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An Emerging Regulatory Landscape For Skeletal Development

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Abstract

Skeletal development creates the physical framework that shapes our body and its actions. In the past two decades, genetic studies have provided important insights into the molecular processes at play, including the roles of signaling pathways and transcriptional effectors that coordinate an orderly, progressive emergence and expansion of distinct cartilage and bone cell fates in an invariant temporal and spatial pattern for any given skeletal element within that specific vertebrate species. Genome-scale studies have provided additional layers of understanding, moving from individual genes to the gene regulatory landscape, integrating regulatory information through cis-regulatory modules into cell type-specific gene regulatory programs. This review discusses our current understanding of the transcriptional control of mammalian skeletal development, focusing on recent genome-scale studies.

Keywords

skeletal development; cell specification; chondrocytes; osteoblast; transcriptional regulators; gene regulatory networks

Two cell types in the skeletal lineages: chondrocytes and osteoblasts

Skeletal development is a well-organized sequential process that involves cell fate specification and the differentiation of skeletal cells (Box 1; Fig. 1). Cartilage matrix-secreting chondrocytes and bone matrix-producing osteoblasts are the main components of skeletal tissues. These two cell types are derived from common skeletal progenitors, and their specification and differentiation have been extensively studied using genetics and biochemical approaches, which have highlighted the importance of specific signaling pathways in these events [1, 2]. Genetic studies conducted predominantly in mice and

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humans have identified key transcriptional regulators directing the progression of chondrocyte and osteoblast fates [1, 2].

Recent advances in next-generation sequencing are providing new insights into these transcriptional control processes through genome-scale analysis. A picture is starting to emerge of the skeletal regulatory mechanisms controlled by key transcriptional regulators within the epigenetic landscape of skeletal development. In this review, we first summarize the function and properties of these key skeletal determinants, and we then discuss their modes of action in terms of the gene regulatory landscape recently revealed by genome-scale studies.

Key transcriptional regulators in skeletal development

Transcriptional regulators engaging the specification and differentiation of chondrocytes

Sox9 is a high-mobility group (HMG) domain containing transcription factor, closely related to the Y chromosome-encoded testis-determining factor SRY. Human mutations in Sox9 are associated with campomelic dysplasia, in which both sexual development and skeletal development are affected [3]. In skeletal development, *Sox9* is initially expressed in condensed mesenchyme that contributes to both chondrocytes and osteoblasts, but later *Sox9* expression is restricted to the chondrocyte lineage.

The Sox9 domain in cartilage encompasses the expression domains of genes encoding key cartilage matrix components including *Col2a1*, *Col9a1*, and *Acan* (Fig. 1). At later stages, Sox9 is present within both the articular cartilage (though levels are low in superficial cells lining the joint) and the growth plate, which drives skeletal growth until puberty in humans.

Mouse genetic studies have shown that Sox9 activity is essential throughout chondrocyte development [4, 5]. Analyses of *Sox9*^{-/-}::WT chimeras demonstrated that *Sox9*^{-/-} cells do not contribute to mesenchymal condensation [6]. The removal of *Sox9* within both proliferating and prehypertrophic chondrocytes prevented hypertrophic progression in mice [7-9], indicating a broad requirement for Sox9 throughout the chondrogenic program. Thus, Sox9 is necessary for mesenchymal condensation and the survival, proliferation and differentiation of chondrocytes, and for the maintenance of the growth plate. In this chondrocyte program, the *Sox9* dosage is critical; haploinsufficiency of the *Sox9* gene results in skeletal bowing and lethality in both humans and mice [10].

Sox9 acts in conjunction with two other Sox family members, Sox5 and Sox6, which serve as cofactors for Sox9-dependent transcription. Their expression is dependent on Sox9, and their expression domain is similar to that of Sox9 in the developing limb [11]. Loss of both *Sox5* and *Sox6* activity results in severe chondrodysplasia, demonstrating the importance of their actions in chondrocyte programs [12].

Although Sox9 is required throughout the chondrocyte lineage, additional regulators control the hypertrophic progression. The MADS box-containing transcription factors Mef2c and Mef2d are highly expressed in prehypertrophic and hypertrophic chondrocytes. Studies of mouse genetics revealed that Mef2c is a positive regulator of chondrocyte hypertrophy, and

Col10a1 is a direct target of Mef2c; Mef2d augments Mef2c's action [13]. In addition, Mef2c also acts in concert with Sox9. They bind to adjacent sites in a *Col10a1* regulatory element to drive the hypertrophic chondrocyte-restricted expression of this extracellular matrix-encoding gene [8].

The runt-related transcription factors Runx2 and Runx3 are weakly expressed in columnar proliferating chondrocytes, whereas their expressions are markedly elevated in prehypertrophic chondrocytes, and their expressions remain high in hypertrophic chondrocytes. These transcription factors are required for chondrocyte hypertrophy and have redundant roles in the process [14]. In addition to the factors described thus far, several proteins have been reported to function with Sox9 in the transcriptional control of targets. However, their precise mechanisms of action in Sox9-regulated programs requires further studies. Members of this group include Wwp2, HDAC4, SIK3, and Arid5a (see reviews [2, 15]).

Transcriptional regulators specifying osteoblasts

Runx2 and Sp7/Osterix are required for osteoblast specification. *Runx2* is expressed in perichondrial cells adjacent to columnar proliferating chondrocytes as well as osteoblasts in the bone collar and primary spongiosa in endochondral bones, whereas *Sp7* expression is more restricted to cells associated with the bone collar and primary spongiosa (Fig. 1) [16, 17]. *Runx2* and *Sp7* null mutant mice lack both intramembranous and endochondral bone, and functional osteoblasts are absent from both mutants [17, 18]. In humans, heterozygous loss-of-function mutations of *RUNX2* cause cleidocranial dysplasia, an autosomal dominant disease characterized by skeletal anomalies including delayed closure of the cranial sutures, rudimentary clavicles, and short stature as well as dental abnormalities [19-21]. An *SP7* homozygous mutation have been linked to recessive osteogenesis imperfecta [22].

Several lines of evidence point to a sequential action of these osteoblast determinants in early osteoblast development. Skeletal progenitors are initially committed to *Runx2*⁺ osteoblast progenitors, which then transition to *Runx2*⁺ and *Sp7*⁺ osteoblast precursors. *Runx2* is required upstream of *Sp7*, as *Runx2* is expressed in *Sp7* null mutant mice, but no *Sp7* expression is observed in the absence of Runx2 [17].

The key transcriptional regulators that mediate the gene regulatory landscape, and their modes of action in skeletal development

Transcriptional components act as part of a regulatory network that regulates cell type-appropriate programs of gene expression through cis-acting enhancer modules that may be positioned hundreds of kilobases from the transcription start sites (TSSs) of target genes. Genome-scale comprehensive studies of the binding profiles of transcriptional regulators determined by chromatin immunoprecipitation and high-throughput sequencing (ChIP-seq) have been combined with similar approaches probing the associated chromatin landscape to provide new insights into the developmental logic of regulatory programs in many model systems [23-28]. Similar integrated studies of the skeletal cell genome are key to a deeper understanding of the dynamic formation of different cell types in mammalian skeletal

development. Several recent studies have started to expand our knowledge of skeletal cell programming at the genomic level (summarized in the Key Table and Fig. 2).

Modes of action of the Sox trio in chondrocytes

Prior to recent genome-scale ChIP-seq analyses [29-31], various approaches revealed Sox9-associated enhancers around Sox9 itself plus several genes encoding cartilage growth factors or extracellular matrix proteins, including *Ctgf*, *Col2a1*, *Col9a1*, *Col10a1*, *Col11a2*, *Col27a1*, *Hapln1*, *Matn1*, and *Acan* [15, 32]. Sox9 is known to dimerize in activating target genes through a quite variable inverted repeat sequence [33, 34]. Three groups recently reported ChIP-seq analyses of Sox9-DNA interactions directly within the primary chondrocytes of mouse skeletal tissues [30] and in a rat chondrosarcoma (RCS) cell line [29, 31]. A comparison of the data sets obtained by the three studies is provided in Key Table, including the cells, species, peak number, enhancer validation assay, and more.

As expected, all of these studies documented a strong association of Sox9 binding around genes that are involved in skeletal development and chondrocyte differentiation. Binding was observed at known enhancer elements defined through transgenic reporter assays [30], and motif recovery predicts dimer interactions through Sox motifs oriented head-to-head with a 3- or 4-nucleotide spacer. These Sox9 targets have been termed Class II targets to distinguish them from non-enhancer mediated interactions [30] (see below). Cooperative interaction between Sox9 and Sox6 was also evident; two-thirds of Sox9-bound regions were also associated with Sox6, which confirmed interactions between Sox9 and Sox5/6 on cartilage enhancers [31] that were observed in a large number of biochemical assays [15, 32, 35] (Fig. 2). An analysis of *de novo* motif recovery and subsequent biochemical studies showed that Sox6 favors Sox monomer motifs that are distinct from Sox9 binding dimer motifs [31].

Gene targets that are expressed at high levels such as Sox9 itself and matrix-encoding genes are associated with large numbers of putative cis-regulatory modules spanning considerable distances (hundreds of kilobases) from the TSS. Two studies [30, 31] revealed that many of these enhancers group together to form “super-enhancer-like” clusters, which were significantly associated with highly expressed genes compared to typical enhancers [36] (Fig. 2). A number of putative target genes were identified, and some of them were verified by an *in vivo* reporter assay in fish [30] or a gene expression analysis in Sox9-deficient mice [31]. These include signaling-pathway components such as *Fgfr2* and *Fgfr3* and transcriptional regulators such as *Arid5a*, *Runx2*, *Runx3* and *Sim2*, indicating that they are components of the Sox9-mediated network in chondrocytes. These findings help further clarify Sox9 functions in chondrogenesis, although it is still a challenge to identify which downstream genes are responsible for different aspects of the functions of Sox9.

Interestingly, motif recovery predictions of the favored Sox9 dimer sequence and biochemical studies of Sox9 DNA interactions *in vitro* all suggest that Sox9 regulation favors multiple, low-affinity binding sites [30]. Weaker interactions may favor flexibility in the response. The actual level of target gene expression is likely to be determined not by the strength of individual binding sites but rather by the summation of multiple enhancer actions and by the clustering of enhancers into super-enhancer modules. A comparison of Sox9

binding in neural crest-derived facial chondrocytes with that in somite-derived rib chondrocytes suggest largely similar regulatory programs despite the distinct origins of each skeletal population [30].

A ChIP-seq study provided additional insights into the mode of Sox9 action [30]. First, Sox9 showed a significant interaction around the TSSs of any gene that was highly expressed within the chondrocyte, including the house-keeping genes expressed in most cells. At these Class I Sox9 targets [30], Sox9 association was correlated with the level of that gene's expression and likely reflected protein-protein interactions between Sox9 and components of the basal transcriptional complex such as p300 [37, 38] (Fig. 2). Since this engagement is positively correlated with the target gene expressions, Sox9 could have an additional role beyond conventional DNA binding-driven transcriptional regulation in broadly enhancing transcription in chondrocytes.

Second, the analysis of *de novo* motifs enriched in Sox9 ChIP-seq studies predicted the co-engagement of several other regulatory factors in conjunction with Sox9; most prominently, a highly enriched AP-1 motif suggested interactions by members of the Fos-Jun AP-1 family [30]. These interactions have been confirmed and functionally analyzed in a recent report that showed that most Sox9 binding occurs in conjunction with Jun, in which Jun binds either directly at AP-1 target sites or indirectly in a complex with Sox9 [9].

In summary, all studies support one mode of Sox9 action, i.e., engagement in super-enhancer-like clusters around chondrocyte-related genes via dimer motifs, whereas the additional depth of analysis may point to the potential of additional Sox9 interactions cooperating with other transcriptional regulators and the basal transcriptional machinery.

Runx2-mediated gene regulatory networks in osteoblasts

Runx2 was originally identified for its binding to an osteoblast-specific cis-acting element in the promoter region of the *Bglap* gene [16, 39]. Runx2-associated cis regulatory elements have been identified in analyses of non-coding regions flanking a number of osteoblast genes including *Alpl*, *Ibsp* and *Spp1* [40]. In these, the DNA binding domain, i.e., the runt domain, recognizes the Runx consensus sequence. Three groups have independently explored Runx2 osteoblast interactions in genome-scale ChIP-seq studies in *in vitro* settings [41-43]. These studies not only confirmed known osteoblast genes as Runx2 targets, but also identified new Runx2 target genes by combinatorial analyses with *in vitro* transcriptional profiling obtained from either a Runx2 loss-of-function analysis or a time-course analysis in cell cultures representing different stages of osteoblast differentiation. These new Runx2 target genes include *Ezh2*, an epigenetic regulator; *Crabp2*, a retinoic acid signaling component; and *Adamts4* and *Tnfrsf19*, remodelers of the extracellular matrix [42]. Further functional analyses of these new targets are needed to reveal their contributions to Runx2-mediated skeletal development.

One of the three above-mentioned studies included a clustering analysis of Runx2 ChIP-seq peak distribution at different time points in the induction of osteoblasts in MC3T3E1 cells [42]. Only a subset of Runx2 peaks was highly associated with genes related to osteoblast differentiation that underwent the induction of osteoblast differentiation. These included

known targets including *Runx2* itself, *Ibsp* and *Sp7*. Another cluster of *Runx2* peaks that were lost upon osteoblast induction were related to biological functions in other cell lineages including fat cell differentiation, leukocyte migration, and erythrocyte differentiation.

These findings suggest *Runx2* may have broader interactions prior to osteogenesis or that binding at non-osteoblast targets may suppress non-osteogenic pathways of cell commitment [42]. Further functional analyses and additional histone modification ChIP-seq analyses are required to study the inhibitory action of *Runx2*. Differences in the range of *Runx2* and *Sp7* targets may reflect the earlier action of *Runx2* at the outset of osteoblast specification. As noted earlier, *Runx2* is critical in chondrocytes for hypertrophic development. This raises an important question of how *Runx2*-directed regulatory programs differ between chondrocytes and osteoblasts. The mechanisms underlying cell-type specific regulatory outcomes also remain to be identified.

De novo motif analyses revealed a consensus *Runx* motif as the most enriched motif in the *Runx2* peaks consistent with direct DNA binding [41-43]. One of these studies also revealed the co-engagement of C/EBP β at enhancer modules, and C/EBP β ChIP-seq studies provided experimental confirmation [43]. *Runx2* and C/EBP β form a transcriptional complex and function together in transactivation [44, 45] (Fig. 2). In addition to C/EBP β a number of other regulators of transcription are thought to cooperate with *Runx2*, including Cbfb, Twist, Stat1, Schnurri 3, SATB2, TAZ, and Zfp521 (see reviews [2, 46, 47]). Additional genome-scale approaches will help place these in the context of osteoblast programs.

Sp7/Osterix-mediated gene regulatory networks in osteoblasts

A recent study addressed *Sp7* interactions in primary calvaria-derived osteoblasts and the pre-osteoblast cell line MC3T3E1 [48]. The *Sp7*-DNA association profile was obtained in primary osteoblasts, and the analysis of *Sp7* peaks revealed that longer-range interactions (>5 kb) were the predominant feature of the *Sp7* regulatory program. The *Sp7* peaks were well conserved among different vertebrate species and strongly associated with genes related to biological processes in skeletal system development and osteoblast differentiation. An unexpected finding from the same study was demonstrated in an analysis of the primary ChIP-seq datasets used to predict *Sp7* DNA interactions [48]. *Sp7* belongs to the *Sp* family of C2H2 zinc finger-containing transcription factors. Members of this family, including the well-studied family founder member *Sp1*, bind DNA at an *Sp* consensus GC box [49] (Fig. 2).

Published data have indicated that *Sp7* acts through a GC box [17]. However, *de novo* motif recovery failed to identify an enriched GC-box consensus sequence in *Sp7* ChIP-seq data. Rather, an analysis suggested an *Sp7* association with DNA targets through an AT-rich motif [48]. Consistent with the functional significance of this motif, mutations in this sequence abrogated transcription from *Sp7*-dependent osteoblast enhancers. However, direct binding studies showed no interaction of *Sp7* with this motif.

Several lines of evidence suggest the AT-rich motif is targeted by members of the *Dlx* family of homeodomain-containing transcription factors — specifically, *Dlx3*, 5 and 6 — and protein-protein interactions between *Dlx* and *Sp7* result in co-occupancy on osteoblast

enhancers to activate their targets [48] (Fig. 2). Indeed, that study's comparative analysis of Sp7 and Dlx5 binding in the osteoblast cell line MC3T3E1 showed highly overlapping DNA association profiles at osteoblast enhancers. As expected from this model, targeted knock-down of Dlx3, Dlx5, and Dlx6 attenuated Sp7 engagements at osteoblast enhancers, and reduced target gene expressions [48]. Involvement of Dlx5 and Dlx6 in osteoblast development is also supported by phenotypes of Dlx5/6 compound mutants, where bone formation is impaired [50]. However, potential redundancy among these factors and an absence of conditional alleles has precluded a more extensive investigation of the Dlx members in *in vivo* osteoblasts.

Functional binding studies indicated that specific mutations within the Sp7 zinc finger domain contribute to the loss of GC-box binding and associate with Sp7's unique role as an osteoblast determinant [48]. A sequence comparison among different vertebrate and non-vertebrate chordate species showed that the closest non-boney vertebrates (e.g., lampreys), the cephalochordates (e.g., amphioxus), and ascidians (e.g., tunicate) all lack an Sp7-type zinc finger variant in their genome [48]. In contrast, an Sp7 gene is present in all boney vertebrate groups and in cartilaginous fish that arose from a boney ancestor and show evidence of non-endochondral cranial bone [48]. These data together suggest that the acquisition of this particular Sp-family variant was closely coupled to the origin of bone and may be a key evolutionary switch in the cartilage-to-bone transition. Dlx 3-, 5- and 6-related genes were likely already present based on the identification of paralogs in non-vertebrate chordates. Thus, Sp7 may have acted to stabilize or enhance Dlx's transcriptional roles in distinct targets within the skeletal regulatory genome.

These findings highlight the value of genome-scale approaches to identify genomic interactions in investigations of the underlining regulatory mechanisms (Box 2). A motif analysis of Sp7 ChIP-seq data also showed an enrichment of consensus motifs for Runx2, Nfatc1, and other key bone regulatory factors [48], suggesting that these sequences will be useful for determining how osteoblast regulatory information is integrated through the identified cis-regulatory modules.

The epigenetic landscape in skeletal cells

The epigenetic landscape in chondrocytes

Gene regulatory networks can be interpreted by not only TF-binding sites, but also the epigenetic landscape in given tissues or cell types. This landscape is created by histone modification and DNA methylation and forms different enhancer states by regulating the DNA accessibility of transcription factors and their binding stabilities [51, 52]. Global comparative analyses among different tissues have provided tissue-distinct putative active enhancer profiles [26, 53]. By using datasets obtained from mouse embryonic limbs, putative regulatory elements to control Sox9 transcription were identified [54, 55]. Some of these elements were further confirmed by *in vivo* reporter assays [54, 55]. Importantly, some of the confirmed regions are located in the genomic regions that have been associated with campomelic dysplasia and the Pierre Robin sequence in which genomic translocation, deletion or duplication is frequently observed, suggesting a link involved in the disease.

Understanding the epigenetic landscape will also contribute to the further elucidation of transcription factor-mediated gene regulatory networks. One *in vitro* study using human bone marrow cells suggested that histone modifications, rather than DNA methylation, provide the primary epigenetic control of the early differentiation of progenitor cells toward the chondrogenic lineage [56]. Comparative analyses of Sox9 binding to the epigenetic landscape showed high species conservation and a predominant open chromatin signature (which are suggestive of active enhancer regions) in mouse primary chondrocytes [30] and the rat chondrosarcoma cell line RCS [31], indicating that Sox9 acts predominantly on active enhancer modules in these contexts.

However, one current limitation would be that the cells used in these studies are a potentially heterogeneous population which might include mitotic chondrocytes, post-mitotic pre-hyperchondrocytes or some different cell types. Since Sox9 has distinct biological functions in different stages of chondrocyte differentiation [4, 8], the next challenge would be to identify the dynamic epigenetic landscape in chondrocyte specification and differentiation. Detailed analyses with distinct cell types purified by using reporter mice or (if available) reliable *in vitro* cell cultures at various time points are necessary to dissect the developmental stage-distinct Sox9 actions. Recently developed methods to analyze small number of cells, such as indexing-first ChIP-seq [57] and ATAC-seq [58], are powerful tools for these analyses.

In addition, detailed transcriptional profiling obtained from distinct cell types could be linked to the epigenetic actions during skeletal formation. Recent single-cell RNA-seq analyses with growth plate chondrocytes [59] and with skeletal progenitors cultured from *in vitro* sequential differentiation [60], as well as an ATAC-seq analysis with the *in vitro* dynamic differentiation of skeletal progenitors [60] could provide insight into the dynamic molecular link at the single-cell level.

The epigenetic landscape in osteoblasts

Several lines of study examined the dynamic epigenetic landscape in osteoblast specification and differentiation *in vitro*. One study revealed few epigenetic changes in osteoblast differentiation [41], whereas another investigation showed significant changes in a subgroup of selected epigenetic profiles that are highly associated with the osteoblast signature [61]. This discrepancy may have occurred because the two studies used different cell lines: the former study used an immortalized human mesenchymal stromal cell line, and the latter study used a human fetal osteoblastic cell line called hFOB 1.19. However, it is more likely that the discrepancy is the result of the difference in location of peaks analyzed; the latter study used only distal peaks in the analysis by removing peaks located around the TSS. In accord with the current consensus that distal enhancers specify cell types [27, 62], the removal of TSS-associated peaks from the analyses may be useful to extract cell type-specific epigenetic signatures. Interestingly, study [61] also suggested a potential interaction between dynamic epigenetic signatures and a signaling pathway including Wnt and AP-1. Further detailed investigations will connect the epigenetic signatures with the gene regulatory networks.

In addition, given that Runx2 is expressed from initial osteoblast precursors to mature osteoblasts and that Runx2 functions in a differentiation stage-dependent manner [63], one can expect dynamic changes in the Runx2-DNA binding track accompanied by changes in the profiles of histone modifications and transcriptions. However, two independent *in vitro* studies indicated that this does not appear to be the case [41, 43]. It remains to be determined whether this reflects artifacts from the *in vitro* system or biologically relevant mechanisms such as the requirement of cooperative interactions with other factors for altering Runx2 functions without affecting its DNA association.

A recent paper provided further insights into the plasticity of the gene regulatory network in the cell-fate specification of bone marrow cells, which have the potential to differentiate into osteoblasts and adipocytes *in vitro* [64]. Strikingly, the epigenetic patterns representing the osteoblast signature were reversible to those of adipocytes when the culture conditions were changed. This may be similar to phenomena seen in macrophages; the tissue-distinct epigenetic landscape of macrophages is defined by the microenvironment, as the macrophages were reprogrammed when they were transferred into a new microenvironment in *in vivo* conditions [65]. Understanding the plasticity of skeletal cells could be informative in attempts to define the potency of not only the skeletal progenitors but also differentiated cells in both developmental and regenerative stages.

Concluding remarks

Emerging genome-scale studies have provided insight into the regulatory landscape of key transcriptional regulators as well as epigenetic regulations in skeletal development. However, the current knowledge is limited to the genomic landscape programmed by a few key transcriptional regulators *in vivo*, and the epigenetic landscape has been studied mainly *in vitro* or with potentially heterogeneous populations *in vivo*. The entire picture of gene regulatory networks in skeletal development remains to be established, and several important questions in the field have not yet been addressed (see the ‘Outstanding Questions’). The identification of cell type-distinct signatures will be central to a better understanding of gene regulatory networks in osteoblast, chondrocyte and sequential stages of cell-fate specification and differentiation from the progenitors. Further integrative analyses with osteogenic or chondrogenic signaling pathway-related transcription factors and cooperative factors of the key transcriptional regulators are required to understand the proper tuning of spatial and temporal gene regulatory networks in the dynamic developmental process (Box 2).

Despite the power of genome-scale ChIP-seq analyses, practical and interpretative difficulties persist. Working with the best cell sources *in vivo* is hampered by difficulties in obtaining enough cells to obtain good ChIP-seq data, and an additional problem is the mix of cell types within the population. From an interpretative perspective, it is unlikely that all binding events have biological significance, and determining which events to focus on is somewhat arbitrary. As a ChIP-seq analysis gives a “snapshot,” multiple replicates providing more evidence than what is observed in each snapshot may have lasting relevance. Each ChIP-seq snapshot reflects a statistical probability based on a number of determinants (concentration, affinity, accessibility, etc.) that a transcriptional regulator is bound to one of

its DNA target sites in the genome. However, determining which of these DNA binding sites are relevant by simple ‘sequence gazing’ is not easy, and validating all of the obtained putative enhancers by functional assays is a daunting task. Distilling the regulatory genome for a given skeletal cell type will likely benefit from identifying the enhancers that broadly integrate input from a swath of additional regulatory factors (Fig. 3).

One of these inputs could be the three-dimensional chromatin architecture, which nowadays can be uncovered by recently developed chromosome conformation capture analyses such as Hi-C [66]. Such analyses can define chromatin subdomains called ‘topologically associated domains (TADs).’ Within TADs, distant gene regulatory elements interact with proximal promoters of their target genes for transcription. TADs are generally conserved among cell types and across species, whereas dynamic chromatin architectures are observed within the TADs representing distinct cell types. Importantly, destructions of the boundary cause genetic disease due to ectopic enhancer interactions and gene expression. For example, the structural variations disrupting TAD structures were observed to cause malformation of limb formation [67]. Since there has not yet been an accurate method to link individual enhancers to the target gene, the TAD information could be useful to define the functional chromatin modules associated with transcription factor bindings.

In addition, extensive genome-wide association studies (GWAS) have identified links between the human genome and skeletal development and disease [68-71]. Since more than 90% of the identified loci in GWAS are localized outside of the protein-coding regions, variants in the regulatory genome that influence enhancer-driven transcription are the most plausible targets of interest. Clearly, defining the regulatory component of the genome (and more precisely, distinct interaction sites for key regulatory factors) will facilitate the identification of non-coding sequence variants that are associated with human development and disease regulatory mechanisms [72].

Lastly, endochondral bone repair has been proposed to recapitulate development based on the appearance of a cartilage template that is subsequently ossified. However, the adult repair processes have not been explored to the same depth as skeletal development [73]. It will be interesting to determine whether seemingly similar cellular events have a conserved regulatory underpinning. Insights here may also provide new avenues for therapeutic intervention. Recent lineage tracing studies obtained evidence of the conversion of a subset of hypertrophic chondrocytes to osteoblasts in normal skeletal development and during the bone repair process [74-76]. Although further studies are necessary to elucidate the molecular mechanism, these observations suggest an intriguing plasticity in the regulatory programs, the understanding of which could have broader significance beyond skeletal modeling.

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Text Box 1**Overview of Skeletal development**

The mammalian skeleton is derived from three distinct cellular origins and formed through two different modes of action [1, 2]. In the head, neural crest cells originating from the dorsal neural tube form the facial bones and the cranium. The paraxial, presomitic and somitic mesoderm gives rise to the parietal bone, base of the skull, and the axial vertebral skeleton and associated ribs. The lateral plate mesoderm gives rise to the skeletal elements of the limbs. Skull and facial bones develop through intramembranous ossification, where condensed mesenchymal cells directly differentiate into bone-forming osteoblasts. Most of the remainder of the skeleton develops through endochondral ossification, in which an initial cartilage model is replaced by mineralized bone tissue with the exception of the joints that are capped by articular cartilage.

In endochondral ossification, the specification and differentiation of cartilage matrix-secreting chondrocytes and bone matrix-producing osteoblasts are closely coordinated (Fig. 1). Initially, skeletogenic mesenchyme cells condense, and the first proliferating chondrocytes appear within the center of these aggregates. Growth, cell proliferation, and chondrocyte differentiation result in distinct zones of cartilage representing different stages of the chondrogenic program. In a typical early long bone in the limb, quiescent and proliferating chondrocytes make up the articular epiphyseal region; in the diaphysis or shaft, proliferating chondrocytes line up in columns and transition to postmitotic, pre-hypertrophic chondrocytes which grow and swell to form mature hypertrophic chondrocytes. The most mature hypertrophic chondrocytes induce mineralization of the surrounding extracellular matrix.

Osteoblasts first emerge from multipotent, perichondrial progenitors surrounding the cartilaginous element in response to osteogenic signaling inputs initially from adjacent early hypertrophic chondrocytes [77-79]. Here, maturing osteoblasts secrete the bone matrix of the bone collar. Osteoblast precursors also invade the mineralized cartilage with vascular tissues, replacing the mineralized cartilage with a bone matrix, creating the mineralized bone shaft in the long bones [80]. In addition to bone matrix-secreting osteoblasts, there are osteoblast-derived osteocytes embedded in the bone matrix. These sensory cells measure mechanical and structural properties of the bone matrix structure, and regulate osteoblast activity, integrating environmental input into their osteoblast-regulating actions [81]. In addition to synthetic and sensory cell types of the osteoblast lineage, hematopoietic lineage-derived osteoclasts play a central role in removing and remodeling the bone matrix [82]. Bone undergoes continuous synthesis and removal throughout life.

Text Box 2**Involvement of signaling pathways in skeletal development**

Sequential and/or developmental stage-specific activations of signaling pathways are essential for skeletal development. As examples, in the chondrocyte lineage, bone morphogenetic protein (BMP) signaling and the downstream Smads 1, 4, and 5 are essential for mesenchymal condensation and later chondrocyte differentiation [83-86], while an Indian hedgehog (Ihh) and parathyroid hormone-related peptide (PTHrP) negative feedback loop is crucial for the appropriate expansion and differentiation of chondrocytes in the growth plate [78, 87, 88]. In the osteoblast lineage, Ihh initiates osteoblast differentiation by specifying skeletal progenitors into *Runx2*-positive osteoblast precursors [79, 89]. Canonical Wnt signaling regulates the proliferation of osteoblast precursors as well as their maturation into osteoblasts [90].

Importantly, Hh and Wnt signaling in the osteoblast lineage prevents chondrocyte differentiation [89-93]. These results suggest that transcriptional regulators acting downstream of these signaling pathways are likely to be main regulators of gene regulatory networks in each lineage. Given that Gli activators, which are transcription factors responsible for the transcription of Hh target genes, can initiate the osteoblast program at least partially in a *Runx2*-independent manner [94], the Gli activators may initially establish osteoblast regulatory modules in this program.

It would be interesting to determine the hierarchical relationships between these signal-responsive transcription factors (Signal TFs) and master transcription factors (Master TFs) in the skeletal cell specification. One scenario is that a Master TF has a pioneering action to modify the epigenetic landscape that allows a Signal TF to exert its cell type-distinct action later. Other scenarios are that Signal TFs define the DNA accessibility for Master TFs, or simultaneous actions of these are required.

Outstanding question box

- Two cell types, osteoblasts and chondrocytes, are derived from a common skeletal progenitor and function in a cell type-dependent manner through different gene regulatory networks. What are the distinct signatures representing each cell type? How and when do these signatures arise and how to they change during distinct phases of skeletal development? What determines the distinct regulatory actions of Runx2 in cartilage and bone programs?
- Sequential and/or developmental stage-specific activity of signaling pathways is essential for skeletal development. How do these signaling actions interface with the regulatory pathways controlled by key skeletal determinants?
- Key transcriptional regulators are believed to form core regulatory networks, or so-called “hotspots,” to define specific cell types. What are the roles of these hotspots in skeletal development?

Trends box

- Recent progress afforded by genome-scale studies has provided new insights into the actions of key transcriptional regulators in the epigenetic landscape regulating mammalian skeletal development.
- ChIP-seq analyses for skeletal cell determinants including Sox9, Runx2 and Sp7 identified their putative targets and modes of action in chondrocytes and osteoblasts
- Other genome-scale studies have revealed the molecular link between the determinants at the single cell-level.
- Integrative analyses with these genome-scale data will be the key to learning spatial-, temporal- and cell type-distinct signatures (which represent the nature of skeletal development) and to gaining molecular-level insights into pathological mechanisms of skeletal diseases.

Fig. 1. Representative structure of the growth plate in long bones of mouse embryos
The expression domain of marker genes and the location of different populations are indicated for chondrocytes in cartilage and osteoblasts in the perichondrium-bone collar.

Fig. 2. Gene regulatory networks (GRNs) mediated by key transcriptional regulators in chondrocytes and osteoblasts

GRNs that have been revealed by recent genome-scale studies are summarized with distinct modes of actions of key transcriptional regulators in each population. In chondrocyte GRNs (upper panel), Sox9 homodimers and Sox6 recognize Sox dimer motifs and monomer motifs, respectively, which are located nearby at distal enhancer elements. They form “super-enhancer-like” clusters, engaging in transcription of chondrocyte related genes (Cho gene). Biochemical and genetic studies have shown that Sox6 works with Sox5, although ChIP-seq data for Sox5 have not been available yet. Specifically in the chondrocyte transition to the hypertrophic program, AP-1 provides additional inputs to the transcription by binding to AP-1 motifs adjacent to the Sox9-bound sites or by protein-protein interactions. Sox9 also engages in transcription of non-Cho genes through an association with the basal transcriptional complex at TSS. In osteoblast GRNs (lower panel), a transcriptional complex of Runx2 and C/EBP β , each of which recognizes consensus motifs at distal enhancer elements, function in transcription of osteoblast related genes (Ob gene). Sp7 is indirectly associated with the osteoblast genome through Dlx factors bound at AT-rich motifs, acting as a co-factor for the Dlx factors in transcription of Ob gene.

Fig. 3. A workflow for comprehensive studies of regulatory landscape

ChIP-seq analyses have great potential for identifying cell type-distinct functional enhancers by the genome-scale mapping of transcription regulator bindings and histone modifications. However, the identification of the target genes requires multiple additional approaches; peak intensity, the distribution of the consensus motif in the peak, the expression of nearby genes, and the conservation of candidate regions among species all provide a level of information. Interaction maps are also important, especially for long-distance enhancer/promoter engagement (over one megabase) where intervening genes are skipped, and for interchromosomal interactions.

Chromosome conformation capture (3C, 4C, 5C and Hi-C) and chromatin interaction analyses by paired-end tag sequencing (ChIA-PET) are powerful tools to confirm the potential interactions between enhancer candidates and their target genes [95]. Verifications of the enhancer candidates have relied on an *in vitro* reporter assay for individual candidates. *In vivo* enhancer assays using reporter mice remain a challenge in terms of the number of testable candidates, although such assay can validate enhancer activities in appropriate physiological contexts.

High-throughput enhancer validation in massively parallel reporter assays [96], coupled to CRISPR-Cas9 genetic screening [97] can be applied to screen a large number of enhancer candidates when robust *in vitro* cell culture models are present. Once we identify the cell type-distinct enhancers, we can further investigate the molecular mechanisms underlying the interactions between proteins and the enhancer. In addition, cross-species analyses are attractive to identify the evolutionary consequences of the identified gene regulatory elements on the exertions of their biological functions.

Key Table

Summary of ChIP-seq analyses of transcriptional regulators in chondrocytes and osteoblasts

Transcriptional regulator	Tissue type	Organism	No. of peaks	Peak caller	Functional validation assay(positive/all)	Enriched motifs	Refs.
Sox9	P1 rib proliferating and prehypertrophic chondrocytes	Mouse	27,656	Cisgenome [98]	In vivo zebrafish transgenic reporter (14/17)	Sox dimer motif, AP-1 motif	[30]
	E18.5 nasal chondrocytes	Mouse	17,440	Cisgenome		No tested	
	P4 rib cultured chondrocytes	Mouse	2,364	No information	In vitro reporter with 293T cells (1/1)	Sox dimer motif	[29]
	RCS cell line	Rat	3,254	No information		Sox dimer motif	
Sox6	RCS cell line	Rat	9,143	MACS [99]	In vitro reporter with RCS cells (8/8)	Sox dimer motif	[31]
			12,072			A-rich motif, Sox monomer motif	
Sp7	P1 calvaria osteoblasts	Mouse	2,112	Cisgenome	In vitro reporter with MC3T3E1 cells (12/12), In vivo mouse transgenic reporter (1/1)	AT-rich motif containing homeobox response elements	[47]
	Overexpression in MC3T3E1 cell line, Cultured for 7 days	Mouse	5,187	Cisgenome		AT-rich motif containing homeobox response elements	
Sp1	Overexpression in MC3T3E1 cell line, Cultured for 7 days	Mouse	9,450	Cisgenome	-	GC-box	[41]
Dlx5	Overexpression in MC3T3E1 cell line, Cultured for 7 days	Mouse	24,365	Cisgenome	-	AT-rich motif containing homeobox response elements	[42]
Runx2	hMSCs, cultured for 28 days	Human	10,212	Custom R/ Bioconductor	-	Runx motif	[41]
	MC3T3E1 cell line, cultured for 0 / 9 / 28 days	Mouse	25,457/60,596/40,330	MACS	In vitro reporter with MC3T3E1 cells (4/5)	Runx motif	[42]
CEBP β	MC3T3E1 cell line, Cultured for 0 / 15 days	Mouse	12,674/ 6,272	Quest [100]/ HOMER[101]	In vitro cell line reporter with MC3T3E1 cells (6/9) Loss of function with Crispr/Cas9 (3/3)	Runx motif	[43, 102, 103]
		Mouse	15,622/ 10,847	Quest/HOMER		CEBP motif, Runx motif,	