MicroRNAs and post-transcriptional regulation of skeletal development

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Abstract

MicroRNAs (miRNAs) have become integral nodes of post-transcriptional control of genes that confer cellular identity and regulate differentiation. Cell-specific signaling and transcriptional regulation in skeletal biology are extremely dynamic processes that are highly reliant on dose-dependent responses. As such, skeletal cell-determining genes are ideal targets for quantitative regulation by miRNAs. So far, large amounts of evidence have revealed a characteristic temporal miRNA signature in skeletal cell differentiation and confirmed the essential roles that numerous miRNAs play in bone development and homeostasis. In addition, microarray expression data have provided evidence for their role in several skeletal pathologies. Mouse models in which their expression is altered have provided evidence of causal links between miRNAs and bone abnormalities. Thus, a detailed understanding of the function of miRNAs and their tight relationship with bone diseases would constitute a powerful tool for early diagnosis and future therapeutic approaches.

Key Words
- miRNAs
- osteoblasts
- osteoclasts
- chondroblasts
- cell differentiation
- bone
- BMPs
- Wnt
- signal transduction

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Introduction

Skeletal development is a process that involves a complex sequence of events, which are regulated by a wide range of signaling pathways (Karsenty 2008). Yet, it mainly involves only three specific types of cells: chondrocytes in cartilage and osteoblasts and osteoclasts in bone. In recent years, considerable efforts have been devoted to understanding the mechanisms that mediate the transition from mesenchymal stem cells (MSCs) to osteoblast and chondroblast lineages. It is well known that osteoprogenitor maturation is controlled by several extracellular signals including bone morphogenetic proteins (BMPs), hedgehogs, WNTs, and fibroblast growth factors, the actions of which lead to the expression of chondroblast- or osteoblast-specific genes (Karsenty 2008). Osteoclasts arise from hematopoietic cells and are essential for bone resorption during skeletal development, homeostasis, and regeneration (Duong & Rodan 2001, Horowitz *et al.* 2001). Furthermore, there is a strong crosstalk between them: osteoblasts are involved in the regulation of osteoclast differentiation through the receptor activator of nuclear factor κB ligand (RANKL)–RANK pathway, essential for a satisfactory balance between bone deposition and bone resorption throughout life (Duong & Rodan 2001, Karsenty & Wagner 2002).

Recently, numerous studies have shown that microRNAs (miRNAs) are important post-transcriptional regulators in virtually all biological processes (Hobert 2008). The miRNA field has advanced so rapidly that it has become an integral component of the way we think gene expression is regulated in cartilage and bone development. Cell-specific signaling and transcriptional regulation in skeletal biology are extremely dynamic processes that are highly reliant on dose-dependent responses. As such, they are ideal targets for quantitative regulation by miRNAs.

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Moreover, the multigene regulatory capacity of miRNAs enables them to cooperatively balance the final precursor cell fate. Thus, different miRNAs can act as either positive or negative determinants within multiple pathways involved in skeletal development processes. The expression of miRNAs is finely orchestrated, being upregulated and downregulated to control the differentiation stage of each bone cell, leading to a characteristic temporal miRNA signature in bone development and homeostasis. Nevertheless, despite all the information available about miRNAs and skeletogenesis, few *in vivo* studies have been conducted to validate each miRNA and it remains unclear how *in vivo* changes in specific miRNAs compromise normal bone development. The purpose of this review is to summarize the current knowledge of miRNA function in skeletal cell lineages and to discuss the main miRNA-related skeletal disorders and the therapeutic perspectives that they provide.

miRNAs: biogenesis and function

miRNAs are short, single-strand, noncoding RNAs approximately 20–25 nucleotides long that have emerged as novel tools capable of post-transcriptionally modifying the expression of mature mRNAs and proteins (Bartel 2004, Mattick & Makunin 2006, Hobert 2008; Fig. 1).

The transcription of miRNAs is mostly mediated by RNA polymerase II, but it can also be mediated by RNA polymerase III (Borchert *et al.* 2006). Sequences encoding miRNAs are found around the genome as separate transcriptional units, although a minority of these sequences are located within the introns of coding genes (generally as clustered miRNAs; Kapinas & Delany 2011). miRNAs are first transcribed as long primary units called pri-miRNAs, which contain characteristic secondary loop structures (Starega-Roslan *et al.* 2011). Various miRNAs can be co-transcribed in a single pri-miRNA, possibly inducing additional effects on a single pathway or gene or allowing crosstalk between different pathways (He *et al.* 2010). The characteristic hairpin of pri-miRNAs helps the microprocessor complex containing Drosha (RNAse III) and some cofactors, including the double-strand RNA-binding protein DGCR8 (DiGeorge syndrome critical region gene), to recognize them from among similar structures present in the nucleus (Han *et al.* 2006, Seitz & Zamore 2006). As a result, a 60–80-nucleotide double-strand miRNA precursor (pre-miRNA) is generated. Pre-miRNAs maintain their stem–loop configuration and have a two-nucleotide extension at their 3′-end. However, some precursors arising from short introns (mirtrons) are capable of bypassing Drosha cleavage and are exported (as regular pre-miRNAs) by exportin 5 to the cytoplasm, where they continue canonical miRNA processing (Lund *et al.* 2004). miRNA precursors are cleaved by a second endonuclease (Dicer), resulting in a double strand of about 21–24 nucleotides. Thanks to argonaute 2 (AGO2), a protein present in the RNA-induced silencing complex (RISC), one of the strands is recruited and guides the complex to its target, whereas the other strand (miRNA*) is degraded.

The 5′-end of mature miRNAs contains the seed region (nucleotide positions 2–7 or 2–8), which has the capacity to identify the complementary bases of the 3′-UTR of the

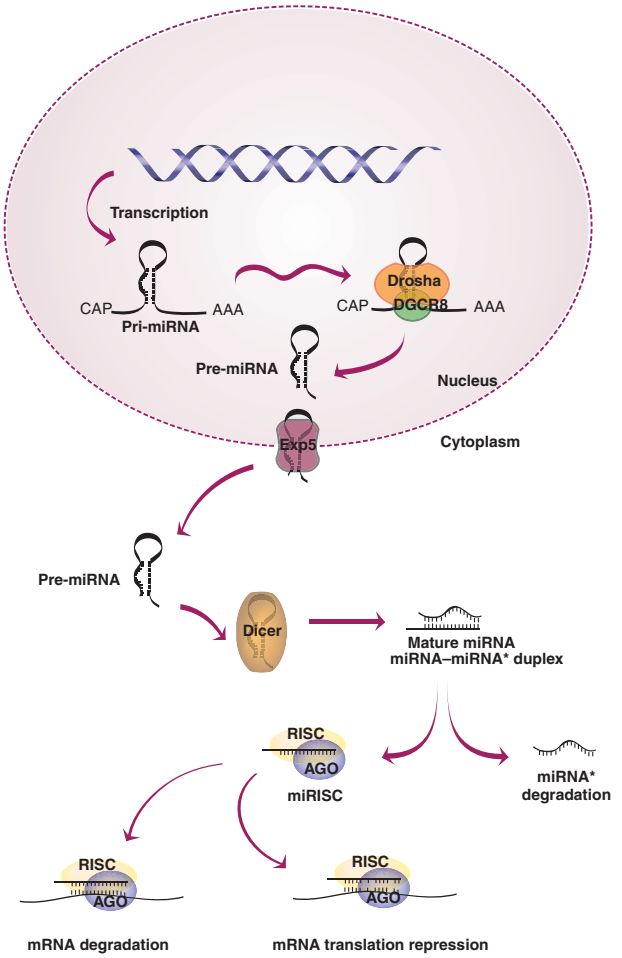

Figure 1
Biogenesis and function of miRNAs. miRNAs are transcribed as long primary transcripts (pri-miRNAs) in the nucleus and are later recognized by Drosha and its cofactor DGCR8 to generate a pre-miRNA. Pre-miRNAs reach the cytoplasm through exportin 5 and are processed by Dicer into a mature miRNA–miRNA* duplex. RNA-induced silencing complex (RISC) recruits the selected miRNA strand and targets the miRNA–RISC complex to the specific 3′-UTR mRNA, while the miRNA* strand is degraded. Depending on the miRNA–mRNA complementarity, miRNA degrades the target mRNA or inhibits its translation. AGO2, argonaute 2.

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target mRNAs and trigger their cleavage and degradation (Guo *et al*. 2010). Nevertheless, there is usually an imperfect complementarity, and the final effect of miRNA activity is a decrease in protein expression due to translational suppression. Interestingly, miRNAs have also been found to target the 5′-UTRs of mRNAs (Lytle *et al*. 2007, Lee *et al*. 2009) and to induce target translation (Vasudevan *et al*. 2007).

miRNAs and skeletal cell specification

It is well known that miRNAs play an important role in chondrogenic and osteogenic differentiation during cartilage and bone formation (Hobert 2008, Kapinas & Delany 2011). The first *in vivo* approach used to study this was implemented through the conditional ablation of the *Dicer* (*Dicer1*) gene under the control of the *Col2a1* promoter (Kobayashi *et al*. 2008). Mutant mice were found to display severe skeletal growth defects due to a reduction in the number of proliferating chondrocytes, leading to premature death. Evident skeletal phenotypes were similarly observed in mice with *Dicer* deficiency in osteoprogenitor cells (using Cre under the control of the 2.3 kb fragment of the *Col1a1* promoter). The ablation of *Dicer* in progenitors prevents their differentiation and compromises fetal survival (Gaur *et al*. 2010). In addition, Mizoguchi *et al*. (2010) have demonstrated that osteoclast *Dicer* is also crucial for normal osteoclast resorption and osteoblast activity. Osteoclast-specific *Dicer* knockout mice were generated by crossing Cathepsin K-cre mice with Dicer flox mice. These mice were found to exhibit higher bone mass and a decrease in osteoclast surface and number. Additionally, the expression of not only osteoclast-related genes but also osteoblast-related ones (*Col1a1*, *Bglap*, and runt-related transcription factor 2 (*Runx2*)) was found to be downregulated (Mizoguchi *et al*. 2010). These data indicate that miRNAs are important not only during bone development but also for bone homeostasis throughout life.

miRNAs and osteoblast differentiation

Lineage commitment from MSCs to osteoblasts has been well characterized. During embryogenesis, bone development occurs by either endochondral or intramembranous ossification. In both mechanisms, osteoblasts and chondrocytes arise from mesenchymal cell condensations, and the factors that contribute to the transcriptional control of these differentiation processes have been widely studied (Nakashima & de Crombrugghe 2003, Karsenty 2008). Osterix (*Osx*), *Runx2*, distal-less homeodomain-containing family 5 (*Dlx5*), *Msx2*, and *Atf4*, among others, have been identified as essential transcription factors for osteoblast differentiation (Nakashima & de Crombrugghe 2003, Karsenty 2008; Fig. 2).

From a common osteochondroprogenitor, the osteoblast differentiation process encompasses different well-characterized stages. The above-mentioned transcription factors (*Osx*, *Runx2*, and *Atf4*) are indispensable for inducing progression to the osteocyte fate, the most mature form of differentiation. Furthermore, each step of osteoblast progression can be clearly recognized by a cohort of molecules that are differentially expressed. *Runx2* plays an essential role in the first step of differentiation into pre-osteoblasts. Pre-osteoblast-specific markers include alkaline phosphatase and low levels of type 1 collagen. Later, they require *Osx* to reach the mature osteoblast stage and to be able to synthesize extracellular matrix (ECM) proteins. Functional osteoblasts additionally express osteocalcin and bone sialoprotein markers and they are responsible for the future mineralized bone matrix. Although the majority of the cells of the osteoblast population undergo apoptosis, a small fraction will differentiate into osteocytes, the main bone population. Osteocytes are matrix-embedded cells and are important mechanosensors controlling bone formation. Recent studies have also indicated osteocytes to be the main source of RANKL (TNFSF11) and therefore closely related to bone resorption.

Runx2 is the first to be expressed in mesenchymal cell condensations, and from the perspective of molecular biology, it is one of the key transcription factors involved in osteoblast differentiation, together with *Osx* (Karsenty *et al*. 1999, Ducy 2000). *Runx2* is expressed as early as day E10.5 and is necessary and sufficient to identify cells as osteochondroprogenitors, as *Runx2*-null mice are unable to produce mature osteoblasts (Ducy *et al*. 1997, Komori *et al*. 1997, Nakashima & de Crombrugghe 2003). From this stage to chondroblast commitment, Runx2 levels decrease until it almost disappears at day E16.5, whereas during osteoblast differentiation Runx2 levels remain stable and induce osteocalcin (*Bglap*) expression at around day E15.5. Thus, *Runx2*-targeting miRNAs simultaneously modulate osteogenesis and chondrogenesis.

The expression of *Runx2* is regulated by several signaling pathways, including vitamin D3 ($1,25(OH)_2D_3$), transforming growth factor β (TGFβ)/BMP2, and Wnt, among others, and it regulates the expression of numerous osteoblastic genes such as *Osx* (*Sp7*), *Alpl* (alkaline phosphatase), *Col1a1*, *Spp1* (osteopontin), *Ibsp* (bone

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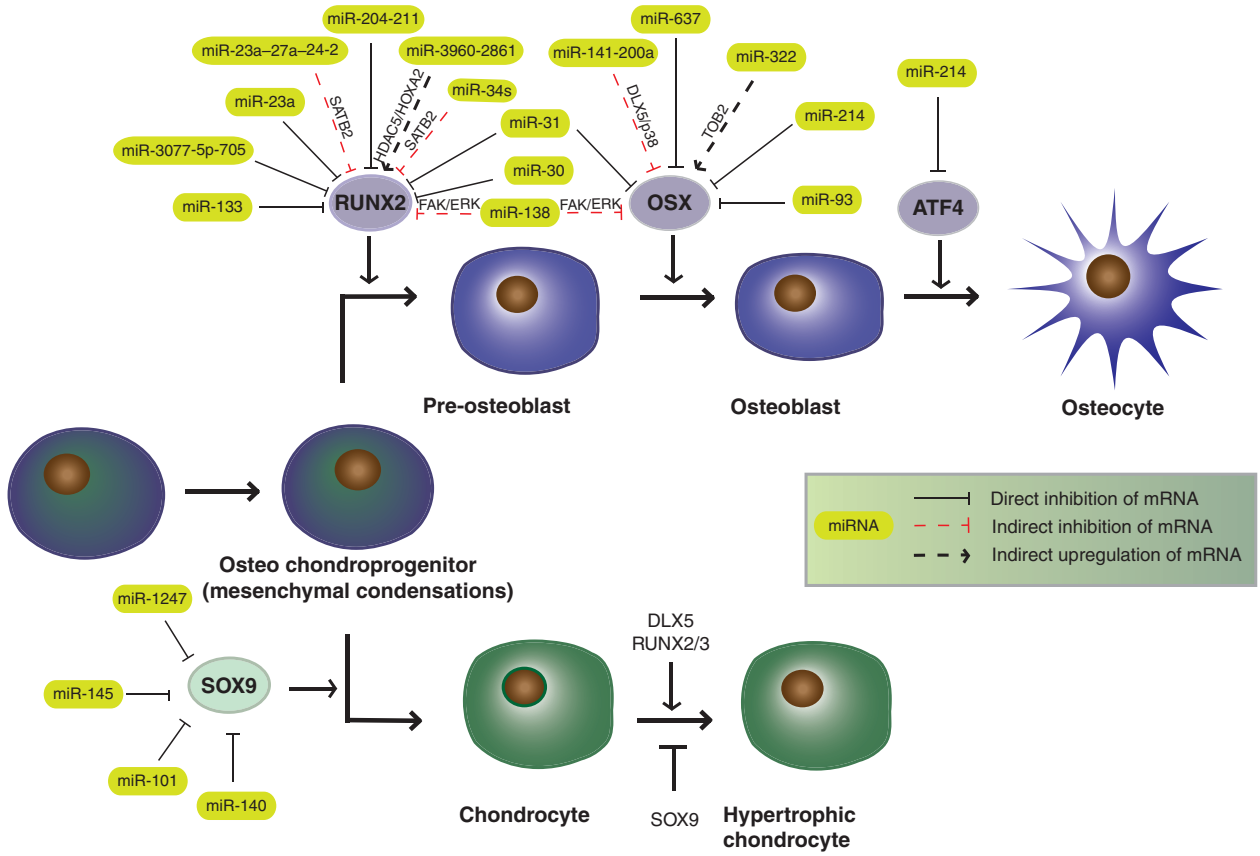

Figure 2

Schematic summary of miRNA role in osteoblast and chondroblast differentiation. A cohort of transcription factors tightly regulates osteoblast and chondroblast commitment from osteochondroprogenitors during skeletal development. miRNAs are important players during this commitment regulating the expression of these transcription factors, therefore allowing or blocking the differentiation process.

sialoprotein), and *Bglap* (osteocalcin). *Runx2* mRNA has a very long 3′-UTR, which probably contains multiple regulatory elements (Huang *et al*. 2010), and it is therefore not surprising that several examples of post-transcriptional *Runx2* mRNA regulation through miRNAs have been described.

miR-204/-211 specifically binds to the 3′-UTR of *Runx2* and inhibits osteoblast differentiation by promoting adipocyte commitment from mesenchymal progenitors (C3H10T1/2, ST2, and hMSCs; Huang *et al*. 2010). Furthermore, as Runx2 has the capacity to regulate the expression of *Bglap* from day E15.5 onwards (Ducy & Karsenty 1995), miR-204 accumulation leads to the repression of *Bglap* expression (Huang *et al*. 2010).

miR-133 also inhibits *Runx2* translation, and its expression is downregulated by BMPs in C2C12 cells (Li *et al*. 2008). Studies carried out by independent groups have reported controversial results on the function of miR-31 during osteoblast commitment of human MSCs. Gao *et al*. (2011) have described miR-31 to be a downregulated miRNA during osteoblast differentiation *in vitro*, indicating that *Runx2* is one of its physiological targets. However, miR-31 was later identified as an upregulated miRNA in a similar study of hMSC differentiation and osterix was confirmed to be one of its targets, indicating a regulatory network (Baglio *et al*. 2013). Other *in vitro* studies have elucidated a regulatory loop involving miR-31, *Runx2*, and *Satb2* (special AT-rich sequence-binding protein 2): downregulation of miR-31 expression by Runx2 in differentiating bone marrow MSCs (BMMSCs) facilitates osteogenic commitment due to an increase in SATB2 protein expression (Deng *et al*. 2013). Additionally, miR-30 family members have been widely studied as regulators of osteoblast differentiation, mainly through the suppression of the expression of *Smad1* and *Runx2* transcription factors (Zhang *et al*. 2011*a*, Wu *et al*. 2012, Eguchi *et al*. 2013).

SATB2 belongs to the family of special AT-rich sequence-binding proteins, members of which are present in the nuclear matrix and can bind to AT-rich sequences,

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activating the transcription of particular genes (Britanova *et al*. 2005). *In vivo* studies have shown that Satb2 physically interacts with and enhances the activity of Runx2 and Atf4 (Dobreva *et al*. 2006, Conner & Hornick 2013). Coupling these osteoblast-specific transcription factors, Satb2 increases the transcription of *Bglap* by binding to its promoter (Dobreva *et al*. 2006) and can also increase the expression of *Ibsp* by direct attachment to an osteoblast-specific promoter element (Dobreva *et al*. 2006). In addition, although Dobreva *et al*. did not find changes in *Osx* expression, others have reported that Satb2 acts in cooperation with Runx2 to upregulate *Osx* expression (Zhang *et al*. 2011*b*). The miR-23a–27a–24-2 cluster inhibits osteogenesis *in vitro* by downregulating the expression of *Satb2* through the direct binding of the three miRNAs to its 3′-UTR. Moreover, Runx2 directly suppresses the expression of the cluster, whereas complementarily miR-23a targets *Runx2* (Hassan *et al*. 2010). Interestingly, other clusters have also been studied, such as the auto-regulatory feedback loops controlling *Runx2* expression. miR-3960/-2861 is transactivated by Runx2 *in vitro*, thereby maintaining its own levels of expression by blocking the expression of *Hoxa* and *Hdac5*, negative regulators of osteoblast differentiation (Kanzler *et al*. 1998, Dobreva *et al*. 2006, Li *et al*. 2009*a*, Hu *et al*. 2011). Furthermore, an *in vivo* approach has demonstrated that *Satb2* is also targeted by miR-34s, affecting osteoblast proliferation mainly by means of miR-34b and miR-34c. Mice with osteoblast-specific deletion in miR-34bc from day E16.5 (using Cre under the control of the 2.3 kb fragment of *Col1a1* promoter) were found to exhibit increased cortical bone volume, bone mineral density, and cortical thickness of long bones (Wei *et al*. 2012).

Following the expression of *Runx2* in osteoprogenitors, *Osx* further strengthens the establishment of bone cell phenotype. *Osx* belongs to the Sp/Kruppel-like family of transcription factors because of its characteristic DNA-binding domain consisting of three tandem C2H2-type zinc finger motifs at the C-terminus. *Osx* is located downstream of Runx2 and, in fact, Runx2 directly binds to the *Osx* promoter (Nakashima *et al*. 2002, Nishio *et al*. 2006). The expression of *Osx* begins at around day E13.5 and it promotes the expression of osteoblast markers such as *Alpl*, *Ibsp*, and *Bglap*. The expression of *Osx* has been shown to be positively regulated by BMP, insulin-like growth factor 1 (IGF1), and MAPK signaling pathways in undifferentiated MSCs (Celil & Campbell 2005, Celil *et al*. 2005, Ortuno *et al*. 2010), and it can also regulate its own expression by interacting with its own promoter (Yoshida *et al*. 2012).

Obviously, *Osx* can also be post-transcriptionally regulated by miRNAs. Shi *et al*. (2013) described miR-214 as a downregulated miRNA during BMP2-induced osteoblast differentiation in C2C12 cells. miR-214 antagomirs lead to the overexpression of *Osx* and other related osteoblast markers such as *Alpl*, *Col1a1*, and *Bglap*. In addition, it has also been reported that miR-214 inhibits Twist (which inhibits the activity of Runx2 as a transcription factor) in intrahepatic cholangiocarcinomas and is overexpressed in elderly patients with fractures, in whom it directly targets *ATF4* (Li *et al*. 2012, Wang *et al*. 2013*a*). As has been stated above, miR-204/-211 targets *Runx2* mRNA *in vitro*. Furthermore, *in vivo* studies comparing differentially expressed miRNAs in calvaria from day E18.5 *Osx*-deficient and WT embryos have revealed that *Osx*-deficient osteoblasts display miR-204/-211 overexpression. As it is known that *Osx*-deficient calvaria exhibit an increase in *Runx2* expression (Zhou *et al*. 2010), Chen *et al*. (2013*a*) have suggested that miR-204/-211 accumulation would dampen *Runx2* overexpression and that Osx coordinately regulates the levels of this miRNA to maintain correct *Runx2* expression.

A regulatory loop for *Osx* expression involves miR-93 in primary osteoblasts. During osteoblast mineralization, Osx can bind to the miR-93 promoter to repress its transcription and, as miR-93 also targets *Osx* mRNA, this facilitates the maintenance of osterix levels (Yang *et al*. 2012). Shi *et al*. (2013) have also reported the downregulation of miR-93 expression during the differentiation of C2C12 cells under BMP stimulation. Fine-tuning of *Osx* expression by miRNAs is also observed in the miR-322/Tob2 feedback mechanism *in vitro*. The Tob2 protein specifically controls the decay of *Osx* mRNA by regulation of its mRNA deadenylation, while BMP2 represses miR-322 expression and reduces miR-322 binding to the *Tob2* 3′-UTR; thus, higher Tob2 protein levels would control *Osx* levels (Gamez *et al*. 2013).

Changes in miR-637 levels have the capacity to maintain the balance between osteoblast and adipocyte differentiation in hMSCs by the inhibition of *OSX* expression and activation of adipogenic markers such as peroxisome proliferator-activated receptor γ (*PPARγ* (*PPARG*)) and CCAAT/enhancer-binding protein α (*c/EBPα* (*CEBPA*)) (Zhang *et al*. 2011*c*). Other miRNAs have also been shown to determine osteoblast–adipocyte balance *in vitro* (Li *et al*. 2013, Liao *et al*. 2013, Wang *et al*. 2013*b*). For instance, miR-3077-5p and miR-705, which work together as negative regulators of osteoblast differentiation through the suppression of *Runx2* and *Hoxa10* expression, eventually lead to a positive regulation of

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adipogenic differentiation (Liao *et al*. 2013). As has been mentioned above, it has been reported that miR-31 forms part of a regulatory loop linking *Runx2* and *Satb2*, but it can also directly regulate the 3′-UTR of *Osx*. It has been suggested that an increase in *Osx* expression due to low expression of miR-31 can play a role in osteosarcomas, as observed in the MG-63 osteosarcoma cell line (Baglio *et al*. 2013).

Dlx5 is a BMP-responsive gene activated through a responsive element in its proximal promoter. Moreover, Dlx5 interacts with the *Osx* promoter and mediates BMP2-induced *Osx* expression independently of Runx2 (Lee *et al*. 2003, Ulsamer *et al*. 2008). miR-141 and miR-200a have been found to act as repressors of *Dlx5* and *Osx* expression, and it has been demonstrated that both bind to *Dlx5* mRNA *in vitro* (Itoh *et al*. 2009). Moreover, miR-141 and miR-200a target *p38α* (*Mapk14*) in mouse models of ovarian cancer, in agreement with previous screenings in human ovarian adenocarcinomas (Mateescu *et al*. 2011). Positive effects of p38 phosphorylation have been observed on Dlx5 transcriptional activity and on Osx stability (Ulsamer *et al*. 2008, Ortuno *et al*. 2010); consequently, the activity of miR-141 and miR-200a in osteoblast lineages is also probably mediated by changes in p38 phosphorylation of Dlx5 and Osx.

Recent studies on *Bglap2*–miR-214 transgenic mice overexpressing miR-214 have revealed its inhibitory role in the regulation of bone formation. *In vitro* manipulation of miR-214 revealed that direct targeting of *Atf4* was required to inhibit osteoblast activity (Wang *et al*. 2013*a*). As the expression of *Osx* and *Runx2* is tightly related, miRNAs targeting proteins involved in their upstream regulatory pathways will affect the expression of both. This is the case with miR-138, inhibited during human MSC osteoblast differentiation, which targets the focal adhesion kinase (FAK) and ERK1/2 pathways, leading to decreased phosphorylation of Runx2 and a lower expression of *Osx* (Eskildsen *et al*. 2011). Other studies have elucidated additional mechanisms connecting miRNAs to osteoblast differentiation by means of targeting different osteoblast differentiation pathways (Mizuno *et al*. 2008, Inose *et al*. 2009, Li *et al*. 2009*b*, Kapinas *et al*. 2010, Wang & Xu 2010, Zhang *et al*. 2012*a*).

miRNAs and chondroblast differentiation

In contrast to adipogenic and osteogenic differentiation-related miRNAs, fewer studies have been conducted on chondrogenic differentiation-related miRNAs. The *Runx2* transcription factor also plays an important role in chondrocyte commitment. Runx2 (and Runx3) are transiently necessary for pre-hypertrophic chondrocytes to reach the hypertrophic state (Yoshida *et al*. 2004). *Sox9* (Sry-related HMG box) is one of the main drivers of chondrocyte differentiation and its absence leads to a failure in chondrocyte commitment in $Sox9^{-/-}$ MSCs or knockout mice (Bi *et al*. 1999, 2001, Mori-Akiyama *et al*. 2003). *Sox9* is required for the commitment of osteochondroprogenitors and for *Runx2* expression in mesenchymal cell condensations (Akiyama *et al*. 2002, 2005). Other members of the Sry family, such as *Sox6* and *Sox5*, also play important roles (Lefebvre *et al*. 2001; Fig. 2).

Several miRNAs (miR-1247, miR-145, miR-140, and miR-199a) have been reported to exert an effect on chondrogenesis by eventually affecting *Sox9* expression positively (Karlsen *et al*. 2013) or negatively (Laine *et al*. 2012, Martinez-Sanchez *et al*. 2012, Martinez-Sanchez & Murphy 2013). Of all the miRNAs affecting chondroblast differentiation, miR-140 has received the most research attention to date (He *et al*. 2009, Nakamura *et al*. 2011, Nicolas *et al*. 2011, Yang *et al*. 2011, Gibson & Asahara 2013, Karlsen *et al*. 2013, Papaioannou *et al*. 2013). The results of these studies indicate that miR-140 is one of the main regulators of chondroblast differentiation through its effects on the expression of not only *Sox9* (Karlsen *et al*. 2013), but also several other targets (*Hdac4*, *Sp1*, *Smad3*, and aggrecan) (Pais *et al*. 2010, Nakamura *et al*. 2011, Yang *et al*. 2011, Karlsen *et al*. 2013). Moreover, independent groups have developed miR-140-null mice, which displayed a concordant phenotype with major growth defects of endochondral bones (Miyaki *et al*. 2010, Nakamura *et al*. 2011, Papaioannou *et al*. 2013). Interestingly, Sox9, L-Sox5, and Sox6 have been proved to cooperatively activate the miR-140 promoter *in vivo* and *in vitro* (Miyaki *et al*. 2010, Yang *et al*. 2011, Yamashita *et al*. 2012), as well as other chondrogenic differentiation-related miRNAs (Guerit *et al*. 2013, Martinez-Sanchez & Murphy 2013).

miR-181a is highly expressed in chondrocytes, and it has been suggested that it works as a negative feedback system to preserve the homeostasis of cartilage by targeting *Ccn1* (*Ccna2*; which promotes chondrogenesis) and *Acan* (encoding the protein aggrecan, the major proteoglycan in the cartilage ECM) (Sumiyoshi *et al*. 2013). miR-181b has also been reported to regulate *Col2a1* expression, and its expression is elevated in human osteoarthritic chondrocytes *in vitro* (Song *et al*. 2013*a*). Other miRNAs regulate cell differentiation by targeting chromatin epigenetic modifiers (Tuddenham *et al*. 2006, Guan *et al*. 2011). For instance, miR-365 stimulates chondrocyte differentiation through *Hdac4*

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repression, thereby increasing the levels of *Ihh* and *Col* X (markers of pre-hypertrophic chondrocytes and hypertrophic chondrocytes respectively; Guan *et al*. 2011).

As has been mentioned above, it should be noted that one particular miRNA may act as a switch for the selection of different cell commitment processes. miR-96, miR-124, and miR-199a have been studied in human BMMSCs and have been found to be differentially expressed during osteogenic, adipogenic, or chondrogenic induction: whereas miR-124 is expressed exclusively in adipocytes, the expression of miR-199a is upregulated in osteoblasts and chondrocytes (Laine *et al*. 2012).

miRNAs and osteoclast differentiation

In contrast to osteoblasts and chondrocytes, osteoclasts arise from hematopoietic cells and are the primary bone-resorbing cells. The transition from mononuclear pre-osteoclasts to mature osteoclasts is dependent on cell–cell fusion and is controlled by sequential exposure to signaling molecules (Fig. 3). Macrophage colony-stimulating factor (M-CSF) and RANKL are the two main cytokines involved in osteoclast differentiation (Manolagas 2000). M-CSF activates the c-Fms receptor, present in early osteoclast precursors, and acts as a survival/proliferation factor by activating Akt, microphthalmia transcription factor (Mitf), or the anti-apoptotic protein B-cell leukemia/lymphoma-associated gene 2 (BCL2). Moreover, M-CSF also stimulates the expression of *Rank* (*Tnfrsf11a*). *Rankl* is a member of the tumor necrosis factor α (TNFα) superfamily present in osteoblasts and stromal cells and can be a membrane-anchored molecule but can also be released as a soluble molecule following proteolytic cleavage. Lately, osteocytes have emerged as an important source of RANKL, indicating a key role for osteocytes in osteoclastogenesis (Nakashima *et al*. 2011, Xiong *et al*. 2011). The RANK–RANKL signaling system links osteoblast lineage and hematopoiesis-derived cells for osteoclast differentiation and activation. Together with M-CSF, RANK signaling is the main signaling pathway involved in osteoclast maturation (Tanaka *et al*. 2005). RANK stimulation leads to the recruitment of TNF receptor-associated

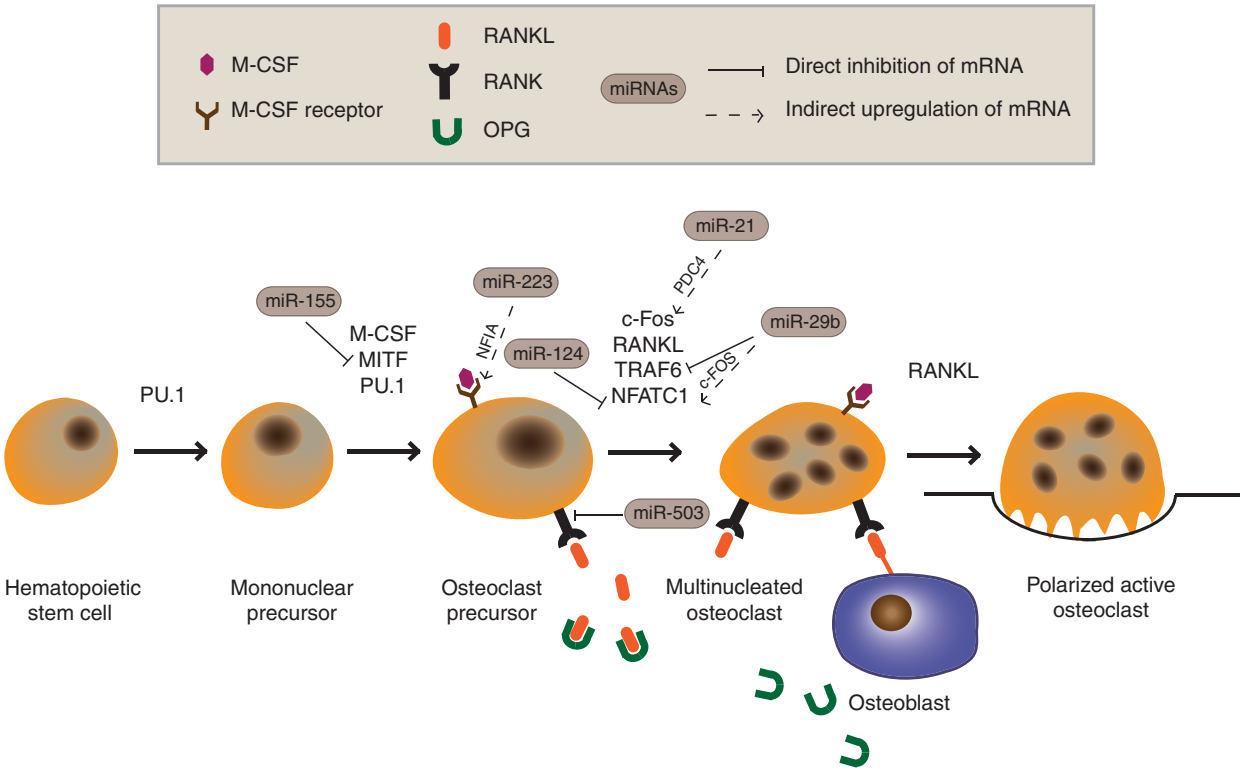

Figure 3
Effects of miRNAs on osteoclast differentiation. miRNAs affect different molecules related to osteoclast commitment such as RANK or M-CSF receptor, among others. miRNA expression leads to changes in osteoclast activity *in vitro* and also to alterations in bone resorption *in vivo*. OPG, osteopontegrin.

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cytoplasmic factors (TRAFs), mainly TRAF6, leading to the activation of several pathways, including the nuclear factor (NF) and activator of transcription NFATc1. Nfatc1 is widely accepted to be the key transcription factor involved in osteoclast differentiation (Kobayashi *et al.* 2001, Gohda *et al.* 2005). Nfatc1 eventually regulates several osteoclast-specific genes in cooperation with other transcription factors: AP1, PU.1, Mitf, and c-Fos (Tondravi *et al.* 1997, Takayanagi *et al.* 2002, Crotti *et al.* 2008). Their transcriptional targets are osteoclast-specific genes such as tartrate-resistant acid phosphatase (*TRAP* (*ACP5*)), cathepsin K, calcitonin receptor, and dendritic cell-specific transmembrane protein (Kukita *et al.* 2004). RANK can be blocked by osteopontegrin (OPG), therefore inhibiting osteoclast differentiation due to the suppression of RANKL stimuli. OPG is produced by osteoblasts and acts as a decoy receptor, preventing the coupling of RANKL to RANK and therefore reducing osteoclast resorption. Thus, the RANKL:OPG ratio must be accurately balanced to control osteoclastogenesis.

There are relatively few reports on the role of miRNAs in osteoclastogenesis. As in the case of osteoblasts, osteoclast-specific *Dicer* alteration has been shown to profoundly affect osteoclast activity *in vivo* and *in vitro* (Sugatani & Hruska 2009, Mizoguchi *et al.* 2010). In these models, a reduction in the expression of osteoclast markers (the expression of *Trap* and *Nfatc1* mRNA was downregulated) and an increment in the values of bone parameters such as bone volume, trabecular thickness, and trabecular number have been observed, all leading to a mild osteopetrotic phenotype as a consequence of decreased osteoclast number and surface.

Of all the miRNAs involved in osteoclast differentiation, miRNA-223 has been studied the most. It was first identified as being specific to the CD11b-positive myeloid cell line (Chen *et al.* 2004). Sugatani and colleagues further confirmed miR-223 expression in the mouse osteoclast precursor cell line RAW 264.7 and showed that the modulation of pre-miR-223 alters osteoclast differentiation. Moreover, the levels of miR-223 in mouse bone marrow macrophages (BMMs) were found to be higher than those in osteoclasts, indicating that it must be repressed for appropriate osteoclast differentiation to occur (Sugatani & Hruska 2007). In the same study, miR-223 was shown to target *Nfia*, an osteoclastogenesis suppressor that eventually negatively regulates the M-CSF receptor. Later, other groups validated these effects of miR-223 on osteoclastogenesis and revealed PU.1-binding sites in the miR-223 promoter (Fukao *et al.* 2007, Sugatani & Hruska 2009, Shibuya *et al.* 2013). Sugatani *et al.* posited the existence of a feed-forward network whereby M-CSF induces PU.1 in osteoclast precursors, and PU.1 stimulates pri-miR-223 transcription, which, by downregulating the expression of *Nfia*, ultimately increases the levels of M-CSF receptor.

As with several other miRNAs affecting osteoclastogenesis, miRNA-223 has also been studied as a marker gene for rheumatoid arthritis (RA; Shibuya *et al.* 2013). As a result of the *in vitro* miR-223 studies mentioned above, it has been suggested that the increased levels of miR-223 found in RA synovium could be related to the inhibition of osteoclastogenesis. Furthermore, miR-21 has also been identified by Sugatani *et al.* (2011) as a miRNA upregulated during RANKL-induced osteoclastogenesis. Moreover, c-Fos and AP1 were found to be associated with its promoter. miR-21 loss-of-function experiments in a model of RANKL induction of BMMs showed a decrease in c-Fos phosphorylation and lower *Nfatc1* and cathepsin K expression, all due to increased levels of programmed cell death 4 (PDCD4). Taken together, these findings indicate the existence of a new positive loop mechanism involving c-Fos/miR-21/PDCD4 (Sugatani *et al.* 2011). In addition, Mann *et al.* performed a differential miRNA screening using the RAW 264.7 cell line under RANKL and M-CSF treatment to induce osteoclastic differentiation. miR-155 was described in this study as an early inhibitor of *MITF*, a nuclear effector that integrates M-CSF/RANKL signals to initiate the expression of osteoclast-specific genes (Mann *et al.* 2010). The RAW 264.7 cell line can differentiate into either macrophages or osteoclasts, and the results of this study suggest that the upregulation of miR-155 expression facilitates macrophage commitment, therefore inhibiting osteoclast differentiation (by targeting *MITF*). These data indicate that miR-155 is involved in the commitment switch of hematopoietic precursors (Mann *et al.* 2010). Zhang *et al.* (2012*b*) revealed that miR-155 is inhibited by IFNβ during osteoclast differentiation, and they identified the effect of miR-155 on the 3′-UTR of *MITF* and suppressor of cytokine signaling 1 (*SOCS1*). Moreover, miR-155 has been observed to be involved in the pathogenesis of autoimmune arthritis in mice, being proposed as a novel target for the treatment of RA (Bluml *et al.* 2011). Other examples include miR-124, which has been shown to directly target *Nfatc1* expression in BMMs (Lee *et al.* 2013), and miR-503, which targets *Rank* expression (Chen *et al.* 2013*b*).

Some of the miRNAs involved in osteoclast function have been shown to affect osteoclast cytoskeleton or migration. The expression of miR-31 has been found to increase in BMMs under RANKL stimulation. Moreover,

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it tightly controls cytoskeleton organization in osteoclasts by targeting *Rhoa*, essential for actin ring formation and bone resorption (Mizoguchi *et al*. 2013). Franceschetti *et al*. (2013) reported that all miR-29 family members (miR-29a, miR-b, and miR-c) are induced during osteoclast differentiation of mouse BMMs and RAW 264.7 cell line. However, Rossi *et al*. (2013) reported a decrease in miR-29b expression during human osteoclast differentiation from circulating human precursors. They also demonstrated the inhibition of osteoclastogenesis by miR-29b through the downregulation of *c-FOS* or *NFATC1* expression, while Franceschetti *et al*. (2013) reported new targets such as *Nfia*, *Cdc42*, and *Srgap2*, among others, indicating that miR-29 positively maintains migration and cell commitment to osteoclasts.

Reciprocal interplay between miRNAs and signaling pathways in skeletal biology

Regulation of miRNA expression

Although several osteogenic differentiation-related miRNAs have been identified in the last decade, little is known about their transcriptional regulation. Numerous screenings have been performed to characterize the miRNA expression scenario during different stages of cell differentiation, but fewer studies have attempted to describe the molecular linkage between the stimuli and their regulatory effect on miRNA expression in full detail. miRNA processing and maturation can be regulated through the interaction of additional proteins with the Drosha complex. For instance, SMAD proteins interact with Drosha to specifically regulate the expression of some miRNAs, as in the case of miR-21 (Davis *et al*. 2008). Specific sequences are required in the loop of pri-miRNAs for them to be post-transcriptionally regulated by the SMAD–Drosha complex (Davis *et al*. 2010). Using several different models, it has been shown that miRNA expression can be regulated through several mechanisms, including the regulation of pre-miRNA nuclear export and Dicer cleavage, regulation of promoter activity by methylation and histone modification, or direct regulation of RNA polymerase II recruitment (Davis-Dusenbery & Hata 2010). However, much less is known about the influence of osteogenic signaling inputs on these mechanisms. To summarize the main themes in what is known about the interplay between signaling and miRNAs, we focus on two important pathways in skeletal development: BMP and Wnt.

The interplay between miRNAs and BMP signaling

BMPs form the largest subfamily of the TGFβ superfamily and are profoundly involved in skeletogenesis (Shi & Massague 2003, Miyazono *et al*. 2010). Early events in BMP signaling are initiated through the phosphorylation of specific BMP receptor-regulated SMAD proteins, namely R-SMAD1, R-SMAD5, and R-SMAD8. After phosphorylation, R-SMADs form heteromeric complexes with the common mediator SMAD4, which then migrate to the nucleus and activate the transcription of specific target genes (Shi & Massague 2003). Two additional SMADs are known as inhibitory SMADs, SMAD6 and SMAD7. Furthermore, BMPs can also activate noncanonical, SMAD-independent pathways, mainly MAPK pathways (Erk, p38), the LIMK pathway, or the PI3K pathway, which are also involved in osteoblast differentiation (Shi & Massague 2003, Gamell *et al*. 2008, Ulsamer *et al*. 2008, Ortuno *et al*. 2010).

Several miRNAs negatively or positively regulate BMP signaling (Fig. 4) and, in turn, BMPs coordinate a wide range of changes in miRNA expression (Inose *et al*. 2009). BMP biology has been widely studied in C2C12 cells, switching the differentiation pathway from a myoblastic to an osteoblastic phenotype (Katagiri *et al*. 1994), and several miRNA studies have been performed using this model (Li *et al*. 2008, Inose *et al*. 2009). For instance, Li *et al*. (2008) conducted a miRNA screening during BMP2-induced osteogenesis in C2C12 cells and found that the expression of almost all miRNAs was downregulated during osteoblast differentiation. These data have been confirmed by other groups (Gamez *et al*. 2013). The same authors also described miR-133 and miR-135 as negative regulators of osteogenesis that act by directly targeting *Runx2* and *Smad5* respectively, thereby inhibiting osteogenic differentiation (Li *et al*. 2008). In addition, it has recently been reported that under BMP stimuli, C2C12 inhibits the processing of myomiRs (muscle-specific miRNAS and myogenic miRNAs) due to the association of phosphorylated R-SMADs and Co-SMAD with phosphorylated KH-type splicing regulatory protein (KSRP (KHSRP)) in the nucleus (Pasero *et al*. 2012). KSRP is a single-strand RNA-binding protein that is essential for the maturation of myomiRs and for the establishment of the myogenic lineage in C2C12 cells (Briata *et al*. 2012); thus, KSRP sequestering by SMADs blocks myogenic differentiation in favor of the osteoblast lineage.

Another well-studied miRNA regulated by BMPs is miR-206 (Inose *et al*. 2009, Sato *et al*. 2009). The expression of miR-206 is downregulated by BMPs in C2C12 cells and

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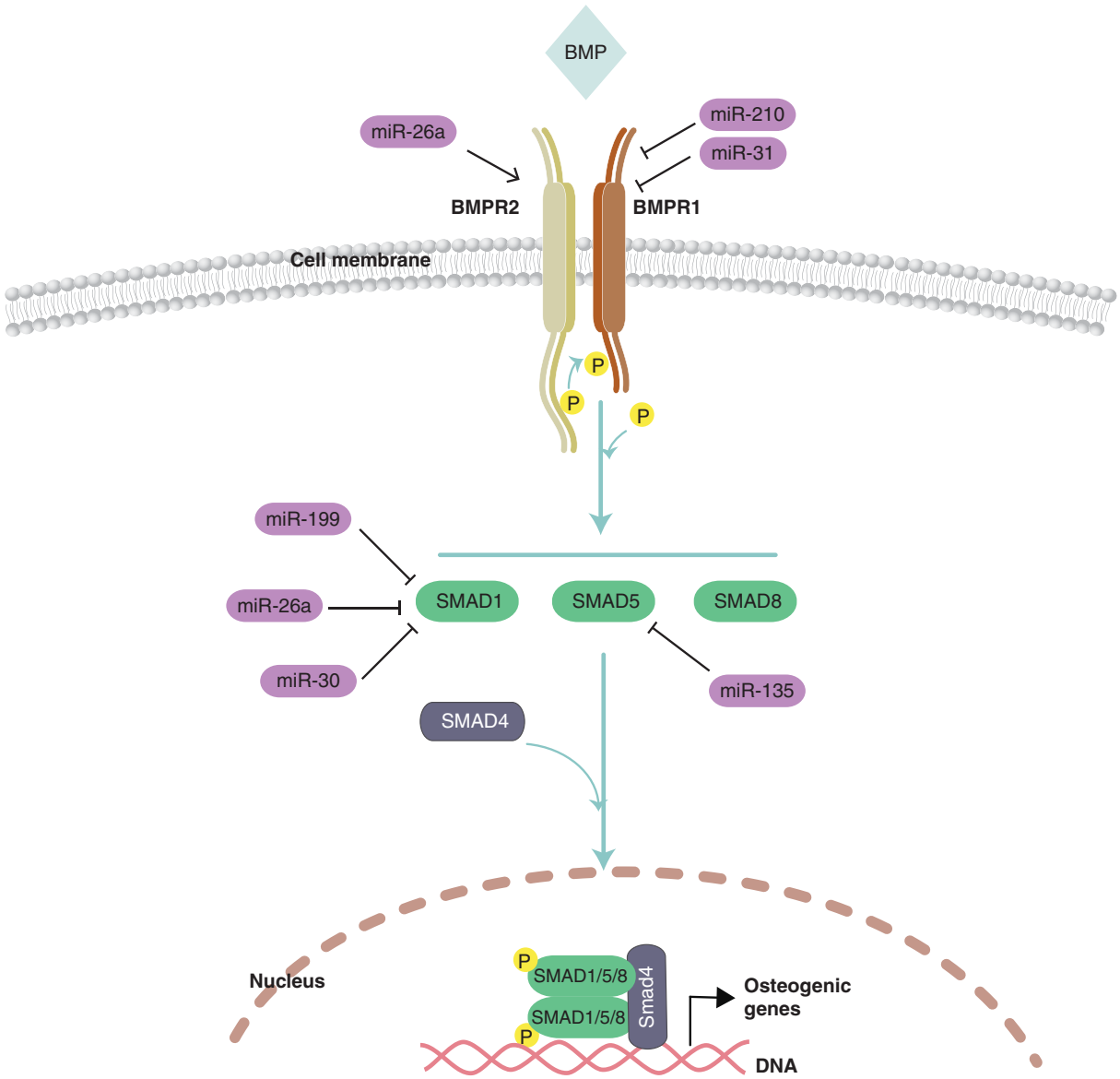

Figure 4
Interplay between the BMP pathway and miRNAs. miRNAs act at different steps of BMP signaling: from those involving BMP receptors to those involving SMADs.

its overexpression blocks osteoblast differentiation due to its effect on connexin43 mRNA, required for osteoblastic gene expression and function (Lecanda *et al.* 1998, Plotkin & Bellido 2013). Sato *et al.* (2009) have also suggested that BMP control of miR-206 occurs post-transcriptionally in C2C12 cells by the repression of pri-miR processing, and other studies have indicated that miR-206 is also required for myogenic differentiation in the same model (Kim *et al.* 2006). Moreover, miR-206 transgenic mice (2.3 kb *Col1a1* promoter) suffer from reduced bone mass because of a decrease in bone formation (Inose *et al.* 2009). TGFβ inhibits both miR-206 expression and myogenic differentiation *in vitro* through an increase in HDAC4 protein expression (Winbanks *et al.* 2011).

miR-125b inhibits the proliferation of ST2 cells (murine MSCs) and BMP4 stimulation attenuates miR-125b expression in these cells. Thus, miR-125b inhibits osteoblast differentiation, possibly regulating the early stages of osteoblastogenesis (Mizuno *et al.* 2008). miR-141/-200a, mentioned above as *Dlx5* and *Osx* repressors, are also regulated by BMP2 in the MC3T3-E1 cell line. Under BMP treatment, the expression of both miRNAs is

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downregulated, thus avoiding *Dlx5* and *Osx* miRNA-related repression (Itoh *et al.* 2009). The expression of miR-322 is also downregulated by BMP2 and has been shown to indirectly repress *Osx* expression to facilitate further osteogenic differentiation (Gamez *et al.* 2013).

BMP2 also controls miRNAs involved in chondrogenic differentiation: for instance, the expression of miR-199a* is upregulated by BMP2 treatment, and its overexpression in pre-chondrogenic cells (ATDC5) or in the multipotential murine C3H10T1/2 cell line represses the expression of chondroblast markers *Sox9* and *Col2a1*. miR-199a* also represses the 3′-UTR *Smad1* transcript. Taken together, these data indicate that BMP2 reduces the expression of miR-199a*, avoiding *Smad1* post-transcriptional miRNA regulation and repressing chondrogenic differentiation-specific markers (Lin *et al.* 2009).

miRNAs can also regulate R-SMAD expression. In addition to the above-mentioned examples, Smad1 has also emerged as a target of miR-26a in osteogenic differentiation of human adipose-tissue-derived stem cells (Luzi *et al.* 2008). These studies thus indicate that miR-26a restrains osteoblast commitment when reaching terminal differentiation (Luzi *et al.* 2008). Other miRNAs also target BMP receptors. For instance, the expression of miR-210 is upregulated during BMP4-induced osteoblast differentiation of mouse mesenchymal ST2 cells. miR-210 positively regulates osteoblast commitment by targeting the *Acvr1b* receptor (type 1 receptor; Mizuno *et al.* 2009). The 3′-UTR of the *ACVR1/ALK2* gene has recently been studied *in vitro* to elucidate miRNAs that when expressed induce BMP signaling alterations in fibrodysplasia ossificans progressiva (Mura *et al.* 2012). Several additional BMP-modulated miRNAs have been identified (Li *et al.* 2008, 2009*a*, Lin *et al.* 2009, Bae *et al.* 2012, Gamez *et al.* 2013), generally as a result of high-throughput expression analysis but without precise information on their transcriptional control and function.

The interplay between miRNAs and Wnt/β-catenin signaling

Wnt signaling encompasses canonical and noncanonical pathways depending on the implication of β-catenin. Canonical Wnt/β-catenin signaling initiates by binding WNT1 class family members to Frizzled (Fzd) and the co-receptors LDL receptor-related proteins 5 and 6 (Lrp5/6). In the absence of stimuli, β-catenin is normally retained in the cytoplasm by a protein complex involving GSK3β (GSK3B), casein kinase 1a (CK1A (CSNK1A1)), APC, and axin, which is also finally responsible for its ubiquitination and degradation. When the Fzd–LRP receptor complex is stimulated by Wnt binding, recruitment of Disheveled leads to the inhibition of GSK3β activity. Thus, β-catenin accumulates and eventually enters the nucleus, where it binds to Tcf/Lef1 and regulates transcription. Several molecules negatively affect the Wnt pathway at different points and have been shown to be highly important for bone biology. Dickkopf (Dkk) family members or SOST antagonizes Wnt signaling by binding to LRP5 and 6, whereas SFRPs sequester Wnts away from binding to the receptors (Bafico *et al.* 2001, Wu & Nusse 2002; Fig. 5).

In vitro studies have generated controversial results about the effects of Wnt signaling on osteoblast differentiation, and theories exist about either a positive or a negative influence depending on the state of specification of the target cell. Most data indicate that β-catenin acts positively to maintain stem cell pluripotency and self-renewal; however, once MSCs reach commitment to osteochondroprogenitors, β-catenin promotes osteoblast progression. Moreover, Wnt signaling leads to *Runx2* expression due to a Tcf regulatory element in its promoter (Gaur *et al.* 2005) and has been proved to work cooperatively with BMP signaling to induce other osteogenic genes such as *Osx*, *Dlx5*, and *Msx2* (Rodriguez-Carballo *et al.* 2011).

One of the main mechanisms whereby miRNAs affect Wnt signaling is through the inhibition of Wnt/β-catenin pathway repressors (Fig. 5). The expression of miR-218 is upregulated during osteoblast differentiation, leading to an increase in the expression of osteoblast markers such as *Alpl*, *Runx2*, and *Bglap*. These effects correlate with a decreased expression of *Sfrp2*, *Sost*, and *Dkk2*. Moreover, BMP and Wnt stimuli induce higher miR-218 levels, leading to the upregulation of β-catenin and *Tcf1* (*Hnf1a*) expression and therefore linking miR-218 to a positive loop mechanism involving Wnt signaling (Hassan *et al.* 2012). *Dkk1* is also a miRNA target, leading to enhanced Wnt signaling. For instance, miR-29a negatively regulates the expression of not only *Dkk1* but also *Kremen2* (a decoy receptor of Wnt signaling) and *Sfrp2*, thereby potentiating the β-catenin pathway and promoting osteoblast differentiation of hMSCs (Kapinas *et al.* 2010). The expression of miR-29a and miR-29c is induced after osteoblast differentiation of MC3T3-E1, human fetal osteoblastic cells (hFOBs), and human primary osteoblasts. It has been suggested that TCF/LEF-binding sites present in miR-29 promoter are required for Wnt induction of miR-29 expression (Kapinas *et al.* 2009, 2010). *Dkk1* is also targeted by miR-335-5, which binds directly to its 3′-UTR and decreases Dkk1 protein levels

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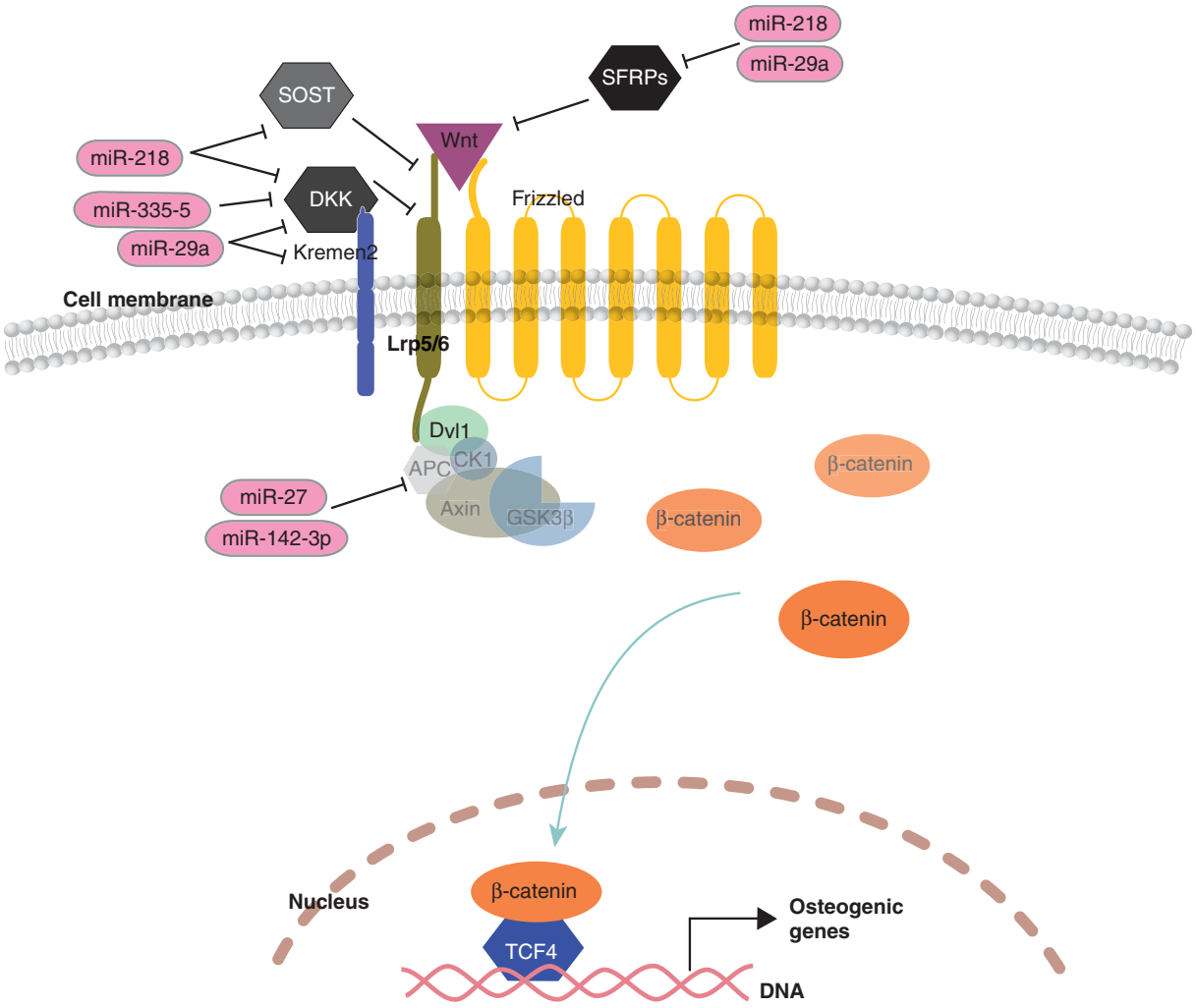

Figure 5
miRNAs involved in the Wnt/β-catenin pathway. miRNAs target several molecules of the Wnt/β-catenin pathway: LRP co-receptors, proteins of the β-catenin degradation complex, or several inhibitory molecules such as DKK or SOST.

during osteoblast differentiation (Zhang *et al.* 2011*d*). It has also been suggested that *Dkk1* levels are post-transcriptionally downregulated by miRNAs, allowing Wnt signaling to support lineage commitment. Ultimately, in the most mature form of osteocytes, the levels of miRNAs affecting *Dkk1* decrease to allow Dkk1 to fine-tune the intensity of Wnt signaling, thereby avoiding disproportionate mineralization (Zhang *et al.* 2011*d*). Another target of miRNA activity is the scaffold protein APC, which is part of the destruction complex of β-catenin. The expression of miR-27 and miR-142-3p is induced in hFOBs and, in turn, these induce osteoblast differentiation. Both promote β-catenin accumulation by targeting APC (Wang & Xu 2010, Hu *et al.* 2013).

Several other pathways are involved in osteoblast differentiation (Notch, IGFs, hedgehogs, etc.), and these are also post-transcriptionally regulated by miRNAs. It is clear nowadays that not only can the expression of a single miRNA fluctuate during cell fate commitment but also a specific miRNA can target different mRNAs depending on the cellular stage. Moreover, a single miRNA can affect several signaling pathways simultaneously, allowing a cooperative effect or the fine-tuned expression of specific mRNAs. One example is miR-34c. Its effect on osteoblasts has been studied *in vitro* and *in vivo*, and it has been shown that it targets not only different factors in the Notch signaling pathway but also *Satb2* and *Runx2* somehow, in a Notch-independent manner (Bae *et al.* 2012). Determining

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how miRNAs regulate and are regulated by different pathways and how they interact to orchestrate a singular scenario for each differentiation step remains a tremendous challenge. In addition, bone-specific *in vivo* approaches have occasionally yielded controversial results compared with *in vitro* information, probably due to the influence of the cell environment and alterations in osteoblast–osteoclast communication.

miRNAs and skeletal disorders: therapeutic perspectives

miRNAs have emerged as important players in a wide range of pathologies. Multiple screenings have been performed in an attempt to determine miRNA signatures for several skeletal diseases. Osteoarthritis (OA) is the main degenerative articular disease caused by an imbalance between cartilage synthesis and degradation, leading to a progressive loss of movement, functional disability, and joint pain and inflammation. At present, therapy is usually based on symptomatic treatment, mainly using non-steroidal anti-inflammatory drugs (NSAIDs), but these fail to slow articular degeneration and disease progression. Studies on OA have demonstrated that miRNA expression is regulated during this process, indicating the possibility of future miRNA therapies (Iliopoulos *et al*. 2008, Jones *et al*. 2009, Akhtar *et al*. 2010, Diaz-Prado *et al*. 2012). One of the main characteristics of osteoarthritic chondrocytes is the secretion of the pro-inflammatory cytokines interleukin 1β (IL1β) and TNFα. Chondrocyte death occurs in OA and the remaining chondrocytes express *IL1β* (*IL1B*), leading to the upregulation of matrix degradation enzymes such as metalloproteinases (MMPs), particularly MMP13, and the aggrecanases ADAMTS4 and ADAMTS5 (a disintegrin and metalloproteinase with thrombospondin motifs). MMPs and ADAMTS are involved in ECM degradation and OA progression due to their capacity to cleave type 2 collagen or aggrecan respectively.

Several miRNAs are involved in the regulation of IL1β downstream mediators and, in turn, IL1β has been used *in vitro* in human chondrocytes as a model for the study of OA. Numerous miRNAs have been shown to be regulated by IL1β action (Miyaki *et al*. 2009, Dai *et al*. 2012, Matsukawa *et al*. 2013). *MMP13* is regulated directly by miR-27b and miR-127-5p (Akhtar *et al*. 2010, Park *et al*. 2013) or indirectly by miR-27a, miR-9, miR-488, or miR-22 (Iliopoulos *et al*. 2008, Jones *et al*. 2009, Tardif *et al*. 2009, Song *et al*. 2013*b*), among others.

As has been mentioned above, ADAMTS aggrecanases are also important miRNA targets in OA patients and *in vitro* models. Besides its known role in chondrocyte differentiation, miR-140 also plays a central role in OA (Araldi & Schipani 2010). *In vivo*, miR-140 and miR-146a target *ADAMTS5* (Miyaki *et al*. 2009, 2010, Tardif *et al*. 2009, Li *et al*. 2011), while miR-125b targets *ADAMTS4* (Matsukawa *et al*. 2013). Moreover, miR-101 directly inhibits the expression of collagen type II and aggrecan genes through the downregulation of *SOX9* expression (Dai *et al*. 2012). miR-34a is also involved in OA through the regulation of chondrocyte apoptosis and migration (Abouheif *et al*. 2010, Kim *et al*. 2011). Inhibition of miR-34 activity leads to a reduction in IL1β-induced apoptosis in osteoarthritic rat chondrocytes (Abouheif *et al*. 2010). The expression of miR-149 is also downregulated in human primary osteoarthritic chondrocytes and in sw1353 chondrocytes under IL1β/TNFα stimulation and, in turn, miR-149 strongly downregulates the levels of pro-inflammatory cytokines such as IL6, TNFα, and IL1β, thus eventually acting as a feedback loop for cytokine expression (Santini *et al*. 2013). Moreover, it has been shown that *Cox2* (*Ptgs2*) expression requires p38 activity (Susperregui *et al*. 2011) and IL1β stimulation induces p38 activation, leading to a negative regulation of miR-199a* that directly controls the expression of *Cox2* by binding to its 3′-UTR (Akhtar & Haqqi 2012).

Another bone-related disease with an important impact is osteoporosis (OP). In both men and women, loss of bone mass typically starts between 40 and 50 years of age, but there is a major loss in women due to the decrease in estrogen levels after menopause. More importantly, OP leads to a higher risk of fractures among elderly women due to bone fragility, although surprisingly the risk of mortality after a hip fracture is higher in men.

Studies of miR-503 inhibition in mouse models of ovariectomy have shown that miR-503 regulates bone resorption *in vivo*, inhibiting osteoclastogenesis by targeting *Rank* (Chen *et al*. 2013*b*). It has also been suggested that a decrease in miR-2861 expression contributes to OP. miR-2861 has already been shown to be a BMP-induced miRNA, targeting *Hdac5* and therefore involved in Runx2 degradation (Li *et al*. 2009*a*, Hu *et al*. 2011). Li *et al*. (2009*a*) have shown that inhibition of miR-2861, using a specific antisense oligonucleotide introduced by a single tail vein injection, leads to decreased bone mass, reduced osteoblast activity, and osteoblast marker alteration in mice. miR-3077-5p and miR-705 are overexpressed in osteoporotic MSCs. The levels of both miRNAs decrease normally during osteogenic induction to allow the expression of their targets *HOXA10* and *RUNX2*. Liao *et al*. (2013) have shown that knockdown of miR-705 and

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miR-3077-5p in osteoporotic MSCs is sufficient to restore osteoblast differentiation and mineralization. Moreover, 17β-estradiol injections in ovariectomized mice were found to lead to the recovery of the osteoporotic phenotype and a reduction in miR-705/-3077-5p expression (Liao *et al*. 2013).

miRNAs are also involved in tumor emergence and progression. Nowadays, the use of miRNAs constitutes a novel strategy to improve tumor detection and to predict patient prognosis. One example is miR-132, which was detected as a miRNA expressed at lower levels in osteosarcoma samples. Lower expression of miR-132 is observed in patients with advanced-stage cancer and presenting a poor response to chemotherapy, identifying miR-132 as an indicator of osteosarcoma prognosis (Yang *et al*. 2013). The downregulation of miR-145 expression is also related to poor prognosis in osteosarcoma patients (Tang *et al*. 2013). However, the molecular mechanisms of miR-145 and miR-132 in relation to osteosarcomas are unknown, and further studies are needed to fully understand their involvement in carcinogenesis. miRNA screening has been performed in chondrosarcoma biopsies (Yoshitaka *et al*. 2013), and recently, a study elucidating miRNA changes in osteolytic bone metastasis has been published (Ell *et al*. 2013). During osteolytic bone metastasis, downregulation of the expression of specific miRNAs leads to increased expression of important osteoclastic genes such as *MITF*, *CALCR*, *TRAF6*, and *MMP14*. Furthermore, ectopic overexpression of some miRNAs (pre-miR-141 and pre-miR-219) leads to a reduction in osteolytic bone metastasis (Ell *et al*. 2013).

Thus, a detailed understanding of the function of miRNAs and their tight relationship with bone diseases would constitute a powerful tool for early diagnosis and future therapeutic approaches. Pre-miR or antago-miR therapies have emerged as a novel way to target dysregulated pathways; however, several questions about safety as well as tissue-specific targeting still remain to be answered before clinical applications can be developed.

Concluding remarks

Microarray expression data have provided evidence for the role of miRNAs in several skeletal pathologies. Moreover, mouse models in which their expression is altered have provided evidence of causal links between miRNAs and bone abnormalities. The current identification of a vast number of skeletal-cell-specific miRNAs, each of them with a list of putative targets, has yielded the present challenge of understanding their biological functions. From the data obtained to date, we know that most miRNAs exert their functional effects via multiple target mRNAs, usually by cooperatively targeting genes in the same pathway. Similarly, there is also redundancy, as the same mRNA is targeted by different miRNAs. The very sensitive nature of developmental programs and signaling pathways renders them the perfect candidates for techniques utilizing the dose-dependent effects of miRNAs. In bone physiology, miRNAs are extremely useful nodes, acting as feedback or feed-forward devices that allow buffering effects that confer robustness to skeletal development programs. miRNAs serve as finely tuned precision regulators of the expression of those genes that confer cellular identity and promote differentiation. Moreover, individual miRNAs may even operate as switches to induce differential cell fates. These properties clearly support the notion that the mutation or dysregulation of miRNAs would profoundly affect expression patterns and contribute to skeletal pathologies. Fortunately, these properties also open up opportunities for designing smart therapies based on miRNAs and small RNAs. miRNAs, which are easy to deliver into cells, are intrinsically highly specific and could regulate several targets at the same time. Thus, it is highly feasible that miRNAs, as well as antago-miRNAs, may be used in the future as drugs to treat skeletal pathologies.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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