

## Molecular mechanisms of mesenchymal stem cell differentiation towards osteoblasts

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Received: June 24, 2013 Revised: August 30, 2013

Accepted: September 14, 2013

Published online: October 26, 2013

### Abstract

Bone is a dynamic tissue that is constantly renewed by the coordinated action of two cell types, *i.e.*, the bone-resorbing osteoclasts and the bone-forming osteoblasts. However, in some circumstances, bone regeneration exceeds bone self repair capacities. This is notably often the case after bone fractures, osteolytic bone tumor surgery, or osteonecrosis. In this regard, bone tissue engineering with autologous or allogenic mesenchymal stem cells (MSCs) is been widely developed. MSCs can be isolated from bone marrow or other tissues such as adipose tissue or umbilical cord, and can be implanted in bone defects with or without prior amplification and stimulation. However, the outcome of most pre-clinical studies remains relatively disappointing. A better understanding of the successive steps and molecular mechanisms involved in MSC-osteoblastic differentiation appears to be crucial to optimize MSC-bone therapy. In this review, we first present the important growth factors that stimulate osteoblastogenesis. Then we review the main transcription factors that modulate osteoblast differentiation, and the microRNAs that inhibit their expression. Finally, we also discuss articles

dealing with the use of these factors and microRNAs in the development of new bone MSC therapy strategies. We particularly focus on the studies using human MSCs, since significant differences exist between osteoblast differentiation mechanisms in humans and mice for instance.

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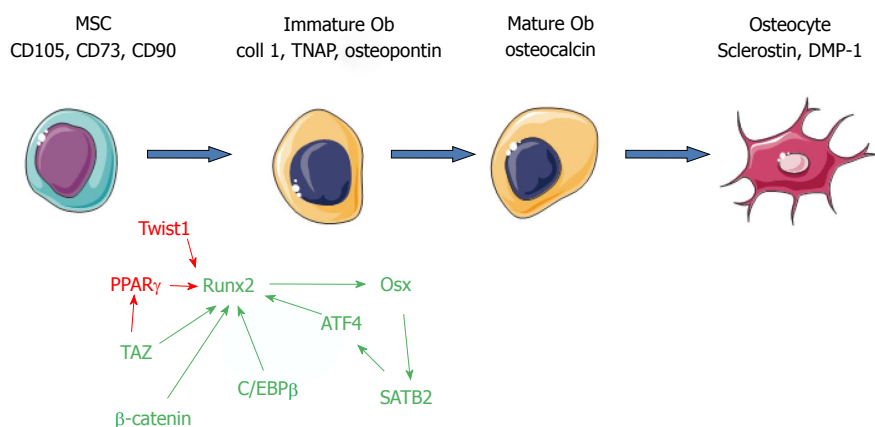
**Key words:** Mesenchymal stem cells; Osteogenesis; Runt-related 2; Wnt; MiRs

**Core tip:** Several excellent reviews on the transcription factors involved in osteoblast differentiation have recently been published, but none also presented the microRNAs that control the expression of these transcription factors. Moreover, most of these reviews mainly reported mouse studies but important differences are well acknowledged between humans and mice. For instance vitamin D<sub>3</sub>, an important hormone controlling bone homeostasis, has very different effect in these species. Therefore, in the present review we particularly focus on human cells to present the transcription factors and MiRs controlling mesenchymal stem cells-osteoblastic differentiation.

Fakhry M, Hamade E, Badran B, Buchet R, Magne D. Molecular mechanisms of mesenchymal stem cell differentiation towards osteoblasts. *World J Stem Cells* 2013; 5(4): 136-148 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v5/i4/136.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v5.i4.136>

### BONE REPAIR WITH MESENCHYMAL STEM CELLS

Historically, Friedenstein *et al*<sup>[1]</sup> were the first to report the presence of fibroblastoid cells in the adult bone marrow that can make bone and reconstitute a hematopoietic



**Figure 1** transcription factors involved in osteoblast differentiation from mesenchymal stem cells. Markers of differentiation are shown in black, stimulatory transcription factors in green, and inhibitory ones in red. MSCs: Mesenchymal stem cells; ATF4: Activating transcription factor 4; C/EBP: CCAAT/enhancer-binding proteins; Coll: Collagen; DMP-1: Dentin matrix protein-1; Ob: Osteoblast; Osx: Osterix; PPAR: Peroxisome proliferator activated receptor; SATB2: Special AT-rich sequence binding protein 2; TAZ: Transcription coactivator with binding capacity to PDZ motifs; TNAP: Tissue-nonspecific alkaline phosphatase; Runx2: Runt-related 2.

microenvironment when transplanted subcutaneously. These mesenchymal stem cells (MSCs) were later reported to contribute to various musculoskeletal tissues such as bone, cartilage, fat, muscle, ligament and tendon<sup>[2]</sup>. In 2006, the International Society for Cellular Therapy proposed that cells with the following characteristics should be considered as MSCs, (1) cells adherent to plastic in culture; (2) presence of CD105, CD73 and CD90 but absence of CD34, CD45, CD14 or CD11b, CD79 $\alpha$  or CD19, and HLA-DR molecules; and (3) cells with the capacity to differentiate into osteoblasts, chondrocytes and adipocytes<sup>[3]</sup>. However, although these criteria are widely accepted, they may still be imperfect. Indeed, the three markers are co-expressed in a wide variety of cells, and may therefore not be able to indentify a single MSC population *in vivo*<sup>[4]</sup>.

MSCs represent less than 0.01% of the bone marrow cell population. At birth, the frequency of MSCs has been reported as 1 MSC/10<sup>4</sup> BM-mononuclear cells, decreasing to 1 MSC/10<sup>5</sup> BM-mononuclear cells in teenagers to 1 MSC/2  $\times$  10<sup>6</sup> BM-mononuclear cells in 80-year-old individuals<sup>[5]</sup>. To overcome the drawbacks associated with MSC isolation from bone marrow, other sources have been contemplated. MSCs can indeed be recovered from several different locations such as adipose tissue<sup>[6]</sup>, dental pulp<sup>[7]</sup> and umbilical cord<sup>[8]</sup>. Recently, Sacchetti *et al*<sup>[9]</sup> reported CD146 high pericytes surrounding bone marrow vascular sinusoids can be considered as MSCs as they are self-renewing osteoprogenitors capable of ectopic bone formation. Finally, differences appear to exist between MSC populations from different tissues, which represents an additional challenge to devise a universal definition<sup>[10]</sup>.

MSC differentiation into osteoblasts can be achieved by adding vitamin D<sub>3</sub>, ascorbic acid and  $\beta$ -glycerophosphate to the culture medium<sup>[11]</sup>. Several laboratories use dexamethasone, a synthetic glucocorticoid, instead of vitamin D<sub>3</sub>. Dexamethasone appears to optimize differentiation from MSCs, but not specifically to the osteoblast lineage<sup>[12]</sup>. In osteogenic conditions, human MSCs secrete a matrix enriched in type I collagen which will be mineralized with apatite crystals upon activation of tissue-nonspecific alkaline phosphatase (TNAP) (Figure 1)<sup>[13]</sup>. Osteoblasts also secrete a tissue-specific protein, osteocalcin,

recently shown to act as a circulating hormone involved in the control of insulin secretion and sensitivity<sup>[14]</sup>. However, although this protein is a useful marker of osteoblast differentiation, it doesn't seem to impact bone formation. Eventually, some osteoblasts will become surrounded by a mineralized collagen matrix and further differentiate into bone-residing osteocytes, which secrete different proteins such as sclerostin, a canonical Wnt signaling inhibitor, and dentin matrix protein-1, a molecule controlling phosphatemia<sup>[15]</sup>.

MSCs have been implanted in association with different scaffolds to rebuild bone<sup>[16,17]</sup>. Injection of MSCs has also been shown to correct bone defects. Notably, allogenic bone marrow transplants or injection of isolated MSCs in children with osteogenesis imperfecta (OI) have improved bone formation and function<sup>[18,19]</sup>. However, although promising data were reported, many others led to contrasting if not disappointing results<sup>[20]</sup>. In this regard, it appears crucial to better understand the molecular mechanisms of osteoblast differentiation from human MSCs. This will allow us to improve the bioactivity of injected MSCs or MSC-containing hybrid materials by stimulating their osteoblast differentiation. This may be achieved through genetic modification of MSCs. For instance, autologous MSCs may be modified to correct the abnormal collagen synthesis in patients with OI<sup>[21]</sup>. Several excellent reviews on osteoblast differentiation have been published in recent years. To our knowledge however, none has focused on the interactions between transcription factors and microRNAs in human mesenchymal stem cells specifically. We believe that it is particularly important since significant differences are well acknowledged between osteoblastogenesis of human and mouse MSCs. For instance, while vitamin D<sub>3</sub> binds to a vitamin D response element (VDRE) in the osteocalcin promoter in humans and rats, the mouse osteocalcin promoter is devoid of any VDRE and vitamin D<sub>3</sub> exerts an indirect inhibitory effect on osteocalcin transcription<sup>[22,23]</sup>.

## GROWTH FACTORS STIMULATING MSC-OSTEOBLASTIC DIFFERENTIATION

Two families of growth factors appear to stimulate osteo-

blast differentiation from MSCs: the Wnt (a portmanteau of Wingless and integration 1) family and the bone morphogenetic proteins (BMPs).

### Wnt family members

Wnt proteins are a family of 19 highly conserved secreted glycoproteins that play essential roles during development and tissue homeostasis<sup>[24]</sup>. Some Wnt proteins such as Wnt3a and Wnt10b bind to Frizzled receptors, and recruit the LRP5/6 coreceptors to activate the canonical signaling pathway, leading to glycogen synthase kinase- $\beta$  inhibition,  $\beta$ -catenin stabilization, translocation into the nucleus and regulation of T-cell factor/lymphoid enhancer factor (TCF/LEF) transcriptional activity. Binding of Wnt proteins to LRP5/6 is inhibited by secreted factors such as Dickkopf-related protein 1 (Dkk1)<sup>[24]</sup>. Dkk1 binds to LRP5/6 causing the receptor to attract Kremen, and this interaction promotes clathrin-mediated internalization thereby inactivating LRP5/6.

The importance of the canonical Wnt signaling in bone is well-acknowledged. Genetic reports established that Wnt/ $\beta$ -catenin activity is essential for bone development<sup>[25]</sup>. Deficiency of Dkk1 is associated with increased bone formation in mice and humans<sup>[26]</sup>. Wnt10b may be particularly important for bone formation. Wnt10b is expressed in the bone marrow by osteoblast progenitors<sup>[27]</sup>, and transgenic overexpression of Wnt10b in mesenchymal cells leads to increased bone density and accelerated osteoblastogenesis *in vitro*, whereas Wnt10b<sup>-/-</sup> mice have reduced trabecular bone<sup>[28]</sup>. Moreover, Wnt10b seems to stimulate osteoblast functions through a positive autocrine loop<sup>[29]</sup>. On the other hand, other recent findings indicate that canonical Wnt signalling inhibits osteoblast differentiation in human MSC cultures<sup>[30-32]</sup>. These contrasting findings have been reconciled recently by Liu *et al*<sup>[33]</sup> who found that Wnt/ $\beta$ -catenin signalling favours osteogenic commitment in basal medium by inhibiting MSC commitment into adipocytes, but inhibits osteoblast differentiation in osteogenic conditions. This was confirmed by Kang *et al*<sup>[34]</sup> who reported that Wnt10b induction of osteogenesis in mouse progenitors was due to inhibition of peroxysome proliferator-activated receptor (PPAR) $\gamma$  and CCAAT/enhancer-binding protein (C/EBP) $\alpha$  activity. The mutual inhibition between  $\beta$ -catenin and PPAR $\gamma$  will be discussed below.

Alternatively, non-canonical Wnt members may also be involved in the effects of TNF- $\alpha$  on ossification. In particular, Wnt5a seems to be the predominant Wnt variant expressed during osteoblastic differentiation of human MSCs<sup>[35]</sup>. Wnt5a<sup>+/-</sup> mice present a reduced bone mass phenotype with decreased osteoblast number<sup>[36]</sup>. Wnt5a appears to stimulate osteoblast differentiation through an autocrine loop in human MSCs<sup>[37,38]</sup>. Another non-canonical Wnt with a potential interest in bone repair is Wnt4. In two different models of craniofacial bone injury, Chang *et al*<sup>[39]</sup> observed that human MSCs genetically engineered to express Wnt-4 enhanced osteo-

genesis and improved the repair of craniofacial defects in nude mice.

### Bone morphogenetic proteins

BMPs are growth factors that belong to the transforming growth factor beta (TGF- $\beta$ ) superfamily<sup>[40,41]</sup>. The term, bone morphogenetic protein was first introduced to describe the components in demineralized bone matrix that can induce ectopic bone formation when implanted intramuscularly or subcutaneously into rodents<sup>[42,43]</sup>. To date, more than 20 BMP members have been characterized. As TGF- $\beta$ , BMPs trigger cellular responses mainly through the Smad pathway<sup>[44]</sup>, although they can also activate the mitogen-activated protein kinase pathway<sup>[45]</sup>. In the Smad pathway, type II and type I receptors with serine/threonine kinase activity and intracellular Smad proteins relay the signal from the cell surface to the nucleus. Three type II receptors can bind BMPs: type II BMP receptor, and type II and II B activin receptors (ActR-II and ActR-II B)<sup>[40]</sup>. Three type I receptors for BMPs have also been characterized: type I A and I B receptors (BMP1A or ALK3 and BMP1B or ALK6), and type I A activin receptor (ActRIA or ALK2). The receptors activated by ligand binding phosphorylate a subgroup of receptor-regulated Smads (R-Smads including Smad 1, 5 and 8). The phosphorylated R-Smads then disassociate from their receptor and form complexes with the common partner Smad 4. Smad heterodimers then migrate into the nucleus where they associate with transcription factors to regulate gene transcription. This Smad signal is inhibited by Smad 6 and Smad 7, which block phosphorylation of R-Smads.

BMP factors are important in skeletogenesis<sup>[40]</sup>. BMP-2 is expressed in areas surrounding cartilage condensations<sup>[46,47]</sup>, while BMP-4 is expressed in perichondrium<sup>[47]</sup>. BMP-2 is also expressed in periosteal and osteogenic zones<sup>[46]</sup>. Due to their effect on runt-related 2 (Runx2) and osterix expression<sup>[48]</sup>, BMPs are very potent inducers of mesenchymal progenitor cell differentiation into osteoblasts<sup>[49]</sup>. Recombinant BMPs can be added in different materials such as in collagen sponges and calcium phosphate ceramics to be delivered *in situ* for clinical practice<sup>[50-52]</sup>. In humans, recombinant human BMP-2 and BMP-7 have been approved for clinical use in orthopedic surgery for long bone open-fractures treated with intramedullary fixation and non-union fractures, and in spine surgery for spinal fusion in place of iliac crest bone graft<sup>[53]</sup>. BMPs do not seem to accelerate fracture healing but tend to increase healing rates without requiring a secondary procedure<sup>[54]</sup>. Nevertheless, several concerns today complicate the use of BMPs, such as heterotopic ossifications, immunogenic reactions or hardware failure<sup>[54,55]</sup>. Moreover, the clinical interest of BMPs is limited to local applications, and BMPs may not represent an alternative treatment to systemic bone diseases such as osteoporosis. Systemic use of BMPs is limited by their non-skeletal effects, mitogenicity, and short half-life.

## TRANSCRIPTION FACTORS INVOLVED IN MSC-OSTEOBLAST DIFFERENTIATION

### Stimulatory transcription factors

**$\beta$ -catenin:** As detailed above,  $\beta$ -catenin is potently activated in the canonical Wnt signaling pathway<sup>[24]</sup>. In this pathway, unphosphorylated  $\beta$ -catenin molecules accumulate in the cytoplasm, translocate to the nucleus, and activate the transcription of downstream genes by binding to LEF/TCF transcription factors. Conditional deletion of  $\beta$ -catenin gene in Dermo-Cre or Prx1-Cre transgenic mice reveals its essential role in osteoblast differentiation<sup>[25,56]</sup>. In addition, conditional deletion of  $\beta$ -catenin gene in Wnt1-Cre transgenic mice, in which Cre is expressed in neural crest cell precursors, results in loss of cranial bones derived from neural crest cells<sup>[57]</sup>. Interestingly Runx2 is expressed in  $\beta$ -catenin deficient cells<sup>[25,56]</sup>, but is strongly enhanced by  $\beta$ -catenin/TCF1. It is required for osterix expression and osteoblast differentiation<sup>[58]</sup> (Figure 1).

**Runx2:** Runx2 belongs to the Runx family, which consist of Runx1, Runx2 and Runx3. These transcription factors form heterodimers with Cbfb and bind to the consensus sequence TGPYGGPyPy<sup>[59]</sup>. Runx2 is considered as the master osteoblast transcription factor (Figure 1). It was identified as a factor binding to an osteoblast specific cis-acting element in the promoter of the genes encoding for osteocalcin<sup>[60]</sup>. Runx2 deficiency in mouse leads to the formation of a skeleton devoid of osteoblasts<sup>[61,62]</sup>. In man, inactivating mutations in Runx2 leads to a skeletal dysplasia called cleidocranial dysplasia<sup>[63]</sup>. Runx2 regulates many genes that determine the osteoblast phenotype. Runx2 is sufficient to induce the expression of many osteoblast markers, such as osteocalcin, in non-osteoblastic cells<sup>[60]</sup>. However, Runx2 overexpression in osteoblasts severely reduces osteocalcin expression and osteoblast maturation<sup>[64,65]</sup>. Therefore, whereas Runx2 is required to commit undifferentiated cells towards the osteoblast lineage, it appears to maintain these cells in an immature stage<sup>[66]</sup>.

In murine fibroblasts, the forced expression of Runx2 is sufficient to induce expression of osteoblast markers such as collagen type I, osteocalcin or bone sialoprotein. Adenoviral overexpression of Runx2 in mouse MSCs generated substantially more bone than control MSCs when implanted in subcutaneous tissue or in calvarial defects<sup>[67]</sup>. Similarly, rat bone marrow stromal cells transduced with *Runx2* retroviral vector seeded onto 3D-fused deposition-modeled polycaprolactone scaffolds, produced biologically-equivalent mineralized matrices at nearly 2-fold higher rates than control cells<sup>[68]</sup>. In human MSCs isolated from adipose tissue, electroporation of Runx2 stimulated osteoblast differentiation *in vitro* with increased expression of alkaline phosphatase and osteocalcin<sup>[69]</sup>.

**Transcription coactivator with binding capacity to PDZ motifs:** Transcription coactivator with binding capacity to PDZ motifs (TAZ) was originally identified during a series of control experiments in a proteomic screen

looking for 14-3-3-interacting proteins<sup>[70]</sup>. TAZ contains a 14-3-3-binding motif, a single WW domain, an extended coiled-coiled region within a larger transcriptional regulatory domain, multiple sites of phosphorylation, and a C-terminal motif that can interact with PDZ-containing proteins<sup>[71]</sup>. The WW domain of TAZ binds to the sequence motif Pro-Pro-X-Tyr. This motif can be found within the regulatory regions of a large number of transcription factors, including Runx2 and PPAR $\gamma$ , as well as members of the Sox, and SMAD families, suggesting that TAZ may be involved in the regulation of MSC commitment and differentiation into osteoblasts, adipocytes and chondrocytes<sup>[71]</sup>. The WW domain-containing molecule TAZ directly interacts with Runx2 and co-activates Runx2-dependent gene transcription<sup>[72]</sup>. In contrast, TAZ binds to, and markedly inhibits, the ability of PPAR $\gamma$  to drive the expression of adipocyte-associated genes such as adipocyte protein 2, and depletion of TAZ increases their adipocyte differentiation<sup>[72]</sup>. The processes through which TAZ is induced and/or activated are poorly understood<sup>[71]</sup>. TAZ levels increase substantially in MSCs induced to differentiate into osteoblasts with BMP-2, whilst conversely, they decrease during adipocyte differentiation<sup>[71]</sup>. It was also reported that TNF- $\alpha$  stimulates osteogenesis in hMSCs from adipose tissue through NF- $\kappa$ B activation and TAZ expression<sup>[73]</sup>. However, the pathophysiological significance of this finding remains obscure. In mouse mesenchymal cells, high-throughput screening allowed to identify a chemical compound, so-called TM-25659, that enhances TAZ nuclear localization and osteoblast differentiation at the expense of adipocytes<sup>[74]</sup>. Moreover, TM-25659 suppressed bone loss *in vivo* and decreased weight gain in an obesity model. Although this compound seems to have a favorable pharmacokinetic profile, work remains to be done to demonstrate its possible interest in clinical application.

**Special AT-rich sequence binding protein 2:** Special AT-rich sequence binding protein 2 (SATB 2) is a member of the family of special AT-rich binding proteins that binds to nuclear matrix attachment regions (MARs) and activates transcription in a MAR-dependent manner. SATB2 inactivation in man results in cleft palate<sup>[75]</sup>. SATB 2<sup>-/-</sup> osteoblasts are characterized by a decreased differentiation, illustrated by reduced bone sialoprotein (BSP) and osteocalcin expression<sup>[76]</sup>. SATB2 can physically interact with both activating transcription factor 4 (ATF4) and Runx2 and enhance the transactivation function of both proteins<sup>[76]</sup>. Overexpression of SATB 2 in mouse bone marrow stromal cells stimulates expression of osterix and BSP<sup>[77]</sup>. Transplanted SATB 2-overexpressing adult stem cells genetically double-labeled with BSP promoter-driven luciferase and  $\beta$ -actin promoter-driven enhanced green fluorescent protein into mandibular bone defects accelerated new bone formation<sup>[77]</sup>. In addition, SATB 2-overexpressing murine induced pluripotent stem cells<sup>[78]</sup> show increased mineral nodule formation and elevated mRNA levels of key osteogenic genes, osterix, Runx2,

Bsp and osteocalcin<sup>[79]</sup>. SATB 2-overexpressing induced pluripotent stem cells combined with silk scaffolds and transplanted into critical-size calvarial bone defects created in nude mice induced enhanced bone repair<sup>[79]</sup>.

**Osterix:** Besides Runx2, the second transcription factor absolutely required for osteoblast differentiation is Osterix (Osx, also known as Sp7). Osx is a zinc finger-containing transcription factor belonging to the SP family of transcription factors. Osx is specifically expressed in osteoblasts, and is required for bone formation<sup>[80]</sup>. The fact that Runx2 is expressed in Osx-deficient mice combined with the absence of Osx in Runx2 null mice places Osx downstream of Runx2<sup>[80]</sup>. Actually, Runx2 may induce Osx expression, through direct binding on its promoter<sup>[81]</sup>. Interestingly, Osx binds to the promoter of *Satb 2* to increase the transcription of the *Satb 2* gene<sup>[82]</sup>. Thus, part of the effects of Osx may rely on SATB 2 activity. Murine bone marrow stromal cells overexpressing Osx associated with type I collagen sponge as a carrier exhibited five times more amounts of newly formed calvarial bone than that the control group in adult mice<sup>[83]</sup>. In addition, overexpression of Osx in human umbilical cord-derived MSCs result in increased alkaline phosphatase activity and osteocalcin expression, and enhanced bone regeneration in nude mice using poly(lactic-co-glycolic acid) as a carrier<sup>[84]</sup>.

**Smads:** Runx2 cooperates with Smad (a portmanteau of Sma in *Drosophila* and Mad in *C. elegans*) 2 and Smad 5 to regulate bone-specific genes<sup>[85,86]</sup>. These interactions appear to be important *in vivo*<sup>[87-89]</sup>. Whilst Runx2 alone does not induce osteoblast differentiation, it synergizes with Smad 2 and Smad 5 to achieve this event. Mutant Runx2 with a truncated transcription activation domain fails to interact with Smad1 and consistently blocks BMP/Smad-induced osteoblast differentiation<sup>[86]</sup>. In addition to Runx2, menin, the product of the multiple endocrine neoplasia type 1 gene, is required for BMP-induced osteoblast differentiation<sup>[90]</sup>. Menin interacts with both Runx2 and Smad 1/5 in multipotential mesenchymal cells. When menin is knocked down, the cells fail to differentiate into the osteoblast lineage.

**CCAAT/enhancer-binding proteins  $\beta$ :** CCAAT/enhancer-binding proteins (C/EBPs) belong to the group of basic leucine zipper transcription factors. They are known to modulate both adipocyte and osteoblast differentiation. C/EBP $\beta$  forms a homodimer or heterodimer complex with other C/EBP family members. C/EBP $\beta$  is expressed before PPAR $\gamma$  and induces it<sup>[91,92]</sup>. More precisely, two main protein forms of C/EBP $\beta$ , induced by alternative translation initiation, present opposite effects on adipogenesis<sup>[91]</sup>. Whereas LAP, the main long isoform, is proadipogenic, the short one, LIP, acts as a dominant negative inhibitor of LAP. In murine mesenchymal cells, LIP inhibits adipocyte differentiation and preferentially induces osteoblast differentiation<sup>[93]</sup>. C/EBP $\beta$  promotes

osteoblast differentiation of mesenchymal cells in Runx2-dependent and -independent mechanisms<sup>[93]</sup>. C/EBP $\beta$  up-regulates Runx2 expression by directly binding to the Runx2 P1 promoter in mesenchymal, pre-osteoblastic, and osteoblastic cells<sup>[94]</sup>. In addition, C/EBP $\beta$  interacts with Runx2 and activates the transcription of the osteocalcin gene<sup>[95]</sup>. C/EBP $\beta$  heterodimerizes with activating transcription factor 4 (ATF4, presented below), another basic leucine zipper transcription factor crucial for osteoblast maturation. This complex transactivates osteocalcin-specific element 1 of the osteocalcin promoter<sup>[96]</sup>. Absence of all C/EBP $\beta$  isoforms results in decreased bone mass in mice, associated with impaired osteoblast differentiation and functional deficiency<sup>[96]</sup>. These data suggest that C/EBP $\beta$  activates osteoblastogenesis. However, before commitment C/EBP $\beta$  may act as a transcriptional repressor of Runx2 and of osteoblast differentiation<sup>[91,97]</sup>. Mechanistically, it has been proposed that once osteogenic differentiation is initiated, Smad3 expression increases, binds to C/EBP $\beta$ , and blocks its inhibitory action on Runx2<sup>[98]</sup>.

**Activator protein 1 proteins:** Activator protein 1 represents heterodimeric transcription factors composed of members of the Jun and Fos family of basic leucine zipper proteins. Overexpression of  $\Delta$ FosB or Fra1 leads to enhanced bone formation. Osteopetrosis in  $\Delta$ FosB overexpressing mice is due to the inhibition of mesenchymal cell differentiation into adipocytes, leading to an increased number of osteoblasts<sup>[99]</sup>. Moreover, conditional Fra1<sup>-/-</sup> mice display reduced levels of several matrix proteins, such as osteocalcin<sup>[100]</sup>. Finally conditional deletion of JunB causes bone defects with reduced osteoblast proliferation, and expression of osteocalcin and bone sialoprotein<sup>[101]</sup>.

**ATF4:** Mice deficient in ATF4 display a decreased bone formation, leading to a severe low bone mass phenotype<sup>[102]</sup>. At the molecular level, ATF4 directly binds to the promoter of osteocalcin to activate transcription<sup>[102]</sup>. This activation appears to rely on the physical interaction between ATF4, SATB2 and Runx2 at the promoter level<sup>[103]</sup>. ATF4 may also cooperate with C/EBP $\beta$  to activate transcription of the osteocalcin gene<sup>[96]</sup>. Finally, ATF4 also plays indirect effects through its activation of amino acid transport<sup>[104]</sup>. Indeed, osteoblasts from ATF4<sup>-/-</sup> mice do not synthesize normal levels of type I collagen unless nonessential amino acids are added to the culture<sup>[102]</sup>.

### Inhibitory TFs

**Peroxisome proliferator-activated receptor  $\gamma$ :** PPAR $\gamma$  proteins are expressed in mice and humans as two different isoforms, PPAR $\gamma$ 1 and PPAR $\gamma$ 2, due to alternative promoter usage and alternative splicing. PPAR $\gamma$ 1 is ubiquitously expressed whereas PPAR $\gamma$ 2 expression is restricted to adipocytes<sup>[105,106]</sup>. Homozygous PPAR $\gamma$ -deficient ES cells fail to differentiate into adipocytes, but spontaneously differentiate into osteoblasts<sup>[107]</sup>. Heterozygous

PPAR $\gamma$ -deficient mice exhibit a high bone mass phenotype but normal osteoblast functions<sup>[107]</sup>. PPAR $\gamma$ 2 has been reported to bind to Runx2 and inhibit its transcriptional activity<sup>[108]</sup>. Inhibition of PPAR $\gamma$  with the pharmacological inhibitor GW9662 in human MSCs stimulates mineralization and bone formation *in vitro* and *in vivo*<sup>[109,110]</sup>. Besides the inhibition of Runx2, PPAR $\gamma$  inhibitory effects may also include  $\beta$ -catenin. Indeed, activated PPAR $\gamma$  in mesenchymal cells induces the proteasomal degradation of  $\beta$ -catenin following direct interaction<sup>[111]</sup>. Moreover, Lu *et al*<sup>[112]</sup> reported that the PPAR $\gamma$  inhibitor GW9662 significantly activates TCF reporter plasmid activity. Furthermore, Krause *et al*<sup>[110]</sup> reported that GW9662 treatment of hMSCs resulted in  $\beta$ -catenin accumulation in the nucleus and PPAR $\gamma$  nuclear export. However, it was recently suggested that whereas PPAR $\gamma$ 2 pro-adipocytic activity relies on  $\beta$ -catenin inhibition, its anti-osteoblastic activity is independent of this interaction<sup>[113]</sup>.

On the other hand, a stimulatory role for PPAR $\gamma$  in osteoblast differentiation has been reported. Overexpression of PPAR $\gamma$ 2 in C3H10T1/2 mouse mesenchymal precursors do not only promote adipogenic differentiation, but also enhances osteogenic differentiation upon BMP-2 stimulation<sup>[114]</sup>. Conversely, MSCs with PPAR $\gamma$ 2 knockdown or mouse embryonic fibroblasts derived from PPAR $\gamma$ 2<sup>-/-</sup> mice exhibit a decrease in adipocyte differentiation, coupled with reduced osteoblastogenesis and decreased mineralization<sup>[114]</sup>. In mouse MC3T3-E1 osteoblasts, activation of PPAR $\gamma$ 1 with low doses of agonists stimulated alkaline phosphatase activity and mineralization<sup>[115]</sup>. In hMSCs, two PPAR $\gamma$  antagonists, BADGE and GW9662, as well as lentiviral knockdown of PPAR $\gamma$  inhibited adipogenesis but had no effect on osteoblastogenesis<sup>[116]</sup>.

In conclusion, while most data seem to demonstrate an inhibitory effect of PPAR $\gamma$  on osteoblastogenesis, several articles suggest that PPAR $\gamma$  action on osteoblasts may actually be more ambiguous. Several mechanisms may account for these discrepancies. For instance, PPAR $\gamma$  directly binds and inhibits Runx2<sup>[108]</sup>, and therefore inhibits MSC commitment into osteoblasts. On the other hand, since Runx2 appears to maintain osteoblasts in an immature stage<sup>[66]</sup>, PPAR $\gamma$  may participate in osteoblast maturation. Besides Runx2, PPAR $\gamma$  has also been shown to bind and inhibit  $\beta$ -catenin pro-osteogenic function<sup>[111]</sup>. However,  $\beta$ -catenin and PPAR $\gamma$  may not be systematically inhibitory because an elegant article recently showed that BMP-2 activated  $\beta$ -catenin/PPAR $\gamma$  dimers have their specific transcriptional targets in endothelial cells<sup>[117]</sup>. Since BMP-2 is a potent osteogenic factor, PPAR $\gamma$  roles in osteoblasts may therefore be more subtle than commonly accepted.

Finally, PPAR $\gamma$  activity is also dependent of a wide number of factors, such as 1,25(OH)<sub>2</sub> vitamin D<sub>3</sub> receptor, PPAR coactivator (PGC-1), the histone acetyltransferase p300, CREB binding protein, and steroid receptor coactivator-1<sup>[118]</sup>; its effects on osteoblasts may thus vary as a function of cell differentiation, species and mode of

activation or inactivation. For instance, it was suggested that full but not partial agonist activation inhibits expression of osteoblast markers in human MSCs<sup>[119]</sup>.

**Twist1:** In mouse, there is a 4-5 d delay between the appearance of Runx2 and that of its target, osteocalcin. This delay seems to be due to the co-expression of Twist1<sup>[120]</sup>. Twist1 is a basic helix-loop-helix transcription factor. Haploinsufficiency at the Twist1 locus causes Saethre-Chotzen syndrome, a form of craniosynostosis, *i.e.*, an increase in bone formation in the skull<sup>[121,122]</sup>. Molecularly, Twist1 binds to the DNA binding domain of Runx2, and inhibits its transcriptional activity. Similarly, Twist1 also interacts with ATF4 and decreases its binding to the Osteocalcin promoter<sup>[123]</sup>. As a consequence, osteoblast differentiation during development proceeds when and where Twist1 expression drops. In C3H10T1/2 mouse cell progenitors, silencing of Twist1 using short hairpin RNA expression enhanced osteoblast gene expression and matrix mineralization *in vitro*<sup>[124]</sup>. In human MSCs, overexpression of Twist1 and Dermo-1 was associated with a decrease in the gene expression of osteoblast-associated markers, bone morphogenic protein-2, bone sialoprotein, osteopontin, alkaline phosphatase and osteocalcin<sup>[125]</sup>.

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## MICRORNAS INVOLVED IN MSC OSTEOBLASTIC DIFFERENTIATION

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### MicroRNAs

MicroRNAs (miRs) are small (19-23 nt) endogenous non-coding single-stranded RNA transcribed from both intergenic and genic regions of the genome<sup>[126,127]</sup>. They are highly conserved molecules that control gene expression post-transcriptionally by binding to the 3'UTR of target mRNA. Near-perfect complementarity between the sequence of miR and its target results in the cleavage of target mRNA, whereas partial complementarity results in its translational inhibition<sup>[128]</sup>. The biogenesis of these small regulatory RNA molecules starts out as primary transcripts termed pri-miR. The pri-miR is first processed in the nucleus by the RNase III enzyme DROSHA to produce pre-miRNAs. Once in the cytoplasm, pre-miRs are further processed by a second RNase III enzyme, DICER1 resulting in dsRNA miR complex, which unwound by the helicase activities of the Argonaute multiprotein complex known as the RNA-induced silencing complex (RISC). The preferred guide strand is incorporated into the RISC complex<sup>[129]</sup>. MiR expression has both spatial and temporal specificity as well as tissue or cell specificity<sup>[130]</sup>. Strikingly, bioinformatics analysis suggests that up to 30% of human genes may be regulated by miR<sup>[131]</sup>. MicroRNAs act as key regulators in diverse biological processes, such as early development, cell proliferation, differentiation, apoptosis, cancer and have the potential to control the expression of virtually any gene<sup>[132]</sup>. Some miRs are directly involved in the formation of the human skeletal system. Thus, miRs have

**Table 1** Non-exhaustive list of microRNAs that have been reported to inhibit or stimulate osteoblast differentiation in mouse or human

miR	Species	Target	Effect	Ref.
MiR-206	Mouse	Connexin 43	Inhibitory	[129]
MiR-34	Mouse	Satb 2	Inhibitory	[130]
MiR-27a	Human	TNAP	Inhibitory	[131]
MiR-204	Mouse	Runx 2	Inhibitory	[132]
MiR-204/211	Human	Runx 2	Inhibitory	[143]
MiR-133	Mouse	Runx 2	Inhibitory	[133]
MiR-135	Mouse	Smad 5	Inhibitory	[133]
MiR-433	Mouse	Runx 2	Inhibitory	[134]
MiR-335	Human	Runx 2	Inhibitory	[142]
MiR-138	Human	FAK	Inhibitory	[144]
MiR-2861	Mouse	HDAC 5	Stimulatory	[136]
MiR-335-5p	Mouse	Dkk 1	Stimulatory	[137]
MiR-29a	Human	Dkk 1, Kremen 2	Stimulatory	[138]

MiRs: MicroRNAs; FAK: Focal adhesion kinase; HDAC 5: Histone deacetylase 5; TNAP: Tissue-nonspecific alkaline phosphatase; Dkk 1: Dickkopf-related protein 1; Runx 2: Runt-related 2; Satb2: Special AT-rich sequence binding protein 2; Smad5: Portmanteau of Sma in *Drosophila* and Mad in *C. elegans*.

the great potential to become a research focus for the prevention and treatment of skeletal diseases<sup>[130]</sup>.

### MicroRNAs and osteoblast differentiation

Conditional deletion of the miR processing enzyme Dicer in osteoblasts, chondrocytes, and osteoclasts has revealed their essential role in normal skeletal development and bone homeostasis<sup>[133]</sup>. Differential expression of miRs has a major impact on the regulation of osteoblast differentiation<sup>[134]</sup>, where by various signaling pathways/transcription factors responsible for osteoblast differentiation can be modulated by miRs. An increasing number of miRs have been identified to negatively regulate osteoblast differentiation and bone formation by targeting important osteogenic factors and positively affect it by targeting negative regulators of osteogenesis.

**Negative regulators:** Many miRs were shown to act as inhibitors of osteoblast differentiation (Table 1). These include miR-206 by targeting *connexin 43* gene (*Cx43*)<sup>[135]</sup> or MiR-34 that decreases *SATB2* accumulation<sup>[136]</sup>. Additionally, Hsa-miR-27a and has-miR-489 down-regulate differentiation through repression of TNAP expression<sup>[137]</sup>; miR-204 a negative regulator of Runx2 inhibits osteogenesis and promotes adipogenesis of mesenchymal progenitor cells and BMSCs<sup>[138]</sup>. MiR-133 and miR-135 target *Runx2* and *Smad1/5* respectively in C2C12 mouse mesenchymal progenitors<sup>[139]</sup>. MiR-433 suppresses BMP2-induced osteoblast differentiation via direct targeting of *Runx2* mRNA in C3H10T1/2 cells<sup>[140]</sup>. Finally, some under-expressed miRs (hsa-miR-31, hsa-miR-106a, hsa-miR-148a and hsa-miR-424) in MSCs undergoing osteoblast differentiation have been predicted to target the mRNAs of *Runx2*, *Cbfb*, and *BMP3*; whereas hsa-miR-30c, hsa-miR-15b and hsa-miR-130b have been predicted to target MSC markers<sup>[141]</sup>.

**Positive regulators:** MiRs that may induce osteoblast differentiation include miR-2861, which promotes BMP2-induced ST2 osteoblast differentiation by repressing histone deacetylase 5 expression<sup>[142]</sup> (Table 1). MiR-335-5p also enhances osteogenic differentiation by inhibiting *Dkk1* expression, and consequently by activating Wnt signaling<sup>[143]</sup>. Moreover, Kapinas *et al*<sup>[144]</sup> have shown that miR-29a promotes osteoblast differentiation by down regulating the inhibitors of canonical Wnt signaling such as *Dkk1*, *Kremen2*, and secreted frizzled related protein.

### MicroRNAs and hMSC

Several MiRs appear to significantly modulate osteoblast differentiation in mesenchymal precursors<sup>[145]</sup>. Dicer or Drosha knockdown in human MSCs inhibits osteogenic differentiation (reviewed in<sup>[146]</sup>). MiR expression patterns differ in MSC progenitors and fully differentiated cells, *e.g.*, osteoblasts, adipocytes and chondrocytes suggesting that these miRs are important in MSC lineage decisions. Indeed, high or low expression of particular miRs may be a prerequisite for the commitment and differentiation of MSCs into specific lineages (reviewed in<sup>[147]</sup>). For instance, undifferentiated hMSCs isolated from various tissues were shown to express high levels of miR-335 while their differentiation resulted in a reduced expression of miR-335. The same miR as well as miR-204/211 impaired hMSC osteoblast differentiation by targeting *Runx-2*<sup>[148,149]</sup>. In human MSCs, decreased expression of miR-138 has also been associated with osteogenesis, possibly by targeting focal adhesion kinase<sup>[150]</sup>. Finally, and as presented above, MiR-148b, -27a, and -489 were found to play a critical role in early osteogenic differentiation of hMSC<sup>[137]</sup>.

## CONCLUSION

In the last decade, we have considerably increased our knowledge on the molecular contributors to osteoblast commitment and maturation. Since the discovery of the key role played by *Runx2* in 1997<sup>[60]</sup>, several other transcription factors have been demonstrated to modulate osteoblastogenesis. In addition, an increasing number of papers now indicate that the expression of these transcription factors is modulated by microRNAs, themselves being expressed under the control of the transcription factors they regulate<sup>[151]</sup>. Many of the results that had been obtained with murine models have now been confirmed with human MSCs. Collectively, the better understanding of the interaction between transcription factors and miRs, and of their effect on osteoblast to genesis and osteoblast function, will help develop new strategies to improve diagnosis and treatment of bone diseases.

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**P- Reviewers** Goebel WS, Ma T, Kita K, Tamama K, Izeta A  
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