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Genetic and molecular control of Osterix in skeletal formation

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Abstract

Osteoblast differentiation is a multi-step process where mesenchymal cells differentiate into osteoblast lineage cells including osteocytes. Osterix (Osx) is an osteoblast-specific transcription factor which activates a repertoire of genes during differentiation of preosteoblasts into mature osteoblasts and osteocytes. The essential role of Osx in the genetic program of bone formation and in bone homeostasis is well established. *Osx* mutant embryos do not form bone and fail to express osteoblast-specific marker genes. Inactivation of *Osx* in mice after birth causes multiple skeletal phenotypes including lack of new bone formation, absence of resorption of mineralized cartilage, and defects in osteocyte maturation and function. Since *Osx* is a major effector in skeletal formation, studies on *Osx* gained momentum over the last five-seven years and implicated its important function in tooth formation as well as in healing of bone fractures. This review outlines mouse genetic studies that establish the essential role of *Osx* in bone and tooth formation as well as in healing of bone fractures. We also discuss the recent advances in regulation of *Osx* expression which is under control of a transcriptional network, signaling pathways, and epigenetic regulation. Finally we summarize important findings on the positive and negative regulation of *Osx*'s transcriptional activity through protein-protein interactions in expression of its target genes during osteoblast differentiation. In particular, the identification of the histone demethylase NO66 as an *Osx*-interacting protein which negatively regulates *Osx* activity opens further avenues in studying epigenetic control of *Osx* target genes during differentiation and maturation of osteoblasts.

Keywords

Osterix; Osx; NO66; gene expression; osteoblasts; osteocytes

Introduction

Bone formation occurs through two developmental processes – intramembranous and endochondral ossification. Intramembraneous ossification which mainly forms craniofacial bone is mediated by the differentiation of the condensed mesenchyme into osteoblasts. In endochondral ossification, mesenchymal cells in the condensation differentiate into chondrocytes, forming a cartilage template that is later replaced by bone [Fang and Hall, 1997]. In skeletogenesis, Sox9, Runx2 and Osterix are three essential transcription factors that have important roles in the cell-fate decision process by which mesenchymal cells become chondrocytes and osteoblasts, through activation of cell type-specific genes. Sox9 is the earliest expressed transcription factor in prechondrogenic mesenchymes, which differentiate into chondrocytes. Runt-domain-containing Runx2, another early differentiation factor, is expressed in the condensed mesenchymes. *Runx2*-expressing cells

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segregate from osteochondroprogenitors to form precursor osteoblasts. Expression of *Osx* in *Runx2*-expressing precursors induces these cells to differentiate into mature and functional osteoblasts, and finally into osteocytes during bone formation [Karsenty, 2001; Nakashima and de Crombrughe, 2003; Nakashima et al., 2002; Nishio et al., 2006; Yang and Karsenty, 2002; Zhou et al., 2010; Zou et al., 2006]. Several other transcription regulators including Wnt/ β -catenin, homeodomain protein *Dlx*, *Msx2*, *ATF4*, and bone morphogenetic proteins have been known to play important roles in controlling gene expression during differentiation and maturation of osteoblasts [Hassan et al., 2009; Karsenty, 2008; Rodda and McMahon, 2006; Yang et al., 2004]. In this review we will primarily focus on the role of *Osx* in control of genetic and molecular programs of osteoblast differentiation

1. Control of *Osx* in the genetic program of bone formation

Over a decade ago, the lab of deCrombrughe identified Osterix (*Osx*/sp7) cDNA using a differential hybridization approach from BMP2-treated and untreated C2C12 cells [Nakashima et al., 2002]. BMP2 treatment induces differentiation of C2C12 cells into osteoblasts by stimulating expression of osteoblast genes. Molecular characterization of this cDNA led to the identification of *Osx*, which contains a proline-rich activation domain at the N-terminus spanning aa 1–289 and three C2H2-type zinc finger DNA-binding domains at the C-terminus. In DNA transfection studies, *Osx* containing aa 1–172 is sufficient for transcriptional activation. Osterix is sometimes referred to as Sp7 because of the striking homology with zinc finger DNA-binding domains of Sp1, Sp3 and Sp4 transcription factors. During mouse embryonic development, *Osx* expression begins around E13.5 in calvaria and perichondrium of long bones and is robustly expressed at E15.5 in all osseous elements. *Osx* is also expressed at a lower level in pre- and hypertrophic chondrocytes. It is clear that *Runx2* is required to prime the prechondrogenic mesenchyme segregate into the precursor osteoblast lineage, whereas *Osx* is subsequently required to accomplish the osteoblast differentiation pathway (see Figure 1).

Osx-null cells are arrested in their differentiation

Osx-null embryos either do not express osteoblast markers or express them at very low levels, despite the normal expression of *Runx2*. Expression of *Col1a1*, which is expressed at higher levels in mesenchyme of wild-type mice, is significantly reduced in *Osx* mutants. Other osteoblast markers including *Osteonectin*, *Osteopontin* and *Bonesialo protein* are undetectable in endochondral and membranous skeletal elements in *Osx* mutants. *Osteocalcin*, the very late and specific osteoblast marker normally expressed at E18.5, is also absent from prefigured skeletal zones in *Osx*-null embryos. Expression of *Runx2* is also normal in the condensed mesenchyme of membranous and endochondral skeletal zones in *Osx*-null embryos and long bones, but *Osx* is evidently not expressed in skeletal parts of *Runx2*-null embryos, indicating that *Runx2* is an upstream regulator of *Osx*. These studies strongly indicate that *Osx* triggers *Runx2*-precursors to develop into osteoblasts by activating expression of osteoblasts genes [Nakashima et al., 2002].

Is *Osx* a negative regulator of chondrocytes?

Osx-null embryos, unlike *Osx*-expressing embryos, do not form bone but apparently chondrocyte differentiation and cartilage formation are not affected in null embryos. *Runx2*-positive *Osx*-null cells express chondrocyte-specific genes such as *Sox9* and *Col2a1* and acquire a chondrocyte-type cell fate [Nakashima et al., 2002]. These observations lead to speculation that *Osx* inhibits chondrocyte differentiation in *Runx2*-expressing precursor osteoblasts. *Runx2* is expressed early on in prechondrogenic mesenchyme cells, which express high levels of *Sox9*. Later on, *Osx* expression is stimulated, *Runx2*-positive cells exit as precursor osteoblasts and *Sox9* expression is down-regulated, and thereby suggesting

that expression of *Sox9* and *Osx* is mutually exclusive. Those *Runx2/Osx*-expressing cells strictly differentiate into mature and functional osteoblasts in which *Sox9* is not expressed. The *Runx2*-expressing precursors, prior to *Osx* expression, remain in the chondrogenic lineage and do not progress into the preosteoblast stage. The spatial and temporal expression of *Osx*, studied by Kaback et al (2008), also indicates that the increase in *Osx* expression specifically coincides with the onset and progression of osteoblast differentiation, but occurs after the maturation of chondrocytes [Kaback et al., 2008]. In addition, the levels of *Osx* mRNA remain high after cartilage is formed and remodeled into bone. These studies suggest that *Osx* plays dual roles in inhibition of chondrocyte formation and in promoting osteoblast differentiation (Table 1).

Essential role of Osterix in bone homeostasis in adults

Osx also plays an essential role in formation of adult bones and in expression of osteoblast genes in the adult. Zhou et al. demonstrated that inactivation of *Osx* in adult mice, using LoxP/inducible Cre activity, causes multiple skeletal phenotypes including lack of new bone formation, abnormal cartilage accumulation beneath the growth plate, and defects in osteocyte maturation and function. In addition to down-regulation of osteoblast genes in *Osx*-ablated adult mice, expression of osteocyte-specific marker genes including *Sost*, *Dkk1*, *Dmp1*, *Phe* is decreased. These studies reveal a new role of *Osx* in resorption of cartilage matrix eventually replaced by bone matrix. Note that this strategy was used to delete *Osx* in all cell types using *pCMV Cre-ERT2* [Zhou et al., 2010]. Role of *Osx* in degradation of cartilage matrix was further implicated by another study indicating that *Osx* plays important role in calcification of cartilaginous matrix by directly targeting activation MMP-13 in the hypertrophic chondrocytes of the growth plate during endochondral bone formation. Conditional and global ablation of *Osx* caused the loss of MMP-13 expression and arrest of endochondral ossification at hypertrophy stage [Nishimura et al., 2012]. When *Osx* is inactivated specifically in osteoblasts using 2.3kb *Col1a1-Cre* during embryonic development, no apparent skeletal defects were observed in new-born mice. However, in growing adult stage mice 8 weeks old or older, *Osx* mutant mice displayed reduced bone volume in trabecular and lumbar vertebra and decreases in thickness and number of trabecular bone, thus exhibiting human skeletal defects similar to osteopenia [Baek et al., 2009]. Further, inactivation of *Osx* in osteoblasts of adult mice with inducible Cre recombinase driven by *Col1a1-Cre ERT2* also causes such skeletal phenotypes as reduced expression of osteoblast genes, reduced osteoblast differentiation and function, and mild reduction in bone loss [Baek et al., 2010]. Taken together, these studies depending upon time of *Osx* inactivation clearly indicate that *Osx* continues to play a role as an anabolic regulator that supports the homeostasis of bone in adults.

Although ablation of *Osx* in mice causes inhibition of bone formation, transgenic mice that over-express *Osx* by 2.3kb *Col1a1* promoter show osteopenia, characterized by woven bone structure in the cortical bone, as well as decreased expression of osteoblast genes, especially *Oc*. In *Osx*-expressing transgenic mice, osteoblast differentiation is inhibited and immature osteoblasts are accumulated. These results indicate that proper dosages of *Osx* are required to promote osteoblast differentiation and bone formation in adults [Yoshida et al., 2012].

2. Mutations in *Osx* are associated with human skeletal diseases

Genetic analyses using GWAS, SNP, and linkage mapping are used to identify genes that cause human diseases. Skeletal disorders such as osteoporosis and osteogenesis imperfecta (OI) are associated with mutations in causative genes, including type I collagen gene, which causes low bone mass, and bone mineral density and makes the animal susceptible to multiple bone fractures. Lapunzina et al identified a homozygous single nucleotide deletion (1052delA) mutation in *OSX* gene of an Egyptian child who had been clinically diagnosed

with OI. This patient had repeated fractures, mild bone deformities, delayed tooth eruption etc. *OSX* mutation occurred at the third zinc finger of the DNA-binding domain, causing a frameshift mutation that produced a truncated *OSX* protein. The reduced activity of *OSX* may have altered expression of the *OSX* target bone-specific genes including *type1 collagen* in this patient [Lapunzina et al., 2010]. Genome-wide association studies further revealed a number of genetic variants associated with bone mineral density (BMD) and osteoporosis. Among several GWAS-derived loci, *OSX* is the only locus containing genetic variants over-represented in female childhood obesity for increased BMD. Further SNPs in the *OSX* region indicate a strong correlation between genetic variants at the *OSX* locus with adult lumbar spine and BMD [Timpson et al., 2009; Zhao et al., 2011]. These genetic, clinical and epidemiological studies indicate the significance of *Osx* for overall bone health in adults. Further studies are required to identify mutations in the *OSX* gene from patients with bone disorders to solidify the correlation between *Osx* mutations and bone diseases.

3. *Osx* is needed for odontoblast differentiation and tooth development

The process of tooth formation is quite similar to that of bone formation. Dental mesenchyme cells differentiate into odontoblasts, which produce and secrete collagenous and non-collagenous matrix protein during dentin formation. Dentin sialo phospho protein is a major component of non-collagenous dentin matrix protein and essential for dentin formation. Mouse genetic and biochemical studies demonstrated that both *Runx2* and *Osx* have important roles in tooth formation and function independently in activation of specific genes required for odontoblast differentiation [Chen et al., 2009; Hirata et al., 2009; Zhao et al., 2007]. Chen et al showed that expression of *Dsp* is positively correlated with that of *Osx*, but not with *Runx2*. *Runx2* and *Osx* expression overlap in alveolar bone osteoblasts, odontoblast, amyoblasts, and dental pulp cells in E16 mouse embryos, when *Dsp* is weakly expressed in preodontoblast. By the later stage of differentiating and mature odontoblasts at E18, expression of *Runx2* dramatically decreases, whereas *Osx* expression remains intense in odontoblast and dental pulp cells in which *Dsp* expression is strongly expressed. It appears that *Osx* is needed for differentiation and maturation of preodontoblast to odontoblast through activation of specific late marker genes. This situation of *Osx* function is reminiscent of osteoblast differentiation where *Osx* is needed for differentiation of preosteoblast to osteoblasts. Overlapping expression of *Osx* and *Dsp* is also observed at the postnatal stage of tooth development. Forced expression of *Osx* in mouse odontoblast-like cells stimulates expression of *Dsp* mRNA, indicating that the *Dsp* gene could be a direct target of *Osx* activation [Chen et al., 2009]. Future studies such as RNASeq in combination with ChIP sequencing are required to validate indications that *Osx* directly activates expression of odontoblast genes during tooth formation.

In the last five years *Osx* has generated much attention in bone as well as dental research. Recently, Cao et al used mouse models to elucidate for the first time *Osx*'s role in the formation of cellular cementum – a mineralized tissue that supports tooth development. Transgenic mice that overexpress *Osx* driven by the 3.6kb *Col1a1* display an increase in cementum formation, whereas tissue and stage-specific deletion of *Osx* in mice *2.3 Col1a1-Cre* in embryos or with *CMV-Cre ERT2* in adults showed sharp declines in cementum formation and expression of *Dmp1*. These data further support the important function of *Osx* in tooth formation [Cao et al., 2012].

4. Potential use of Osterix in bone fracture and repair

Given that Osterix is a classic osteoblast marker and essential for bone formation, exploitation of *Osx* as an anabolic molecule is an appealing avenue in the repair of bone fractures and injuries. Bone repair is a complex process that includes spatial and temporal

expression of multiple molecules and interactions of these molecules in formation of new bone during fracture healing. A review on gene therapy approaches in bone regeneration with recent works on molecules that are required for bone healing is summarized [Franceschi, 2005]. Studies involving controlled bone injuries followed by healing in animals have demonstrated that *Osx* is expressed intensely during regeneration of bone cells at fracture sites. Implanting of *Osx*-expressing BMSc (bone marrow stroma cells) at the surgically incisor of rat calvaria accelerates bone healing at the injured calvaria much more than implanted control BMS cells by forming new bone at the injured calvaria as well as by inducing expression of bone-specific genes [Tu et al., 2007]. Further, *Osx*-expressing BMSc cells implanted into nude mice also induced bone formation through acceleration in osseous integration of implants [Tu et al., 2006; Xu et al., 2009]. Kaback et al showed that in mice with controlled bone fracture, the fracture callus and cartilage began to form at day 7 after fracture and continued at day 10 with an increase in *Sox9* mRNA. *Osx* was expressed in the osteoblast of the woven bone of the fracture callus at day 10, but was excluded from cortical bone, hypertrophic chondrocytes and blood vessels. At day 14 post-fracture, *Osx* was mostly expressed in osteoblasts surrounding the healed sites whereas expression of *Sox9* decreased and cartilage of the callus remodeled into bone [Kaback et al., 2008]. In the same study, treatment of PTHrP or BMP2 to osteochondro progenitors which were derived from mouse limb buds that expressed high levels of *Sox9* induced expression of *Osx* but PTHrP or BMP2 decreased that of the chondrocyte markers *Sox 9* and *Col2a1*. Over-expression of *Osx* in these progenitors decreased *Sox9* level, suggesting that *Osx* might inhibit chondrocyte differentiation while promoting osteoblast differentiation [Kaback et al., 2008].

The mechanism of migration of precursor osteoblasts at the primary ossification site during normal bone formation or fracture healing was further demonstrated by lineage tracing approach using two mouse strains, harboring *Osx*-CreERT and *Col1a1*-ERT transgenes, and crossing these mice with *Rosa26R*-LacZ reporter [Clarkin and Olsen, 2010; Maes et al., 2010]. Injection of 4-OH tamoxifen to transgenic mice at specific time during development activates expression of Cre recombinase which subsequently induces *LacZ* expression [Maes et al., 2010]. In this study, authors reported that two populations of osteoblast-lineage cells exist at the perichondrium; the precursor osteoblasts which are *Osx*-positive, and the differentiated osteoblasts which are *Col1a1*-positive. These experiments showed that *Osx*/*LacZ* expressing precursor osteoblasts were sparsely present in the cortex but were abundantly present inside the trabecular bone whereas *Col1a1*/*LacZ* cells were enriched at the cortex and engaged into cortical bone formation, and were hardly present in the trabecular bone. This study proposes a mechanism by which precursor osteoblasts (*Osx*/*LacZ* positive cells) enter at the site of primary ossification center along with blood vessels because *Osx*-expressing precursors are intimately associated with endothelial cells of blood vessels. Additionally, bone fracture studies performed in *Osx*-Cre:GFP mice that allowed tracing of GFP positive cells further revealed that before fracture these *Osx*-GFP positive cells were present in the thin periosteal layer of the cortical bone, but after fracture these cells along with blood vessels were massively localized to the woven bone callus at the healing regions. These studies indicate the potential application of *Osx* as bone regenerative medicine in healing of bone fractures.

5. *Osx* expression is dependent on or independent of *Runx2* activity

In *Osx*-null embryos *Runx2* is expressed whereas in *Runx2*-null embryos, *Osx* is not expressed, and therefore, *Osx* can be placed downstream of *Runx2*, where it activates *Osx* expression (see Figure). Sequence analysis of the *Osx* promoter reveals that several putative *Sox9*, VDRE, *Runx2*, *Dlx5* and *Sp1* response elements are present within upstream regulatory sequence of the *Osx* gene [Nishio et al., 2006]. In a reporter assay, *Runx2* was able to activate the *Osx* promoter through direct interactions with *Runx2*-binding element

present within 1 kb of the *Osx* promoter. Other studies also indicated that *Osx* expression is not exclusively dependent on Runx2 activity. In *Runx2*-null calvarial cells in which *Osx* is normally not expressed, BMP2 treatment induced *Osx* expression through upregulation of Dlx5, which interacted with *Osx* promoter containing Dlx5 binding elements [Lee et al., 2003]. BMP signaling did not directly induce expression of *Osx* but through phosphorylation of Dlx5 enabled the interactions of Dlx5 with the *Osx* promoter. These studies indicate that *Osx* expression is mediated through alternate pathways independently of Runx2. It would be reasonable to conclude that, although Runx2 alone may not be sufficient for *Osx* expression, it functions in tandem with other signaling pathways to mediate Runx2-independent *Osx* expression during osteoblast formation. Interestingly, *Osx* regulates its own expression through a feedback mechanism by interacting with its own promoter [Yoshida et al., 2012]

***Osx* expression under control of signaling pathways**

Several studies elucidated the role of bone morphogenetic protein 2 (BMP2) and insulin-like growth factor (Igf1) signaling pathways in activation of osteoblast genes during osteoblast differentiation. These growth factor-ligand complexes elicit a cascade of signal transduction pathways followed by phosphorylation of downstream substrates to control gene transcription [Huang et al., 2007; Jadowiec et al., 2004]. BMP2 is known to induce *Osx* expression in a Runx2-dependent manner. BMP2/Smad pathway targets activation of *Runx2*, which in turn activates *Osx* expression. Igf1 also up-regulates *Osx* expression without altering *Runx2* expression. Although Igf1 response in *Osx* expression is not as strong as BMP2, a combination of BMP2/Igf1 synergistically increases *Osx* expression in human mesenchymal stem cells (hMSC) compared to Igf1 alone. This suggests that *Osx* expression requires other signaling pathways beyond BMP2/Smad/Runx2. The synergistic activation of *Osx* expression is most likely due to common convergent mitogen-activated protein kinase (MAPK) and protein kinase D (PKD) pathways, activated by the BMP2/Igf1 signaling [Celil and Campbell, 2005; Celil et al., 2005]. MAPK has been reported to participate in induction of Alp activity and Ca⁺⁺ deposition during bone formation. There are four members of the MAPK super family--extracellular-signal-regulated kinase 1/2 (Erk1/2), c-Jun amino-terminal kinase (JNK), p38, and Erk5. BMP2 treatment of hMSC activates MAPK components p38 and Erk1/2 through phosphorylation of p38 and Erk1/2, which mediate BMP2-induced *Osx* expression. Inhibition of p38 and Erk1/2 signaling using specific inhibitors down-regulates BMP2-mediated *Osx* expression. Further over-expression of dominant-negative form of p38 or Erk in hMSC completely abolished *Osx* expression under osteogenic media, thereby strongly indicating that *Osx* expression involves the MAPK pathway rather than BMP2/Smad/Runx2 signaling, because MAP/Erk does not inhibit *Runx2* expression. However, activated MAP/Erk does phosphorylate Runx2 to increase the stability, and the transcriptional activity of Runx2 in expression of *Osx*. A recent report further suggests a novel mechanism of *Osx* expression during osteoblast differentiation under osteogenic media by ascorbate-dependent prolyl hydroxylase domain protein (PHD) and proteasome pathway, which targets degradation of yet-to-be-defined transcriptional repressors of the *Osx* gene [Xing et al., 2011]. Ascorbate effects in *Osx* expression are independent of BMP2, Igf1, ECM-mediated integrin signaling, and MAPK pathways, because blockade of either pathway does not affect *Osx* expression. However, inhibition of PHD by the specific inhibitors dimethyloxallyl glycine and ethyl 3, 4-dihydroxybenzoate strongly affects ascorbate-induced *Osx* expression and OB differentiation.

Function of tumor-suppressor p53 has also been documented in osteoblast differentiation and bone formation by regulating *Runx2/Osx*, supported by both gain of function and loss of functions in mouse models [Liu and Li, 2010]. Wang et al showed that tumor-suppressor p53 is able to inhibit *Osx* expression in DNA transfection assays as well as in vivo using

mouse genetic approaches [Wang et al., 2006]. Inhibition by p53 of *Osx* expression does not occur through direct binding on the *Osx* promoter, because there is no p53 binding sequence present in the *Osx* gene. It was suggested that like other target genes, p53 represses *Osx* expression by preventing recruitment of p300 and other activators to the target chromatin. In *p53*-null mice, expression of *Osx*, but not *Runx2*, is up-regulated and so are the other *Osx* target genes. As a result, the loss of p53 induces accelerated osteoblast differentiation and high bone mass phenotype in *p53* mutant mice [Wang et al., 2006].

ER-stress responds to *Osx* induction

Mature osteoblasts produce high levels of extracellular matrix (ECM) proteins during skeletal development, often resulting in accumulation of misfolded proteins that cause endoplasmic reticulum stress (ER stress) [Boot-Handford and Briggs, 2010; Murakami et al., 2009; Park et al., 2012]. To circumvent the ER stress so that ECM proteins may be secreted by osteoblasts, the unfolded protein response pathway is activated by ER stress transducers including IRE1 α (inositol-requiring endonuclease 1 α), which is involved in processing of transcription factor *XBPI* mRNA [Tohmonda et al., 2011]. *XBPI* then binds to UPR elements and activates many genes to alleviate ER stress. The *Osx* promoter also contains the recognition sequence for *XBPI* and UPR. Tohmonda et al showed that upon treatment of thapsigargin, an ER stress inducer, the *Osx* gene is activated by *XBPI* through interaction with *Osx* promoter during BMP-2-induced osteoblast differentiation of mouse embryonic fibroblasts [Tohmonda et al., 2011]. Overexpression of *XBPI* stimulates *Osx* expression but not *Runx2*, indicating a functional role of the IRE1 α -*XBPI* pathway in *Osx* expression during differentiation of preosteoblasts to mature osteoblasts.

Negative regulation of *Osx* expression

Epidermal-growth factor receptor (EGFR) and tumor-necrosis factor (TNF) oppose osteoblast differentiation by negatively regulating expression of osteoblast genes. EGFR inhibits *Runx2* expression and consequently *Osx* expression through stimulation of HDAC levels in cells [Zhu et al., 2011]. Several HDACs including HDAC 3–7 are known to inhibit osteoblast differentiation by inhibiting *Runx2* activity [Bradley et al.; Bradley et al., 2011; Jensen et al., 2007; Jensen et al., 2008; Kang et al., 2005]. TNF α promotes bone loss and delayed osteoblast differentiation through upregulation of *Prx1* (paired related homeodomain). *Prx1* interacts with the *Osx* promoter and represses *Osx* expression [Lu et al., 2011]. Characterization of *Osx* gene revealed that two transcription start sites, each driven by independent promoters, give rise to two isoforms of *Osx*; *Osx1* being the more abundantly expressed form compared to alternatively spliced *Osx2*. TNF-response elements are present in both promoters, and the binding of TNF at the *Osx* promoter inhibits *Osx* expression in preosteoblasts MC3T3 cells.

Epigenetic control of *Osx* expression by DNA methylation and miRNAs

DNA methylation is a repressive epigenetic mark that is associated with silencing of the key genes required for cellular differentiation [Minard et al., 2009; Ong and Corces, 2012]. Demethylation of those key genes is essential for activation of gene expression that allows cell-type differentiation during development. A study using cell line based experiments reported that the promoters of the osteoblast genes including *Dlx5*, *Osx*, *Oc* and *Alp* are hypomethylated in osteoblast cells that express these genes and are hypermethylated in non-osteogenic cells, which do not express these genes [OLee et al., 2006]. Treatment with azacytidine (5Aza), an inhibitor of DNA methylation, activates expression of silenced osteoblast genes including *Osx* and *Dlx5* in C2C12 mesenchymal cells. This suggests that DNA methylation restricts expression of those genes to prevent the differentiation of multipotent precursors into specific cell lineages [Hupkes et al., 2011]. Hupkes et al further reported that the majority of 5Aza-treated C2C12 cells differentiate into myoblasts and to a

lesser extent into osteoblasts because 5Aza treatment stimulated expression of the key transcription factors *MyoD* and *Osx* through DNA demethylation [Hupkes et al., 2011]. It is likely that hypermethylation of the *Osx* promoter plays a crucial role in imposing a barrier in expression of this gene in pluripotent mesenchymes. Hence, demethylation of the *Osx* promoter might begin to occur in precursor osteoblasts, which results in restriction of its expression to osteoblasts. Along this line, a recent report indicates that an osteocyte-specific marker *Sost* gene is hypermethylated in osteoblasts that do not express *Sost* gene. In osteocytes, the *Sost* promoter is hypomethylated and is expressed [Delgado-Calle et al., 2012]. Note that *Osx* is needed for the maturation and function of osteocytes by activating osteocyte-specific *Sost*, *Dmp1*, and *Phex* genes [Zhou et al., 2010]. We should speculate that DNA methylation of *Osx* and its target genes is lost at specific developmental stages to ensure transcription of these genes in osteoblasts and osteocytes during bone formation.

MicroRNAs are small noncoding RNAs that negatively regulate gene expression at post-transcriptional and post-translational level by directly targeting mRNA of the key genes, and then subsequently control cellular activities including proliferation and differentiation. A comprehensive review on the role of several miRNAs in skeletal development recently appeared in literature [Lian et al., 2012]. Among those miRNAs, miR-93, 125, 135, 138, 637 have been reported to function in regulation of *Osx* and to inhibit osteoblast formation [Eskildsen et al., 2011; Goettsch et al., 2011; Schaap-Oziemlak et al., 2010; Yang et al., 2012]. Yang et al showed that lentivirus-mediated overexpression of miR-93 suppressed osteoblast differentiation and mineral deposition through inhibition of *Osx* protein, but not *Osx* mRNA, by directly binding to coding region of *Osx* gene. Interestingly, this study also revealed that expression of miR-93 gene is under the control of *Osx* activity because *Osx* interacted with the promoter of miR-93 gene and overexpression of *Osx* suppressed miR-93 expression. These data indicate that miR-93 regulates differentiation program of osteoblast through *Osx*/Sp7 feedback mechanism [Yang et al., 2012]. Another miR-637 is overexpressed in adipocytes and suppressed *Osx* expression in these cells by directly targeting the 3'UTR of the *Osx* gene. Thus, miR-637 promotes transdifferentiation of mesenchymes cells into adipocytes by inhibiting *Osx* expression and then subsequent osteoblast differentiation [Zhang et al., 2011]. Others miRNA-125b, 135b and 138 have also been reported to negatively regulate *Osx* expression directly or indirectly in non-osteoblasts such as vascular smooth muscle cells, unrestricted somatic cells or human stromal mesenchymal cells, all of which have potential to differentiate into osteoblasts in which these miRNAs are down-regulated.

6. Control of *Osx* transcriptional activity

Given that *Osx* was discovered over a decade ago and *Osx* is a recognized key regulator of bone formation, we are dismayed that we have such limited knowledge of the molecular mechanisms of *Osx* activity in activation of osteoblast genes. In this section, we will update our knowledge from recent studies of the regulation of *Osx* activity.

NFATc, TFII, p300 and Brg1 promote *Osx* activity

Nuclear factor of activated T cells (NFATc), an essential transcription factor for osteoclast differentiation, also plays important role in osteoblast differentiation through positive regulation of *Osx* activity. NFATc induces *Osx*-dependent activity of its target promoter 2.3kb *Col1a1* in reporter assays. NFATc interacts with the activation domain of *Osx* and this interaction is needed for recruitment of NFATc at the *Osx*-target *Col1a1* promoter. In *Osx* knocked-down cells (by siRNA), occupancy of NFATc at the *Col1a1* promoter is sharply decreased. Further, there is no activation of the 2.3kb *Col1a1*-reporter in *NFATc*^{-/-} primary osteoblasts, suggesting that NFATc acts positively in osteoblast differentiation by stimulating *Osx* activity [Koga et al., 2005]. Other study shows that the *Osx* fragment with

aa 141–210 is sufficient for activation and interacts with TFII and that the C-terminus of Osx (280–428aa) interacts with Brg1, a catalytic component of SWI/SNF complexes of ATP-dependent chromatin remodeling activity [Hatta et al., 2006].

NO66 histone demethylase inhibits Osx activity

NO66 is a jumonji C containing protein identified as Osx-interacting polypeptides using a proteomic approach and mass spectrometry. NO66 exhibits demethylase activity *in vitro* with dual specificity for lysine 4 and 36 of histone H3 [Sinha et al., 2010]. NO66 is a part of the pre-ribosome complex, localized mainly in nucleoli but also in nucleoplasm, ubiquitously expressed in all tissues, and evolutionarily conserved among vertebrates [Eilbracht et al., 2004]. Histone demethylases control the levels of histone methylation in chromatin and regulate the transcriptional state of the gene in various cellular activities including proliferation, differentiation, senescence etc [Benevolenskaya, 2007; Bernstein et al., 2006; Cloos et al., 2008; Klose et al., 2006; Martin and Zhang, 2005; Tsukada et al., 2006]. Along these lines, the interactions of NO66 demethylase with Osx should be considered physiologically significant in regulating osteoblast differentiation through modulation of Osx activity [Sinha et al., 2010].

Interactions between Osx and NO66 occur through the activation domain of Osx and JmjC domain, including the C-terminus of NO66, in vitro and in vivo. In DNA transfection assays, NO66 inhibits the Osx-dependent activation of the Osx-target *Bsp* and *Oc* promoters. Knock-down of NO66 by shRNA in preosteoblast MC3T3 cells accelerates osteoblast differentiation in these cells by stimulating both *Alp* activity and extracellular matrix deposition. Expression of *Col1a1*, *Bsp* and *Oc* genes is significantly up-regulated in NO66-depleted MC3T3 cells. NO66 inhibits expression of Osx target osteoblast genes likely through interaction with Osx and through regulating histone methylation levels in the chromatin of these genes. Chromatin immunoprecipitation (ChIP) assays indicate that there is an inverse relationship between occupancy of NO66 histone demethylase, and that of Osx as well as H3K4me3 or H3K36me3 [Sinha et al., 2010]. This supports the hypothesis that interactions of NO66 with the chromatin cause demethylation of these histone lysines for the maintenance of gene repression in the preosteoblast state. These studies open new insights into the regulation of osteoblast genes through epigenetic mechanisms involving histone methylation of the osteoblast-specific chromatin and provide clear evidence for a physiological role of NO66 in osteoblasts.

7. Phosphorylation of Osx regulates its transcriptional activity

Post-translational modification by phosphorylation elicited by signaling pathways is a key cellular event in controlling the activities of transcription factors during gene activation. Numerous studies have implicated for phosphorylation of Osx triggered by signaling pathways such as BM2 and MAPK in the regulation of Osx activity will be discussed here. Osx is phosphorylated by p38 MAPK at Ser-73 and Ser-77 to increase protein stability and to interact with transcriptional activators including p300 at the target *Bsp* and *Fmod* genes during osteoblast differentiation. BMP-2-induced Osx or ectopically expressed Osx exists as doublet polypeptide bands due to phosphorylation. Inhibition of p38 MAPK inhibits Osx expression at both mRNA and protein level [Ortuno et al., 2010]. Wang et al showed that Spata4 (spermatogenesis associated 4 gene) interacts with Erk1/2 in osteoblasts and enhances phosphorylation of Erk1/2, which then phosphorylates Osx to increase protein stability and activity of Osx during activation of Osx target genes [Wang et al., 2011].

Based on converging evidence, phosphorylation of Osx is indispensable for Osx activity for expression of osteoblast genes. Akt is a serine/threonine-specific protein kinase that phosphorylates Osx at threonine residues present within Akt recognition element RXXS/T

near the C-terminus of Osx. BMP-2 treatment of C2C12 cells induces Osx protein in an Akt-dependent manner. Further, Akt stimulates Osx-dependent activation of the *Oc*, *Alp* and *Bsp* promoters in transfection assays, indicating that phosphorylation by Akt increases protein stability and Osx activity during osteoblast differentiation [Choi et al., 2011]. Control of Osx expression and the transcriptional activity of Osx are outlined in the figure.

8. Concluding remarks

In this review, we have compiled many of the important research findings in genetic and molecular control of Osx in bone and tooth formation. Osx is a major effector and essential for activation of bone-specific genes that support bone formation in embryos and adults. Runx2 is a critical in formation of precursor osteoblasts from mesenchymes and Osx is a critical for differentiation of *Runx2*-expressing precursors into mature and functional osteoblasts. Since Osx is a classic osteoblast marker, the question remains to be addressed whether Osx once expressed takes control of transcriptional program of osteoblast target genes independently of Runx2 function. Animal models and cell culture studies indicate the potential for therapeutic use of Osx in bone injury repair. The published literature we have reviewed gives a brief assessment of Osx's role in control of bone formation, but unlike Runx2 which has been extensively characterized till date, we do not have explicit knowledge of the mechanisms underlying Osx expression or the regulation of Osx activity, which is essential for osteoblast formation. A list of suggested articles on detailed study of Osx is included in this review for the reader. Osx and its interactions with other proteins and post-translational modifications by phosphorylation and methylation, signaling pathways, miRNAs, and DNA methylation, any and all of which may regulate Osx expression and activity, should be considered for future work to better understand how Osx controls chromatin structure of its target genes during osteoblast differentiation. Finally we leave a big question to our readers and for future studies – is Osx a slave of Runx2 or the master of skeletogenesis?

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Figure 1. Osteoblast differentiation is a multi-step process

Multipotent mesenchymal precursors in condensed mesenchymes differentiate into *Sox9*-expressing osteochondroprogenitors, which segregate into chondrocytes that express high levels of *Sox9*, and preosteoblasts that express high levels of *Runx2* and low level of *Col1a1* and *Akp*. *Osx* expression is needed to accomplish the differentiation of preosteoblasts to mature and functional osteoblasts that express high levels of osteoblast markers including *Col1a1*, *Akp*, *Bsp*, and *Oc*. *Osx* controls the genetic program of osteoblast differentiation (shown in light blue area). Functional osteoblasts produce extracellular matrix molecules which form a calcified bone matrix. *Osx*-null cells are arrested in their differentiation and acquire chondrocytes phenotype by expressing chondrocyte markers. The molecular control of *Osx* is depicted in light yellow area. Expression of *Osx* mRNA and protein is positively regulated by signaling pathways (BMP2, MAPK, Igf1), *Runx2*, *Dlx5*, ER-stress pathway (IRE1a/XBP1), and negatively regulated by DNA methylation and microRNAs, p53, and TNF. The transcriptional activity of *Osx* is documented by NFATc as positive regulator and by NO66 histone demethylase as negative regulator.

Table 1

Following publications are suggested for further information on Osterix studies

Description of studies	Experimental models	Authors
Discovery & function of <i>Osx</i> in osteoblast differentiation and bone formation during embryonic development	mouse KO studies	Nakashima et al (2002)
Role of <i>Osx</i> in bone homeostasis in adult and in formation of osteocytes	Conditional mouse KO	Zhou et al (2010)
Frameshift mutation in <i>OSX</i> gene	Osteogenesis imperfecta	Lapunzina P et al (2010)
Function of <i>Osx</i> in cementogenesis (tooth formation)	Conditional mouse KO	Cao et al (2012)
Expression patterns of <i>Osx</i> during tooth formation	Mouse studies	Hirata et al (2009)
<i>Osx</i> -mediated inhibition in differentiation and maturation of chondrocytes	Mouse and cell culture	Kaback et al (2008)
Runx2 independent activation of <i>Osx</i> through <i>Dlx5</i>	Cell culture studies	Lee et al (2003)
Runx2-mediated activation of <i>Osx</i>	Cell culture studies	Nishio et al (2006)
Requirement of precursor osteoblast (<i>Osx</i> ⁺) in primary ossification as well as in bone healing process	Mouse studies using lineage tracing	Maes et al (2010)
ER-stress response in activation of <i>Osx</i> during osteoblast differentiation	Cell culture studies	Tohmonda et al (2011)
Function of <i>Osx</i> in bone-related injuries	Mouse studies	Tu et al (2006, 2007)
Inhibition of bone formation by p53 through negative regulation of <i>Osx</i>	Mouse KO studies	Wang et al (2006)
Inhibition of osteoblast differentiation by NO66 histone demethylase through interactions with <i>Osx</i>	Cell culture studies	Sinha et al (2010)
p38-mediated <i>Osx</i> phosphorylation	Cell culture studies	Ortuno et al (2010)
BMP-2 dependent and independent activation of <i>Osx</i> through MAPK	Cell culture studies	Celil et al (2005) Celil & Cambell (2005)
Akt and Erk1/2 dependent <i>Osx</i> activity	Cell culture studies	Choi et al (2011)
mir-93 controls <i>Osx</i> protein	Cell culture studies	Yang et al (2012)