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Roles and regulation of SOX transcription factors in skeletogenesis

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Abstract

SOX transcription factors participate in the specification, differentiation and activities of many cell types in development and beyond. The 20 mammalian family members are distributed into eight groups based on sequence identity, and while co-expressed same-group proteins often have redundant functions, different-group proteins typically have distinct functions. More than a handful of SOX proteins have pivotal roles in skeletogenesis. Heterozygous mutations in their genes cause human diseases, in which skeletal dysmorphism is a major feature, such as campomelic dysplasia (*SOX9*), or a minor feature, such as LAMSHF syndrome (*SOX5*) and Coffin-Siris-like syndromes (*SOX4* and *SOX11*). Loss- and gain-of-function experiments in animal models have revealed that SOX4 and SOX11 (SOXC group) promote skeletal progenitor survival and control skeleton patterning and growth; SOX8 (SOXE group) delays the differentiation of osteoblast progenitors; SOX9 (SOXE group) is essential for chondrocyte fate maintenance and differentiation, and works in cooperation with SOX5 and SOX6 (SOXD group) and other types of transcription factors. These and other SOX proteins have also been proposed, mainly through *in vitro* experiments, to have key roles in other aspects of skeletogenesis, such as SOX2 in osteoblast stem cell self-renewal. We here review current knowledge of well-established and proposed skeletogenic roles of SOX proteins, their transcriptional and non-transcriptional actions, and their modes of regulation at the gene, RNA and protein levels. We also discuss gaps in knowledge and directions for future research to further decipher mechanisms underlying skeletogenesis in health and diseases and identify treatment options for skeletal malformation and degeneration diseases.

1. Introduction

The vertebrate skeleton is an edifice of many structures varying in composition, size, shape and anatomical position. Its development involves the specification and coordinated actions of highly specialized progenitor and differentiated cells (Berendsen & Olsen, 2015). Progenitors arise from the cranial neural crest, paraxial mesoderm and lateral plate mesoderm. Upon migrating to their destined locations, they form skeletogenic mesenchymal condensations. They then engage in multi-step differentiation programs to become chondrocytes, osteoblasts, synovial fibroblasts or tenocytes, which build the skeleton and ensure its growth and maturation. Subsets of progenitors persist throughout development within and around skeletal structures to produce new waves of differentiating cells and

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participate in intense patterning and differentiation cross talk with them. All cells' phenotypes rely on the implementation of specific genetic programs, and thus on proper expression and utilization of unique sets of transcription factors. The discovery three decades ago that forced expression of the transcription factor MYOD was sufficient to convert mesenchymal cells into myoblasts led to the proposition that each cell type would be governed by a single master transcription factor. Since then, it has been well proven that transcription factors work in sets rather than solo and that many families of transcription factors participate in cell type-specific functions. Each family is characterized by a unique DNA-binding domain, which typically recognizes a precise DNA sequence. Most transcription factors also feature domains that confer specific transcriptional activities. Pioneer transcription factors physically interact with naive chromatin and recruit chromatin-modifying enzymes to displace nucleosomes and poise gene loci for transcriptional activation (Iwafuchi-Doi & Zaret, 2016). Transactivators bind specific DNA sequences at open enhancers or promoters and recruit co-activators that contact the basal transcription machinery to effect transcription. Transrepressors, in contrast, recruit co-repressors to inhibit the basal transcriptional machinery. Architectural factors promote the assembly of enhanceosomes (protein complexes bound to enhancers) by binding DNA near other factors and facilitating their physical and functional interactions.

The family of SOX proteins are key members of cell type-specific transcription factor sets in many lineages (Kamachi & Kondoh, 2013). We here review current knowledge and gaps in knowledge regarding skeletogenic SOX proteins. We introduce them in the context of their family, describe human diseases due to mutations in their genes, and review their roles and modes of regulation. We conclude with suggestions for future research on the SOX family to deepen understanding of skeletogenesis and related diseases.

2. Shared and distinctive features of SOX proteins

SOX proteins belong to the super-family of HMG (high-mobility-group) domain-containing proteins, as do the TCF/LEF WNT signaling targets and mediators (Kamachi & Kondoh, 2013). The HMG domain comprises three α -helices that bind DNA in the minor groove and force a 30–100° DNA bent (Fig. 1A). The latter property allows LEF1 to promote the assembly of enhanceosomes and might therefore be a property of SOX proteins too (Giese, Amsterdam, & Grosschedl, 1991).

SRY was the first SOX protein to be discovered. Encoded by the Sex-determining Region on the Y chromosome in mammals, it initiates a cascade of genetic events that lead to testis development and thus to male differentiation. Subsequently, all genes found to encode a protein with at least 50% identity with SRY in the HMG domain were called SOX, for SRY-related HMG box. SOX proteins share only 20% identity in this domain with other super-family members, but have conserved the residues necessary for characteristic DNA binding and bending. SOX genes exist in animals only, with a handful of them in invertebrates and 20 of them in humans and most mammals (Phochanukul & Russell, 2010; Schepers, Teasdale, & Koopman, 2002).

SOX proteins are distributed into eight groups (Fig. 1B). Those involved in skeletogenesis include the SOXB-group SOX2; the SOXC-group SOX4, SOX11, and SOX12; the SOXD-group SOX5 and SOX6, and the SOXE-group SOX8 and SOX9. Members of the same group share almost 100% identity in the HMG domain, but only about 50% with other-group proteins. Most SOX proteins exhibit at least one additional functional domain, conserved among group members only (Fig. 1C). SOX2, SOXC and SOXE proteins feature group-specific transactivation domains and SOXD and SOXE proteins possess group-specific homodimerization domains (Barrionuevo & Scherer, 2010; Dy et al., 2008; Hoser et al., 2008; Lefebvre, Li, & de Crombrughe, 1998; Sarkar & Hochedlinger, 2013).

All SOX proteins bind DNA motifs matching or resembling the $C^A/TTG^A/T/T$ sequence (Kamachi & Kondoh, 2013). They thus have the potential to select and compete for the same target genes. This mechanism was proposed to explain competition between SOXD and SOXE proteins in non-skeletal cell types, such as glial cells (Reiprich & Wegner, 2015), and sequential actions of SOX2 and SOXC proteins on the same targets in the neuronal lineage (Bergsland et al., 2011). SOX proteins, however, often select other targets than their family members do and distinct targets in different cell types or cell differentiation stages because of cooperativity with separate partners. This has been best documented for SOX2 (Sarkar & Hochedlinger, 2013) and is starting to be uncovered for other SOX proteins too.

Evolution has diversified the coding as well as regulatory sequences of SOX genes, such that each gene is expressed in a discrete set of cell types. These cell types can be far removed from each other. SOX9, for instance, is expressed in chondrocytes, Sertoli cells, neuronal cells, and many progenitor cell types. Same-group members often overlap in expression and thus functions, and different-group members can overlap in expression, but generally have distinct functions.

3. Skeletal dysmorphism due to SOX mutations

Mutations in 10 SOX genes, including skeletogenic ones, are known to date to cause a human developmental syndrome. The diseases are rare and most often due to *de novo* heterozygous mutations that result in gene or protein inactivation and thus reflect gene haploinsufficiency.

Mutations affecting *SOX9* cause Campomelic Dysplasia (CD), a severe skeletal malformation syndrome associated with XY sex reversal (Unger, Scherer, & Superti-Furga, 2008). Features include limb (melic) bending (campo), low-set ears, a flat nasal bridge, small jaw, cleft palate, and narrow chest. Most cases die neonatally from respiratory distress due to skeletal malformations. Survivors have short stature, flat face, micrognathia, kyphoscoliosis, hypoplastic nails, and hypotonia (Corbani et al., 2011). Most also have global developmental delay, mild mental retardation, hearing impairment, cardiac defects and hydronephrosis. CD-causing mutations abolish SOX9 protein production or function, or affect SOX9 gene expression. Chromosomal translocations occurring up to 700kb upstream of *SOX9* generally cause CD; up to 1Mb cause acampomelic dysplasia, a mild form of CD; and up to 1.5 Mb cause Pierre Robin Sequence, featuring micrognathia and cleft palate (Gordon et al., 2009).

Heterozygous mutations in *SOX5* cause LAMSHF syndrome, a global developmental delay disorder manifested by marked speech and locomotor retardation, hypotonia, strabismus and skeletal malformations (Lamb et al., 2012; Nesbitt et al., 2015). The latter include short stature, frontal bossing, microretrognathia, clinodactyly, butterfly vertebrae, and scoliosis.

A balanced translocation (t(9;11)(q33;p15) disrupting *SOX6* (11p15) was found in a child with a complex craniofacial dysostosis including craniosynostosis (Tagariello et al., 2006). A child with a 9q32–q34 deletion had a similar phenotype, but without craniosynostosis, and a child with a missense mutation in *SOX6* only had craniosynostosis. These cases suggest that *SOX6* mutations could cause craniosynostosis, but additional cases are needed to definitively link *SOX6* mutations to this disease.

SOX11 heterozygous mutations were described to cause a mild CoffinSiris-like syndrome (Hempel et al., 2016; Tsurusaki et al., 2014). Affected children had developmental delay, intellectual disability, short stature, microcephaly, fifth-finger clinodactyly, 2–3 toe syndactyly, nail hypoplasia, scoliosis, a wide mouth and prominent lips. One child also had a cleft palate (Khan, Study, Baker, & Clayton-Smith, 2018). Very recently, *SOX4* heterozygous mutations were found to cause a similar syndrome (Zawerton et al., 2019).

These autosomal-dominant diseases reveal the importance of SOX gene dosage in skeletogenesis and other processes, but do not reveal the full spectrum of SOX gene activities. The higher severity of campomelic dysplasia compared to the LAMSHF and Coffin-Siris-like syndromes does not mean that only SOX9 has key skeletogenic functions, but rather reflects the fact that SOX9, unlike SOX5/SOX6 and SOX4/SOX11, has no redundant partner in skeletogenesis. Reaching deep understanding of the SOX gene functions is necessary to better understand developmental and adult diseases directly or indirectly due to SOX deficiencies.

4. SOX genes and the control of skeletal progenitors

Embryogenesis involves a cellular hierarchy, whereby embryonic pluripotent stem (ES) cells give rise to progenitor cells with progressively more restricted lineage potential. ES cell programming, self-renewal, and activity are governed by a quartet made of the transcription factors SOX2, OCT3/4 (POU-domain protein), KLF4 (zinc-finger protein), and c-Myc (a basic-helix-loop-helix protein) (Sarkar & Hochedlinger, 2013). The quartet has both pioneer and transactivation functions. The proteins bind their targets on adjacent recognition sites in enhancers and promoters. They remain expressed and essential in several types of multipotent progenitors. Relevant to skeletogenesis, SOX2 controls the delamination of neural crest cells from the neural tube (Mandalos & Remboutsika, 2017). It is expressed *in vitro* in a subset of newborn mouse calvarium cells (Basu-Roy et al., 2010). These presumptive osteoblast stem cells rely on SOX2 for self-renewal. Other SOX genes are also expressed and have important roles in skeletal progenitors.

The SOXE genes are expressed in the cranial neural crest and redundantly specify these cells (Haldin & LaBonne, 2010). Only *SOX10* remains expressed during cell migration to

definitive anatomic sites. It is then turned off in skeletal progenitors, but stays on and directs cell differentiation in the neuronal, glial and melanocyte lineages (Sommer, 2011).

The SOXC genes, primarily *Sox4* and *Sox11*, are expressed in many progenitor types, including skeletal progenitors (Dy et al., 2008). They are needed for cell survival at the time of mesenchyme formation and act at least in part by activating the gene for TEAD2, a HIPPO pathway mediator (Bhattaram et al., 2014, 2010). They may also act in skeletal progenitors as in cancers by promoting cell migration and epithelial-to-mesenchymal transition by upregulating genes for essential AKT, p53, WNT, and NOTCH signaling components (Kuo et al., 2015; Lourenco & Coffey, 2017).

Sox8 and *Sox9* are co-expressed with the SOXC genes in skeletogenic mesenchyme (Akiyama et al., 2005). Their single inactivation has no obvious effect before osteoblasts and chondrocytes are due to differentiate, respectively (Akiyama, Chaboissier, Martin, Schedl, & de Crombrughe, 2002; Mori-Akiyama, Akiyama, Rowitch, & de Crombrughe, 2003; Schmidt et al., 2005). Whole-transcriptome and whole-epigenome profiling in mouse limb buds before pre-cartilaginous condensation have revealed limited differences between wild-type and *Sox9*-null embryos (Liu, Angelozzi, Haseeb, & Lefebvre, 2018). While these findings suggest limited transcriptional activity of SOX9 in progenitor cells, other studies have suggested important non-transcriptional actions. For instance, competition between SOX9 and β -catenin, an anti-chondrogenic protein, contributes to specifying the chondrocytic versus non-chondrocytic cell fate (Akiyama et al., 2004; Topol, Chen, Song, Day, & Yang, 2009). Complexes formed between the two proteins are degraded through the proteasomal pathway, resulting in elimination of the less abundant protein (Akiyama et al., 2004; Topol et al., 2009). SOX9 can also physically interact with RUNX2 and may thereby delay the master osteogenic actions of this RUNT-domain transcription factor (Zhou et al., 2006).

Once skeletal tissues overtly develop, progenitor/stem cells are maintained in specific locations and involve SOX proteins in their regulation and activities. The SOXC genes are highly expressed in perichondrium and presumptive joint cells. They set the boundaries of individual skeletal elements by keeping these cells non-chondrogenic, and they also establish cross talk with chondrocytes to initiate growth plate formation (Bhattaram et al., 2014). They may favor articular cartilage development by upregulating *Gdf5* (growth and differentiation factor-5) in presumptive joint cells (Kan et al., 2013), and may induce growth plate formation and organization by upregulating *Wnt5a* (non-canonical WNT ligand) in perichondrial cells (Kato, Bhattaram, Penzo-Mendez, Gadi, & Lefebvre, 2015). They also efficiently bind to and stabilize β -catenin, aborting thereby chondrogenesis in perichondrium and presumptive joints (Bhattaram et al., 2014).

Pthlh (parathyroid hormone-related signaling factor)-positive cells have been identified as chondrocyte stem cells in mouse epiphyseal growth plates (Mizuhashi et al., 2018), and multipotent skeletal stem cells in mouse and human growth plates based on cell surface markers (Chan et al., 2018, 2015; Mizuhashi et al., 2018). The expression and roles of SOX genes in these cell types are likely, but remain undocumented.

Based on all data described above and for neurogenesis (Reiprich & Wegner, 2015), a SOX hierarchy might exist in skeletal progenitors. SOX2 would govern stem cell specification and self-renewal, SOXC proteins would ensure cell survival and cross talk with the environment, and SOXE proteins would be involved in controlling downstream lineage commitment (Fig. 2A).

5. Roles of SOX genes in chondrogenesis

SOX9 and SOX5/SOX6 have long been known to be essential for chondrogenesis (Hata, Takahata, Murakami, & Nishimura, 2017; Kozhemyakina, Lassar, & Zelzer, 2015; Lefebvre & Dvir-Ginzberg, 2017). Yet, several outstanding questions on their specific actions were answered only recently and others remain unanswered. Their genes are active in the chondrocyte lineage from the progenitor mesenchymal stage until the prehypertrophic stage in growth plates or throughout adulthood in permanent cartilages (Dy et al., 2008; Henry, Liang, Akdemir, & de Crombrughe, 2012; Lefebvre et al., 1998; Ng et al., 1997) (Fig. 2B). None is necessary for progenitor specification and colonization of skeletogenic sites (Akiyama et al., 2002; Bi, Deng, Zhang, Behringer, & de Crombrughe, 1999; Smits et al., 2001), and although SOX9 was postulated to specify the chondrogenic fate of progenitors, it was recently found dispensable for this event (Liu et al., 2018). The pioneer factors that poise progenitors for chondrogenesis remain unknown, but it is possible that SOX9 is one of them and acts in redundancy with its co-expressed relative, SOX8 (Sock, Schmidt, Hermanns-Borgmeyer, Bosl, & Wegner, 2001). In its first or next role, SOX9 ensures prechondrocyte condensation and survival (Akiyama et al., 2002; Bi et al., 1999), likely by upregulating genes for cytoskeleton assembly, homotypic cell-cell adhesion and heterotypic cell-cell repulsion, such as *Sema3c* and *Sema3d* (Liu et al., 2018). SOX5 and SOX6 are dispensable at that stage (Smits et al., 2001).

SOX9 and SOX5/SOX6 are called the chondrogenic trio because they are essential for early chondrocyte differentiation. When *Sox9* is inactivated at the onset of chondrogenesis in mice, prechondrocytes fail to produce a cartilaginous extracellular matrix because they are unable to robustly express chondrocyte-specific genes, such as *Col2a1* (collagen type 2) and *Acan* (aggrecan) (Akiyama et al., 2002). Mice lacking either *Sox5* or *Sox6* have modest skeletal defects, whereas mice lacking both genes have severely underdeveloped cartilage primordia, indicating a large degree of gene redundancy (Smits et al., 2001). *Sox5/Sox6*-null chondrocytes weakly express cartilage-specific genes, despite expressing *Sox9* normally. These loss-of-function studies have thus demonstrated that the SOX trio is necessary for early chondrocyte differentiation. Complementing them, gain-of-function studies have demonstrated that the trio is also sufficient to convert progenitor cells into chondrocytes (Ikeda et al., 2004). The SOX trio has similar roles in the notochord. This embryonic structure secretes key morphogens for organogenesis and features a cartilage-like sheath that provides axial support. Without *Sox9* or *Sox5/Sox6*, notochord cells fail to produce this sheath and die before converting into intervertebral disc nuclei pulposi (Barrionuevo, Taketo, Scherer, & Kispert, 2006; Smits & Lefebvre, 2003).

The SOX trio is also indispensable for cartilage growth plate formation (Akiyama et al., 2004; Dy et al., 2012; Ikegami et al., 2011; Smits, Dy, Mitra, & Lefebvre, 2004). It

continues to ensure extracellular matrix production as chondrocytes line up in longitudinal columns. Its expression increases in these columns, likely delaying cell proliferation arrest and prehypertrophic differentiation. It is abruptly turned off in hypertrophic chondrocytes, but the SOX9 protein survives its RNA (SOX5 and SOX6 have not been tested) and the trio is required for chondrocyte enlargement and expression of specific markers, such as *Col10a1* (Dy et al., 2012; He, Ohba, Hojo, & McMahon, 2016; Smits et al., 2004). Sox9-null prehypertrophic chondrocytes die or prematurely convert into osteoblasts. Conversely, chondrocytes overexpressing SOX9 in the *Col10a1* domain remain hypertrophic longer than normally (Hattori et al., 2010). SOX9 thus maintains the chondrocyte fate throughout hypertrophy. It remains unknown whether SOX5/SOX6 are needed for this activity.

The approach of chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq) has greatly helped provide detailed information on the transcriptional actions of the SOX trio. In non-hypertrophic chondrocytes, the trio binds enhancers and super-enhancers (enhancer clusters) associated with hundreds of cartilage-specific genes (Liu & Lefebvre, 2015; Ohba, He, Hojo, & McMahon, 2015). These genes encode extracellular matrix macromolecules (e.g., the collagen types II, IX and XI, aggrecan, and link protein), regulators of this matrix (e.g., chondroitin 4-sulfotransferase-11), and key signaling pathway components (e.g., fibroblast growth factor receptor-3). The trio also positively feedbacks its own genes. SOX9 binds most enhancers at pairs of SOX motifs oriented head-to-head and separated by 3–4 nucleotides. It may also contact enhancers through protein-protein interactions. This indirect mechanism was also proposed for its binding to promoters of broadly expressed genes. SOX5/SOX6 preferentially bind tandem pairs of SOX-like motifs close to SOX9. They thereby strengthen SOX9 binding to DNA and gene transactivation. Recognition motifs for forkhead, RUNT-domain, NFAT, zinc-finger and AP1 family members are enriched in SOX trio-bound enhancers. Accordingly, there is *in vivo* and *in vitro* evidence that GLI factors, which are zinc-finger proteins mediating Hedgehog signaling, functionally interact with SOX9 in proliferating and prehypertrophic chondrocytes, that the JUN and FOSL2 AP1 factors functionally interact with SOX9 in the transition to hypertrophy, and that competition between SOX9 and the forkhead FOXA2 factor may be determining in regulating hypertrophic markers, including *Col10a1* (He et al., 2016; Tan et al., 2018).

SOX2 was recently shown to be expressed, along with its stem cell partners OCT4 and NANOG, in bone fracture callus where hypertrophic chondrocytes transition into osteoblasts and to be involved in this event (Hu et al., 2017). This finding, along with evidence of *Sox2* expression in cartilage growth plates, suggests that SOX2 could have the same role in developmental endochondral ossification and that chondrocytes do not transdifferentiate into osteoblasts but rather revert to a progenitor state before undergoing osteogenesis.

As described earlier, SOXC expression is needed in progenitor cells around cartilage primordia to establish tissue boundaries and induce growth plate formation (Bhattaram et al., 2014). In addition, the SOXC trio also has cell-autonomous roles in growth plate chondrocytes. Its inactivation in chondrocytes results in short growth plates and mild dwarfism (Kato et al., 2015). Chondrocytes form partially disorganized columns and die at a low, but significant rate. The SOXC trio does not appear to control chondrocyte

differentiation genes, but rather controls growth plate dynamics by promoting expression of signaling pathway mediators, including non-canonical WNT pathway components.

In conclusion, several SOX proteins are essential at multiple steps of chondrogenesis. SOX9 maintains the chondrocyte fate from progenitors to hypertrophic chondrocytes. It cooperates with SOX5/SOX6 to effect early chondrocyte differentiation and interacts with different transcription factors during growth plate chondrocyte maturation. The SOXC trio ensures proper patterning, growth and maturation of cartilage structures, and SOX2 may ensure growth plate chondrocyte transition into osteoblasts.

6. Roles of SOX genes in osteogenesis

Osteoblasts form bone and osteoclasts resorb bone. Their coordinated actions help ensure proper bone development and adult homeostasis. While no SOX gene is known to be expressed and critical in osteoclasts, several SOX genes are expressed in the osteoblast lineage and may functionally interact with master regulators, namely, RUNX2 (RUNT-domain protein) and OSX/SP7 (zinc-finger protein) (Liu & Lee, 2013).

As described earlier, SOX2 maintains a population of osteoblasts with stem cell properties *in vitro* (Basu-Roy et al., 2010) (Fig. 2C). Mice with inactivated *Sox2* in *Col1a1*[2.3kb]Cre-positive cells, which include osteoblasts, are small and osteopenic, suggesting important roles for SOX2 in osteoblasts too. However, since these mice also have non-skeletal phenotypes that might affect bones, further studies are warranted to definitively ascertain the roles of SOX2 in osteoblasts.

Sox8 and *Sox9* are co-expressed in osteochondroprogenitors (Akiyama et al., 2005; Schmidt et al., 2005). *Sox8*-null mice are osteopenic, likely because of reduced proliferation of progenitor cells and upregulation of *Runx2* leading to precocious differentiation of osteoblasts (Schmidt et al., 2005). Forced expression of SOX8 in differentiating osteoblasts in *Col1a1*-SOX8 transgenic mice leads to drastic downregulation of *Runx2* and deficient bone formation. Similarly, *Col1a1*-SOX9 transgenic mice are osteopenic and weakly express osteoblast markers (Zhou et al., 2006). *In vitro* experiments led to the proposition that SOX9 inhibits RUNX2 activity through physical interaction. Like SOX8, SOX9 may delay osteoblast differentiation of osteochondroprogenitors, but it remains unknown whether *in vivo* deletion of *Sox9* in these cells causes osteopenia and whether combined deletion of *Sox8* and *Sox9* has more severe consequences.

SOXC genes likely have important roles in osteoblastogenesis too. *Sox11*^{-/-} mice die at birth with underdeveloped intramembranous and endochondral bones (Sock et al., 2004), *Sox4*^{+/-} mice are osteopenic (Nissen-Meyer et al., 2007), and combined inactivation of all SOXC genes in mesodermal progenitors severely impairs skull formation (Bhattaram et al., 2014). *In vitro* studies have shown that *Sox11* and *Sox4* are highly expressed in osteoblast progenitors and that *Sox11*, but not *Sox4*, is downregulated during osteoblastogenesis (Gadi et al., 2013; Nissen-Meyer et al., 2007). *Sox4* knockdown in primary osteoblasts reduces progenitor cell proliferation and delays osteoblast differentiation without affecting *Runx2* expression (Nissen-Meyer et al., 2007). Similarly, *Sox11* knockdown in MC3T3-E1 cells

reduces cell numbers and delays osteoblastogenesis (Gadi et al., 2013). As for other SOX genes, additional investigations are needed to pinpoint the specific activities of SOXC proteins in osteoblastogenesis.

7. Regulation of SOX genes and RNAs in skeletal cells

Each SOX gene is expressed in a discrete number of cell types. This pattern is specific to each one, and likely involves complex regulatory mechanisms.

Sox2 expression is upregulated downstream of fibroblast growth factor signaling in calvarium osteoblast progenitors *in vitro* (Basu-Roy et al., 2010). This result is consistent with the importance of FGF signaling in the development of skull and other bones (Ornitz & Marie, 2015), but it remains to be validated *in vivo*.

Disease-causing genomic alterations occurring in the 2-Mb region around *SOX9*, the analysis of topologically associated domains in this region, and transgenic mouse reporter assays with yeast artificial chromosomes have compellingly suggested that *SOX9* transcription is regulated by multiple, widely spread regulatory modules (Franke et al., 2016; Gordon et al., 2009; Wunderle, Critcher, Hastie, Goodfellow, & Schedl, 1998). Accordingly, a dozen enhancers active in chondrocytes at discrete differentiation stages and in other cells have been identified (Bagheri-Fam et al., 2006; Benko et al., 2009; Gonen et al., 2018; Mead et al., 2013; Mochizuki et al., 2018; Yao et al., 2015). *SOX9* and *SOX5/SOX6* regulate several of these enhancers and *STAT3*, a transcription factor activated downstream of many pathways, may control a rib cartilage-specific enhancer. The *SOX9* promoter region is insufficient to drive reporter expression in chondrocytes (Bagheri-Fam et al., 2006; Mead et al., 2013), but may nevertheless be involved in *SOX9* upregulation or downregulation in response to signaling pathways. This is suggested by evidence of HIF-1 α (hypoxia-inducible factor-1 α) (Amarilio et al., 2007), CREB (cyclic-AMP response element-binding protein) (Piera-Velazquez et al., 2007) and *STAT3* (Hall, Murray, Valdez, & Perantoni, 2017) binding to specific recognition motifs. In addition, two long non-coding RNAs may upregulate *SOX9* in chondrocytes. DA125942 (encoded by *CISTR-ACT*) interacts with *PTH1LH* in *cis* and with *SOX9* in *trans* (Maass et al., 2012). *RORC* (regulator of chondrogenesis RNA), located 94kb upstream of *SOX9*, is critical for *SOX9* expression in mesenchymal stem cells *in vitro* and successful differentiation of the cells into chondrocytes (Barter et al., 2017). Finally, the *SOX9* RNA level may also be directly regulated by several microRNAs, whose roles remain untested *in vivo* (Lefebvre & Dvir-Ginzberg, 2017).

SOX5 and *SOX6* do not require *SOX9* for activation at the onset of chondrogenesis (Liu et al., 2018). Multiple enhancers are active and bound by the SOX trio within and around them in chondrocytes, suggesting positive feedback regulation (Liu & Lefebvre, 2015). Other transcription factors involved in their expression remain unknown. Among signaling pathways, bone morphogenetic protein signaling is required for their expression as well as *Sox9* expression at the onset of chondrogenesis *in vivo* and *in vitro*, but no direct link has been established yet (Uusitalo et al., 2001; Yoon et al., 2005). *Mir-194* and *miR-146b* downregulate the *SOX5* RNA level and affect chondrogenic differentiation of human adipose stem cells and human bone marrow-derived skeletal stem cells *in vitro*, respectively,

but their roles are unknown *in vivo* (Budd, de Andres, Sanchez-Elsner, & Oreffo, 2017; Xu, Kang, Liao, & Yu, 2012).

The *cis*-acting elements controlling *SOX4* and *SOX11* expression in skeletogenic cells are uncharacterized. Whereas signaling pathways regulating *SOX11* remain unknown too, *SOX4* expression was shown to be stimulated by parathyroid hormone in osteoblastic cells *in vitro* (Reppe et al., 2000). *SOX4* is upregulated by TGF β signaling in cancer (David et al., 2016; Lourenco & Coffey, 2017; Vervoort et al., 2018) and its expression in skeletogenic cells that are dependent upon TGF β signaling suggests that it could be a target of this pathway in skeletogenesis too. Several lncRNAs and miRNAs have been linked to *SOX4* in cancers and the findings suggest that they could also control *SOX4* expression in skeletogenesis. For instance, *LncSOX4* was shown to promote liver tumorigenic cell self-renewal by binding to the *SOX4* promoter and recruiting STAT3 to transactivation (Chen et al., 2016). This lncRNA was proposed to promote cell proliferation and migration in osteosarcomas through stabilizing β -catenin, a property also attributed to *SOX4* (Tian et al., 2017). *miR-188* and *SOX4* expressions inversely correlate in osteosarcomas (Pan, Meng, Liang, & Cao, 2018). The miRNA directly targets *SOX4*, and its inhibition of cell survival, proliferation and migration is restored by *SOX4* overexpression. Similarly, *MiR-129-5p* targets the *SOX4* RNA in chondrosarcomas and may thereby inhibit canonical WNT signaling, cell survival, proliferation and migration (Zhang, Li, Song, & Wang, 2017).

Further studies are clearly needed to fully uncover the mechanisms controlling *SOX* genes in skeletogenesis. These mechanisms are likely more sophisticated than currently appreciated. Their deciphering and the analysis of genetic variants in *cis*-regulatory elements may help uncover the basis of skeletal malformation diseases as well as the complexity of the skeleton, including size and shape differences, among vertebrate species and among human individuals.

8. Post-translational regulation of SOX proteins in skeletal cells

Various types of post-translational modifications have been shown to affect *SOX* protein stability, intracellular localization, or activity, but few, reviewed below, have been validated in skeletogenesis *in vivo* to this date.

PKA (cAMP-dependent protein kinase A) increases *SOX9* activity *in vitro* by phosphorylating the protein at Ser64 (upstream of the dimerization domain) and Ser181 (C-terminal to the HMG domain) (Huang, Zhou, Lefebvre, & de Crombrughe, 2000). The latter event occurs in growth plate chondrocytes downstream of PTHrP signaling, where it could help delay chondrocyte hypertrophy (Huang, Chung, Kronenberg, & de Crombrughe, 2001). These phosphorylation events, however, remain untested functionally *in vivo* and could be driven by several kinases downstream of many pathways (Lefebvre & Dvir-Ginzberg, 2017). Interestingly, *SOX9* phosphorylation at Ser181 was found necessary for SUMOylation at Lys398 (N-terminal to the transactivation domain) downstream of BMP and canonical WNT signaling (Liu et al., 2013). SUMOylation enhances the ability of overexpressed *SOX9* to promote neural crest delamination in chicken embryos. *Ptpn1* (protein-tyrosine phosphatase SHP2) inactivation in limb bud skeletal progenitors was

shown to perturb cartilage patterning, growth and endochondral ossification and to increase SOX9 protein, but not RNA level (Zuo et al., 2018). SHP2 was proposed to limit SOX9 protein level and activity by preventing Ser181 phosphorylation and hence Lys398 SUMOylation. In other studies, SOX9 ubiquitination was proposed as an important mechanism to limit SOX9 protein level by inducing proteasomal degradation downstream of canonical WNT signaling (Akiyama et al., 2002). DDRGK1 (DDRGK domain-containing protein 1) was shown to physically interact with SOX9 and thereby to inhibit SOX9 ubiquitination and proteasomal degradation at the onset of chondrogenesis (Egunsola et al., 2017). This mechanism may explain why humans with a homozygous loss-of-function mutation in *DDRGK1* have Shohat-type spondyloepimetaphyseal dysplasia (SEMD).

Many types of post-translational modifications have been described for SOX2 (Ramakrishna, Kim, & Baek, 2014; Sarkar & Hochedlinger, 2013), but their relevance to skeletogenesis remains unknown. In contrast, few modifications have been reported for SOX5, SOX6, SOX8, and the SOXC proteins, and none is directly relevant to skeletogenesis. There is little doubt, however, that these proteins are subjected to post-translational regulation. Supporting this concept, inhibition of SOXC proteasomal degradation was proposed as a major mechanism whereby inflammatory cytokines mediate synovial fibroblast transformation in arthritis (Bhattaram, Muschler, Wixler, & Lefebvre, 2018). A post-translational mechanism was postulated but remains to be identified. The involvement of a similar mechanism for developmental pathways remains to be tested.

These studies and others carried out *in vitro* infer that adequate post-translational modifications of SOX proteins by various mechanisms must occur to ensure proper skeletogenesis. Their identification could help decipher mechanisms underlying skeletal malformation diseases and design treatments for diseases dependent directly or indirectly upon changes in SOX activities.

9. Conclusions and perspectives

The efforts of many research teams over the last three decades have uncovered or postulated central roles, distinct and complementary, for several SOX family members in pivotal cell fate determination and differentiation events in skeletogenesis. The SOXC proteins—SOX4 and SOX11—control progenitor cell survival, undifferentiated status, and ability to cross talk with other cells to properly pattern, grow and mature skeletal structures. SOX8 maintains osteoblast progenitors at the proliferating, undifferentiated stage. SOX9 is mandatory to keep the chondrocyte fate from early to late differentiation stages and cooperates with SOX5 and SOX6 in driving robust expression of most early chondrocyte differentiation markers. It also cooperates with transcription factors from several families in the activation of stage-specific markers during growth plate chondrocyte maturation. Other roles have been proposed, but remain to be documented *in vivo*. They include roles for SOX2 in the maintenance of skeletal progenitor/stem cells and for SOXC proteins in osteoblast differentiation. This review has focused on skeletal progenitors, chondrocytes, and osteoblasts, but additional cells contribute to skeletogenesis, namely, osteoclasts, synovial fibroblasts, tenocytes, and intervertebral disc cells. Further studies are necessary to determine whether and how SOX proteins govern these cells. The deciphering of the

molecular actions of SOX9 and SOX5/SOX6 in chondrogenesis has significantly progressed in recent years largely thanks to high-throughput sequencing approaches. Further studies are needed to complete the knowledge of the actions of SOX proteins in skeletogenesis, to better understand functional interactions with other transcription factors, to determine whether they have pioneer roles to poise genomes for cell fate changes, and to define non-transcriptional activities. To date, our understanding of modes of regulation of SOX genes and proteins remains in its infancy. Several pieces of a certainly large and complex puzzle have been uncovered and assembled, but more systematic and wide-ranging proteomic and genetic approaches *in vitro* and *in vivo* are warranted to identify all pieces and completely assemble the puzzle. Current knowledge and constantly evolving experimental approaches allow us to predict that ongoing and future research efforts on SOX genes and proteins will help identify key nodes in the normal process of skeletogenesis, the basis of normal and pathological skeletal variations among individuals, and targets for the development of efficient treatments for skeletal malformation disorders as well as adult-onset degeneration diseases and cancers involving similar mechanisms.

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Fig. 1.

General and specific properties of SOX transcription factors. (A) Rendering of the SOX4 HMG domain (rainbow-colored) bound to DNA (gray). The HMG domain has three α -helices that fold into an L-shape. It contacts DNA in the minor groove and induces a strong bend. Its N- and C-termini are indicated. This cartoon was generated by SWISS-MODEL using published data (Jauch, Ng, Narasimhan, & Kolatkar, 2012). (B) Phylogenetic tree of the human SOX family members established based on sequence conservation in the HMG domain. It was generated using the UPGMA method in MacVector software (version

16.0.8). Skeletogenic SOX proteins are highlighted in blue. (C) Schematics of the domain structure of skeletogenic SOX proteins. HMG, DNA-binding domain; TAD, transactivation domain; D, dimerization domain. The same colors are used for the dimerization and transactivation domains of same-group proteins because of high conservation. Different colors are used for these domains for distinct-group proteins to reflect the lack of homology.

Fig. 2.

Schematics summarizing current knowledge of the roles of SOX proteins in cell fate determination and differentiation during skeletogenesis. See the main text for detailed information.