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Review Article

Genome-scale actions of master regulators directing skeletal development

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ABSTRACT

The mammalian skeleton develops through two distinct modes of ossification: intramembranous ossification and endochondral ossification. During the process of skeletal development, SRY-box containing gene 9 (Sox9), runt-related transcription factor 2 (Runx2), and Sp7 work as master transcription factors (TFs) or transcriptional regulators, underlying cell fate specification of the two distinct populations: bone-forming osteoblasts and cartilage-forming chondrocytes. In the past two decades, core transcriptional circuits underlying skeletal development have been identified mainly through mouse genetics and biochemical approaches. Recently emerging next-generation sequencer (NGS)-based studies have provided genome-scale views on the gene regulatory landscape programmed by the master TFs/transcriptional regulators. With particular focus on Sox9, Runx2, and Sp7, this review aims to discuss the gene regulatory landscape in skeletal development, which has been identified by genome-scale data, and provide future perspectives in this field.

1. Introduction: the process of skeletal development

The skeletal system supports our bodies and works as an organ fundamental to our locomotion. It also plays pivotal roles in calcium and phosphate homeostasis and provides a bone marrow microenvironment for hematopoiesis. The mammalian skeleton develops from three distinct origins: the neural crest, the paraxial mesoderm, and the lateral plate mesoderm [1]. The neural crest, which originates in the neuroectoderm, gives rise to facial bones, the frontal bone, the squamous part of the temporal bone, and the anterior part of the sphenoid bone [2,3]. The paraxial mesoderm forms somites, which differentiate into the sclerotome and dermomyotome; the sclerotome cells then generate the parietal bone, the occipital bone, the petrous part of the temporal bone, the posterior part of the sphenoid bone, the vertebral column, and the ribs [2,3]. The lateral plate mesoderm gives rise to the sternum and the appendicular skeleton [2,3]. From these origins, the mammalian skeleton develops through two distinct modes of ossification: intramembranous ossification and endochondral ossification. Both processes begin with condensation of mesenchymal cells at places where future bones develop. Intramembranous ossification, in which the condensed mesenchymal cells directly differentiate into bone-forming osteoblasts, underlies formation of the cranial vault, most of the facial bones, and the body of the clavicle [3,4]. In endochondral ossification, the condensed mesenchymal cells first differentiate into chondrocytes to form a cartilage model. Following the formation of bone-forming osteoblasts in and around the model, the cartilage is replaced by mineralized bone tissues. The cranial base, the mandibular condyle, the vertebral column, the sternum, the ribs, the end of the clavicle, and the appendicular skeleton are formed by this type of ossification [3,4].

Chondrogenesis and osteogenesis are well coordinated in endochondral ossification. In the center of the condensed mesenchyme, mesenchymal cells initially differentiate into chondrocytes; they proliferate to enlarge the cartilage model and subsequently become hypertrophic. Hypertrophic chondrocytes calcify their surrounding matrix and undergo apoptosis. The perichondrium, a thin layer of cells located at the periphery of cartilage, is thought to contain osteo-chondroprogenitors. These progenitor cells are specified into osteoblast precursors in response to osteogenic factors mainly secreted from hypertrophic chondrocytes [5,6]. The precursors differentiate into osteoblasts that deposit the bone matrix and form the bone collar. They also invade into the mineralized cartilage with vascular tissues, forming the primary spongiosa [7].

During the process of skeletal development, SRY-box containing gene 9 (Sox9), runt-related transcription factor 2 (Runx2), and Sp7 work as master transcription factors (TFs) or transcriptional

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regulators, underlying the cell fate specification of the two distinct populations. Mutations in *SOX9* were found to cause campomelic dysplasia (CD), which is characterized by skeletal malformation and sex reversal [8,9]. Mouse genetic studies further revealed the crucial roles of Sox9 in endochondral ossification. First, *Sox9*$^{-/-}$ cells did not contribute to mesenchymal condensation in *Sox9*$^{-/-}$/WT chimeric mice [10]. Second, limb bud-specific *Sox9* deletion resulted in mice without limbs, and its deletion in early chondrocytes led to severe chondrodysplasia [11]. Third, removal of *Sox9* in proliferating and prehypertrophic chondrocytes prevented their hypertrophy [12,13], whereas overexpression of *Sox9* in chondrocytes caused its delay [14]. Sox9 was also shown to repress the expression of *Col10a1* in immature/proliferating chondrocytes, in order to restrict the expression to hypertrophic chondrocytes [15]. Recently, Haseeb et al. demonstrated that Sox9 plays key roles in maintaining the growth plate and protecting articular cartilage from osteoarthritis stimuli at postnatal stages [16]. These data indicate that Sox9 is necessary for (1) mesenchymal condensation, (2) survival, proliferation, and proper differentiation of chondrocytes, and (3) maintenance of the growth plate and articular cartilage.

Runx2 and Sp7 (also known as Osterix) are required for osteoblast specification and maturation, acting as master regulators of osteoblast development. *Runx2*$^{-/-}$ mice and *Sp7*$^{-/-}$ mice were completely devoid of functional osteoblasts in both intramembranous and endochondral bones [17–19]; phenotypes of mice homozygous for the *Runx2* C-terminal deletion also indicate that subnuclear targeting of Runx2 is required for its function in osteogenesis [20]. The two factors sequentially regulate osteoblast differentiation; skeletal progenitors are initially specified into Runx2-expressing osteoblast precursors, which then become Runx2- and Sp7-double-positive precursors. The double-positive precursors eventually differentiate into functional osteoblasts. Sp7 is thought to act genetically downstream of Runx2 in osteoblast development, as *Runx2* is expressed in *Sp7*$^{-/-}$ mice, whereas no *Sp7* expression is observed in *Runx2*$^{-/-}$ mice [19]. Runx2 also regulates chondrocyte hypertrophy together with Runx3 [21]. The requirement of Runx2 and Sp7 for skeletal development is further supported by human diseases arising from their mutations. Cleidocranial dysplasia, an autosomal dominant disease characterized by hypoplasia/aplasia of the clavicle, patent fontanelles, supernumerary teeth, and short stature, is caused by heterozygous loss-of-function mutations of *RUNX2* [18,22,23]. *SP7* mutations are associated with a recessive osteogenesis imperfecta [24,25].

Thus, Sox9, Runx2, and Sp7 act as master regulators in skeletal development; they are also involved in the development of craniofacial structures including teeth as well as bones and cartilages. *Runx2* is highly expressed in the condensed dental mesenchyme from the bud to early bell stages of the tooth germ [26]. The development of the tooth germ was arrested at the cap stage in *Runx2*-deficient mice, where odontoblast and ameloblast differentiation was blocked, indicating that Runx2 is required for odontoblast development and epithelial-mesenchymal interactions in tooth development [27]. Runx2 was also shown to inhibit the terminal differentiation of odontoblasts [28]. Although *Sp7* expression overlapped with *Runx2* in the dental mesenchyme [29], the function of Sp7 is distinct from that of Runx2; conditional knockout studies using several Cre driver strains in mice suggest that Sp7 plays critical roles in cementogenesis and root dentin formation [30,31]. Regarding Sox9, it is indispensable for cranial neural crest (CNC)-derived endochondral ossification in the craniofacial region [32]. Sox9 expression was detected in the dental epithelium and mesenchyme during mouse incisor development [33]. Although the Sox9 expression pattern, as well as its involvement in CNC development, may support its roles in tooth development, further functional studies remain to be performed.

Core transcriptional circuits underlying skeletal development have mainly been identified through mouse genetics and biochemical approaches. Next-generation sequencer (NGS)-based analyses are further enabling us to confirm the identified core circuits, and also to gain genome-scale views on the mode of TF actions, the functional interactions between TFs and regulatory elements on the genome, and epigenome dynamics. From this point of view, this review aims to discuss the mode of actions of the master TFs/transcriptional regulators, Sox9, Runx2, and Sp7, and the gene regulatory landscape in skeletal development, based on recent genome-scale data obtained by NGS-based chromatin immunoprecipitation sequencing (ChIP-seq; profiling of TF binding sites and histone modifications), RNA sequencing (RNA-seq; profiling of gene expression), and assay for transposase-accessible chromatin sequencing (ATAC-seq; profiling of open chromatin regions).

2. Overview of transcriptional control

Two concepts, the “epigenetic landscape” and the “gene regulatory network”, are keys to understanding how the genome encodes and executes the program to specify cell fates during developmental processes. The former, proposed by C.H. Waddington in 1957 [34], describes the cell fate determination process as a ball (i.e., the cells) rolling down through a landscape of bifurcating valleys (the developmental landscape). The latter, proposed by R.J. Britten and E.H. Davidson in 1969 [35], explains how the developmental landscape is constructed: DNA sequence-specific regulation of gene expression underlies the genomic control of developmental processes.

The promoter region and *cis*-regulatory elements on the genome act in concert to initiate and subsequently control the transcription of target genes. In particular, *cis*-regulatory elements enable spatiotemporal regulation of transcription, i.e., transcription of proper gene sets in proper cells with the appropriate timing. These elements include enhancers, silencers, and insulators, which function independently of the orientation and location of the sequence [36]. Among the three types of *cis*-regulatory elements, this review focuses on enhancers, which serve as target sites for TFs and “enhance” the rate of transcription. Sequence-specific binding of TFs and their co-factors to enhancers triggers changes of the local chromatin structure, which make the regulatory elements more accessible, and facilitates the promoter-enhancer interaction to initiate the transcription of target genes.

3. Genome-scale views on the regulatory action of Sox9 in the chondrocyte program

Sox9-dependent gene regulation via *cis*-regulatory elements was identified in the context of cartilage development by means of traditional, random and bioinformatically driven analyses of non-coding regions flanking *Sox9*, *Ctgf* (connective tissue growth factor), and eight genes encoding cartilage matrix proteins: *Col2α1*, *Col9α1*, *Col10α1*, *Col11α2*, *Col27α1*, *Hapln1*, *Matn1*, and *Acan* [37,38]. In addition, Sox9 was shown to bind to DNA via the inverted repeat of a quite variable Sox recognition sequence, and Sox9 dimerization was shown to be required for activation of Sox9 [39,40]. However, it remained unknown if there was any functional interaction among Sox9-bound enhancers and how general the proposed mode of Sox9 action was over the chondrocyte genome.

Three independent groups, including ours, answered these questions by performing a ChIP-seq analysis for Sox9 in either mouse *in vivo* chondrocytes or the rat chondrosarcoma (RCS) cell line [41–43]. A common finding of these studies was that Sox9 peaks were highly associated with skeleton- and chondrocyte-related genes. These targets include cartilage matrix proteins, such as *Col2a1*, *Acan*, *Col9α1* and *Matn1*, and transcriptional regulators in

chondrogenesis, such as *Sox5*, *Sox6*, *Sox9*, and *Wwp2*. Importantly, Sox9-bound regions were accompanied by H3K4me2, H3K27ac, and p300 [42], indicating that Sox9 utilized distally-located, tissue-specific active enhancer elements. In addition, the Sox9-bound enhancers form clusters, likely functioning as "super-enhancers" to drive robust expression of genes crucial for chondrocyte identities [42–44].

de novo motif analysis on Sox9-bound distal enhancers revealed enrichment of Sox dimer motifs [42,43], providing genome-scale support for previous findings that Sox9 preferentially acts as a homodimer in chondrocytes [39,40]. The recovered Sox dimer motif had extensive sequence degeneracy/variations; the motifs that were actually present and bound by Sox9 on the chondrocyte genome showed lower affinity to Sox9 than the optimal motif, which was predicted by the *de novo* motif analysis as the most representative set of nucleotides in each position under the Sox9 binding regions. Why does Sox9 prefer lower affinity to the genome? We speculate that the weaker interaction may enable a flexible response and/or cooperative engagement in the chondrocyte program. Despite the preference, Sox9 is likely to assure the high expression level of target genes by utilizing enhancer clusters, i.e., super enhancers, in order to define the chondrocyte lineage.

Sox9 also engages around transcriptional start sites (TSSs) of non-chondrocyte genes, including those related to general cell activities. In this mode, (1) two basal transcriptional components, p300 and RNA polymerase II, are colocalized with Sox9, and (2) Sox motifs are not enriched at the Sox9-associated regions. These facts suggest that Sox9 regulates general cell activities of the chondrocyte lineage, probably through indirect interaction with DNA via basal transcriptional machinery [42]. Since this engagement is positively correlated with the target gene expressions, Sox9 may act on the transcriptional machinery to enhance the expression of the non-chondrocyte genes [42]. Thus, Sox9 has two distinct modes of action on the chondrocyte genome: indirect binding to the TSS regions of non-chondrocyte genes, and direct binding to multiple distal enhancer elements, which form super-enhancers, around chondrocyte-related genes via sub-optimal, low-affinity Sox dimer motifs (Fig. 1).

4. Genome-scale cooperation between Sox9 and other TFs in the chondrocyte program

Sox9 is known to work together with Sox5 and Sox6 to maximize its transcriptional outputs in chondrogenesis [45,46]. The cooperative action was also confirmed in a genome-wide manner. Liu et al. found that two-thirds of Sox9 ChIP-seq peaks highly overlapped with Sox6 peaks, with a particularly large number of overlaps for super-enhancers, indicating that they work together on super-enhancers in the chondrocyte program [43]. *de novo* motif analysis and subsequent biochemical assays demonstrated that, unlike Sox9, Sox6 favors pairs of tandem Sox motifs. Because no physical interaction has yet been reported between Sox9 and Sox5/Sox6, they are thought to separately bind to their own target sites that are closely situated and may form transcriptional complexes [47] (Fig. 1).

Enrichment of other TF motifs provided a clue to the cooperative actions among Sox9, Sox5/6, and multiple TFs in the chondrocyte program. Interaction between Sox9 and activator protein-1 (AP-1) family members was initially predicted by *de novo* motif analysis on Sox9 ChIP-seq data [42] and subsequently verified by additional ChIP-seq and biochemical analyses [48]. Sox9 binding regions mostly overlapped with those of Jun on the chondrocyte genome [48]. Sox9 dimer motifs and/or AP-1 motifs were enriched in the shared regions [48], suggesting that direct binding of Sox9, AP-1, or both to DNA was the primary mode of their interaction on the chondrocyte genome. Here two distinct interacting modes were predicted: direct co-binding of both factors to their own target sites within an enhancer and protein-protein interactions of Sox9 and AP-1 (Fig. 1). Biochemical analyses indicated that the direct co-binding to DNA enhanced target gene transcription, whereas the protein-protein interactions attenuated their transactivation potential [48]. Prehypertrophic chondrocyte-specific expression of *Jun* and *Fosl2*, and positive actions of AP-1 and Sox9 on chondrocyte hypertrophy support the idea that the combined action of the two interacting modes on multiple enhancers contributes to fine-tuning of target gene expression in the process.

Despite the accumulated knowledge of genome-scale Sox9 actions on its target genes, regulatory mechanisms underlying Sox9 expression itself in chondrocytes remained to be elucidated; enhancer modules critical for *Sox9* expression had not been identified, although some data suggested that critical regions were located at a 2-Mb non-coding region upstream of Sox9 gene. In 2018, Mochizuki et al. identified a costal and sternum cartilage-specific enhancer regulating Sox9 expression [49]. They took advantage of a CRISPR/Cas9-based capture of distal enhancer regions interacting with the Sox9 promoter. By utilizing multiple CRISPR/Cas9 technologies, they further confirmed the *in vivo* function of the identified enhancer and found Stat3 as a trans-acting factor on the enhancer [49]. Thus, their CRISPR/Cas9-based combinatorial approach provides a sophisticated platform to identify functional enhancers and their regulatory mechanisms.

5. Genome-scale views on the regulatory action of Runx2 in the osteoblast program

Runx2-associated *cis*-regulatory elements were identified at non-coding regions around osteoblast genes such as *Bglap*, *Alpl*, *Ibsp*, and *Spp1* [50–52]. Recent Runx2 ChIP-seq studies performed by three different groups further provide genome-scale, broader views on how Runx2 executes the osteoblast program [53–55].

First, osteoblast differentiation is accompanied by dynamic changes in the regulatory actions of Runx2 on the genome. Runx2 binding to distal genomic regions is a general feature of the Runx2 action, but the functional outcome of the binding appears to be highly complex [55]. When Runx2 genomic distribution was analyzed in an *in vitro* osteoblast induction culture, Runx2 binding to osteoblast-related gene regions, including *Runx2* itself, *Ibsp*, and *Sp7*, appeared upon osteoblast induction; Runx2 binding was in turn lost in another set of genes associated with fat cell differentiation, leukocyte migration, and erythrocyte differentiation upon the induction [55]. Thus, Runx2 may mask genomic regions related to non-osteogenic lineages in order to suppress non-osteogenic differentiation in progenitors; once Runx2 turns on the osteoblast program by activating osteoblast-related genes, the masking may no longer be required and somehow depleted.

Second, binding to Runx motifs is the primary mode of the action of Runx2 on the genome, as the motifs are enriched in Runx2 ChIP-seq peaks [53,54] (Fig. 2). This is consistent with a large number of previous studies showing Runx2 binding to the motifs. The high enrichment of C/EBPβ motifs in the Runx2 ChIP-seq peaks and the subsequent C/EBPβ ChIP-seq also indicated that C/EBPβ was a possible Runx2 co-regulator in the osteoblast program [54]. Runx2 and C/EBPβ are likely to share osteoblast enhancer modules and form a transcriptional complex [56,57] (Fig. 2). In addition, Runx2, C/EBPβ, VDR, and RXR binding were enriched around osteoblast-related genes in an osteogenic culture of mouse mesenchymal stem cells (MSCs), suggesting the formation of TF "hot spots" for osteoblast identity [58] (Fig. 2). Core binding factor beta (Cbfb) [59,60], Twist [61], Stat1 [62], Schnurri 3 [63], SATB2 [64], TAZ [65], MED23 [66], Nell-1 [67], and Zfp521 [68] have also been identified as cooperative factors of Runx2. Further genome-scale interaction studies will elucidate whether a bona-fide hotspot, where key TFs bind cooper-

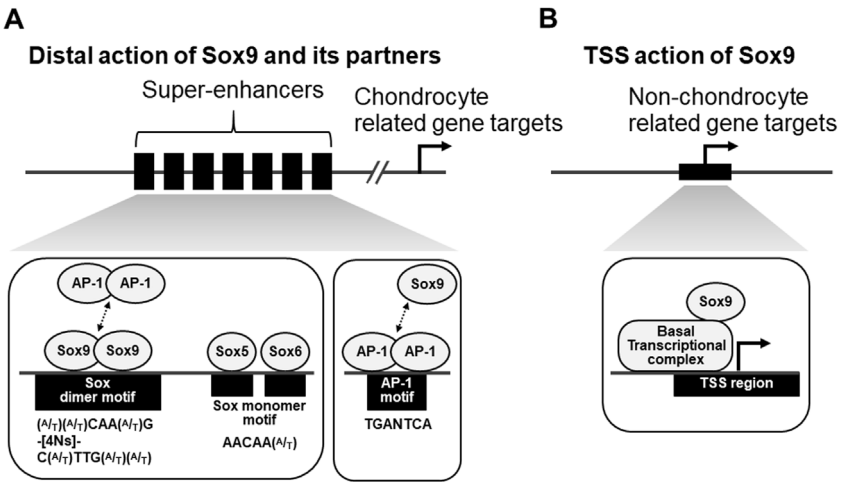

Fig. 1. A model for the action of Sox9 and its partners on the chondrocyte genome.
Sox9 has two distinct modes of action. (A) A distal action of Sox9 and its partners. In distal regions far from the transcriptional start sites (TSSs) of target genes, Sox9 utilizes multiple enhancer clusters, i.e., super-enhancers, where Sox9 dimers bind to DNA via sub-optimal, low-affinity Sox dimer motifs. Sox5 (and probably Sox6 as well) binds to Sox monomer motifs at regions adjacent to Sox9-bound ones. AP-1 binds to its consensus motif within Sox9-bound enhancers and also physically interacts with Sox9 on the genome. The distal action of Sox9 and its partners on super-enhancers elicits high expression levels of chondrocyte genes in order to specify and maintain chondrocyte identity. Consensus motifs for the Sox dimer, Sox monomer, and AP-1 are shown. (B) A TSS action of Sox9. In the TSS regions of non-chondrocyte genes including those related to general cell activities, Sox9 is indirectly associated with the genome through interactions with basal transcriptional machinery. Such interaction is likely to enhance the expression of the target genes.

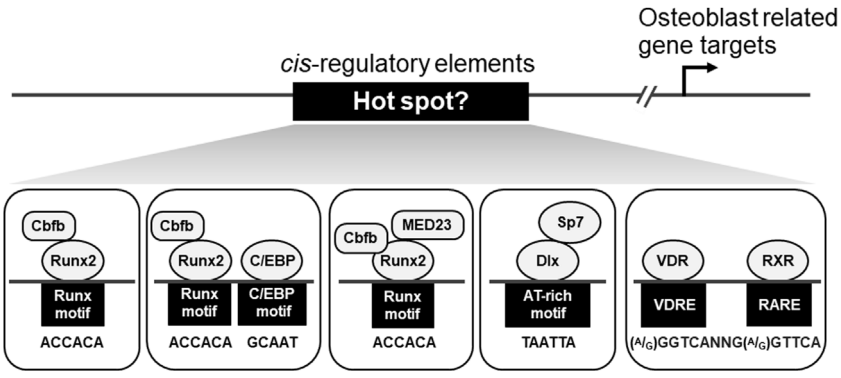

Fig. 2. Presumable transcriptional hot spots for the osteoblast program.
Master TFs/transcriptional regulators, their cofactors, and epigenetic regulators might be co-localized at certain genomic regions to form transcriptional hot spots, activating the transcription of key genes for osteoblast identity. Interactions obtained from ChIP-seq studies are illustrated. Consensus motifs for Runx2, C/EBP, Dlx, and VDR/RXR are shown. VDRE, vitamin D response element; RARE, retinoic acid response element.

atively to form core regulatory networks defining cell identity [69], is present on the osteoblast genome.

Lastly, Runx2 binding profiles were not well correlated with the dynamic signatures of histone modifications and transcription during osteoblast differentiation [54]. Although the *in vitro* settings may be less biologically relevant, this result suggests that Runx2-DNA interactions are stable during osteoblast differentiation, and other factors may modify Runx2 functions without altering Runx2-DNA interactions. However, there is still the possibility that Runx2 itself may determine epigenetic status at an initial phase of osteoblast differentiation. Analysis of epigenetic changes upon deletion or ectopic expression of Runx2 could at least partly verify or rule out this possibility.

6. Genome-scale views on the regulatory action of Sp7 in the osteoblast program

Sp7/Osterix is a member of the Sp family of transcription factors. Thus, it has been thought to function as a TF by binding to the Sp consensus motif, GC-box, on the genome [19]. Our Sp7 ChIP-seq study on primary mouse calvarial osteoblasts demonstrated a novel action of Sp7 on osteoblast enhancers and an evolutionary relationship between Sp7 and bone-forming vertebrates [70].

Sp7 ChIP-seq peaks were predominantly located at distal genomic regions. The Sp7-bound regions were associated with genes related to skeletal development and expressed in bone tissues. The sequences of the Sp7-bound regions were well conserved among different vertebrate species. These data indicate that the longer-range interaction (> 5 kb) between Sp7-bound enhancers and promoters is a well-conserved, primary mode of Sp7-mediated transcription of osteoblast-related genes [70].

de novo motif analysis of Sp7 ChIP-seq peak regions provided an unexpected result. An AT-rich motif, not the GC-box, was enriched in the Sp7 ChIP-seq peak regions [70]. The AT-rich motif resembled a homeodomain-responsive element. This result was not consistent with previous understanding of the Sp7-DNA interaction, because Sp family members are known to bind to the GC-box, which is mainly located in promoter regions on the genome, using their highly conserved zinc finger domains [71]. The mode of action has been confirmed by ChIP-seq analysis for Sp1, Sp2, and Sp5 [72–74] and high throughput screening of protein-DNA bindings for Sp1, Sp3, and Sp4 [75,76].

In comparative analysis of Sp1 and Sp7 ChIP-seq in the pre-osteoblastic cell line MC3T3-E1, the AT-rich motif was again enriched in Sp7 peaks, whereas the GC-box was enriched in Sp1 peaks [70]; Sp1 mainly engaged in proximal regions of housekeeping genes, whereas Sp7 was engaged mostly in distal regions of osteoblast-related genes [70]. Thus, Sp7 is indeed distinct from Sp1 (and probably other Sp family members) in terms of its interacting modes and genomic targets in the osteoblast lineage.

Sp7-binding affinity to the GC-box was even weaker than the Sp1-binding affinity, and mostly below detection limit in biochemical analyses [70]. Since Sp7 acquired the GC-box preference by substitution of three amino acids in its zinc finger domain into their Sp1 counterparts, the amino acid differences are likely to be the cause of the loss of its GC-box preference. How, then, does Sp7 interact with the AT-rich motifs on the osteoblast genome? Since direct binding of Sp7 to the AT-rich motifs was not detected [70], Sp7 was likely to associate with the AT-rich motif indirectly through partner TFs. Homeodomain-containing Dlx transcription factors were identified as partner TFs, based on the following results: (1) they were highly expressed in osteoblasts and contributed to osteoblast biology [77–79]; (2) there was a high rate of overlap (almost 80%) between the Sp7-bound regions and Dlx5-bound ones on the MC3T3-E1 genome [70]; (3) physical interaction between Dlx5 and Sp7 was detected [70]; (4) binding of the Sp7-Dlx5 complex to the AT-rich motif was detected [70]; and (5) the Sp7 association with osteoblast enhancers was attenuated, leading to decreases in the Sp7 target gene expression, by knock-down of Dlx factors [70].

These data support the idea that Sp7 executes its osteoblast program by acting as a co-factor of Dlx rather than a TF (Fig. 2). This also raises the possibility that Sp7 interacts with other homeodomain-containing TFs, such as *Msx1/2*, *Satb2* and *Alx4*, which are all highly expressed in osteoblasts [70]. In addition, consensus motifs for Runx2 [80] and other key bone regulatory factors are enriched in Sp7 ChIP-seq peaks [70]. Several studies have shown that Runx2 and Sp7 physically interact and synergistically function in the transcription of osteoblast genes [81,82]. Although Runx2 is required for initial *Sp7* expression, Sp7 may in turn cooperate with Runx2 on the genome to execute the osteoblast program. Thus, multiple regulatory inputs are likely integrated on *cis*-regulatory elements, probably forming transcriptional hot spots on the osteoblast genome. Integrative analysis of ChIP -seq data for multiple TFs and transcriptome data will verify the regulatory mechanism and its biological significance.

The Sp7 amino acid variants causing its distinct characteristics suggested an evolutionary linkage between the appearance of Sp7 and establishment of an osteoblast gene regulatory network. Sp7 is present in all bone-forming vertebrates [70]. In contrast, Sp7-type zinc finger variants are lacking in the genomes of the closest non-boney vertebrates (e.g., lampreys), the cephalochordates (e.g., amphioxus), and ascidians (e.g., tunicate). Thus, the appearance of Sp7-like variants in the Sp family is likely coupled to the evolution of bone-forming vertebrates. Sp7 may act as a key evolutionary switch in the cartilage-to-bone transition. Given that Dlx paralogs pre-exist in non-vertebrate chordates, Sp7 may emerge as a stabilizer or enhancer of the roles of Dlx in executing the osteoblast program.

7. Pioneer factors in skeletal development

Accessibility of specific genomic regions is a key for the cell type-distinct transcriptional program directing cell fate specification. Certain TFs called “pioneer factors” have been proposed to bind to naïve chromatin, recruit factors that make the regions more accessible to transcriptional machinery by inducing epigenetic changes, and assemble transcriptional complexes [83]. FoxA, one of the forkhead-domain (Fox) family transcription factors, and GATA transcription factors have been extensively studied as pioneer factors in endodermal development, especially liver development [83].

Given its crucial roles in chondrogenesis, one can imagine that Sox9 may function as a pioneer factor in chondrocyte development, and indeed, Sox9 was shown to function in this manner in hair follicles [84]. However, Liu et al. demonstrated that Sox9 assisted in the chromatin remodeling process, but was not necessary for initiation of the process at *cis*-regulatory elements around chondrocyte-related genes [85], suggesting the presence of other TFs possessing the pioneering function for chondrocyte development. Recently, Xu et al. showed that Foxc1 established chromatin accessibility at chondrogenic enhancers in cranial neural crest cell (CNCC)-derived cartilage of the zebrafish face [86]. Those enhancers, which were enriched for Fox motifs as well as Sox9 motifs, became accessible during CNCC-derived cartilage development [86]. More importantly, chromatin accessibility was globally decreased in zebrafish Foxc1 mutant facial chondrocytes [86]. Given that Foxc1 mutant mice showed extensive abnormalities in skeletal elements, including both endochondral and intramembranous bones [87], Foxc1 may work as a pioneer factor in mammalian chondrocytes.

In the osteoblast program, no strong candidate for pioneer factors has yet been proposed. The critical roles of GATA4 in liver development may support its pioneering action in other tissues. Indeed, chromatin accessibility and active enhancer signatures were decreased at the *RUNX2* loci by *GATA4* knock-down in the U2OS osteosarcoma cell line, although this finding was limited to within the local regulation of Runx2 [88]. One may also imagine that Runx2 functions as a pioneer factor in the osteoblast program, given its crucial function in osteogenesis. Systematic investigation remains to be performed from this point of view.

8. Concluding remarks

NGS-based studies have provided genome-scale views on the gene regulatory landscape programmed by master TFs/transcriptional regulators in skeletal development. However, our knowledge is still limited to the landscape made up by a few key transcriptional regulators. In addition, most of the genome-scale studies have so far captured only a snap-shot profile of TF binding and epigenome status in a mass of millions of cells that are potentially heterogenous. Thus, the overall landscape remains unclear, and a cell-type-distinct, dynamic landscape remains to be elucidated. Single-cell RNA-seq, one of the recently emerging single-cell technologies, are uncovering how the gene regulatory networks are established at each stage of skeletal development [89,90], how they change throughout the whole process [91], and how they are disrupted under pathological conditions [92]; single-cell ATAC-seq provides another layer of information in terms of epigenome dynamics at the single-cell resolution. Integrative analysis for multiple factors will further clarify how their regulatory inputs are coordinated and cause distinct outcomes during the process. When combined with the CRISPR/Cas9 technology, such knowledge will enable us to gain insight into the connection of regulatory variants on the genome with human skeletal disorders, and to design genome-oriented therapeutic approaches for those disorders.

Conflict of interest

None.

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