die of starvation shortly after birth owing to excessive growth of gastric endothelial cells [51] with no overt skeletal defects. However, *Runx3* was noted to cooperate with *Runx2* in chondrocyte maturation evident from the lack of hypertrophic chondrocytes or the expression of the hypertrophic chondrocyte marker, *Col10a1*, in the skeleton of a $Runx2^{-/-}Runx3^{-/-}$ mouse embryos [9, 10]. These observations suggest that *Runx2* and *Runx3* play compensatory roles in chondrocyte maturation during endochondral ossification. However, *Runx2* dominates in advancing chondrocyte maturation over *Runx3* as chondrocyte maturation was more impeded in $Runx2^{-/-}$ mice than in $Runx3^{-/-}$ mice [9].

MicroRNA control of *Runx2* and human disease

Recently a host of miRNAs have been discovered as a form of intermediate regulatory mechanism employed by the *Runx2* transcription factor. The miR23a~27a~24-2 cluster was found to bind to the 3'UTR of *Satb2* to inhibit its activity. *Runx2* directly represses the transcription of the miR23a~27a~24-2 cluster thus releases the direct inhibition of *Satb2*, a *Runx2* repressor, to retard osteogenesis. There is a feedforward mechanism whereby miR23a binds directly to the 3'UTR of *Runx2* to induce *Runx2* transcription which in turn represses the miR23a~27a~24-2 cluster.

Constitutive *Runx2* expression through the final stages of osteoblast differentiation results in osteopenia in mice. The increase in these miRNAs during the end stages of osteoblastogenesis is thus believed to be one mechanism to interrupt sustained bone formation to prevent osteopenia. [52].

Another recent study, in MC3T3E1 and ATDC5 cells has established that at least 10 miRNAs (miR23a, miR-30c, miR-34c, miR-133a, miR-135a, miR204, miR205, miR217,

miR-218, miR338) directly target the 3'UTR of the *Runx2* mRNA and through that significantly inhibit osteogenic differentiation [53].

A new study has found evidence that miR-3960 directly targets *Hoxa2* which is a repressor of *Runx2* expression and miR-2861 directly targets *Hdac5* to release the inihibition on *Runx2* resulting in an increase in *Runx2* protein production. *Runx2* was also found to bind to the promoter of the miR-3960/miR-2861 cluster to increase its transcriptional activity. Hence, an autoregulatory relationship was described between *Runx2* and the miR-3960/miR-2861 cluster, found clustered at the same loci and transcribed from the same miRNA polycistron [54].

Currently, miRNAs targeting *Runx3* or regulated by *Runx3* in the context of bone formation are yet to be discovered.

Pax1 and *Pax9* in skeletogenesis

The *Pax* gene family constitutes a group of genes encoding transcription factors with a highly conserved DNA-binding domain, the paired-box. Genes within the family are further divided into subfamilies based on the presence of a combination of domains: paired-domain containing two Helix-turn-helix motifs [55], paired-type homeodomain and octapeptide motif (HSVSNILG) [56]; their sequence similarity; and overlapping domains of expression. Identified initially through similarity to the paired-box in the Drosophila gene *paired*, *Pax1* and *Pax9* are two of the *Pax* genes in the same subfamily, essential for the early stages of axial skeleton formation [57].

Of all the nine members of the *Pax* gene family in the mouse, *Pax1* and *Pax9* are the only *Pax* genes that are expressed in sclerotomal cells. They contain only the paired-domain and the octapeptide motif, and share a high protein sequence similarity of 79%, diverging only at their C-terminal ends. Moreover, they share similar expression domains (but not identical), especially in the sclerotome and later in the intervertebral disc anlagen [58].

The Sonic Hedgehog (Shh) morphogen emanating from the notochord and floor plate of the neural tube induces the expression of *Pax1* transcripts at E8.5 in the ventro-medial deepithelializing somites to specify their sclerotomal fates [59]. *Pax9* transcripts are expressed slightly later (E9.0) and restricted to the caudal half of the sclerotome, unlike *Pax1* which is expressed in the rostral half as well. Subsequently *Pax1* and *Pax9* become restricted to the intervertebral disc anlagen by E12.5 [58, 60].

The importance of *Pax1* in the development of vertebral column, scapula and sternum was initially identified through several spontaneous mouse mutants: *undulated* (*un*) [61], *Undulated short-tail* (*Un*^{s}) [62], *undulated-extensive* (*un*^{ex}) [63] and *undulated intermediate* (*un-i*) [64] which consist of either point mutations or the deletion of the entire *Pax1* locus [11, 56, 60]. Subsequent targeted disruption of *Pax1* in mice confirmed its role in the proper formation of these skeletal structures. *Pax1* heterozygotes were externally normal like wild-type mice, but displayed abnormalities of some skeletal elements such as the first two cervical vertebrae, lumbar vertebrae and sternum with an overall penetrance of 88%. *Pax1*^{null} mice were smaller than wild-type mice and had a charactersitic shortened, kinked-tail phenotype. The entire axial skeleton encompassing the vertebral column, scapula, sternum and tail were all defective with reduced or lost ossification centers, fusion of pedicles, loss of acromion process and inappropriate ossification of some of the intersternebra. Deformation in the lumbar region was more severe, with split vertebrae, lack of intervertebral discs and formation of ventral rod-like cartilage structures [11].

Targeted inactivation of *Pax9* surprisingly does not give rise to any vertebral column defects. While *Pax9* heterozygotes are perfectly normal, $Pax9^{null}$ mutants display several defects [12].

$Pax9^{null}$ mice display preaxial polydactyly, cleft secondary palate and lack the derivatives of pharyngeal pouches (parathyroid glands, thymus and ultimobrancial bodies) and all teeth. This phenotype, distinct from that of $Pax1^{null}$, corroborates with the *Pax9* expression sites in the neural crest-derived cells of the craniofacial and tooth mesenchyme [12].

Despite the complete lack of vertebral column defects, and the possession of a distinct set of phenotypic changes in the $Pax9^{null}$ mice, it was postulated that *Pax9* and *Pax1* may have a genetic interaction due to their high sequence similarity and overlapping expression domains in the sclerotome. It was hypothesized that they may have redundant roles in their site of co-expression - the sclerotome. This prompted the generation of the *Pax1/Pax9* double null mice [20].

A study of $Pax1^{null}Pax9^{null}$ (double null) mice revealed that these two closely related TFs indeed have redundant roles in vertebral column development. While *Pax1* can fully compensate for the loss of *Pax9* in the vertebral column, absence of *Pax1* can only be partially compensated for by *Pax9*. In accordance to their redundant roles, there is a gene-dosage effect observed in the compound mutants and the disruption of both *Pax* genes leads to an overt phenotype in the vertebral column where there are no vertebral bodies or intervertebral discs (IVD) and proximal parts of the ribs are also defective. The vertebral column defects in $Pax1^{null}Pax9^{null}$ double mutants were more severe than that in $Pax1^{null}$ single mutants [20].

The development of the axial skeleton itself is a multi-step process beginning with somitogenesis, followed by de-epithelialization of somites, proliferation of the sclerotomal cells which then migrate and condense around the notochord, which subsequently undergoes endochondral ossification [65]. While *Pax1* and *Pax9* are not required for the formation of the sclerotome per se, it is hypothesized that they are needed to maintain the proliferative capacity of sclerotomal cells, sufficient to attain a critical density of cells for mesenchymal condensation to form, upon which chondrogenesis takes place [20]. Indeed, the essential role of *Pax1* in regulating cell proliferation is evident through its genetic interaction with another TF, the mesenchyme forkhead-1 (*Mfh1*). *Mfh1* is also expressed in the sclerotome and has been shown to synergize with *Pax1* to control the mitotic activity of sclerotomal cells [66].

Furthermore, *Pax1* and *Pax9* have been shown in an *in vitro* study to directly bind to the promoter and trans-activate *Bapx1*, another key TF known to be essential for the proper differentiation of prechondroblast into chondrocytes in axial skeletal formation [19, 67]. This lends support that both the *Pax*es are involved in the early stages of axial skeleton formation and are critical for its development.

Therefore, identification of the target genes of *Pax1* and *Pax9* will help illuminate the early events of regulation involved in the commitment of MSCs towards the osteo-chondrogenic lineage.

Human disease associated with the *Pax* gene family

The diseases of *Pax1* and *Pax9* are not limited to that of the mice. In fact, similar phenotypes of malformed vertebral column have been observed in human fetuses suffering from the Jarcho-Levine syndrome, whereby *PAX1* and *PAX9* protein expression was significantly reduced [68]. Similarly, *PAX1* mutations have been associated with certain forms of

Klippel-Feil syndrome [69]. The conserved roles of *Pax1* and *Pax9* in mouse and humans indicates the suitability of mouse as a model system to study such developmental disorders.

CONCLUSION

It is becoming increasingly clear that a broader systems biology approach is required to understand the complex developmental systems of vertebrates and in turn get a clearer picture of developmental diseases. Efforts are underway to elucidate comprehensive gene regulatory networks that will eventually lead to better comprehension of developmental disorders and how to manage them. The mouse will continue to be an indispensible ally in this effort and mapping multiple mutations in important genes will allow us to decipher human phenotypes better and hopefully lead to the development of remedies or cures.

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