Review

MicroRNAs Modulate Signaling Pathways in Osteogenic Differentiation of Mesenchymal Stem Cells

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Abstract: Mesenchymal stem cells (MSCs) have been identified in many adult tissues and they have been largely studied in the last years, especially in view of their potential use for treating diseases, damaged tissues and organs. MSCs are capable of self-replication and differentiation into osteoblasts and are considered an important source of cells in tissue engineering for bone regeneration. Several epigenetic factors are believed to play a role in the osteogenic differentiation of MSCs, including microRNAs (miRNAs). MiRNAs are small, single-stranded, non-coding RNAs of approximately 22 nucleotides able to regulate cell proliferation, differentiation and apoptosis by binding the 3′ untranslated region (3′-UTR) of target mRNAs, which can be subsequently degraded or translationally silenced. MiRNAs control gene expression in osteogenic differentiation by regulating two crucial signaling cascades in osteogenesis: the transforming growth factor-beta (TGF-β)/bone morphogenic protein (BMP) and the Wingless/Int-1(Wnt)/β-catenin signaling pathways. This review provides an overview of the miRNAs involved in osteogenic differentiation and how these miRNAs could regulate the expression of target genes.

Keywords: mesenchymal stem cells; MSCs; microRNA; miRNA; bone; osteogenic differentiation; osteogenesis; cell differentiation; stem cells; miRNAome; bone regeneration

1. Introduction

Mesenchymal stem cells (MSCs) are multipotent cells widely studied for their potential promising properties in regenerative medicine and in clinical practice [1,2]. Isolated for the first time in 1968 from bone marrow (BM-MSCs) [3], MSCs have been obtained from several body districts and evaluated for their ability to differentiate in vitro into osteocytes, chondrocytes, and adipocytes [4,5] (Figure 1).
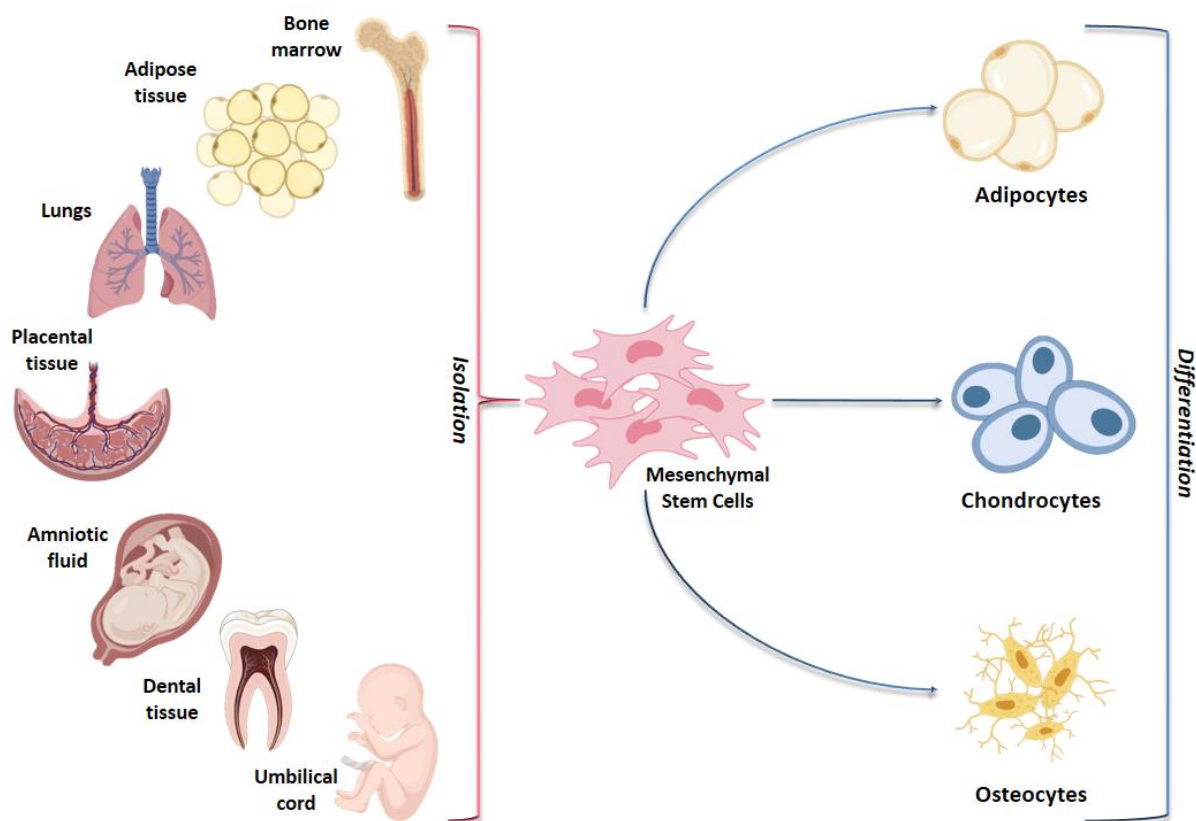
Figure 1. Schematic representation of adult and fetal/neonatal tissue sources of mesenchymal stem cells (MSCs) and their potential of differentiation in various cell lines. MSCs can be isolated from several tissue sources in the body and they may differentiate into adipocytes, chondrocytes and osteocytes.

MSCs play a key role in the natural events leading to bone repair and healing by differentiating into osteoblasts, which secrete a regenerate bone matrix. Because of their biological features such as sustained self-renewal and expansion, ease of availability, ability to differentiate into osteoblasts, MSCs are also attractive candidates for bone tissue engineering approaches. In addition, they show anti-inflammatory and immunomodulatory effects and secrete molecules that can initiate or support tissue regeneration/replacement [5]. A large number of studies has been carried out on the biological properties of MSCs, signaling pathways involved in their differentiation and factors that might facilitate MSC osteogenic differentiation [5,6].

Endogenous growth factors, cytokines and signals from the extracellular matrix, mainly via integrin interactions, represent essential regulators in cell differentiation [7,8]. Further, external stimuli, such as mechanical forces or electromagnetic fields, modulate cell behavior during osteogenesis [9,10].

Biomaterials provide the biological structure that supports MSC osteogenic differentiation [2,11–18]. The addition of ions has been proved to enhance the osteogenic potential of scaffolds [19]. Biological macromolecules such as growth factors [20] and microRNAs (miRNAs) [21], as well as biophysical stimuli [22] can be also associated with scaffolds to increase cell differentiation.

Growing evidence shows that MSCs differentiation is influenced by several epigenetic processes/factors, including histone methylation and acetylation, DNA methylation [23], non-coding RNA molecules such as long non-coding RNAs (lncRNA) [24] and miRNAs [25–28].

MiRNAs are small single-stranded, non-coding RNAs consisting of approximately 21–25 nucleotides (nt) and expressed in many organisms from viruses [29–31] to eukary-

ote cells [32]. MiRNAs are able to negatively regulate gene expression at post-transcriptional level, by interfering with target messenger RNA (mRNA) translation [33], and their functions have been widely studied in several cellular and molecular processes [34,35], including MSC osteogenic differentiation [5,6]. MiRNAs can positively or negatively regulate osteogenic differentiation by targeting transcription factors (TFs) and genes coding for either negative or positive differentiative modulators [27,36–38]. Different computational tools, such as TargetScan, miRanda and DIANA microT have been developed in order to predict miRNA targets [39]. After prediction, targets have to be experimentally validated in laboratory by PCR [40] and western blot analyses [41] to confirm the miRNA functional activity. Knowledge concerning the role of miRNAs is complex as they modulate mRNAs acting in concert with other classes of non-coding RNAs, such as long non-coding RNAs (lncRNA) [27,42]. Further, each miRNA shows multiple activities targeting several mRNAs, thus directly or indirectly modulating the expression of a plethora of genes involved in many cellular functions [43].

This review is addressed to the miRNAs that, by sequence complementarity, bind mRNAs directly regulating the gene expression of molecules involved in the main signaling pathways of MSCs osteogenic differentiation, such as the (TGF-β)/bone morphogenic protein (BMP) and the Wingless/Int-1(Wnt)/β-catenin pathway [44,45].

2. MSCs Signaling Pathways in Osteogenic Differentiation

MSC osteogenic differentiation encompasses three phases: the first, also known as the proliferative phase, consists of the recruitment and expansion of osteoprogenitor cells; the second involves the cell maturation in pre-osteoblasts upon extracellular matrix (ECM) synthesis; and the third phase consists of the matrix mineralization [46].

All of these phases are modulated by several signaling pathways [47]. Among them, the TGF-β/BMP and the Wnt/β-catenin pathways [45] play critical interconnected roles and have been largely investigated also for clinical implications in bone healing [48–50]. TGF-β signaling appears mainly involved in the early phase of osteogenesis allowing osteoprogenitor cells to differentiate into immature osteoblasts [44], whereas it inhibits osteoblast maturation, mineralization, and transition into osteocyte [51,52]. Contrariwise, BMP signalling promotes almost every phase during osteoblast differentiation and maturation [44]. BMPs have been the first proteins identified as inducing bone formation in vivo [53]. Among over 20 BMP proteins, BMP2, 4, 5, 6, 7 and 9 play important roles in osteogenesis [54,55].

TGF-βs and BMPs work through type I and type II heterodimeric kinase receptors and need Sma and Mad related (SMAD) proteins to transduce the signal inside the cells (Figure 2).
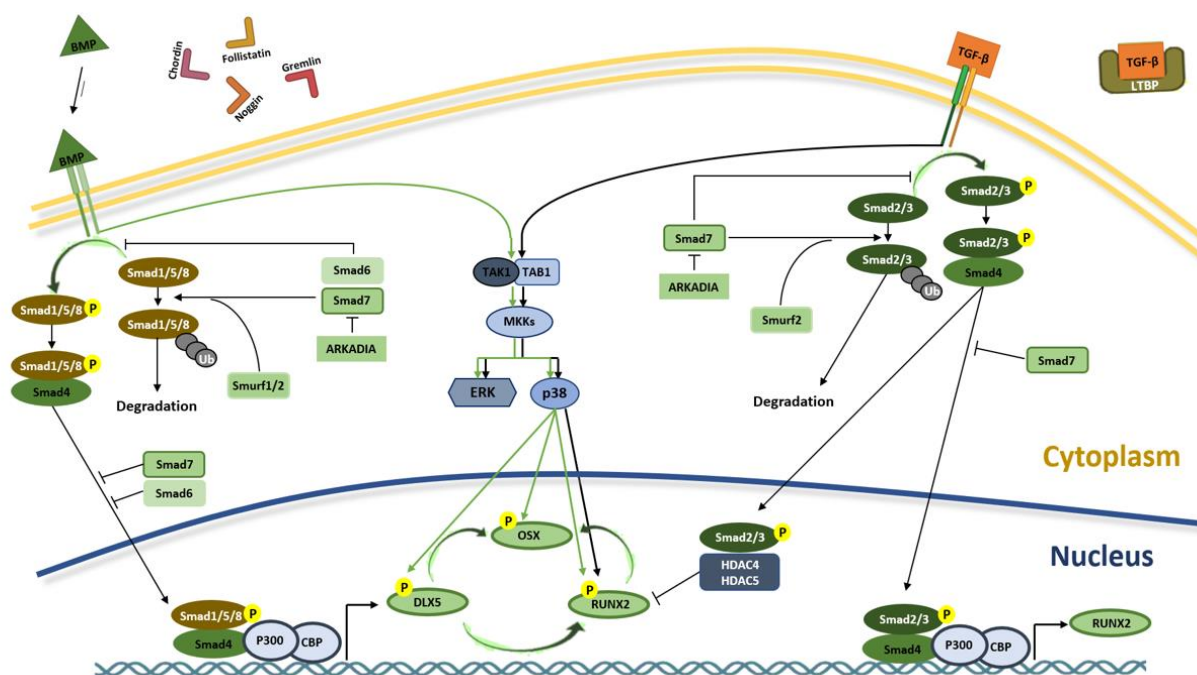
Figure 2. BMPs and TGF-β signaling pathways in osteogenesis. Interaction between TGF-βs and BMPs and receptors initiate SMAD-dependent and Non-SMAD-dependent signaling pathways. In SMAD-dependent signaling pathway, interaction between TGF-β and receptor leads to SMAD2/3 (R-SMAD) phosphorylation. Phosphorylated-R-SMAD (SMAD2/3-Pi) binds SMAD4 protein and migrates to the nucleus of the cell where, alongside P300 and CREB-binding protein (CBP) coactivators, controls *RUNX2* expression. In the nucleus, SMAD2/3-Pi without SMAD4 recruits HDAC4 and HDAC5 and blocks RUNX2 activity. In BMP signaling, R-SMAD (SMAD1/5/8) binds SMAD4 and migrate to the nucleus inducing *RUNX2* and *OSX* expression through *DLX5* activation. In the Non-SMAD-dependent signaling pathway, TAK1 and TAB1 activate the MKKs -p38 MAPK or -Erk cascades inducing DLX5, RUNX2, and OSX phosphorylation. The interaction between TGF-β and its receptor is blocked by the latent TGF-β binding protein (LTBP), which binds to TGF-β. Similarly, in order to prevent the BMP-receptor interaction, Gremlin, Chordin, Follistatin and Noggin proteins bind to BMP. The translocation of SMAD2/3 and SMAD1/5/8 into the nucleus is prevented by SMAD7 that, along with SMURF1/2, induce their proteasome-mediated degradation. In addition, the binding between ARKADIA and SMAD6/7 proteins positively regulates osteoblast differentiation.

Several type I and type II receptors have been identified in transducing the signal of TGF-βs and BMPs ligands [49,56]. The preferential type I receptor of TGF-βs is TβRI/ALK5 while the main type I receptors of BMPs are BMPR1A/ALK3, BMPR1B/ALK6, and AcvR1/ALK2. The type II kinase receptors of TGF-βs and BMPs are mainly TβRII/TGFBR2 and BMP receptor type II (BMPRII), respectively. The interaction between the ligand and the receptor induces the trans-phosphorylation of a receptor subunit, resulting in the activation of the SMAD-dependent (canonical) or Non-SMAD-dependent signaling cascades, leading to the gene expression regulation and activation of the osteogenic transcription factors Distal-less Homeobox 5 (DLX5), RUNX2 and the zinc finger Osterix (OSX), also called SP7 (Figure 2) [44,57]. Finally, this results in the expression of specific osteoblast genes including osteopontin (OPN), osteocalcin (OCN), osteonectin (ON) and collagen type 1 (COL1) and cell differentiation [58].

TGF-β and BMP signaling pathways are regulated by different components, which stimulate and/or inhibit osteoblast differentiation. They comprise BMP binding proteins, such as Noggin, Chordin, Gremlin, and Follistatin that sequester ligands preventing binding to receptors, and intracellular inhibitory SMAD proteins, such as SMAD7, and ubiquitin-proteasomal degradation pathway (Figure 2) [44,49].

The Wnt/β -catenin or canonical Wnt is another signaling pathway with an essential regulatory role in MSCs self-renewal and differentiation [50]. Wnt/β-catenin signaling pathway occurs in the early stage of osteogenesis allowing the MSCs to differentiate into

osteoblast lineage whilst it is downregulated in differentiated cells [57]. Wnt signaling is initiated upon binding of Wnt ligands with the membrane receptor complex known as Frizzled/low-density lipoprotein receptor-related protein 5/6 (FZD/LRP) [59]. Interactions between Wnt ligands and FZD/LRP5/6 trigger disheveled (DSH), that switching off the glycogen synthase kinase 3 β (GSK-3β) enables β-catenin protein to be translocated into the nucleus, whereby it complexes with the binding T cell factor/lymphoid enhancer binding factor (TCF/LEF) to stimulate the expression of target genes involved in the osteogenesis including *RUNX2* [60,61] (Figure 3).
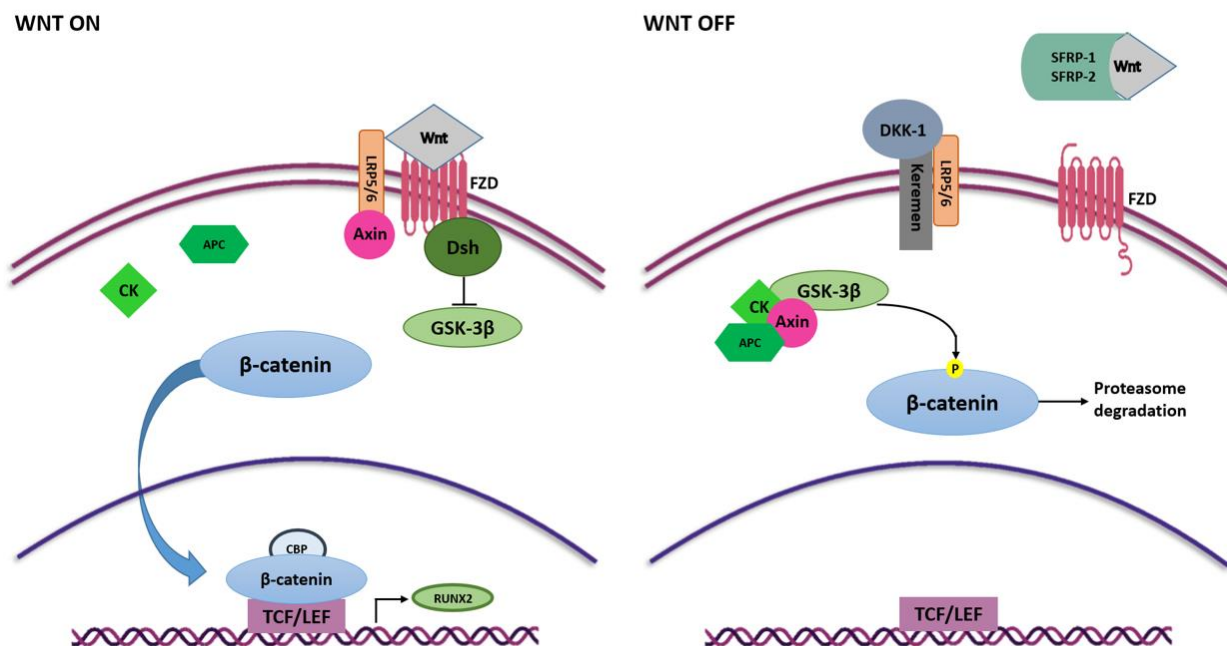

Figure 3. Wnt/β-catenin signaling pathway in osteogenesis. Wnt ligands interact with FZD and activate Wnt signaling pathway. FZD binds Dsh that inhibits GSK3-β activity, thus the phosphorylation of β-catenin. β-catenin translocates into the nucleus, where it binds to TCF/LEF and CBP and induces the expression of *RUNX2*. Interaction between Wnt ligands and SFRP-1/2 and the binding of LRP5/6 and Keremen with DKK-1 antagonist switch off Wnt signaling pathway. In this case, GSK3-β, together with Axin, CK and APC, phosphorylate β-catenin inducing it to proteasome-mediated degradation.

Several Wnt ligands, including Wnt4, Wnt5a, Wnt6, Wnt10a and Wnt10b, are involved in initiating the Wnt/β-catenin cascade and stimulation of osteoblast differentiation [62–64].

Contrariwise, Wnt3a and Wnt16 have negative effects on MSC osteogenic differentiation [65–67]. In addition, extracellular Wnt signaling antagonists can negatively modulate the Wnt/β-catenin signaling pathway, such as the secreted frizzled-related protein 1 and 2 (SFRP1 and SFRP2) [68], that bind Wnt ligands preventing their interaction with receptor [50], and the dickkopf-related protein1 (DKK1) [69], which binds the active site of the LRP5/6 receptors allows proteasome β-catenin degradation through GSK-3β complex activation [70,71] (Figure 3). Another relevant Wnt signaling inhibitor is sclerostin (SOST) which binds the first beta propeller (E1) of the LRP5/6 Wnt co-receptors and mainly inhibits the Wnt1 canonical signaling pathway [50,72].

TGF-β/BMP and Wnt/β-catenin signaling pathways strictly interplay in a very complex way and affect each other in a positive feedback loop allowing osteoblast differentiation in vitro and, thus, a proper skeletal development in vivo. Further, signals from the extracellular (ECM) cooperate with osteogenic signaling pathways, mainly acting through integrins, the mediators of cell adhesion to the ECM and important mechano-transducers involved in both matrix deposition and organization [7,9,73]. Moreover, TGF-β/BMP and

Wnt/β-catenin signaling pathways are components of a more complex molecular network with other different pathways and cytokines such as Hedgehog (Hh), NOTCH, Fibroblast Growth Factor (FGF) and Parathyroid Hormone-related Peptide (PTHrP) that interplay for an appropriate osteoblast differentiation and bone development [44].

3. miRNAs

MiRNAs, are a class of non-coding RNAs (ncRNAs), generally 22 nucleotides long, with essential role in gene regulation. They account for 1–5% of the human genome and regulate 30–60% of protein-coding genes [74]. MiRNAs are generated through a multi-step process (Figure 4).
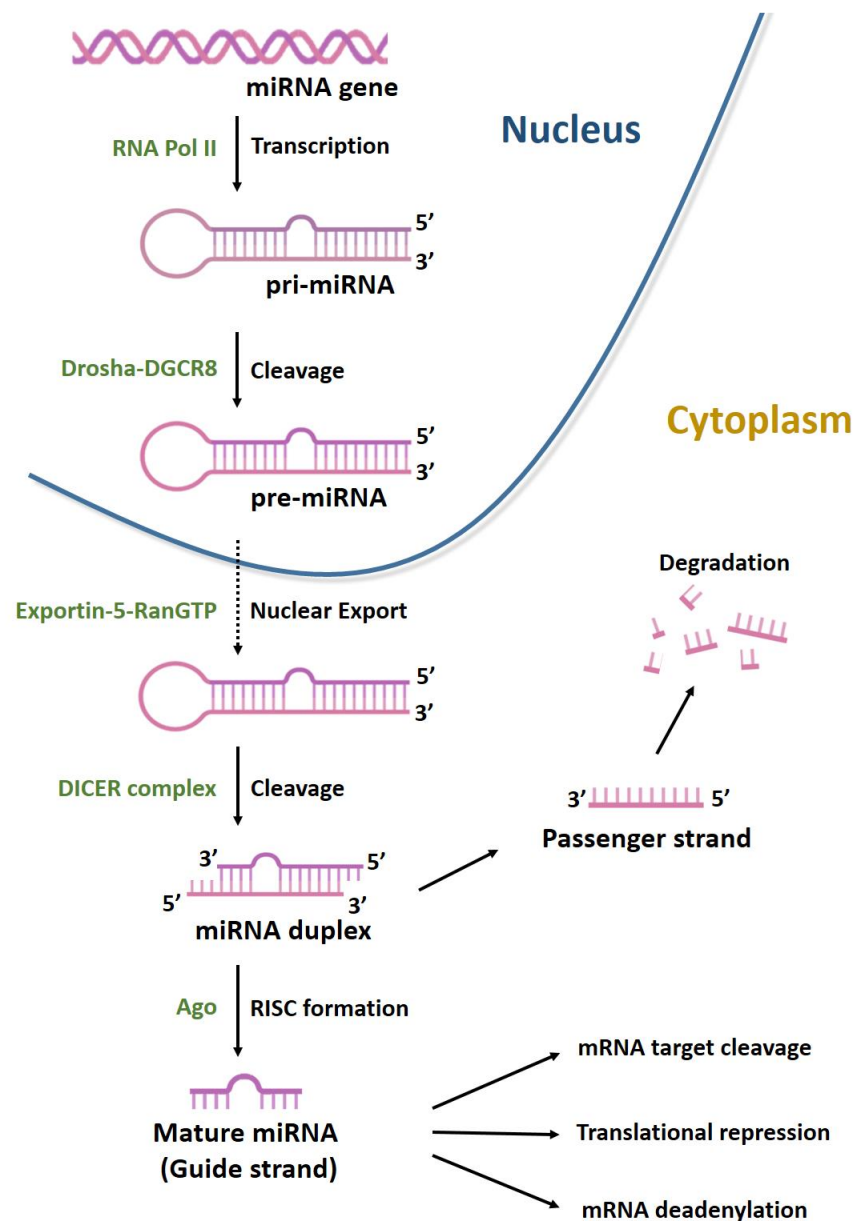
Figure 4. Biogenesis of microRNAs. MiRNAs are transcribed by RNA polymerases II (Pol II) to generate a long precursor transcript named primary microRNA (pri-miRNA). This RNA molecule folds up into a secondary structure (stem-loop) to form a partial double helix, composed of 100–1000 nt with a 5'-cap. MiRNA maturation process can be divided into three phases. In the first, known as cropping, pri-miRNA is converted into the precursor miRNA (pre-miRNA) via the cutting activity of the Drosha enzyme, a nuclear endoribonuclease III. Pre-miRNA has a hairpin structure (stem-loop) and a length of about 70–80 nt. Following cropping, the pre-miRNA has a 5'P and a 3'OH and 2–3 nt at the protruding end with a single strand. In the second phase, the nuclear export factors Exportin-5 and ras-related nuclear protein (RAN-GTP)

mediate the export of pre-miRNAs from nucleus to cytoplasm. In the third phase, known-as dicing, another type of RNA-endonuclease III, known as Dicer, processes pre-miRNA in the cytoplasm by cleaving it into an 18–22 nt double stranded miRNA (miRNA duplex). Lastly, mature miRNA incorporated into the RNA-induced silencing complex (RISC) is able to bind the 3′UTR region of its target mRNA.

The gene silencing mechanism is determined by the degree and nature of complementarity between the miRNA seed site and the 3′-UTR of its target mRNA. When complementarity is complete, the mRNA target undergo degradation, whilst, when it is partial, mRNA target protein levels are reduced [75]. Binding to mRNA represents the main activity of miRNA, although it is known they can also regulate gene expression through other molecular mechanisms. MiRNAs can positively or negatively regulate osteogenic differentiation by targeting either osteogenic negative or positive regulatory genes and TFs, as shown below [76–78]. Due to these activities, miRNAs have been proposed as targets for innovative therapeutic approaches in several diseases, including bone related diseases [79]. In tissue engineering approaches, miRNA mimics or antago-miRNAs are employed as bioactive factors [80] in combination with stem cells [5] or scaffolds [81] in order to improve bone tissue regeneration.

4. Osteogenic Differentiation by miRNA Regulation

TGF-β/BMP and Wnt/β-catenin cascades are post-transcriptionally modulated by different miRNAs that stimulate or inhibit osteogenesis [45,50]. Specifically, several miRNAs can directly increase or reduce the expression of the genes coding for components of the signaling pathways and/or the TFs involved in osteogenic differentiation, ultimately exerting both stimulatory and inhibitory effects on osteogenesis, as reported herein (Table 1).

Table 1. MicroRNAs, their direct target genes and cell types in which they play a role during the osteogenesis.

miRNA	Cell Type	Target Gene	Effect on Osteogenesis
let-7a-5p	PMOP BM-MSCs	TGFβR1	−
miR-9	MSCs	DKK1	+
miR-9-5p	MSCs	Wnt3a	−
miR-10b	hASCs	SMAD2	+
miR-16-2-3p	hBM-MSCs	Wnt5a	−
miR-17-5p	hBM-MSCs	SMAD7	+
miR-21	BM-MSCs	SMAD7	+
miR-21-5p	BM-MSCs	SMAD7	+
miR-21a	C3H10T1/2	SMAD7	+
miR-23a	MSCs, PDLSCs, MC3T3-E1, hBM-MSCs	RUNX2, LRP6	−
miR-23b	MSCs	RUNX2	−
miR-24	BM-MSCs, MC3T3-E1	TCF-1	−
miR-26a	BM-MSCs, hASCs	SMAD1, GSK3β	−/+
miR-26b	BM-MSCs	GSK3β	+
miR-27a	hFOB1.19, BM-MSCs	APC, SFRP1, GREM1	−/+
miR-29a	BM-MSCs, MC3TC-E1	HDAC4	+
miR-29b	BM-MSCs, MC3TC-E1	HDAC4	+
miR-30	MC3T3-E1, MSCs, hASCs, C3H10T1/2	RUNX2, SMAD1	−
miR-30a	MC3T3-E1	RUNX2, SMAD1	−
miR-30b	MSCs, MC3T3-E1	RUNX2, SMAD1	−
miR-30c	MSCs, hASCs, MC3T3-E1	RUNX2, SMAD1	−
miR-30d	MC3T3-E1	RUNX2, SMAD1	−
miR-30e	hBM-MSCs	LRP6	−
miR-34c	MSCs, MC3T3-E1	RUNX2	−
miR-93-5p	MSCs, BM-MSCs	BMP2	−

miR-96	Mice AS cells	SOST	+
miR-98	hMSCs	BMP2	−
miR-100	hMSCs	BMPR2	−
miR-125b	hBM-MSCs	BMPR1B	−
miR-129-5p	MC3T3-E1, C57BL6	TCF-4	−
miR-132	UC-MSCs	β-catenin	−
miR-133	C2C12	RUNX2	−
miR-133a	DPSCs, C2C12	RUNX2, SMAD5	−
miR-133a-5p	MC3T3-E1	RUNX2	−
miR-133b	DPSCs	RUNX2, SMAD5	−
miR-135	C2C12	SMAD1, SMAD5	−
miR-135a	MSCs, MC3T3-E1, ATDC5, C2C12, DPSCs	RUNX2, SMAD5	−
miR-135a-5p	C2C12	RUNX2	−
miR-137	MSCs	RUNX2	−
miR-137-3p	BM-MSCs	RUNX2	−
miR-139-5p	hBM-MSCs	β-catenin, FZD4	−
miR-140-5p	hMSCs	BMP2	−
miR-142-3p	hFOB1.19	APC	+
miR-143	MSCs	RUNX2	−
miR-144-3p	C3H10T1/2	SMAD4	−
miR-145	MC3T3-E1	CBFB	−
miR-146a	hASCs, AS fibroblast	SMAD4, DKK1	−/+
miR-153	hMSCs	BMPR2	−
miR-154-5p	hASCs	Wnt11	−
miR-155	C2C12, MEF	BMPR2	−
miR-195-5p	PDLSCs	BMPR1A	−
miR-199b-5p	hBM-MSCs	GSK3β	+
miR-203	MSCs	RUNX2	−
miR-203-3p	MSCs	SMAD1	−
miR-204	C3H10T1/2, C2C12, ST2, BM-MSCs	RUNX2, BMP2	−
miR-205	MSCs, BM-MSCs	RUNX2, SATB2	−
miR-208a-3p	MC3T3-E1	ACVR1/Alk2	−
miR-214	BM-MSCs	BMP2	−
miR-217	MSCs	RUNX2, DKK1	−/+
miR-218	hASCs, BM-MSCs, MC3T3, hDPSCs	DKK2, SFRP2, SOST, RUNX2	+/−
miR-221	MSCs	RUNX2	−
miR-221-5p	MB D-MSCs	SMAD3	−
miR-222-3p	BM-MSCs	RUNX2, SMAD5	−
miR-335-5p	MSCs, C3H10T-1/2, MC3T3-E1, MLO-A5, -Y4	DKK1	+
miR-338	MSCs	RUNX2	−
miR-338-3p	BM-MSCs mice	RUNX2, FGFR2	−
miR-346	hBM-MSCs	GSK3β	+
miR-370	MC3T3-E1	BMP2	−
miR-378	MSCs	Wnt6, Wnt10a	−
miR-381	hBM-MSCs	Wnt5a, FZD3	−
miR-433-3p	MSCs, hFOB1.19, ROS17/2.8	DKK1	+
miR-505	MC3T3-E1	RUNX2	−
miR-590-3p	hMSCs	APC	+
miR-590-5p	C3H10T1/2, MG63, MC3T3-E1	SMAD7	+
miR-628-3p	MG63	RUNX2	−
miR-708	MSCs	SMAD3	−
miR-1297	hBM-MSCs	Wnt5a	−

4.1. TGF-β/BMP Signaling Pathways

4.1.1. TGF-β/BMP Ligands and Receptors

Few studies have reported on miRNA regulation of TGF-β receptors in osteogenesis. Let-7a-5p targeted the *TGFβRI* inhibiting the osteogenic differentiation of BM-MSCs in postmenopausal osteoporosis (PMOP) mice [82]. Several miRNAs target both BMP receptors and ligands. MiR-100 and miR-153 have been shown to attenuate human MSCs (hMSCs) osteogenic differentiation by targeting *BMP receptor type II* (*BMPR2*) [83,84]. Moreover, BMPR2 is a direct target of miR-155, whose inhibitory activity in the osteogenic differentiation has been proven in C2C12 cells and *mouse embryonic fibroblasts* (*MEF*) treated with bone morphogenetic protein 9 (BMP9) [85]. MiR-195-5p inhibited osteogenesis in periodontal MSCs (PDLSCs) from periodontitis patients by targeting the *BMP receptor type IA* (*BMPR1A*) [86]. In the same study it was also shown that miR-195-5p was regulated by mechanical loading and involved in mechanical loading-induced osteogenic differentiation. In hBM-MSCs miR-125b binds to the 3′-UTR of the BMP receptor type 1B gene and inhibits differentiation [87]. Furthermore, it has been shown that miR-208a-3p targets ACVR1/Alk2 gene inhibiting osteoblast differentiation in MC3T3-E1 cells and in vivo [88]. For what concerns BMP ligands, miR-93-5p, miR-98 and miR-140-5p inhibit osteogenic differentiation of hMSCs by direct targeting BMP2 [89–91]. In BMP2-stimulated murine pre-osteoblast MC3T3-E1 cells, miR-370 attenuates osteogenic differentiation by targeting *BMP2* and *Erythroblastosis virus E26 Oncogene Homolog 1* (*Ets1*) gene [92]. In hBM-MSCs miR-214 reduces BMP2 expression binding to the 3′UTR and when overexpressed it inhibits osteogenic differentiation [93]. Analogously, direct interaction of miR-204 with BMP2 mRNA reduces differentiation of rat bone marrow MSCs [94].

4.1.2. SMAD Cascade

Several miRNAs target the TGF-β signaling-induced SMAD2/3, the BMPs signaling-induced SMAD1/5 and the TGF-β/BMP signaling-induced common SMAD4 affecting osteogenic differentiation. In TGF-β signaling SMADs, miR-10b promoted osteogenesis by targeting *SMAD2* in hASCs and enhanced bone formation in vivo, while its expression correlated with bone formation *marker genes alkaline phosphatases* (*ALP*), *RUNX2* and *OPN* in clinical samples from patients affected by osteoporosis [95]. MiR-221-5p inhibited osteogenesis in myeloma bone disease mesenchymal stem cells (MBD-MSCs) by directly targeting *SMAD3*, which in turn negatively affected the PI3K/AKT/mTOR signaling pathway and consequently osteogenic differentiation [96]. *SMAD3* is also a direct target of miR-708, which suppressed the osteogenesis and adipogenesis in MSCs, and it was found to be overexpressed in MSCs from patients with steroid-induced osteonecrosis of femoral head (ONFH) [97].

In BMP signaling SMADs, miR-26a, a key player of the skeletal muscle differentiation and regeneration [98], inhibited osteogenesis in hASCs by targeting *SMAD1*, while it activated osteogenic differentiation in Wnt signaling-induced BM-MSCs [99]. Members of miR-30 family, such as miR-30a, -30b, -30c, and -30d, inhibited osteogenesis in MC3T3-E1 by targeting *SMAD1* and *RUNX2*, and have been found down-regulated during osteoblast differentiation [100]. Further, miR-222-3p appears as a negative regulator of the osteogenic differentiation, as its overexpression significantly suppressed *SMAD5* and *RUNX2* protein levels whereas its inhibition increased their expression in human BM-MSCs [101]. Similarly, miR-133 and miR-135 suppressed osteogenesis targeting *RUNX2* and *SMAD1/5*, respectively, in C2C12 mouse mesenchymal progenitors [102]. In agreement, the down-regulation of miR-133a -133b and -135a found in human dental pulp stem cells (DPSCs) grown on titanium disks allowed the target genes *RUNX2* and *SMAD5* to be expressed, leading to osteogenic differentiation [103]. Tang et al. reported that miR-203-3p targets the 3′-UTR of *SMAD1* mRNA inhibiting in vitro and in vivo osteogenesis in diabetic rats, suggesting miR-203-3p a potential therapeutic target in diabetic bones for ameliorating osteoporosis and fracture healing [104].

Finally, in TGF-β/BMP signaling-induced common SMADs, miR-144-3p negatively regulated osteogenic differentiation and proliferation in embryo cells from C3H mice (C3H10T1/2) cells by directly targeting *SMAD4* [105]. Similarly, miR-146a negatively regulated osteogenesis and bone regeneration through interaction with 3′-untranslated region (3′-UTR) of *SMAD4* mRNA, both in vitro and in vivo [106].

4.1.3. TGF-β/BMP Signaling Pathway Intracellular and Extracellular Inhibitors: SMAD7, HDAC4, BMP Antagonist Proteins

SMAD7 gene has an antagonistic role in TGF-β/BMP signaling [107] and appears regulated by several miRNAs [108–110]. MiR-17-5p, which is part of the miR-17/92 cluster and has important roles in several cancers, directly targets *SMAD7* in human BM-MSCs increasing their osteoblastic differentiation and cell proliferation [111]. MiR-21, largely known for its regulatory role in bone [112], induces BM-MSC bone regeneration through the SMAD7-SMAD1/5/8-RUNX2 pathway [107]. The link between miR-21a and *SMAD7* was confirmed by a very recent study, demonstrating that phytol, derived from aromatic plants, is able to promote the osteoblast differentiation in C3H10T1/2 cells by down-regulating *SMAD7* through mir-21a, thus leading to the increased expression of RUNX2 [113]. Similarly, the expression of miR-21-5p was increased and associated to a reduced expression of *SMAD7* also during the Pulsed Electromagnetic Fields (PEMFs)-induced osteogenic differentiation of human BM-MSCs, stimulating the TGF-β signaling pathway [114]. Further, in a recent study, high levels of miR-21-5p, as well as of the proosteogenic miR-129-5p and miR-378-5p have been identified in hMSCs differentiated by treatment with serum from runners, after a half marathon. This suggests that miRNAs can regulate MSC osteogenic differentiation also in response to physical exercise [115]. By targeting *SMAD7*, miR-590-5p indirectly protects and stabilizes the RUNX2 in C3H10T1/2 and MG63 cells [116]. Interestingly, MC3T3-E1 cells exposed to elevated glucose conditions showed increased *SMAD7* levels with significant downregulation of miR-590-5p and osteoblastic proteins, e.g., Collagen I, RUNX2, and ALP, suggesting that miR-590-5p could be a potential target for treatment of the diabetic osteoporosis [117].

Several extracellular proteins act as antagonist of BMP signaling. They include Gremlin, noggin, follistatin [118]. To the best of our knowledge, only miR-27a, targeting gremlin 1 (GREM1) has been involved in the regulation of osteogenic and adipogenic differentiation in rat BM-MSCs under steroid treatment [119].

HDAC is a family of key epigenetic factors that regulates the expression of genes in concert with DNA methylation and miRNA expression [120–124]. In TGF-β signaling pathway, the HDAC4/5 complex binds the nuclear phosphorylated R-SMAD SMAD2/3 to repress the *RUNX2* expression, resulting in osteoblast differentiation inhibition [125]. The overexpression of miR-29a and miR-29b blocked *HDAC4*, thus inducing osteoblast differentiation in primary BM-MSCs and MC3TC-E1 cells [126,127]. Due to the miR-29b ability, the R9-LK15/miR-29b nano-complex that promotes osteogenic differentiation with high transfection efficiency has been recently developed [45]. Further, miR-29b level has been increased following exposure of hBMSCs to external stimuli such as PEMFs [128].

4.2. Wnt/β-Catenin Signaling Pathway

4.2.1. Wnt Ligands and Receptors

During osteoblast differentiation, the expression of miR-16-2-3p, also named miR-16-2*, is significantly decreased in human BM-MSCs, whereas its over-expression impaired the osteogenic differentiation blocking the Wnt signal pathway by directly targeting *Wnt5a* mRNA [129]. In another study, Wang et al., reported that *Wnt5a* mRNA can be also a target of miR-1297, which was found highly expressed in sera of osteoporotic patients, and significantly decreased in BM-MSCs after osteogenic induction [130]. Thus, the increased expression of miR-1297 may participate in osteoporosis progression by targeting

Wnt5a. In this context, also miR-9-5p promotes the occurrence and progression of osteoporosis through inhibiting osteogenesis and promoting adipogenesis via targeting *Wnt3a* in MSCs [131]. *Wnt5a* and FZD3 expression has been recently found to be inhibited also by miR-38, that binding to the mRNA 3'UTR hampered osteogenic differentiation of BM-MSCs [132]. The same authors also reported the involvement of miR-139-5p in hBM-MSCs osteogenesis by direct targeting *β-catenin* gene and frizzled 4 (FZD4) [133]. In addition, low-density lipoprotein receptor-related protein 6 (LRP6) and low-density lipoprotein receptor-associated protein 5 (LRP5), critical co-receptors for Wnts, have been shown to be direct target of miR-30e and miR-23a, respectively. It was described that miR-30e reciprocally regulates the differentiation of adipocytes and osteoblasts by directly targeting LRP6 [134] and miR-23a overexpression inhibited osteogenic differentiation of hBM-MSCs [135].

Other Wnt ligands have been identified as targets of specific miRNAs. Wnt6 and Wnt10a were identified as targets of miR-378. In this study the use of miR-378 mimics could suppress osteogenesis in hMSCs, whereas anti-miR-378 allowed their osteogenic differentiation, thus confirming that miR-378 inhibited osteogenesis via inactivating Wnt/β-catenin signaling [136]. Further, miR-154-5p negatively regulates hASCs osteogenic differentiation under tensile stress, through the Wnt/PCP pathway by directly targeting *Wnt11* [137].

4.2.2. Transcription Factors: β-Catenin and TCF/LEF

β-catenin is a fundamental factor in the Wnt/β-catenin signaling pathway which binds to TCF/LEF transcription factors leading to the regulation of genes involved in osteogenic differentiation [138]. Few miRNAs have been involved in osteogenic differentiation though binding to β-catenin and TCF/LEF mRNAs, although several miRNA targeting these genes have been reported in cancer [139]. MiR-132 inhibited the osteogenic differentiation of umbilical cord MSCs (UC-MSCs) targeting β-catenin and resulting in the downregulation OSX expression [140]. By targeting TCF-1, miR-24 overexpression decreased the expression of osteogenic differentiation markers [141]. miR-129-5p targets TCF-4 and its downregulation enhanced osteoblast differentiation in MC3T3-E1 cell line, and in C57BL6 mice ameliorated osteoporosis [142]. Further, it has been shown that miR-26a and miR-26b targeting glycogen synthase kinase three beta (GSK3β) lead to Wnt signaling activation and promote osteogenic differentiation of BM-MSCs [99,143]. Interestingly, miR-26a is increased during osteogenic differentiation induced by PEMFs [128].

4.2.3. Wnt Cascade Inhibitors

As mentioned above, APC, GSK-3 β, CK1α and Axin form the β-catenin destruction complex, which operates modulating β-catenin levels. MiR-27a and miR-142-3p downregulated APC and therefore activated the osteoblastic differentiation in human fetal osteoblastic cell line 1.19 (hFOB1.19) through Wnt signaling pathway [144,145]. In hMSCs, miR-590-3p bound to 3'UTR of *APC* mRNA, leading to osteogenic differentiation via β-catenin stabilization [44]. GSK3β, an osteogenesis suppressor, participates in the silencing of Wnt signaling pathway by phosphorylating cytoplasmic β-catenin. MiR-26a, miR-346 and miR-199b-5p directly target 3'-UTR of *GSK3β* mRNA and prevent its translation. In agreement to their role, these miRNAs are maintained at high expression levels during osteoblastic differentiation of mouse and human BM-MSCs [99,146,147].

Among the several extracellular Wnt signaling antagonists, DKK1, highly expressed in osteoblasts and MSCs, is a pivotal inhibitor of the Wnt cascade [50]. MiR-9, miR335-5p, miR-433-3p and miR-217 promoted osteoblastic differentiation in mouse and human MSCs targeting the 3'-UTR region of *DKK1* mRNA [148–151].

Of note, miR-335-5p was significantly upregulated in bone marrow stem cells during low-magnitude, high-frequency vibration induced osteogenic differentiation [152].

Also, miR-146a inhibited *DKK1* expression by directly targeting its 3′UTR mRNA, and was found upregulated in hip capsule tissues from patients affected by ankylosing spondylitis (AS) [153]. MiR-218 has shown stimulatory effects on Wnt signaling pathway, through the downregulation of different inhibitors, including *DKK2*, *SFRP2*, and *sclerostin* (*SOST*) in mice BM-MSCs, MC3T3, and hASCs [154,155]. Further, miR-96 binds to SOST gene and inhibits its expression. In agreement, the overexpression of miR-96 increase osteoblast differentiation and bone formation in AS-affected mice [156]. During osteoblastic differentiation of hFOB miR-27a was upregulated and targeted the extracellular antagonist of Wnt signaling pathway *SFRP1* [157]. Thus, miR-27a has dual beneficial effects on Wnt signaling pathway by positively regulating it through the downregulation of *APC* and *SFRP1* [144,157].

4.2.4. Transcription Factor RUNX2

CBFA-1/RUNX2 is the master transcription factors for osteogenic differentiation [158]. As reported above, its expression is regulated by several signaling pathways, especially by BMP and Wnt [159]. In addition, RUNX2 can regulate TCF/LEF family genes expression, and the β-catenin-TCF/LEF dimer can bind to the RUNX2 promoter and promote its transcriptional activity. Because of the key role of RUNX2 in the differentiation of MSCs into osteoblasts [160], several studies have been focused on RUNX2 regulation by miRNAs and recently reviewed [159,161].

Zhang et al., found a panel of 11 RUNX2-targeting miRNAs, such as miR-23a, miR-30c, miR-34c, miR-133a, miR-135a, miR-137, miR-204, miR-205, miR-217, miR-218, and miR-338, expressed in a lineage-related pattern in different mesenchymal cell types. A part miR-218, all RUNX2-targeting miRNAs inhibited osteoblast differentiation in osteoblastic cell line MC3T3 [162]. Conversely, miR-218 down-regulated RUNX2 expression in undifferentiated human-derived DPSCs [163]. Moreover, computational analyses have been used to identify miR-23a, miR-23b, miR-30b, miR-143, miR-203, miR-217, and miR-221 with potential direct regulation of RUNX2 during the differentiation of MSCs to pre-osteoblasts [164].

Most of the miRNA reported above have been investigated in different studies, confirming or not their role in osteogenic differentiation [135,151,165–168]. Great interest was shown for miR-23a, which was found over-expressed in mouse non transformed osteoblastic cells MC3T3-E1, inhibiting osteogenic differentiation [162,169]. In PDLSCs, miR-23a prevented osteogenic differentiation, and its expression was suggested to be a potential biomarker of periodontitis [170]. Differently, Park and collaborators reported the limited role of miR-23a in osteogenesis and bone homeostasis in vivo [171]. The activity of miR-30 in the downregulation of RUNX2 by binding its mRNA 3′-UTR sequence influencing osteogenic differentiation has been confirmed in the mesenchymal stem cell line C3H10T1/2 [172] and more recently in hASCs [173]. Also, the role of miR-133, miR-133a, miRNA-133a-5p and miR-135a as miRNAs targeting RUNX2 and inhibitors of osteogenesis has been investigated and confirmed in different osteoblastic cell lines including C2C12 cells [162,174] and MC3T3-E1 cells [162,175]. Notably, the expression of miR-135a-5p resulted overexpressed in osteoporotic post-menopausal females analyzing the RNA extracted from the bone tissue fragments of postmenopausal women with and without osteoporosis [176]. Other miRNAs have been involved in the regulatory mechanisms of osteogenesis in osteoporosis. MiR-338-3p expression was found down-regulated during the osteoblastic differentiation of BM-MSCs, and increased in BM-MSCs derived from osteoporosis mice, whereby miR-338-3p inhibited osteogenic differentiation by targeting RUNX2 and fibroblast growth factor receptor2 (FGFR2) [177]. MiR-205 has been involved in altered osteogenic differentiation of human aortic smooth muscle cells (HASMCs) [178]. Further, it inhibits osteogenic differentiation in female mouse model of type 2 diabetes mellitus (T2DM) and osteoporosis (OP) and in agreement high levels of miR-205 are identified in human female patients. This shows the correlation between the in vitro and in vivo activities of this miRNA [179], which negatively regulates osteoblastic differentiation

of BM-MSCs, via inhibition of RUNX2 as well as special AT-rich sequence-binding protein 2 (SATB2) expression [180].

MiRNAs have been investigated in other bone disorders or alteration of the osteogenic differentiation. Recently, miR-137-3p, directly targeting RUNX2 has been involved in osteonecrosis of the femoral head. It showed the ability to target also CXCL12 coding for SDF-1α, an angiogenic factor and, notably, the silencing of miR-137-3p could favor both osteogenesis and angiogenesis [181]. MiR-204 deficiency has been involved in the increased osteogenic activity associated to calcific aortic valve disease [182]. This result agrees with several studies reporting that in different mesenchymal progenitor cell lines such as C3H10T1/2, C2C12, ST2, and primary BM-MSCs where miR-204, targeting RUNX2, negatively controls osteogenic differentiation [94,183]. The findings of a recent study provided evidence that miR-628-3p is upregulated in patients with atrophic non-union, a serious complication of fractures, and may exert an inhibitory effect on osteogenesis suppressing its target gene RUNX2. Indeed, in vitro experiments on human osteoblast-like cells (MG63) cells transfected with miR-628-3p mimic led to a decreased osteogenic differentiation [184].

Different miRNAs can cooperatively regulate a common signaling pathway by affecting one specific transcription factor complex. For instance, miR-34c and miR-145 cooperate to inhibit osteoblast differentiation of MC3T3-E1 cells by targeting the RUNX2 and core binding factor beta (CBFB), respectively, hampering the transcription factor complex RUNX2/CBFB to be formed and subsequent transcription of RUNX target genes [185]. Recently, miR-505 has been identified as a new regulator of RUNX2 [186]. Specifically, in MC3T3-E1 cells miR-505 resulted downregulated during osteogenic differentiation *in vitro*, whilst its overexpression has been found to inhibit osteogenic differentiation and suppress osteoblast cells growth. Therefore, miR-505 may be a new potential therapeutic target for promoting new bone regeneration [186].

5. Conclusions

MSCs are the key players in bone formation and natural bone repair processes as well as in bone tissue engineering approaches. Dysregulation of MSCs activities is involved in several bone disorders, including osteoporosis [187]. Growing evidence shows that MSCs differentiation is regulated by different epigenetic factors, including miRNAs. Since miRNAs discovery, there have been great advancements in miRNA knowledge by both mechanistic studies and studies evaluating miRNAs as diagnostic and predictive biomarkers and therapeutics. The present review highlighted miRNAs as powerful post-transcriptional regulatory factors of the osteogenic process and was focused on the miRNAs which directly target genes coding for components of the main signaling pathways involved in osteogenesis. Each signaling pathway includes extracellular ligands, membrane receptors and intracellular proteins which acts as mediators of the pathway. Indeed, data collected show that miRNAs positively or negatively regulate osteogenic differentiation by targeting genes involved in TGF-β/BMP and Wnt/β-catenin signaling pathways. A number of miRNAs suppress osteogenesis by directly targeting mRNAs from genes coding for positive osteogenic factors, such as: (i) the extracellular ligands *TGF-β*, *BMP* and *Wnt*; (ii) the TFs *RUNX2*, *TCF-1*. On the other hand, miRNAs can promote osteogenesis by directly targeting mRNAs from genes coding for negative osteogenic factors, such as: (i) *HDAC4*, *SMAD7*, *DKK1*, *GSK-3β*, *APC*, *SFRP1*, *SFRP2* together with *DKK2* and *SOST*. Interestingly, some miRNAs display both stimulatory and inhibitory effects during osteogenesis. As each miRNA can target different genes, these data indicate that the activity of a miRNA can differ in different MSCs which may be characterized by specific regulatory signaling pathways and molecules including other non-coding RNAs [99,188].

Interestingly, some studies begin to show that miRNAs associated to the main osteogenic pathways can be modulated by physical stimuli including electromagnetic field stimulation and mechanical forces [128]. In this context, it has been recently reported that

physical exercise can induce the expression of proosteogenic miRNAs indicating a molecular basis for environmental effects on bone [115]. At the cellular level, the integrins are important mechano-transducers involved in both matrix deposition and organization. In spite of their relevant role also as modulators of TGF-β/BMP and Wnt/β-catenin signaling pathways, to date only few miRNAs targeting integrins have been identified in osteogenic differentiation [189]. Further studies identifying the biological interaction between integrins and miRNAs in osteogenesis are needed [189].

Notably, recent studies begin to identify altered levels of specific miRNAs which target genes of BMP or Wnt pathways in bone disorders including osteoporosis or osteonecrosis [97,176,190]. Since the essential roles of these pathways in the osteogenic process, these results are expected, on the other hand they may be helpful in planning therapeutic approaches.

In conclusion, large evidence supports miRNAs involvement in the osteogenic process and bone tissue formation, although the related mechanisms remain still poorly understood. Understanding the mechanisms of osteoblast differentiation regulated by specific miRNAs, as well as stimuli able to regulate their expression will be significant to develop new therapeutics to the treatment of bone disorders, and guide lifestyles. Further studies aimed to discover novel miRNAs and to understand their complex molecular activities on their gene targets are needed. In this context, knowledge on the tissue-specific MSC biology, the deep understanding of the complex relationships among miRNAs, other classes of non-coding RNAs and their target genes are required. Further, crossing data concerning altered miRNAs in specific pathological conditions with their experimental established functional activity may also direct the clinical approaches based on the use miRNA mimics or antago-miRNAs in bone disorders.

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