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Review Article

Deregulated miRNAs in bone health: Epigenetic roles in osteoporosis

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ABSTRACT

MicroRNA (miRNA) has shown to enhance or inhibit cell proliferation, differentiation and activity of different cell types in bone tissue. The discovery of miRNA actions and their targets has helped to identify them as novel regulations actors in bone. Various studies have shown that miRNA deregulation mediates the progression of bone-related pathologies, such as osteoporosis.

The present review intends to give an exhaustive overview of miRNAs with experimentally validated targets involved in bone homeostasis and highlight their possible role in osteoporosis development. Moreover, the review analyzes miRNAs identified in clinical trials and involved in osteoporosis.

1. Introduction

Bone is a mineralized connective tissue that has two essential biological roles: 1) a mechanical role – supporting body locomotion and protecting brain and splanchnic organs; and 2) a metabolic role - regulating electrolytes balance and energy metabolism in response to different endocrine signals, as parathyroid hormone (PTH), Vitamin D metabolites, sexual hormones, *etc*. [1]. Bone attends to these roles by a continuously modeling and remodeling process through the activities of two main cell populations: osteoblasts (OBs) and osteoclasts (OCs), which are responsible of bone formation and bone resorption, respectively. OBs originate from mesenchymal stem cells (MSCs) precursors through several microenvironment signals [2–5], such as Wingless-Type MMTV Integration Site Family members (Wnt), transforming growth factor-beta (TGF-β), bone morphogenetic proteins (BMPs), differentiating into osteocytes and synthetizing bone matrix. OCs originate from peripheral blood mononuclear cells (PBMCs) precursors differentiating by the effect of Receptor Activator of Nuclear Factor Kappa-B Ligand (RANKL)/osteoprotegerin (OPG) ratio and degrading bone matrix [6].

MicroRNAs (miRNAs) are endogenous small noncoding RNA molecules (of about 15–25 nucleotides), which regulate post-transcriptional gene expression through targeting of mRNA that have partially complementary sequences, inhibiting their translation or enhancing their degradation [7]. Mature miRNAs are generated by the sequential cleavage of precursor transcripts, indicated as pri-miRNAs, that is cleaved in the nucleus by Drosha and the Di George syndrome critical region gene 8 (DGCR8) complex, producing a hairpin of 70–100 nucleotides, named pre-miRNA. Pre-miRNAs are subsequently exported in the cytoplasm by Exportin 5, where it is bound by the ribonuclease Dicer that produces a double-stranded mature miRNA [8]. One miRNA of two strands will bind with Argonaut 2 (AGO2) protein to form a complex, that direct the pairing of miRNA with 3′ Untranslated Region (3′UTR) of target mRNAs, determining inhibition of translation or mRNA degradation [9]. MiRNAs play an important role in many physiological and pathological contexts. In physiological processes, miRNAs regulate bone formation and bone resorption, thus contributing to the maintenance of bone homeostasis. Under pathological conditions, a deregulation of miRNA signaling contributes to the onset and progression of skeletal disorders, *e.g.* osteoporosis. However, despite the high number of studies and research on this issue, the exact mechanisms through which miRNAs regulate the interaction between the different cell types in the bone remodeling unit under physiologic conditions or in bone diseases, are still restricted.

The current review represents an exhaustive overview of all miRNAs with validated targets that have a role in osteogenesis and/or osteoclastogenesis, focusing on their deregulation and establishment of perturbation of bone homeostasis. In addition, in order to highlight the

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role of relevant miRNAs in osteolytic related bone diseases, such as osteoporosis, clinical studies that identified deregulated miRNAs in osteoporotic patient cohorts with the intent of using them as markers of bone status are also reported.

2. Research strategy

The following literature research was carried out in the MEDLINE database (PubMed research engine): ((miRNA) AND (((osteoblast differentiation) OR osteoclast differentiation) OR osteoporosis)), by considering articles with abstracts in English (AND "English" [language]) and published after January 1, 2008 (AND ("2008/01/01"[Date - Entrez]: "2018/09/01"[Date - Entrez])). This search retrieved 619 articles. Reviews (NOT Review [Publication Type]) were then excluded, reducing the number of collected articles to 519. Four reviewers manually assessed the titles and abstracts of the collected articles; those not considered pertinent were excluded (*e.g.* (a) *in vitro* and pre-clinical studies that did not present clear relationship between miRNA and osteoblast or osteoclast differentiations and activities, (b) articles on miRNA, whose targets were not validated, or (c) clinical studies not pertaining to osteoporosis). Finally, an additional 17 references were added manually, as they were considered of interest to up-grade information on some technical aspects, in order to have a major understanding of mechanisms acting in bone regulation and osteoporosis development (Fig. 1).

Tables 1 to 3 report different miRNAs involved in the regulation of bone metabolism by modulating the osteoblastogenesis and osteoclastogenesis or connected to osteoporosis.

3. MicroRNA involved in osteoblast differentiation

The role of several miRNAs in the regulation of OB differentiation by targeting positive or negative regulators of osteogenesis is increasingly evidenced. MiRNAs target transcription factors and signaling molecules of the most knowledge pathways of osteoblastogenesis, as well as of OB functions, while others implicated in OC differentiation are able to modify the balance between bone formation and resorption, determining alterations in bone homeostasis.

Figs. 2 to 4 illustrate Wnt (Fig. 2), BMPs (Fig. 3) and TGF-β (Fig. 4) signal pathways, playing a key role in the regulation of osteoblastogenesis [4,5]. These pathways interact positively or negatively with other signals such as Fibroblast Growth Factor (FGF), Hedgehog (Hh), Notch and Insulin Growth Factor 1 (IGF1) (Fig. 5), in which Runt-related transcription factor 2 (RUNX2), special AT-rich binding transcription factor 2 (SATB2) and Osterix (OSX), key transcriptional regulators of OB activities, represent common regulated factors.

Different evidences highlight as deregulation of miRNA levels that act in these pathways are able to alter bone regulation, determining increasing or decreasing OB differentiation. Table 1 reports, in increasing alphabetical and numerical order, a list of the miRNAs with validated targets involved in osteoblastogenesis.

3.1. MiRNA involved in Wnt signaling pathways

Wnt canonical signal through Frizzled/Low Density Lipoprotein Receptor-Related Protein (LRP)5/6 activation, determines Axin sequestration, disrupting Axin/Adenomatous Polyposis Coli (APC)/Glycogen Synthase Kinase 3β (GSK3β) complex implicated in β-catenin degradation (Fig. 2). Free β-catenin is translocated into the nucleus where, with Transcription Factor (TCF) and Lymphoid enhancer-binding factor (LEF), activates the transcription of TGF-β and BMP2 genes. Furthermore, Wnt signal enhances transcription of both OPG and RANKL, determining an overall reduction of RANKL/OPG ratio, inhibiting OC differentiation and promoting overall bone formation. Wnt non-canonical pathway determines the activation of RUNX2 through co-factor ROR2 or RYK, leading OB differentiation commitment and inhibition of Peroxisome Proliferator Activated Receptor Gamma (PPARG) pathway, which is involved in adipocyte differentiation [4].

Micro-RNAs that target Wnt receptor or co-receptor mRNA determine an attenuation of Wnt signals resulting in decreased OB differentiation. In fact, miR-376c negatively regulates OB differentiation by preventing β-catenin accumulation and transactivation by direct targeting of Wnt3 mRNA, blocking this pathway [10]. Li et al. showed how miR-23a inhibits OB differentiation by direct targeting of LRP5 mRNAs [11]. Similarly, miR-30e positively regulates adipocyte differentiation and negatively regulates OB differentiation by direct targeting of LPR-6 mRNA [12]. MiR-376c also targets ADP Ribosylation Factor - Guanine nucleotide exchange factor-1 (ARF-GEF-1) involved in the activation of small GTPases in different intracellular pathways as Wnt

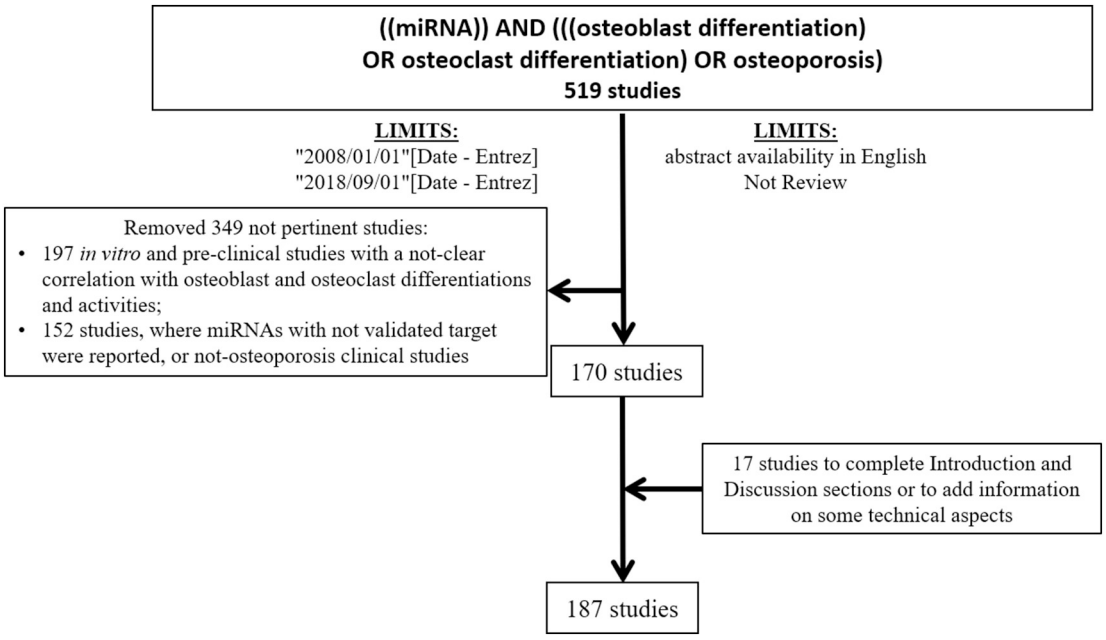

Fig. 1. Flowchart of the research strategy and selection of bibliographic references.

Table 1
List of miRNAs implicated in osteogenesis in *in vitro*, *in vivo* and clinical studies.

miRNA	Target gene	Pathway/enzyme	Study type	Involved cells/ *in vivo* model/ clinical samples	Effect on OB differentiation	References
Let-7b	MMP1	Matrix degradation	*In vitro*	hMSCs	Suppression	[122]
Let-7f	AXIN2	Wnt	*In vitro*	hMSCs	Promotion	[13]
miR-10b	BCL6	Runx2	*In vitro*	MC3T3-E1	Suppression	[96]
miR-15b	SMURF1	Tgf-β	*In vitro*	hBMSCs	Promotion	[68]
miR-16	SMAD5	Bmp	*In vitro*	hBMSCs	Suppression	[41]
	ACVR2A	Tgf-β				
miR-17	BMP2	Bmp	*In vitro*	hADSCs	Suppression	[34]
	SMAD5	Bmp	*In vitro*	C2C12; MC3T3-E		[42]
			In vivo	C57BL/6J mice		
	TCF3	Wnt	*In vitro*	hPDLSCs	Promotion	[21]
	SMURF1	Tgf-β				
miR-20a	BAMBI	Bmp	*In vitro*	hBMSCs	Promotion	[56]
	CRIM1					
	PPARG	Wnt; Fgf				[57]
	KDM6B	Tgf-β		3T3-L1; ST2 cells; C3H10T1/2	Suppression	[63]
	TGFBR2					
miR-21	SMAD7	Bmp; Tgf-β	*In vitro*	mMSCs	Promotion	[58]
	SPRY1	Mapk	*In vitro*	mMSCs;		[73]
				MC3T3-E1		
			in vivo	C57BL/6J mice		
			Clinic	hBMSCs		
			In vitro	hBMSCs		[74]
			In vivo	C57BL/6 mice;		
				miR-21$^{-/-}$ mice		
			Clinic	Serum samples		
	RECK	Matrix degradation	*In vitro*	mMSCs		[124]
			In vivo	C57BL/6J mice		
miR-23a	LRP5	Wnt	*In vitro*	hBMSCs	Suppression	[11]
	RUNX2	Runx2	*Clinic*	Bone samples;		[101]
				blood samples		
			In vitro	C3H10T1/2; C2C12; NIH3T3; 3T3-L1		[86]
	SATB2	Runx2	*In vitro*	rOBs;		[102]
				MC3T3-E1		
			In vivo	C57BL/6 mice;		[103]
				miR-23a Tg mice		
miR-23b	RUNX2	Runx2	*In vitro*	hBMSCs	Suppression	[93]
			In vivo	C57BL/6J mice		
	SMAD 3	Tgf-β	*In vitro*	MC3T3-E1		[65]
miR-24	RUNX2	Runx2	*In vitro*	rOBs;	Suppression	[102]
				MC3T3-E1		
		Runx2		hBMSCs; MG63;		[94]
				rOBs		
	SATB2	Runx2		rOBs;		[102]
				MC3T3-E1		
		Runx2		hBMSCs; MG63;		[94]
				rOBs		
	TCF1	Wnt		hBMSCs;		[22]
				MC3T3-E1		
miR-26a	GSK3B	Wnt	*In vitro*	mBMSCs	Promotion	[14]
	SMAD1	Bmp		mADSCs		
		Bmp		hADSCs		[45]
	TOB1	Bmp	*In vitro*	mBMSCs		[59]
			In vivo	C57BL/6J mice		
miR-27	SFRP1	Wnt	*In vitro*	MG63; hFOBs	Promotion	[25]
	APC	Wnt	*In vitro*	MDPC-23		[16]
			In vitro	C2C12; MC3T3-E1		[17]
	MEF2C	Bmp, Tgf-β	*In vitro*	hMSCs	Suppression	[55]
			In vivo	BALB/c mice		
			Clinic	Blood samples		
miR-29a	DKK1	Wnt	*In vitro*	hFOB;	Promotion	[26]
	KREMEN2	Wnt		primary hOBs		
	SFRP2	Wnt				
	ON	Matrix mineralization	*In vitro*	ABSa15		[125]
	HDAC4	Runx2	*In vitro*	mBMSCs		[91]
			In vivo	FVB mice;		
				FVB/TNar-Tg-29a/PGK mice		

(*continued on next page*)

Table 1 (*continued*)

miRNA	Target gene	Pathway/enzyme	Study type	Involved cells/ *in vivo* model/ clinical samples	Effect on OB differentiation	References
miR-29b	COL1A1	Matrix synthesis	*In vitro*	MC3T3-E1	Promotion	[19]
	COL5A3	Matrix synthesis				
	COL4A2	Matrix synthesis				
	HDAC4	Runx2				
	TGFB3	Tgf-β				
	ACVR2A	Tgf-β				
	CTNNBIP1	Wnt				
	DUSP2	Inflammation				
miR-29c	ON	Matrix mineralization	*In vitro*	hFOB; primary hOBs	Promotion	[26]
miR-30a	SMAD1	Bmp	*In vitro*	MC3T3-E1	Suppression	[44]
miR-30b	SMAD1	Bmp	*In vitro*	MC3T3-E1	Suppression	[44]
miR-30c	RUNX2	Runx2	*In vitro*	MC3T3-E1	Suppression	[87]
		Runx2		C3H10T1/2; C2C12; NIH3T3; 3T3-L1		[86]
	SMAD1	Bmp		MC3T3-E1		[44]
miR-30d	SMAD1	Bmp	*In vitro*	MC3T3-E1	Suppression	[44]
miR-30e	LRP6	Wnt	*In vitro*	mBMSCs; 3T3-L1; MC3T3-E1	Suppression	[12]
miR-31	SATB2	Runx2	*In vitro*	hMSCs	Suppression	[104]
	RUNX2	Runx2	*In vitro*	MCF-7; SaOS2; MG-63; U2OS; hBMSC		[105]
miR-33	HMGA2	Igf	*In vitro*	MC3T3-E1	Promotion	[128]
	SATB2	Runx2	*In vitro*	mBMSCs	Suppression	[100]
miR-34a	JAG1	Notch	*In vitro*;	hMSCs;	Suppression	[80]
	CDC25A	Cell cycle	*In vivo*	hMSC-TERT		
	CDK4	Cell cycle				
	CDK6	Cell cycle				
	CYCLIN D1	Cell cycle				
	E2F3	Cell cycle				
miR-34b	SATB2	Runx2	*In vitro*	mOBs	Suppression	[78]
			In vivo	129Sv/EV mice		
miR-34c	NOTCH1	Notch	*In vitro*	C2C12	Suppression	[79]
			In vivo	FVB/N mice		
	SATB2	Runx2	*In vitro*	C2C12		
			In vivo	FVB/N mice		
	RUNX2	Runx2	*In vitro*	C3H10T1/2; C2C12; NIH3T3; 3T3-L1		[86]
miR-92a	PTEN	Igf	*In vitro*	HAL; HOS; 143B; IOR/MOS; IOR/OS9; IOR/OS10; IOR/OS14; IOR/OS15; IOR/OS18; SARG; KPD; MG-63; MHM; MNNG/HOS; OHS; OSA; Saos-2; U-2OS; ZK-58	Promotion	[84]
miR-93	BMP2	Bmp	*In vitro*	hBMSCs	Suppression	[36]
	OSX	Runx2	*In vitro*	mOBs		[107]
miR-96	OSX	Runx2	*In vitro*	mBMSCs	Suppresses OB differentiation promotion	[111]
			In vivo	C57BL/6J mice		
			Clinic	Human bone marrow samples		
	HBEGF	Inflammation	*In vitro*	MC3T3-E1; mBMSCs		[120]
			In vivo	C57BL/6J mice		
miR-98	SOST	Wnt	*In vitro*	Saos-2; U-2OS; hFOB 1.19	Promotion	[29]
	FIAT		*In vitro*	Primary mOB		[54]
miR-100	BMPR2	Bmp	*In vitro*	hADSCs	Suppression	[38]
	SMAD1	Bmp	*In vitro*	MC3T3-E1		[46]
miR-103a	RUNX2	Runx2	*In vitro*	hFOB 1.19; hBMSCs	Suppression	[88]
			In vivo	C57BL/6J mice		
miR-106	BMP2	Bmp	*In vitro*	hADSCs	Suppression	[34]
			In vitro	Placental MSCs		[35]
			In vivo	C57BL/6 mice		
miR-124	DLX2	Bmp	*In vitro*	hBMSCs; mBMSCs; C2C12; MC3T3-E1	Suppression	[47]
			In vivo	NIH-bg-nu-xid nude mice		
	DLX3	Bmp	*In vitro*	hBMSCs; mBMSCs; C2C12; MC3T3-E1		
			In vivo	NIH-bg-nu-xid nude mice		
	DLX5	Bmp	*In vitro*	hBMSCs; mBMSCs; C2C12; MC3T3-E1		
			In vivo	NIH-bg-nu-xid nude mice		
miR-125b	BMPR	Bmp	*In vitro*	hBMSCs	Suppression	[39]
			In vivo	BALB/c nude mice		
	OSX	Runx2	*In vitro*	hBMSCs		[108]
			Clinic	Bone marrow samples		
miR-133	RUNX2	Runx2	*In vitro*	C2C12	Suppression	[23]
		Runx2		Vascular smooth muscle cells (VSMCs)		[24]
	ZIP1	Micro-elements recovery	*In vitro*	hMSCs; mMSCs		[127]
			In vivo	C57BL/6J mice		
			Clinic	Bone marrow samples		
miR-135a	RUNX2	Runx2	*In vitro*	MC3T3-E1	Suppression	[87]
	SMAD5	Bmp		C2C12		[23]
miR-135b	OSX	Runx2	*In vitro*	USSCs	Suppression	[109]
miR-137	RUNX2	Runx2	*In vitro*	MC3T3-E1	Suppression	[87]

(*continued on next page*)

Table 1 (*continued*)

miRNA	Target gene	Pathway/enzyme	Study type	Involved cells/ *in vivo* model/ clinical samples	Effect on OB differentiation	References
miR-138	FAK	Cell interactions; Wnt	*In vitro*	hBMSCs	Suppression	[20]
miR-140	TGFB3	Tgf-β	*In vitro*	MC3T3-E1	Suppression	[62]
miR-141	DLX5	Bmp	*In vitro*	MC3T3-E1	Suppression	[48]
miR-142	APC	Wnt	*In vitro*	hFOB1.19	Promotion	[18]
miR-148a	KDM6B	Tgf-β	*In vitro*	mBMSCs; ST2 cells	Suppression	[64]
miR-153	BMPR2	Bmp	*In vitro*	hBMSCs	Suppression	[40]
miR-155	SOCS1	Tnf-α	*In vitro*	MC3T3-E1	Suppression	[118]
	SMAD5	Bmp		MC3T3-E1		[43]
miR-181a	TGFB1	Tgf-β	*In vitro*	C2C12; MC3T3-E1; mOBs	Promotion	[61]
	TGFBR1	Tgf-β				
miR-188	HDAC-9	Wnt; Fgf	*In vitro*	mBMSCs; hBMSCs	Suppression	[76]
			In vivo	C57BL/6 mice		
			Clinic	Bone marrow samples		
	RICTOR	Wnt; Fgf	*In vitro*	mBMSCs; hBMSCs		
			In vivo	C57BL/6 mice		
			Clinic	Bone marrow samples		
miR-194	STAT1	Runx2	*In vitro*	mBMSCs	Promotion	[97]
miR-195	VEGF	Inflammation	*In vitro*	hMSCs; MC3T3-E1	Suppression	[121]
			In vivo	Chicken embryo (*Gallus gallus*)		
miR-196a	HOXC8	Bmp	*In vitro*	hADSCs	Promotion	[49]
miR-200a	DLX5	Bmp	*In vitro*	MC3T3-E1	Suppression	[48]
miR-204/211	RUNX2	Runx2	*In vitro*	ST2; C2C12; C3H10T1/2; hMSCs	Suppression	[95]
		Bmp		C3H10T1/2; C2C12; NIH3T3; 3T3-L1	Suppression	[86]
	ATG 14	Autophagy	*In vitro*	hiPSCs	Promotion	[130]
miR-205	RUNX2	Runx2	*In vitro*	MC3T3-E1	Suppression	[87]
miR-206	CX43	Cell interactions	*In vitro*	C2C12	Suppression	[126]
			In vivo	FVB mouse		
miR-208	ETS1	Bmp	*In vitro*	MC3T3-E1	Suppression	[52]
	ACVR1	Tgf-β	*In vitro*	MC3T3-E1	Suppression	[67]
			In vivo	C57BL/6 mice		
miR-210	ACVR1B	Tgf-β	*In vitro*	ST2; NRG	Promotion	[66]
	PPARG	Wnt; Fgf	*In vitro*	hBMSCs	Suppression	[77]
miR-214	ATF4	Bmp	*In vitro*	MC3T3-E1; hFOB 1.19; mBMSCs	Suppression	[53]
			In vivo	C57BL/6J F2 mouse		
			Clinic	Bone tissue samples		
	FGFR1	Fgf	*In vitro*	mBMSCs		[69]
			In vivo	C57BL/6J mice		
miR-216a	c-CBL	Apoptosis	*In vitro*	hADSCs; hucMSCs	Promotion	[83]
			In vivo	NOD/SCID mice		
miR-217	RUNX2	Runx2	*In vitro*	MC3T3-E1	Suppression	[87]
miR-218	SFRP2	Wnt	*In vitro*	MC3T3-E1; mBMSCs	Promotion	[27]
	DKK2					
	TOB1					
	SOST					
	SFRP2			mBMSCs; ADSCs		[28]
	DKK2					
miR-221	DKK2	Wnt	*In vitro*	mOBs	Suppression	[30]
miR-222	SMAD5	Bmp	*In vitro*	hBMSCs	Suppression	[89]
	RUNX2	Runx2				
miR-223	FGF2R	Fgf	*In vitro*	mBMSCs; C3H10T1/2; ST2	Suppression	[70]
	IGF1R	Igf	*In vitro*	MC3T3-E1		[81]
miR-320a	HOXA10	Bmp	*In vitro*	hBMSCs	Suppression	[50]
miR-335	DKK1	Wnt	*In vitro*	C3H10T-1/2; MC3T3-E1; MLO-Y4; mOBs	Promotion	[31]
miR-338	RUNX2	Runx2	*In vitro*	MC3T3-E1	Suppression	[87]
		Runx2	*In vitro*	mBMSCs; MC3T3-E1		[71]
			In vivo	BALB/c mice		
		Runx2	*In vitro*	hOBs		[92]
			In vivo	Wistar rats		
	FGFR2	Fgf	*In vitro*	mBMSCs; MC3T3-E1		[71]
			In vivo	BALB/c mice		
miR-346	GSK3B	Wnt	*In vitro*	hBMSCs	Promotion	[15]
miR-351	VDR	Vitamin D	*In vitro*	rBMSCs	Suppression	[114]
miR-370	BMP2	Bmp	*In vitro*	MC3T3-E1; mOBs	Suppression	[37]
miR-376c	WNT3	Wnt	*In vitro*	mOBs	Suppression	[10]
	ARF/GEF					
miR-377	CDK6	Cell cycle	*In vitro*	MG-63; hFOB1.19	Suppression	[129]
miR-378	CASP	Caspase	*In vitro*	MC3T3-E1	Promotion	[85]
miR-422a	TGFβ2	Tgf-β	*In vitro*	MG-63; Saos-2; U-2 0S; hFOBs	Suppression	[60]
miR-433	DKK1	Wnt	*In vitro*	hFOB1.19; Rat ROS17/2.8; rBMSCs	Promotion	[32]

(*continued on next page*)

Table 1 (*continued*)

miRNA	Target gene	Pathway/enzyme	Study type	Involved cells/ *in vivo* model/ clinical samples	Effect on OB differentiation	References
miR-483	ERK1	Mapk	*In vitro*	hADSCs	Suppression	[75]
			In vivo	Balb/c nude mice		
miR-494	FGFR2	Fgf	*In vitro*	MC3T3-E1	Suppression	[72]
miR-497	IGF1R	Igf	*In vitro*	HOS; U2OS; hFOBs	Suppression	[82]
miR-542	SFRP1	Wnt	*In vitro*	rBMSCs	Promotion	[33]
			In vivo	Sprague–Dawley rats		
miR-628	RUNX2	Runx2	*In vitro*	MG63	Suppression	[90]
miR-637	OSX	Runx2	*In vitro*	hMSCs	Suppression	[110]
			In vivo	Athymic nude mice		
	BMP2K	Bmp	*In vitro*	hOBs; MG-63		[106]
miR-705	HOXA10	Bmp	*In vitro*	mBMSCs	Suppression	[51]
			in vivo	C57BL/6J mice		
miR-764	CHIP/STUB1	Runx2	*In vitro*	MC3T3-E1; L mOBs	Promotion	[98]
miR-1192	HBEGF	Inflammation	*In vitro*	C2C12; MC3T3; C3H10T1/2	Promotion	[119]
miR-1228	BMP2K	Vitamin D, Bmp	*In vitro*	hOBs; MG-63	Suppression	[106]
miR-2861	HDAC5	Runx2	*In vitro*	mOBs; ST2; mBMSCs	Promotion	[99]
			In vivo	C57BL/6 mice		
miR-3077	RUNX2	Runx2	*In vitro*	mBMSCs	Suppression	[51]
			In vivo	C57BL/6J mice		

signaling [10]. The expression of these miRNAs determines a decrease of Wnt signal, resulting in an inhibition of osteoblastogenesis. On the contrary, the expression of miRNAs that targets factors implicated in β-catenin inhibition, determines an induction of osteoblast differentiation. During the early stage of OB differentiation of MSCs, Lethal-7f (Let-7f) expression is up-regulated and determines a reduction of Axin-2 level, a repressor of β-catenin activity and consequently an induction of OB differentiation [13]. In Bone Marrow Mesenchymal Stem Cells (BMSCs), it is demonstrated as miR-26a and miR-346 target GSK3β mRNA, promoting osteogenesis [14,15]. MiR-27a and miR-142 act as potent promoters of differentiation in pre-OBs by affecting a negative regulator of Wnt signal, APC [16–18]. Expression of miR-142 promotes OB differentiation, by targeting APC mRNA and enhancing Wnt pathway through β-catenin action [18]. PTH and E-Caderin pathways help the activation of β-catenin through their stabilization. Catenin Beta Interacting Protein 1 (CTNNBIP1) is another negative regulator of osteogenesis that block the interaction between β-catenin with TCF, inhibiting transcription of downstream effectors of Wnt pathway. MiR-29b supports OB differentiation by inhibiting the expression of CTNNBIP1 mRNA [19]. miR-138 inhibits osteogenic differentiation by direct targeting of focal adhesion kinase (FAK) mRNAs. FAK is implicated in the transduction of integrin signals, enhancing Wnt signal. Mechanical tension load application determines a down-regulation of miR-138 expression by H19 sponge activity, thus promoting osteogenesis [20]. Finally, miRNAs that affect directly the expression of downstream transcription factors of Wnt pathway block osteogenesis. In human periodontal ligament tissue-derived mesenchymal stem cells (PDLSCs), it was found that miR-17 acts by targeting of Transcription Factor 3 (TCF-3) negative regulators of osteoblastogenesis, thus promoting osteogenesis [21]. In the study of Zhao et al., it is shown that miR-24 inhibits osteogenic differentiation through block of translation of TCF-1 mRNA, a key downstream transcription factor of Wnt/β-catenin [22]. Another miRNA, miR-133, is able to suppress osteogenesis by TCF-7 mRNA targeting [23,24].

Wnt signal is blocked by different molecules that bind Wnt molecules or interfere with receptor or co-receptors inhibiting the binding. Dikkopf (Dkk), Kremen and sclerostin factors that inhibit LRP5/6 co-factor, while Secreted Frizzled Related Protein (sFRP) binds Wnt factors. miRNAs that target the mRNA of these negative regulators induce osteogenic differentiation. In fact, miR-27a also affects sFRP1 expression [25] inducing differentiation in pre-OBs. Furthermore, miR-29a targets different negative regulators of Wnt signaling, such as Dkk1, Kremen2, and sFRP2, and its expression promotes osteogenesis [26]. Also, miR-218 is induced by Wnt signal and promotes osteogenesis, targeting different inhibitors of Wnt pathway as Dkk1, Sclerostin (SOST) and sFRP2 mRNA. The expression of these miRNAs creates a positive feedback loop that optimizes Wnt signaling to promote OB differentiation in different MSCs [27,28]. Other miRNAs implicated in the promotion of osteogenesis are miR-98, miR-221, miR-335, miR-433 and miR-542. In particular, miR-98 exerts its functions by targeting of SOST mRNA, thus inducing OB differentiation [29]. The expression of miR-221 attenuates terminal OB differentiation through Dkk2 mRNA targeting, required for OB maturation [30]. Expression miR-335 and miR-433 promotes osteoblastogenesis by targeting of the 3′UTR of Dkk1 mRNA. In particular, miR-335 is robustly expressed in pre-OB during OB differentiation, increasing the response of these cells to Wnt signaling and promoting osteogenesis [31,32]. MiR-542 exerts its activity through sFRP1 mRNA targeting, promoting osteogenesis. In rat, miR-542 expression prevents ovariectomy-induced osteoporosis and is also found down-regulated in postmenopausal osteoporotic patients, thus indicating its involvement in this pathology [33].

3.2. MiRNA involved in BMP signaling pathways

The binding of BMP to its receptor (BMPR) induces the auto-phosphorylation of BMPR, and SMA- And MAD-Related Protein 1/5/8 (Smad1/5/8) protein complex recruitment and activation of signaling cascade, with the binding of Smad 4 and translocation into the nucleus, where it determines the transcription of RUNX2, Distal-Less Homeobox 5 (DLX5) and OSX (Fig. 3). The auto-phosphorylation of BMPR also determines the activation of a kinase signaling cascade ending to p38 kinase, a MAPK family member, which leads to OB maturation through the phosphorylation of RUNX2, DLX5, and OSX [5].

The expression of miR-17, miR-93, miR-106, and miR-370 regulates osteogenic and adipogenic differentiation of human Adipose Stem Cells (ADSCs) by targeting of BMP2 mRNA. This determines the reduction of BMP signaling and Runx-2 factor production with the subsequently inhibition of osteogenic differentiation and increasing adipogenic CCAAT-Enhancer-Binding Proteins (C/EBPα) and PPARγ levels, resulting in a promotion of adipogenic differentiation [34–37]. Three miRNAs, miR-100, miR-125b and miR-153, exert its inhibition on ADSCs and MSCs osteogenesis through BMP receptors (BMPR1b and BMPR2) mRNA targeting [38–40]. In *in vivo* experiments, the inhibition of expression of miR-125b, shows a better capacity of BMSCs to repair bone defects [39]. The expression of miR-153 during osteogenic differentiation of hMSCs resulted significantly down-regulated, promoting

Table 2
List of miRNAs implicated in osteoclastogenesis in *in vitro*, *in vivo* and clinical studies.

miRNA	Target gene	Pathway /enzyme	Study type	Involved cell	Effect on OC differentiation	References
Let-7e	ITGA4	Cell interaction	*In vitro*	hPBMCs	Promotion	[137]
	THBS1	Cell interaction				
miR-7b	DC-STAMP	Cell interaction	*In vitro*	RAW264.7; mOCs	Suppression	[158]
miR-9b	C-CBL	Apoptosis	*In vitro*	RAW264.7; mBMMs; mOCs	Promotion	[161]
			In vivo	C57BL/6 mice		
miR-20a	ATG16L1	Autophagy	*In vitro*	RAW264.7	Suppression	[166]
miR-21	PDCD4	Rank	*In vitro*	hBMMs	Suppression	[74]
			In vivo	C57BL/6 mice; miR-21$^{-/-}$ mice		
miR-26a	CTGF	Rank	*In vitro*	mBMMs	Suppression	[159]
miR-29b	C-FOS	Rank	*In vitro*	hPBMCs	Suppression	[139]
	MMP2	Matrix degradation				
miR-29c	CALCR	Rank	*In vitro*	RAW264.7; mBMMs	Promotion	[150]
	NFIA					
	GPR85					
	CD93					
miR-34a	TGIF2	Tgf-β	*In vitro*	mBMMs	Suppression	[147]
			In vivo	C57BL/6J mice CAG34a-C57BL/6J mice		
miR-99b	IGF1R	Igf	*In vitro*	hPBMCs	Promotion	[137]
miR-124	NFATC1	Rank	*In vitro*	mBMMs	Suppression	[143]
	RAB27A	Osteoclast activity	*In vitro*	mBMMs		[157]
			In vivo	C57BL/6J mice		
	IL11	Inflammation	*In vitro*	RAW264.7; MC3T3-E1		[163]
			In vivo	Nude mice		
miR125a	TRAF6	Rank	*In vitro*	hPBMCs	Suppression	[135]
miR-126	MMP13	Matrix degradation	*In vitro*	mBMMs; spindle-like stromal cells of Giant cell tumor (GCTSC)	Suppression	[154]
miR-133	CXCL11	Rank	*In vitro*	hPBMCs	Promotion	[174]
			Clinic	Blood samples		
	CXCR3	Rank	*In vitro*	hPBMCs		
			Clinic	Blood samples		
	SLC39A1	Micro-elements recovery	*In vitro*	hPBMCs		
			Clinic	Blood samples		
miR-142	GP130	Inflammation	*In vitro*	mPBMCs	Suppression	[165]
miR-145	OPG	Rank	*In vitro*	MG-63	Promotion	[133]
miR-146a	TRAF6	Rank	*In vitro*	hPBMCs	Suppression	[136]
miR-148a	MAFB	Rank	*In vitro*	hPBMCs	Promotion	[144]
			In vivo	C57BL/6 mice		
			Clinic	Blood samples		
miR-155	MITF	Rank; M-Csf	*In vitro*	RAW264.7; mBMMs	Suppression	[140]
			In vivo	C57BL/6 mice		
	SOCS1	Rank; M-Csf	*In vitro*	RAW264.7; mBMMs		
			In vivo	C57BL/6 mice		
miR-181a	C-CBL	Apoptosis	*In vitro*	RAW264.7; mBMMs; mOCs	Promotion	[161]
			In vivo	C57BL/6 mice		
miR-183	HO1	Oxidative stress	*In vitro*	Raw264.7; mBMMs	Promotion	[167]
miR-186	CTSK	Matrix degradation	*In vitro*	RAW264.7; mBMMs	Suppression	[156]
			In vivo	C57BL/6J mice		
miR-204/211	IL11	Inflammation	*In vitro*	MSCs	Suppression	[164]
miR-214	PTEN	M-Csf	*In vitro*	mBMMs; OC-TG214	Promotion	[148]
			In vivo	C57BL/6J mouse; OC-TG214 mouse		
	TRAF3	Rank	*In vitro*	RAW264.7		[149]
			In vivo	C57BL/6J mouse; miR-214-3p flox/ − C57BL/6J mouse		
miR-218	TLR4	Inflammation	*In vitro*	RAW264.7	Suppression	[138]
miR-222	C-SRC	Rank	*In vitro*	RAW264.7	Suppression	[142]
miR-223	NF1A	M-Csf	*In vitro*	mBMMs	Promotion	[151]
			In vitro	RAW 264.7; mBMMs		[152]
			In vivo	DBA/1 mice		
			Clinic	Synovial tissue samples		
Mir-335	RANKL	Rank	*In vitro*	mPBMCs	Suppression	[132]
			In vivo	NOD/SCID mice		
	IGF1R	Igf	*In vitro*	mPBMCs		
			In vivo	NOD/SCID mice; NOD/SCID IL2R$_{\gamma}^{null}$ mice		
miR-338	RANKL	Rank	*In vitro*	mBMMs	Suppression	[131]
miR-365	MMP9	Matrix degradation	*In vitro*	mOBs; MC3T3-E1; RAW 264.7; mBMMs	Suppression	[155]
			In vivo	C57BL/6J mice		
miR-422a	C-CBL	Rank	*In vitro*	hPBMCs	Suppression	[162]
			Clinic	Blood samples		

(*continued on next page*)

Table 2 (*continued*)

miRNA	Target gene	Pathway /enzyme	Study type	Involved cell	Effect on OC differentiation	References
miR-503	RANK	Rank	*In vitro* *In vivo* *Clinic*	hPBMCs C57BL/6 mice Blood samples	Suppression	[134]
miR-532	MMP13	Matrix degradation	*In vitro*	UMR 106–01; rOBs;	Suppression	[153]
miR-618	TLR4	Inflammation	*In vitro*	RAW264.7	Suppression	[138]
miR-1270	IRF8	Rank	*In vitro* *Clinic*	hPBMCs Blood samples	Promotion	[145]
miR-9718	PIAS3	Inflammation	*In vitro* *In vivo*	mBMMs; RAW 264.7 C57BL/6 mice	Promotion	[141]

osteogenic differentiation of hMSCs [40]. The transcription of miR-16, miR-17, miR-135a, miR-155 and miR-222 inhibits osteogenic differentiation through direct targeting of Smad5 mRNA [23,41–43]. Fang et al. observed that miR-17 can suppress osteogenic differentiation of pre-treated BMP2 immortalized mouse myoblast and mouse osteoblast precursor cell lines by targeting of Smad-5 [42]. Additionally, silencing miR-17 in sham-operated and ovariectomized (OVX) mice, increased bone formation and bone mass, resulting in an improvement of trabecular microarchitecture [42].

Increased levels of member miR-30 family (miR-30a, -30b, -30c, and -30d) negatively regulate the response of BMP2 signaling, through Smad1 mRNA targeting [44]. In ADSCs, miR-26a targets Smad1 mRNA, reducing their ability to differentiate in OB [14,45]. Fu et al. showed as miR-100 expression is related to reduced Smad1 levels through direct mRNA targeting [46].

The expression of miR-124 suppresses osteogenic differentiation and matrix mineralization, through direct targeting of the 3′UTRs of Dlx2, Dlx3 and Dlx5, implicated in osteoinductive cascade of BMP [47]. Similar to miR-124, miR-141 and miR-200a are able to inhibit OB differentiation by Dlx-5 direct mRNA targeting [48]. In ADSCs, miR-196a reduces proliferation and enhanced OB differentiation, without affecting adipogenic differentiation, through Homeobox C 8 (HOXC8) mRNA targeting [49]. Dlx factors and HoxC8 are implicated in the enhancing of Osterix activities, increasing osteogenesis and matrix mineralization.

Down-regulation of miR-320a and miR-705 promotes OB differentiation by up-regulation of Homeobox A 10 (HOXA10) level that is a transcription factor implicated in osteogenesis [50]. In particular, miR-705 was found up-regulated in MSCs of OVX mice by activation of Nuclear Factor Kappa B (NF-κB) pathway, inhibiting osteogenesis and positively adipogenesis [51]. Two miRNAs, miR-208 and miR-370, inhibit OB differentiation of pre-OB by targeting of Ets1 mRNA, implicated in the transactivation of OPN, PTH, Runx2, tenascin-C and type I pro-collagen, positive regulators of osteogenesis [37,52]. The level of miR-214 is correlated with a lower degree of bone formation in aged patients with fractures. Experiments in different mouse models revealed the inhibitory effect of miR-214 in bone formation by activating transcription factor 4 (ATF4)-targeting, through inhibition of OB activities and matrix mineralization [53]. Furthermore, miR-98 is implicated in the decreased expression of Factor inhibiting ATF4-mediated transcription (FIAT), a negative regulator of osteoblast differentiation, through direct targeting of its mRNA [54]. In a clinical study, it was found that miR-27 targets Myocyte Enhancer Factor 2C (Mef2c) in MSCs, which is an important factor inducing osteogenesis, enhancing Dlx expression. In MSCs, miR-27 determines then inhibition of OB differentiation [55].

In BMP pathway, several factors act as repressor: Noggin inhibiting BMP binding to its receptor, Smad6 inducing Smad 1/5/8 degradation, and Smad7 inhibiting auto-phosphorylation of BMPR and then Smad 1/5/8 complex phosphorylation. Increased level of miR-20a regulates negatively the expression of PPARγ, Bone Morphogenetic Proteins and Activin Membrane-Bound Inhibitor (BAMBI) and Cysteine-Rich Motor Neuron 1 (CRIM1), negative regulators of BMP signaling, in BMSCs inducing osteogenesis [56,57]. Furthermore, miR-21 negatively regulates the expression of Smad7 involved in the inhibition of OB differentiation, promoting osteogenesis [58]. Li et al. showed that miR-26a is negatively correlated with bone loss in OVX mice, where this miRNA act on inhibition of Transducer of Rb—B2 Receptor Tyrosine Kinase 1 (Tob1) expression, negative regulator of the BMP signaling, leading to the promotion of osteogenesis [59].

3.3. MiRNA involved in TGF-β signaling pathway

TGF-β factors exert a dual role on osteoblastic bone formation: the activation of proliferation and commitment of pre-OB, and the inhibition of late OB differentiation and mineralization (Fig. 4).

TGF-β, through activation of their receptors, determines Smad2/3 activation with Smad4 recruitment. This intracellular signal activation determines transcription of different factors such as Wnt factors that promote proliferation and OB commitment [5]. Expression of miR-29b supports OB differentiation by translation blocking of TGF-β3 mRNAs [19]. While miR-422a inhibits TGF-β2 expression by directly targeting its 3′UTR, a significant decrease of phosphorylation of Smad2/3 and inhibition of OB differentiation occurs [60]. Expression of miR-181a induces OB differentiation by direct targeting of TGF-β signaling molecules, as TGF-β Induced (TGFBI) and Receptor 1 (TGFBR1), inhibiting TGF-β signaling and promoting OB differentiation. Bhushan et al. (2013) demonstrated that miR-181a is up-regulated during BMP mediated OB induction of MSCs *in vitro*, and during physiological development of mouse tibia and calvaria *in vivo* [61]. The transcription of miR-140 is down-regulated under Wnt3a signaling activation determining up-regulation of its validated target TGF-β3, thus promoting OB differentiation [62]. In pre-adipocytes, miR-20a promotes adipogenic differentiation by targeting of TGF-β Receptor 2 (TGFBR-2) and Lysine-specific demethylase 6b (Kdm6b), a positive modulator of osteogenesis, important OB signaling implicated in inhibition of adipocytic differentiation [63]. Expression of miR-148a targets Kdm6b mRNA, inhibiting MSCs proliferation and OB differentiation and promoting adipogenic differentiation [64]. TGF-β pathway improves OB differentiation also with a parallel RUNX2 activation by p38k phosphorylation. The study of Liu et al. demonstrated that miR-23b reduces osteogenic differentiation by Smad3 targeting, then interfering together with TGF-β signal. This miRNA is up-regulated by Lipopolysaccharides (LPS) treatment, leading to an inhibition of osteoblastogenesis [65]. TGF-β signaling and activation of Smad2/3 determines inhibition of late differentiation of OBs, through Histone Deacetylase (HDAC) activation, repressing RUNX2 activities. Activin receptors, belonging to TGF-β receptor superfamily, exert inhibitory effect on osteoblastogenesis, stimulating Smad2/3 activation. In BMSCs, miR-210 promotes OB differentiation of ST2 cells *via* Activin Receptor 1b (AcvR1b) mRNA targeting [66]. Similarly, Arfat et al. found that miR-208 inhibited osteogenesis of pre-OB as well as reversed partially bone loss induced by hindlimb unloading [67]. Also miR-16 inhibits osteogenic differentiation by AcvR-2a mRNA targeting [41].

Table 3
List of miRNAs implicated in osteoporosis and reported in clinical studies.

miRNA	Up or down in osteoporosis	miRNA gene targets indicated in the study	Samples	References
Let-7b	Down	–	Blood samples	[181]
Let-7g	Down	–	Blood samples	[180]
miR-7b	Down	–	Blood samples	[181]
miR-10b	Up	–	Blood samples	[180]
miR-16	Down	–	Blood samples	[181]
	Up	SMAD5, ACVR2A	Bone marrow samples	[41]
miR-19a	Down	–	Blood samples	[181]
miR-19b	Down	–	Blood samples	[181]
miR-21	Down	SPRY1	Bone marrow samples	[73]
	Down	–	Blood samples	[179]
	Up	PDCD4	Blood samples; bone samples	[176]
	Up	–	Blood samples	[180]
	Down	SPRY1	Blood samples	[74]
	Down	SMAD7, SPRY1, DKK2, BMP3	Blood samples	[175]
	Up	PDCD4, SMAD7, SPRY1	Blood samples; bone samples	[177]
miR-22	Down	–	Blood samples	[180]
miR-23a	Up	RUNX2	Blood samples; bone samples	[176]
	Down	RUNX2, SATB2	Blood samples	[175]
	Up	RUNX2, SATB2	Blood samples; bone samples	[177]
miR-24	Up	RUNX2	Blood samples; bone samples	[176]
	Up	RUNX2, SATB2	Blood sample; bone samples	[177]
miR-25	Up	–	Bone samples	[176]
miR-27a	Down	MEF2C	Bone marrow samples	[55]
miR-29b	Down	–	Blood samples	[181]
	Down	COL3A1, COL5A3, PTHLH, DUSP2	Blood samples	[175]
	Down	–	Blood samples	[182]
miR-30b	Down	SMAD1	Blood samples	[186]
miR-30e	Down	LRP6	Blood samples	[181]
miR-34a	Down	TGIF2	Blood samples	[147]
miR-34c	Down	–	Blood samples	[181]
miR-93	Up	OSX	Blood samples; bone samples	[176]
	Up	OSX	Blood samples; bone samples	[177]
miR-96	Up	OSX	Blood samples	[111]
miR-100	Up	BMPR2	Blood samples; bone samples	[176]
	Up	BMPR2	Blood samples; bone samples	[177]
miR-122	Up	–	Blood samples; bone samples	[176]
	Up	–	Blood samples; bone samples	[177]
miR-124	Up	–	Blood samples; bone samples	[176]
	Up	–	Blood samples; bone samples	[177]
	Up	NFATC1	Blood samples	[175]
miR-125b	Up	OSX	Bone marrow samples	[108]
	Up	–	Blood samples; bone samples	[176]
	Up	–	Blood samples; bone samples	[177]
miR-130	Down	–	Blood samples	[181]
	Down	–	Blood samples	[186]
miR-133	Up	CXCL11, CXCR3, ZIP1	Blood samples	[174]
	Up	RUNX2	Blood samples	[179]
	Up	RUNX2	Blood samples	[180]
	Up	ZIP1	Bone marrow samples	[127]
	Up	RUNX2, ZIP1, CXCL11, CXCR3	Bone marrow samples	[185]
miR-140	Down	BMP2	Blood samples	[181]
miR-142	Down	–	Blood samples	[186]
miR-148a	Up	MAFB	Blood samples; bone samples	[176]
	Up	MAFB	Blood samples	[178]
	Up	MAFB	Blood samples; bone samples	[177]

(*continued on next page*)

Table 3 (*continued*)

miRNA	Up or down in osteoporosis	miRNA gene targets indicated in the study	Samples	References
miR-152	Up	DKK1	Blood samples	[181]
miR-186	Down	–	Blood samples	[181]
miR187	Down	IL6, TNFα	Blood samples	[183]
miR-210	Down	PPARG	Bone marrow samples	[77]
miR-214	Up	ATF4	Bone samples	[53]
miR-320a	Up	RUNX2, LEPR, CTNNB1	Bone samples	[184]
	Up	RUNX2, LEPR, CTNNB1	Blood samples	[181]
miR-324	Down	–	Blood samples	[181]
	Down	–	Blood samples	[182]
miR-328	Down	–	Blood samples	[180]
	Down	SFRP1	Blood samples	[186]
miR-335	Up	DKK	Blood samples	[181]
miR-378	Down	–	Blood samples	[181]
miR-422a	Up	CBL, TOB2	Blood samples	[162]
miR-483	Up	IGF2	Bone samples	[184]
miR-503	Down	RANK	Blood samples	[134]
miR-518	Up	–	Blood samples	[183]
miR-532	Down	–	Blood samples	[181]
miR-550	Down	–	Blood samples	[181]
	Down	–	Blood samples	[182]
miR-1270	Up	IRF8	Blood samples	[145]
miR-2861	Down	HDAC5	Bone samples	[99]
	Up	HDAC5	Blood samples	[175]

Smad7 and SMURF act as differentiation repressors of TGF-β pathway: Smad7 inhibiting the nuclear translocation of Smad2/3-Smad4 complex and both Smad7 and SMURF leading Smad2/3 degradation. MiR-21 negatively regulates the expression of Smad7 involved in the inhibition of OB differentiation, promoting osteogenesis. The pre-miR-15b is highly expressed in differentiated OBs. Its target is SMAD Ubiquitination Regulatory Factor 1 (Smurf1), implicated in Runx2 degradation. Up-regulation of miR-15b results in a decreased amount of Smurf1 and a consequent increased level of Runx2, favoring OB differentiation [68]. In hPDLSCs, it was found that miR-17 acts by targeting of Smurf 1, negative regulators of osteoblastogenesis, thus promoting osteogenesis [21]. In a clinical study, it was found that miR-27 targets Myocyte Enhancer Factor 2C (Mef2c) in MSCs, which is an important factor inducing osteogenesis. In MSCs, miR-27 determines inhibition of OB differentiation [55].

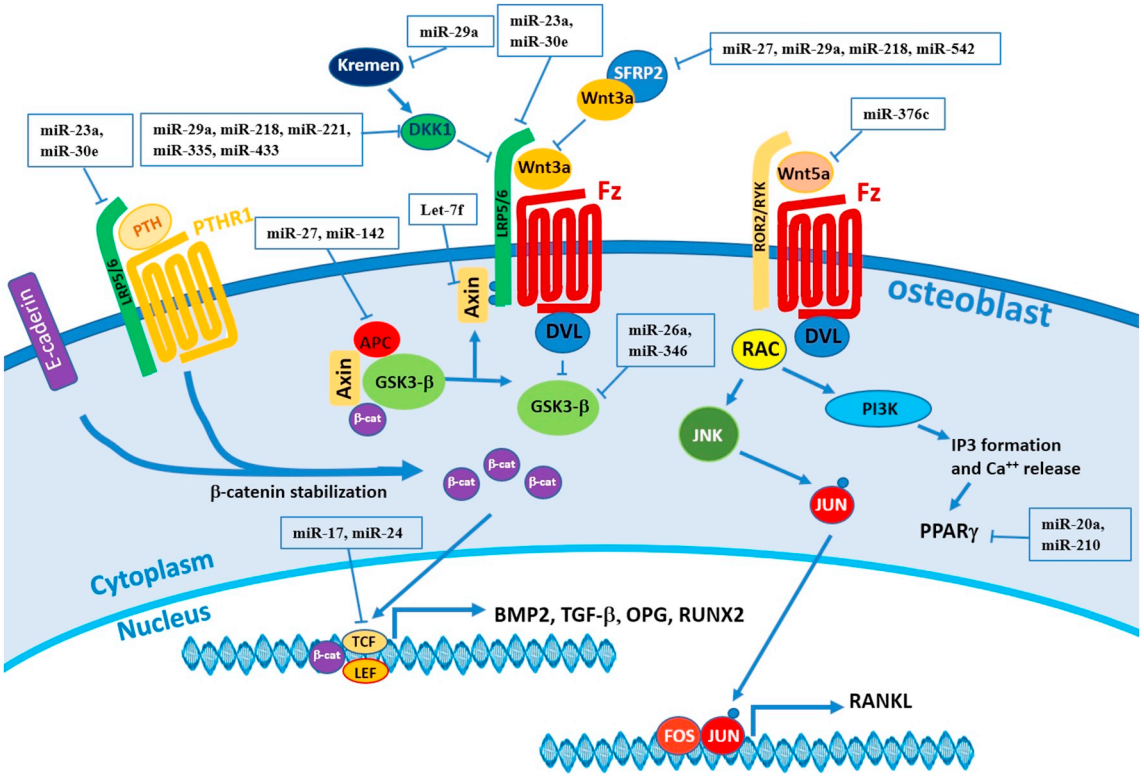

Fig. 2. Schematic drawing of Wnt pathway implicated in osteoblast differentiation, where the miRNA reported are those binding 3′UTR of the mRNA of the target protein.

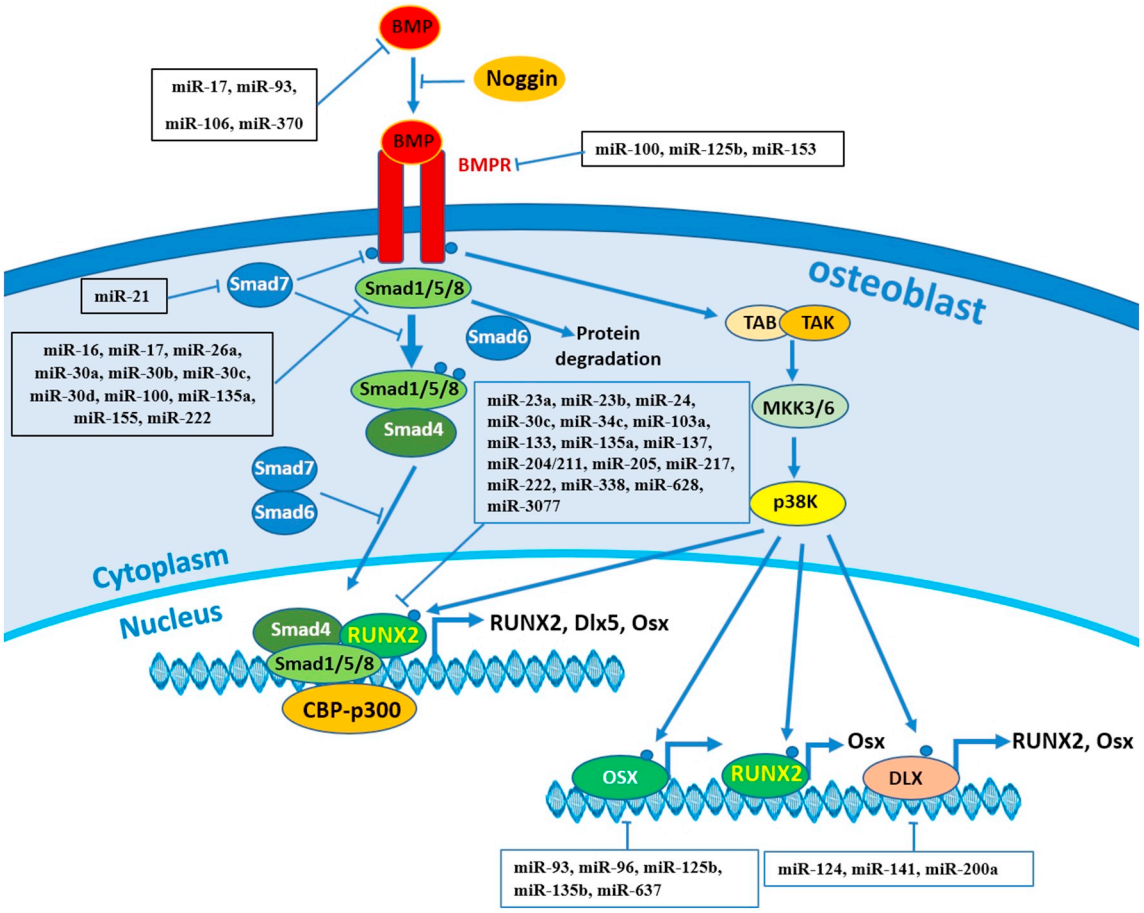

Fig. 3. Schematic drawing of BMP pathway implicated in osteoblast differentiation, where the miRNA reported are those binding 3′UTR of the mRNA of the target protein.

3.4. MiRNA involved in other osteogenic pathways

Several other pathways are implicated in the regulation of osteogenesis such as FGF, Notch, IGF, and Hh (Fig. 5).

FGF receptor (FGFR), activated by its ligand, determines phospholipase C (PLC) activation, catalyzing the formation of diacylglycerol (DAG) and inositol tri-phosphate (IP3). DAG activates protein kinase C (PKC) that increases RUNX2 activities by phosphorylation through MAPK activation. Furthermore, MAPK pathway with Extracellular Signal-Regulated Kinase (ERK) proteins activation determines nuclear exportation of ERF protein, an inhibitor of RUNX2 activities, improving osteogenesis [2].

The expression of miRNAs that targets FGFR, such as miR-214, miR-223 and miR-338, suppresses osteogenesis. In particular, miR-214 exerts its action by negatively regulation of expression of FGFR1 in BMSCs [69], while miR-223 and miR-338 inhibits differentiation of pre-OB cell lines into OB-like cells by targeting FGFR2 mRNA [70,71]. Furthermore, in osteoporotic mice model, miR-338 level is increased respect to sham-operated ones and inhibition of its expression is able to rescue OB differentiation potential of MSCs [71]. The mechano-sensitive miRNA, miR-494, increases in response to pressure solicitations on OBs; Iwawaki et al. described how in response of compressive force, induction of miR-494 represses OB proliferation and differentiation through targeting of Fgfr2 mRNA [72]. The expression of miR-21 regulates negatively Sprouty homolog 1 (SPRY1) mRNA, reducing protein level, preventing activation of MAPK cascade and then inhibiting Runx2 activation and activities [73,74]. Furthermore, miR-483 transcription inhibits osteoblastogenesis by 3′UTR targeting of ERK1 mRNA and promotes adipogenesis by consequent activation of PPARγ [75]. On the contrary, miR-188 promotes adipogenesis by direct targeting of HDAC9 and Rictor. Li et al. showed how BMSCs-specific overexpression of miR-188 reduces bone formation and improves bone marrow fat accumulation. In the aforementioned study, it has shown how the miR188 level in MSCs increases with age, determining a MSCs predisposition in aged organisms to differentiate in adipocytes [76].

On the contrary, miR-20a and miR-210 are able to inhibit adipocytes differentiation through decreases of PPARγ level, inducing OB differentiation of MSCs [56,57,77].

Notch signal activation determines proteolytic cleavage of its receptor, causing Notch intracellular domain (NICD) liberation and translocation into the nucleus, where with Core-Binding Factor α1 (CBFA1) induce transcription of HES and HEY, which negatively regulate RUNX2. Two miRNAs, miR-34b and miR-34c blocks osteogenic differentiation signal in mature OBs through Notch mRNA targeting [78,79]. MiR-34a decreases osteogenic differentiation by down regulation of Notch signaling through Jagged 1 (JAG1) targeting *in vitro* and *in vivo* [80].

Insulin-like growth factor 1 (IGF1) receptor autophosphorylation leads to Phosphatidylinositol 3 Kinase (PI3K) activation catalyzing the phosphorylation of phosphatidyl inositol diphosphate (PIP2) in PIP3, determining Protein Kinase B (Akt) activation that activates mTOR favoring OB survival and inhibits FOXO1 causing OCN, FGF and RUNX2 down-regulation. Two miRNAs, miR-223 and miR-497, target the IGF1 receptor (IGF1R), promoting OB cell apoptosis [81,82]. In particular, miR-223 expression induced by Advanced glycation end products (AGEs) is found to determine OB and endothelial cell apoptosis in diabetes mellitus (DM) [81].

The expression of miR-216a enhances OB differentiation of MSCs

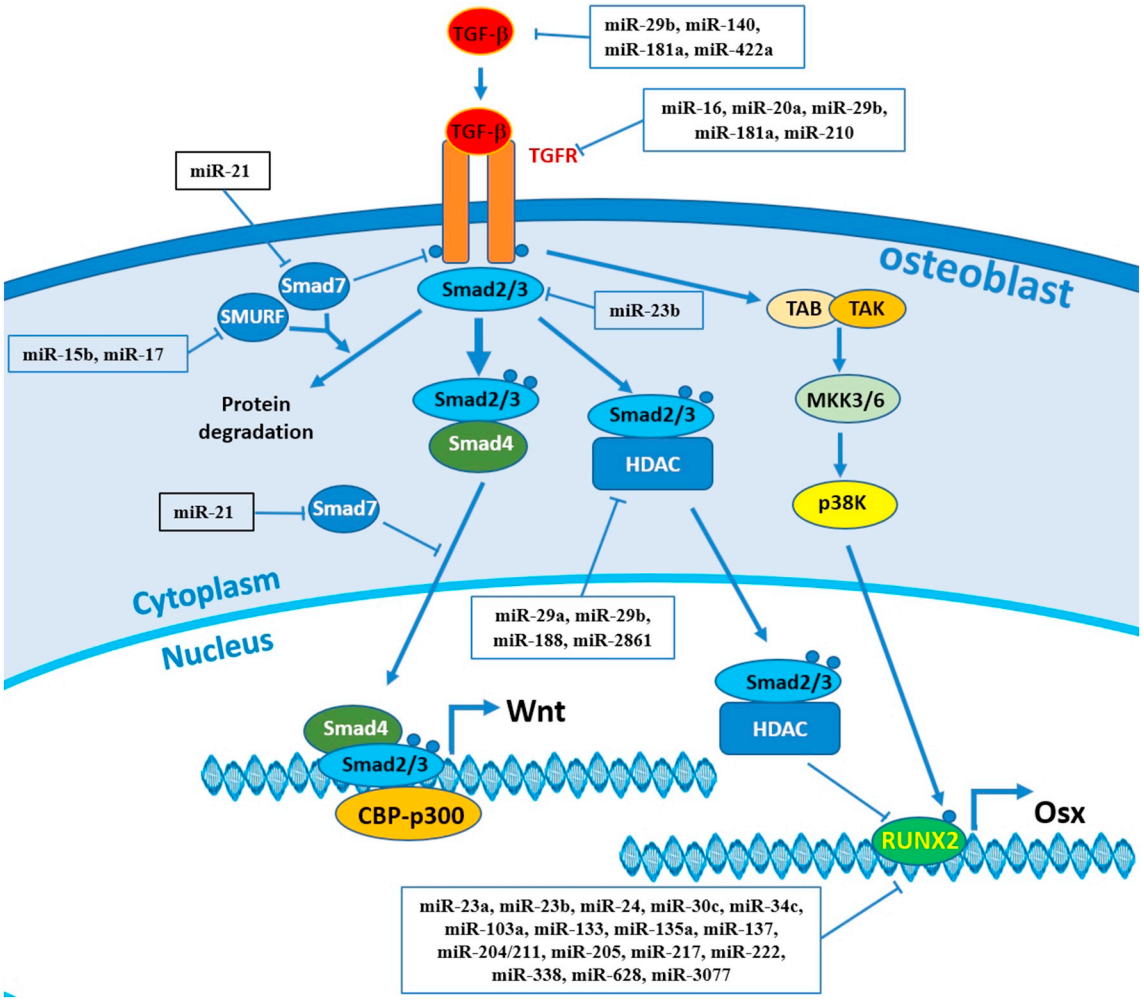

Fig. 4. Schematic drawing of TGF-β pathway implicated in osteoblast differentiation, where the miRNA reported are those binding 3′UTR of the mRNA of the target protein.

through downregulation of cellular-Casitas B-Lineage Lymphoma (c-Cbl) level, which inhibits the PI3K signaling pathway. Furthermore, using an ectopic bone formation mouse model, Li et al. (2015) found that miR-216a promoted bone tissue generation *in vivo* [83]. In osteoprogenitor cells, miR-92a is early up-regulated, activating PI3K/Akt pathway through Phosphatase and Tensin Homolog (PTEN) mRNA targeting, while it is down-regulated in the late stage of OB maturation [84]. The expression of miR-378 attenuates high glucose suppression of OB differentiation through targeting caspase 3 (CASP3) and activating the PI3K/Akt pathway, determining a restoration of Alkaline phosphatase (ALP) activity and the expression of Runx2, Osx, Col I, OCN and ON under high glucose conditions [85].

The binding of Hh to Patched 1 (PTC1) determines smothered (SMO) activation with the translocation into the nucleus of GLI proteins and transcription of BMP2 and WNT. Although Hh pathway influences osteogenesis, no miRNAs have been identified with targets validated in this signaling pathway until now.

3.5. MiRNAs involved in the regulation of master transcription factors of osteoblastogenesis

All pathways converge to Runt-related transcription factor 2 (RUNX2) and its co-factor SATB2 expression and activation, which are transcription factors that represent master regulators responsible for OB differentiation. miRNAs involved in their downregulation obviously determine osteogenesis inhibition. RUNX2 is a bone-specific transcription factor that is able to suppress pre-osteoblast proliferation and promote the expression of crucial genes, committing cells into OBs [2].

Several miRNAs such as miR-23b, miR-30c, miR-103a, miR-133, miR-135a, miR-137, miR-204/211 miR-205, miR-217, miR-222, miR-338, miR-628 and miR-3077, show targeting RUNX2 mRNA, with a consequent reduction of Runx2 mRNA or its translation, determining inhibition of osteogenesis *in vitro* and leading to bone loss *in vivo* [23,51,71,86–95]. In particular, Zuo et al. showed how the tail vein injection of antagomir of miR-103a, a mechano-sensitive miRNA, in hindlimb unloading mice model determines partial rescue of osteoporosis. This study represents one of the first to identify the role of miR-103a in regulating the OB differentiation to mechanical loading and indicates this miRNA as a therapeutic target for treating human bone remodeling disorders related to mechanical loading [88]. The expression of miR-204 and its homolog miR-211 was also positively correlated with adipocyte differentiation [86,95]. Regarding miR-628, its high expression has been observed in the fracture site of patients with atrophic non-union, while it is down regulated in OB differentiation [90]. Liao et al. showed as estrogen deficiency determines up-regulation of miR-3077 in MSCs of OVX mice by activation of NF-κB pathway, that could be associated to its bone loss causes [51].

Other miRNAs that affect RUNX2 activities are: miR-10b, miR-29a, miR-29b, miR-194, miR-764 and miR-2861 [91,96–99]. MiR-10b is the most studied miRNA among those involved in regulation of cell proliferation. It targets B-cell lymphoma 6 (BCL-6) proteins involved in

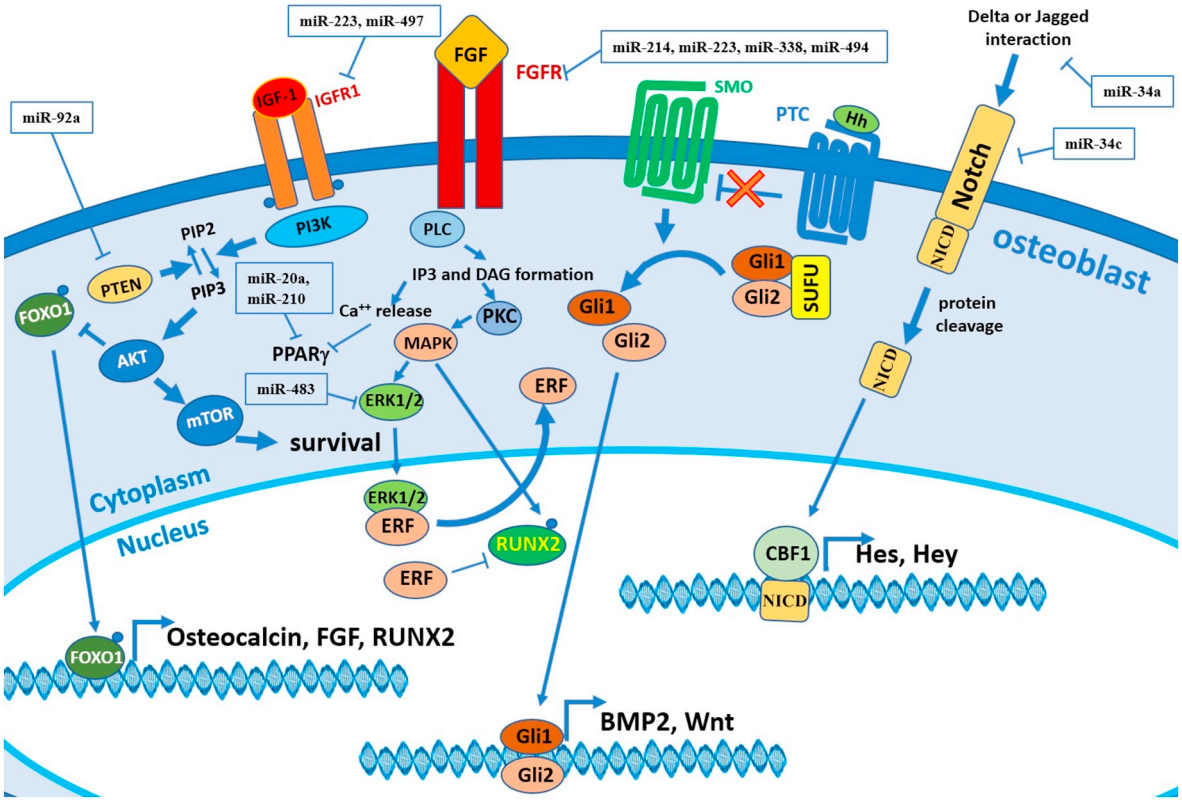

Fig. 5. Schematic drawing of Igf1, Fgf, Hh and Notch pathways implicated in osteoblast differentiation, where the miRNA reported are those binding 3′UTR of the mRNA of the target protein.

inhibition of Signal Transducer and Activator of Transcription 1 (STAT1) pathway. Overexpression of miR-10b determines an enhanced activation of STAT1, a negative regulator of Runx2 that acts blocking its nuclear translocation [96].

On the contrary, miR-194 expression decreases STAT1 level, increasing nuclear translocation of RUNX2 and promoting OB differentiation of BMSCs [97]. The expression of miR-29a and miR-29b down-regulates the level of Histone Deacetylase 4 (HDAC4), increasing OB differentiation through protection of Runx2 deacetylation [19,91]. MiR-764 inhibits the Carboxy Terminus of Hsp-70 Interacting Protein/Stress Induced Phosphoprotein 1 Homology and U-Box Containing Protein (CHIP/STUB) expression, preventing ubiquitination and degradation of Runx2 and thus promoting OB differentiation [98]. Furthermore, miR-2861 promotes BMP2-induced osteoblastogenesis through a decrease of HDAC5 expression, a histone deacetylase involved in Runx2 degradation. *In vivo* studies showed that miR-2861 silencing reduces Runx2 protein expression, with a consequent inhibition of bone formation, and decreased bone mass. Mutation of miR-2861 gene in homozygosis in humans blocks its expression and is shown to be the cause of primary osteoporosis in adolescents [99].

Special AT-rich sequence-binding protein 2 (SATB-2) is a DNA-binding protein that specifically involved in transcriptional regulation of OB differentiation genes, inducing bone regeneration. Expression of miR-33 and miRNA-34b block osteogenic differentiation signal in mature OBs though Satb2 mRNA targeting [78,100]. Different works have demonstrated the implication of miR-23a, miR-24, miR-31 and miR-34c in blocking differentiation signals of OB and osteocytes, through direct targeting of both RUNX2 and SATB2 mRNAs [79,86,101–104].

Runx-2/Satb-2 activation determines Osterix (Osx) expression, a zinc-finger transcription factor, implicated in the expression of effector genes of OB and osteocyte. Additionally, several works established the essential role of Osx in matrix mineralization and bone formation. Expression of miR-31 determines a reduction of stability of Osx mRNA, influencing its protein level, thus inhibiting OB mineralization [105]. In BMSCs, five miRNAs, miR-93, miR-96, miR-125b, miR-135b and miR-637, are also involved in the inhibition of Osx expression, but through direct targeting and degradation of its mRNA [106–111]. In particular, Yang et al. showed that Osx negatively regulates miR-93 transcription, demonstrating a regulatory feedback loop between miR-93 and Osx in OB mineralization [107]. The expression of miR-637 promotes adipogenesis and suppresses osteoblastogenesis, regulating the balance between OB and adipose differentiation of MSCs [110]. Furthermore, miR-637 is up-regulated directly by 1.25$(OH)_2$ vitamin D treatment, without the regulation of host gene Death Associated Protein Kinase 3 (DAPK-3), through the presence of internal promotor activated by Vitamin D Receptor (VDR) binding. In addition, miR-637 determines the down-regulation of collagen IV expression through direct targeting of its mRNA [106].

3.6. MiRNA involved in vitamin D actions and inflammatory status on osteoblastogenesis

Active vitamin D metabolite (1,25$(OH)_2$ vit D) has a very important role on MSCs differentiation into osteogenic lineage, as well as in the activation of bone matrix mineralization by OBs. In fact, 1,25$(OH)_2$ vit D induces the expression of osteoblastic specific genes such as collagen type-I (COL1A1), osteocalcin (OCN or BGLAP), osteopontin (OPN), osteoprotegerin (OPG), RANKL, and the same VDR. Despite active metabolites of vitamin D that promote expression of RANKL, inducing osteoclastogenesis, this process is inhibited by the increased expression of OPG, which prevents interaction of RANKL with Receptor Activator of Nuclear Factor Kappa-B (RANK) on OC precursors [112]. Deregulation in its biogenesis, as well as in its activities, could impair bone formation and bone resorption leading to osteoporosis [113].

MiRNAs that modify positively or negatively activities of vitamin D metabolites influence osteogenesis. MiR-208 and miR-370 inhibit OB

differentiation of pre-OB by targeting of mRNA of transcription factor Ets1, expressed by VDR activities and implicated in the transactivation of OPN, PTH, Runx2, tenascin-C and type I pro-collagen, positive regulators of osteogenesis [37,52]. Furthermore, miR-351 a negative regulator of osteogenesis through VDR mRNA targeting, is down-regulated in MSCs during OB differentiation [114]. Expression of miR-1228 is induced by 1.25$(OH)_2$ vitD treatment, through the up-regulation of its host gene LRP1. The up-regulation of this miRNA determines the translation inhibition of Bone Morphogenetic Proteins 2 Kinase (BMP2K) mRNA and reduction of OB differentiation in response to VDR activities [106].

Other pathways involved in osteoblastogenesis are those of several pro- and anti-inflammatory cytokines. Under inflammatory conditions, as in presence of interleukin (IL)1β or Tumor necrosis factor α (TNFα), OBs synthesize different members of IL6 cytokine family, as IL11, oncostatin M and Ciliary Neurotrophic Factor. The IL6 cytokine family is able to promote RANKL molecule in the concurrent induction of OB differentiation [115]. Through Janus Tyrosine Kinases (JAK)/STAT pathway activation, IL6 determines OB differentiation by up-regulation of RUNX2, ALP and OCN gene expression [116]. Finally, it was found that IL4 inhibits proliferation of OB precursors, induces OB differentiation, stimulates ALP synthesis in a dose-dependent manner, and clearly enhances mineralization rate of OBs [117].

Expression of miR-155 is involved in TNF-α-mediated inhibition of OB differentiation by Suppressor of Cytokine Signaling 1 (SOCS1) mRNA targeting. In fact, miR-155 is expressed under inflammatory condition and inhibits BMP2-induced OB differentiation [118].

Furthermore, miR-1192 is a Runx2-up-regulated miRNA and promotes OB differentiation by targeting of Heparin-Binding EGF-Like Growth Factor (HBEGF) mRNA, preventing activation of Epidermal Growth Factor Receptor (EGFR) and Rb−B2 Receptor Tyrosine Kinase 4 (ErbB4) and their downstream pathways [119]. Furthermore, another study reported an implication of miR-96 in the promotion of OB differentiation at late stage by suppression of HBEGF/EGFR signaling by targeting HBEGF mRNA. It is known that activated EGFR pathway strongly inhibits OB differentiation and mineralization in several OB cells lines [120]. HBEGF pathway is activated by pro-inflammatory cytokines, creating a positive feedback loop of inflammation status, involved in bone remodeling [119,120]. In MSCs, miR-195 determines a reduction of proliferation rate and OB differentiation through Vascular Endothelial Growth Factor (VEGF) targeting, a mediator of both angiogenesis and inflammation [121].

3.7. Other MiRNAs influencing osteogenesis

Various miRNAs influence the synthesis or degradation of extracellular matrix proteins, as well as the availability of micronutrient, which could influence OB differentiation. Overexpression of let-7b suppresses osteogenic differentiation of MSCs by partial targeting of matrix metalloproteinases 1 (MMP1) mRNA and consequently decreases MMP1 protein levels. MMP1 acts in modifying the interactions between cells and extracellular matrix and liberating factors implicated in osteogenic differentiation [122]. The levels of let-7 miRNAs (let-7a, let-7b, let-7c, let-7d, let-7g, let-7i) are strongly reduced in osteocytes compared with OB precursors, and this determines consequent activation of osteocyte-specific genes involved in osteocytic phenotype [123]. MiR-21 negatively regulates the expression of Reversion-inducing cysteine-rich protein with Kazal motifs (RECK), a key suppressor factor of MMP2, MMP9, and MT1-MMP that acts to regulate homeostasis of osteogenic differentiation [124]. MiR-29a and miR-29c promote OB differentiation and accelerate extracellular mineralization by inhibition of osteonectin (ON) expression [26], [125] that is essential for the maintenance of bone mass and for balancing bone formation and resorption in response to PTH, while miR-29b supports OB differentiation by reducing the level of COL1A1, COL5A3 and COL4A2, preventing fibrosis and facilitating mineral deposition [19]. Another miRNA, miR-206, exerts its effects by connexin 43 (Cx43) mRNA targeting. Depletion of Cx43 by miR-206 expression decreases OB differentiation and function [126]. Furthermore, miR-133 is able to inhibit Solute Carrier Family 39 Member 1 (SLC39A1) expression, which codifies for the Zinc Transporter 1 (Zip1), a very important micronutrient in osteogenic differentiation; miR-133 resulted up-regulated in estrogen deficiency induced osteoporosis [127].

Other identified miRNAs that influence the OB differentiation and activities are miR-33, miR-34a and miR377. MiR-33 is a mechanosensitive miRNA that increases in response to pressure solicitations on the cells. In an *in vitro* experiment using simulated microgravity or fluid shear stress, miR-33a promoted OB differentiation by directly targeting of High Mobility Group AT-Hook 2 (Hmga2) mRNA, a repressor of osteogenic differentiation [128]. MiR-34a down-regulates cell cycle regulators as Cell Division Cycle 25A (CDC25A), Cyclin-Dependent Kinase 4 (CDK4), CDK6, cyclin D1 and E2F Transcription Factor 3 (E2F3), inhibiting proliferation [80]. Moreover, miR-377 inhibits dramatically OB proliferation, without affecting cell apoptosis, through direct targeting of CDK6 mRNA [129]. In human induced pluripotent stem cells (hiPSCs), miR-204/211 positively regulates Autophagy-related gene 14 (Atg 14), inducing OB differentiation [130].

4. MicroRNA involved in osteoclast differentiation

As for osteoblastogenesis, several miRNAs are involved in the process of osteoclastogenesis acting on specific targets of the two pathways mainly responsible in this process and briefly summarized in Fig. 6: RANK/RANKL and Macrophage colony-stimulating factor (M-CSF)/colony-stimulating factor-1 receptor (c-FMS) system [6].

4.1. MiRNA involved in RANK/RANKL signaling pathway

RANK/RANKL/OPG system is the first regulator of OC differentiation and activation when osteotrophic factors, such as PTH and calcitriol, induce OBs to produce RANKL. This pathway is contrasted by OPG synthesis that binds RANKL interfering with signal activation [6].

The presence of miRNAs that determines variations of RANKL and OPG levels obviously influences OC differentiation. MiR-335 and miR-338 in pre-OCs are able to regulate negatively RANKL expression by directly targeting of the 3′-UTR portion of its mRNA and thus suppressing OC differentiation [131,132]. In parallel, miR-145 in OBs, decreases OPG synthesis through direct targeting of its mRNA, determining an increase of RANKL/OPG *ratio* with a subsequently increase of OC differentiation signal in pre-OCs [133]. On the contrary, the suppression of miR-145 synthesis by estrogens observed in osteoblast-like cells by Jia et al. (2017) induces OPG expression with a decrease of RANKL/OPG ratio, thus inhibiting OC differentiation. Furthermore, RANK is a direct target of miR-503, whose overexpression in RANKL-induced PBMCs reduces significantly OC differentiation, while the silencing of miR-503 promotes osteoclastogenesis. In *in vivo* experiments using agomiR-503 and antagomirR503, it was found in both sham-operated and OVX mice that miR-503 is implicated in the inhibition of osteoclastogenesis by targeting of RANK. MiR-503 was also identified as the most significantly down-regulated miRNA in circulating progenitors of OCs from osteoporotic patients [134].

The binding of RANKL to RANK stimulates multiple intracellular signal transduction cascades. A major downstream recruited factor is the adaptor protein Tumor Necrosis Factor Receptor Associated Factor 6 (TRAF6), which mediate the activation of Inhibitor of Kappa B Kinase (IKK), Dual Specificity Mitogen Activated Protein Kinase 1 (MAP2K1/MEK1), 6 (MAP2K6/MKK6) and 7 (MAP2K7/MKK7), Proto-oncogene tyrosine-protein kinase Src (SRC) and Phospholipase C (PLC).

Two miRNAs, miR125a and miR-146a, are able to suppress OC differentiation from PBMCs and the formation of multinucleated OCs through direct targeting of TRAF6 mRNA, also under RANKL and M-CSF stimulation [135,136]. Normally, miR-125a is down-regulated

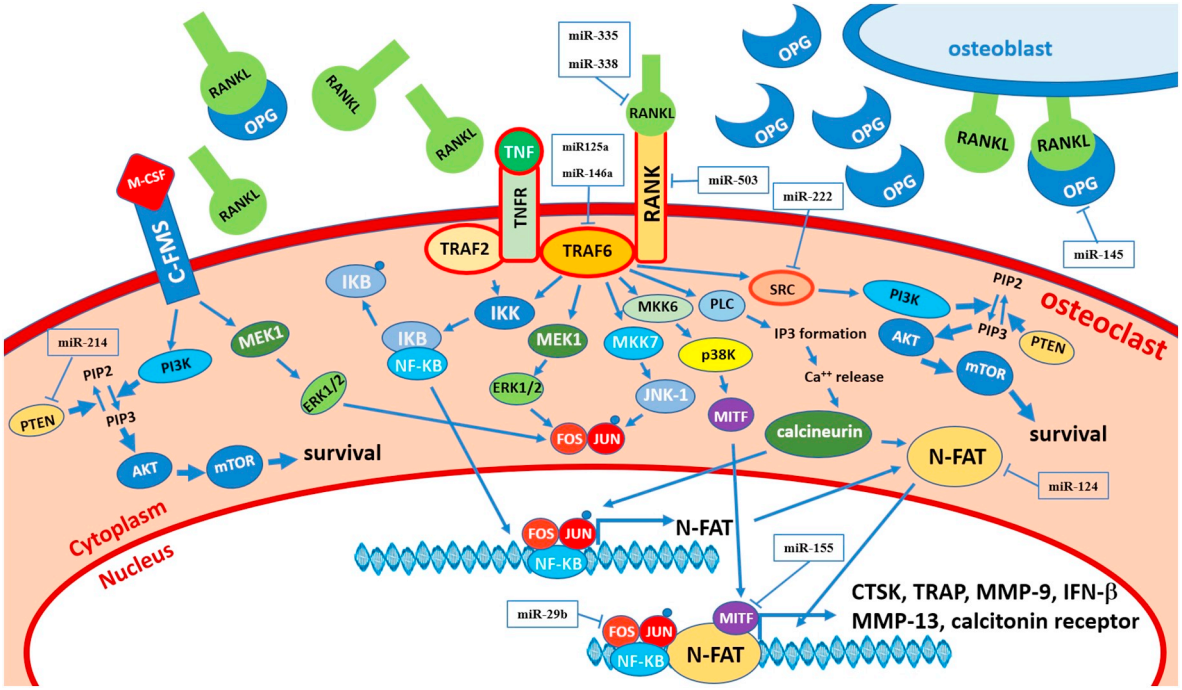

Fig. 6. Schematic drawing of pathways implicated in osteoclastogenesis, where the miRNA reported are those binding 3′UTR of the mRNA of the target protein.

during OC differentiation stimulated by M-CSF and RANKL treatments [135].

IKK is a kinase implicated in the inactivation of NF-kB inhibitor (IKB) by phosphorylation, leading its degradation and permitting NF-kB activation. Let-7e miRNA is up regulated in monocytes under pro-inflammatory stimulation through NF-kB activation, regulating negatively monocytes specific genes, such as Integrin Alpha 4 precursor (ITGA4) and Thrombospondin (THBS), and stimulating OC differentiation [137]. MiR-218 and miR-618, down-expressed in a post-menopausal osteoporosis female, target Toll Like Receptor 4 (TLR4), which has a role in NF-kB activation that is very important in osteoclastogenesis [138].

MEK1 activates ERK and then the transcription factor FOS. The miR-29b inhibits c-Fos expression through targeting of its mRNA and is able to inhibit OC activity. During OC differentiation, NF-kB activation suppresses miR-29b promoter function and miR-29b expression decreased progressively during OC differentiation induced by RANKL and M-CSF [139].

MKK6, activator of p38 kinase, has Microphthalmia-Associated Transcription Factor (MITF) as target, which is fundamental for the expression of genes of OC activities such as Cathepsin K (CTSK) and Tartrate-resistant acid phosphatase (TRAP) enzymes. MITF mRNA is the target of miR-155, and high level of this miRNA results in the inhibition of bone resorption [140]. The mRNA of Protein Inhibitor of Activated STAT3 (PIAS3), a protein that regulates the activities of other transcription factors implicated in the same pathway as MITF and NF-κB, is the target of miR-9718. It was found that miR-9718 is temporally regulated during osteoclastogenesis induced by M-CSF and RANKL signaling pathways activation. *In vivo* experiments in a mice model showed how injection of antagomiR-9718 determined a significant increase in femur bone mineral density (BMD) in comparison with controls [141].

SRC protein activated by TRAF6, determines the activation of PI3K/Akt/mammalian Target of Rapamycin (mTOR) pathway. A miRNA that interferes in this survivor signaling is miR-222, which acts as an inhibitory regulator of RANKL-driven osteoclastogenesis in pre-OCs through down-regulation of c-Src mRNA, and then protein level [142].

Through the formation of a complex that contains dimer Activator Proteins 1 (AP1) (composed by c-Fos and c-Jun) and NF-kB, all these pathways activates NFAT-c1, determining the auto-stimulation of its transcription, which is the master regulator of transcription of osteoclast specific genes such as TRAP, CTSK, MMP9, MMP13, *etc*. Osteoclastogenesis is negatively regulated by miR-124 synthesis *via* suppression of Nuclear Factor of Activated T Cells C1 (NFATC1) expression, through direct targeting of its mRNA [143]. Even miR-21 inhibits Programmed Cell Death Protein 4 (PDCD4) expression, leading to a down-regulation of both mRNA expression of c-Fos and its phosphorylation level. This determines the inhibition of AP1 formation and NFATc transcription, resulting in a decreased OC differentiation [74]. Synthesis of miR-148a induces OC differentiation by repression of Musculoaponeurotic Fibrosarcoma oncogene homolog B (MAFB) expression. This protein is a repressor of transcription factors NFATC1, c-Fos, and MITF, resulting in the inhibition of transcription of effector genes of OC activities [144]. Another miRNA implicated in the regulation of master regulators of osteoclastogenesis is miR-1270, which was found up-regulated in a cohort of postmenopausal osteoporotic women of Mexican Mestizo population. MiR-1270 targets Interferon Regulatory Factor 8 (IRF8) mRNA that encodes for a transcription factor that suppresses OC differentiation, thus inhibiting the NFATC1 activity [146].

Finally, it was demonstrated that TGF-β is able to induce RANK expression, facilitating RANKL induction of osteoclastogenesis [145]. OC differentiation was inhibited by miR-34a, whose expression inhibits the expression of TGFB induced factor homeobox 2 (TGIF2) that is a key regulator of pro-osteoclastogenic differentiation implicated in enhancing NFATC1, NF-κB and c-Jun activation. Furthermore, NFATC1, NF-κB and AP1 were found to bind sites in TGIF2 promoter, indicating that TGIF2 expression is enhanced also by these factors, creating a positive feedback loop that enhance OC differentiation. In an *in vivo* study, the OC-specific over-expression of miR-34a in OVX-induced osteopenia mice reduced bone resorption and bone loss [147].

4.2. MiRNA involved in M-CSF/c-FMS signaling pathway

M-CSF/c-FMS interaction determines activation of MEK1, improving the signal of RANKL in AP1 activation, and of PI3K of PI3K/

AKT/mTOR pathway that guarantees OC survival [6]. This last pathway is enhanced by the expression of miR-214 that is up-regulated in bone marrow macrophages (BMMs) when committed to osteoclastogenesis by M-CSF and RANKL treatment. MiR-214 exerts its functions by targeting of PTEN mRNA and inducing an up-regulation of phosphorylated Akt and NFATC1, after RANKL induction. The use of miRNA inhibitor and mimic showed *in vitro* as TRAP, MMP9, Chloride Channel 7 (CLC7) and CTSK are down-regulated or up-regulated, respectively. Furthermore, in an *in vivo* study, it was observed that transgenic mice with OC specific miR-214 overexpression exhibited a down-regulation of PTEN protein level in OCs that has an increased activity, with a consequent reduction of mice BMD [148]. MiR-214 induces also OC differentiation by targeting of TRAF3 mRNA, in order to activate canonical and non-canonical NF-κB signaling [149]. Moreover, miR-29c and miR-223 promote OC differentiation by inhibiting the expression of Nuclear Factor I A (NFIA), negative regulator of osteoclastogenesis. In fact, this repressor of CCAAT-box binding transcription factor family is implicated in the repression of c-FMS; its inhibition by these miRNAs amplifies M-CSF signaling [150–152].

4.3. Other MiRNAs influencing osteoclastogenesis

MMPs are very important in the differentiation process, determining modification of matrix composition and liberating soluble factors implicated in OC differentiation. MiRNAs that modify MMPs expression could influence OC differentiation and activities: MiR-29b inhibits OC differentiation through modification of MMP2 expression by direct targeting of its mRNA [139]; miR-126 and miR-532 inhibit osteoclastogenesis by targeting of MMP13 mRNA, the principal proteinase expressed by the stromal cells for matrix degradation and implicated in OC differentiation and activities [153,154]. Up-regulation of miR-365 determines inhibition of MMP9 expression in BMMs, thus repressing OC differentiation and activities [155].

Moreover, some miRNAs modify only the expression of genes implicated in OC activities, negatively influencing bone resorption. MiR-186 regulates CTSK expression, leading to CTSK suppression and decrease of OC activities, suggesting its role as a therapeutic target in the management of glucocorticoid-induced bone loss, and calcium homeostasis unbalance [156]. Expression of miR-124 decreases Ras-Related protein 27a (Rab27a) expression, involved in OC activities. In fact, Rab27a-deficient OCs showed a markedly impaired bone resorption activity, indicating its important role in maintaining OC normal functions. In addition, miR-124 expression sensibly reduces osteoclastogenesis dependent on RANKL and M-CSF signaling pathways; miR-124 levels are down-regulated in OVX-dependent osteoporotic mice BMMs [157].

Modification of matrix protein (synthesis or degradation) as well as lacking or presence of mechanical stimulation could influence OC differentiation. The miR-7b expression inhibits osteoclastic Dendritic Cell-Specific Transmembrane Protein (DC-STAMP) expression, implicated in cell-cell fusion for the formation of mature OCs. MiR-7b expression is able to suppress OC bone resorption in preclinical studies [158]. Expression of miR-26a increases in response to RANKL induction; its target is Connective Tissue Growth Factor (CTGF) that promotes osteoclastogenesis *via* induction of DC-STAMP. Aberrant CTGF production induced by TNF-α is responsible of abnormal OC activation [159].

Osteocytes under mechanical loading have a suppressive effect on OC differentiation through IL6 production. The same pathway activated in OB with terminal activation of ERK1/2 and STAT3 determines inhibition of expression of CTSK, TRAP, and MMP9, in respect to unloaded osteocytes. This inhibitory effect is reverted using IL6 blocking antibody, demonstrating the direct effect of this cytokines [160].

MiRNA, which act in cell cycle regulation or survival and apoptotic signals are able to modify OC differentiation. miR-99b and mir-335 suppress OC differentiation by IGF1R mRNA targeting, decreasing cell proliferation and *in vitro* OC induction by affecting pathway critical in OC differentiation [131,137]. miR-9b, miR-181a and miR-422a are negative regulators of osteoclastogenesis [161,162]. Down-regulation of these miRNAs determines an increase of c-Cbl expression, an E3 ubiquitin ligases, promoting B-Cell Lymphoma 2 Interacting Mediator of Cell Death (BIM) degradation and OC survival and activation [161,162].

Cytokines and different other signals and miRNAs that act in these pathways, could influence OC differentiation. For example, miR-29c is implicated also in the promotion of OC differentiation through targeting of calcitonin receptor (CALCR) mRNA, influencing positively Ca^{2+} liberation, triggering OC activity. Furthermore, it is able to inhibit the expression of CD93 and G Protein-Coupled Receptor 85 (GPR85), very important in macrophage differentiation of monocytes, favoring then their OC differentiation [150].

Pro-inflammatory cytokines as IL1β, TNFα, IL17 determine the activation of OC differentiation, triggering local or systemic expression of RANKL in OB and immune system cells. IL1β is one of the key inflammatory cytokines, and it was reported to have an important role in inflammatory bone loss. IL1β receptor, with the binding of its ligand, determines activation of TRAF6 protein, with transduction of osteoclastogenic signals. IL11 and CNTF, produced by OB in pro-inflammatory condition, determine an up-regulation of OC differentiation [115]. MiR 124, miR-204 and miR-211 are key regulators of IL11 expression, by binding its 3′ UTR mRNA portion. Increased levels of these miRNAs determine a decrease of IL11 levels that inhibits OC maturation and activities [163,164]. Expression of miR-142 inhibits OC generation from macrophage and dendritic cells, by targeting of gp130 mRNA, which codifies for IL6 cytokine receptor, important in OC differentiation, through activation of STAT3 factor [165].

Other miRNAs acting on different pathways such as that related to TGF-β activity on BMMs inducing SOCS1 signaling with OC/macrophage commitment and inducing the expression of NFATC1. It is known that SOCS1 block the inhibitory effect of inflammatory cytokines and IFN-β facilitating osteoclastogenesis. Expression of miR-155 that target SOCS1 mRNA, determines suppression of OC differentiation [140].

It is known that autophagy genes regulate hypoxia-induced OC differentiation. HIF-1α induction by hypoxia determines miR-20a inhibition. This miRNA inhibits OC differentiation process by targeting of Atg16L1, a protein involved in Light Chain 3 lipidation for autophagosome formation [166].

Expression of miR-183 in macrophages induces osteoclastogenesis, through inhibition of heme oxygenase-1 (HO-1). In fact, HO-1 is a protein with anti-inflammatory and anti-oxidative activities that is able to down-regulate RANK and c-FMS expression. Inhibition of HO-1 expression by miR-183 promotes osteoclast formation and activities [167].

On the contrary, some anti-inflammatory cytokines (IL4, IL10, IL13) are potent inhibitors of osteoclastogenesis [168], activating JAK and STAT6, resulting in a inhibition of RANK receptor expression and OC differentiation [169]. IL10 has a central role in the modulation of inflammatory response, and its expression is regulated transcriptionally and post-transcriptionally [170]. It suppresses OC differentiation *via* the: (1) up-regulation of OPG expression; (2) down-regulation of RANKL and M-CSF-1 in OBs; and (3) reduction of NFAT-c1 expression in OCs. Furthermore, IL10 determines an inhibition of pro-inflammatory cytokines, resulting in reduction OC differentiation [171].

IFN-β is an anti-inflammatory molecule that regulates bone turnover processes, activating JAK/STAT pathway (Stat1 and Stat2) and IRF9 that inhibit c-Fos activity, resulting in OC inhibition [172]. C-X-C Motif Chemokine Ligand 11 (CXCL11) expression seems to be involved in the INF-β action, inhibiting monocytes osteoclastogenesis also in the presence of M-CSF and RANKL [173]. Expression of miR-133 negatively regulates CXCL11 and CXC Chemokine Receptor 3 (CXCR3), thus inducing osteoclastogenesis. Furthermore, miR-133 is up-regulated in monocytes of osteoporotic post-menopausal women. MiR-133 production is also able to negatively regulate SLC39A1 expression, protein

implicated in zinc recruitment, important micronutrient in osteoclastogenesis [174].

5. Deregulated miRNAs and osteoporosis

Numerous clinical studies on different miRNAs associated with bone metabolism and with altered expression levels in elderly patients highlighted that they might be implicated in osteoporosis. Table 3 reports, in alphabetically order, miRNAs in osteoporotic patients (almost all diagnosed with Dual-energy X-ray absorptiometry - DXA), collected generally by blood sample (serum). Almost all studies were carried out in two investigative steps. In the first step, indicated as 'discovery step', miRNAs were screened through RNA-sequencing or miRNA arrays analysis in small cohorts of osteoporotic and healthy patients. In the second step, the identified miRNAs that showed significantly high differences between osteoporotic and healthy patients were further validated in large cohorts, indicating this phase as 'replication or validation step', through quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). However, most of the miRNAs identified in 'discovery step' were not validated in the 'replication step' due to the small sample size of the selected cohorts.

The first clinical study on osteoporosis to identify miRNAs implicated in OB differentiation was a genomic DNA mutation study carried out in adolescent patients with primary osteoporosis ($n = 10$), in adult osteoporotic patients ($n = 369$) and in healthy children ($n = 357$) and adults ($n = 396$), from 5 to 79 years, coming from the same geographic area. MiR-2861 was identified as novel miRNA that promotes OB differentiation by repressing HDAC5 mRNA expression at post-transcriptional level [99]. The mutation observed in the promotor of miR-2861 gene in adolescent patients with primary osteoporosis represses its expression and determining an Hdac5-mediated inactivation of Runx2 and osteoporosis development [99].

More recently, Yavropoulou et al. screened 14 miRNAs implicated in bone metabolism in 3 cohorts of women: osteopenic/osteoporotic postmenopausal women distinguished on the basis of absence ($n = 35$) or presence ($n = 35$) of vertebral fractures and healthy postmenopausal women ($n = 30$). Five out of 14 screened miRNAs were significantly and differently expressed [175]. Among these five miRNAs, the authors found that miR-2861, together with miR-124, was up-regulated, while miR-21, miR-23a, miR-29a were down-regulated. According to the authors, the up-regulation of miR-2861 together with miR-124 is a result of a response of postmenopausal osteoporotic patients to bone degradation. In fact, the over-expression of miR-124 suppressed NFATC1 expression, thus inhibiting osteoclastogenesis, while the up-regulation of miR-2861, through its target HDAC5, and the down-regulation of miR-23a, a repressor of RUNX2 transcription, improve osteogenesis [175]. Concerning miR-21, it was more down-regulated in cohorts of patients with fractures, compared to those without fractures; it acts on targets SPRY1 in OBs and PDCD4 in OC leading to a decrease in osteogenesis and an increase in osteoclastogenesis, respectively. The down-regulation of miR-29a, which belongs to the miR-29 family, a best-characterized group of miRNAs that repress OB differentiation targeting of negative OB regulators, improved osteogenesis [175]. Different results were instead found by Seeliger et al. that showed an up-regulation of miR-21 and miR-23a in patients with osteoporotic fractures compared to control [176]. However, according to Yavropoulou et al., these differences could be attributed to the selected patients population [175]. In fact, Seeliger et al. identified and validated circulating and bone tissue miRNAs in hip fractured osteoporotic ($n = 33$) and non-osteoporotic ($n = 30$) patients aged over 50 years. The study recognized 83 circulating miRNAs in pooled samples of osteoporotic ($n = 10$; 3 male and 7 female) and non-osteoporotic patients ($n = 10$ female) during the 'discovery step' and 9 of them were validated only in female patients ($n = 30$ osteoporotic and $n = 30$ healthy patients). These validated miRNAs (miR-21, miR-23a, miR-24, miR-93, miR-100, miR-122a, miR-124a, miR-125b, and miR-148a) were significantly up-regulated in osteoporosis and showed a significant sensitivity and specificity in distinguishing osteoporotic patients; 6 of them (miR-21, miR-23a, miR-24, miR-25, miR-100, and miR-125b) were validated also in bone tissue. MiR-21 regulates positively OB differentiation and negatively OC differentiation, while miR-23a, miR-24, miR-100 and miR-125b negatively regulate osteogenesis. Even circulating miR-93, miR-124a and miR-148a negatively regulate OB differentiation; the latter positively regulates OC differentiation [176]. Until now, no targets have been identified for miR-25 and miR-122a [176].

The role of miR-125b was also validated on BMSCs in a small cohort study of elderly osteoporotic patients with fractures ($n = 4$) and healthy donors ($n = 5$), which observed that miR-125b acts as a key regulator in the development and progression of senile osteoporosis [108]. MiR-125b levels were significantly higher in osteoporotic patients determining an inhibition of BMSCs proliferation and their OB differentiation by *OSX* mRNA targeting [108]. Moreover, MiR-125b was validated in a study that found selected circulating miRNA (miR-21, miR-23a, miR-24, miR-93, miR-100, miR-122, miR-124, miR-125b and miR-148a) over-expressed in osteoporotic patients by comparing 4 small cohorts of patients who underwent total hip replacement, with osteoporotic hip fractures ($n = 14$; 7 male, 7 female) or coxarthrosis ($n = 14$; 7 male, 7 female) [177]. Furthermore, in this study the miR-125b and other miRNAs, *i.e.* miR-21, miR-24, miR-93 and miR-100, were also significantly correlated with BMD values, with miR-21 significantly differently expressed in non-osteoporotic, osteopenic and osteoporotic patients. Interestingly, miR-21, miR-93, miR-100 and miR-125b were over-expressed in both OBs and OCs, while others were specifically expressed in OBs (miR-23a and miR-24) or OCs (miR-122, miR-124 and miR-148a). MiR-21 inhibits both OB differentiation by targeting of SMAD7, SPRY1 and RECK mRNAs, and OC differentiation by PDCD4 (mRNA), while miR-93 and miR-100 up-regulation determines inhibition of BMP2 signal as well as impairs mineralization and maturation of OBs through OSX (mRNA) target of miR-93; miR-125b up-regulation determines a decreased OB and OC differentiation by BMP/BMPR and RANKL/RANK signal inhibitions, respectively. MiR-23a and miR-24 interfere with RUNX2 and SATB2 expression, inhibiting osteoblastogenesis of MSCs and osteoprogenitor cells. MiR-122 and miR-124 were found significantly up-regulated in OCs from osteoporotic patients; until now no knowledge on miR-122 role are available, while it has been proven that miR-124 act through suppression of NFATC1 expression, inhibiting osteoclastogenesis. Finally, miR-148a has pro-OC effects in PBMCs, through direct targeting of MAFB mRNA, and its up-regulation enhances M-CSF/RANKL signals and OC differentiation in osteoporotic patients [177]. MiR-148a was significantly expressed in osteoporotic patients also in a clinical study by Bedene et al. that compared osteoporotic postmenopausal ($n = 17$) to healthy women ($n = 57$) to screen 9 miRNAs (let-7d, let-7e, miR-30d, miR-30, miR-126, miR-148a, miR-199a, miR-423 and miR-574) implicated in postmenopausal osteoporosis [178]. Authors evidenced how up-regulated miR-148a had a double negative role in osteoporosis, (1) promoting OC differentiation of circulating monocytes, and (2) inducing adipocyte differentiation of hMSCs, impeding osteogenesis [178].

In addition to the key role of miR-21, miR-23a, miR-124, miR-125b and miR-148a in osteoporosis pathogenesis, many reviewed studies focused their attention also on miR-133 and 133a, as their importance in the development of skeletal muscle. Wang et al. were the first to demonstrate the association of miR-133a expression levels in circulating monocytes with postmenopausal osteoporosis [174]. In this study, 365 miRNAs were screened in small cohorts of postmenopausal Caucasian women with high ($n = 10$) and low ($n = 10$) lumbar spine and total hip BMD. Among 156 identified miRNAs, only miR-133a and miR-382 showed significantly different expression in serum levels between groups, but only miR-133 up-regulation was confirmed in the same cohorts of patients. MiR-133 was significantly expressed in blood of post-menopausal osteoporotic women and it was related to the

promotion of osteoclastogenesis through the down-regulation of the CXCL11, CXCR3, and SLC39A1 [174]. CXCL-11 is a cytokine that inhibits OC differentiation, CXCR-3 is a G protein-coupled receptor, which is significantly decreased during OC differentiation, and finally, SLC39A1 inhibits osteoclastogenesis [174].

Up-regulation of miR-133a was also found in a study performed on Chinese postmenopausal women ($n = 120$) that compared osteopenic and osteoporotic patients to healthy ones [179]. The study highlighted significant differences in the blood levels of miR-133a, but also of miR-21, both miRNAs significantly correlated to BMD values. MiR-21 that negatively regulates OC differentiation and positively regulates OB differentiation was down-regulated, while miR-133a, which negatively regulates OB differentiation, was up-regulated [179]. Similar up-regulation of miR-133a was detected also by Chen et al. who carried out a clinical study on small cohorts of Chinese osteoporotic ($n = 31$) and healthy ($n = 30$) postmenopausal women (aged 50 to 59 years) [134]. In the 'discovery step', RNA extracted from serum of blood samples of osteoporotic ($n = 10$) and healthy ($n = 10$) women showed 4 down-regulated (miR-218, miR-305, miR-503 and miR-618) and 3 up-regulated miRNAs (miR-107, miR-133a, and miR-411). Since miR-503 was dramatically down-regulated in PBMCs of postmenopausal osteoporotic patients compared to healthy ones, they focused their further *in vitro* studies (gain and loss function analysis in patient PBMCs cultures) and clinical validation, on miR-503. MiR-503 was confirmed *in vitro* to negatively regulate osteoclastogenesis by RANK mRNA targeting and its expression was markedly decreased in postmenopausal osteoporotic patients that presented significantly higher RANK levels [134]. Finally, also Lv et al. investigated the specific role of miR-133 in osteoporosis [127]. Differently from the above-mentioned studies on miR-133, they analyzed MiR-133 activity in BMSCs isolated from osteoporotic postmenopausal women ($n = 5$) and healthy premenopausal ones (n = 5). They evaluated miR-133 in BMSCs cultures before and after OB differentiation, showing its over-expression was negatively correlated with OB differentiation by SLC39A1 mRNA target, that plays an important role in the promotion of ALP activity and mineralization [127]. A negatively correlation with ALP in aged women and men, but also with the Bone Gamma-Carboxy-Glutamic Acid-Containing Protein (BGLAP) expression was also found for miR-214 [53]. In this study, performed on fractured patients ($n = 40$) with different age and lumbar spine BMD values, the expression of 33 miRNAs, previously identified in healthy adults ($n = 9$) and regulated in OB differentiation, bone development and bone formation, were evaluated. Among 6 miRNAs varying with age (miR-23b, miR-30a, miR-130a, miR-140 and miR-214), miR-214 was higher in older patients, also identifying *in vitro* its role on OBs through the regulation of ATF4 expression as inhibitor of bone formation [53]. Reviewing the above-mentioned studies, we found that miR-133a in circulating monocytes is upregulated in postmenopausal women with low BMD compared to postmenopausal women with high BMD, thus identifying miR-133a a potential biomarker associated with postmenopausal osteoporosis.

Cao et al., planned to further identify other potential miRNA biomarkers in circulating monocytes for postmenopausal osteoporosis [162]. Three hundred sixty-five miRNAs were screened in two small cohorts of postmenopausal women, aged > 57 years, with high ($n = 10$) and low ($n = 10$) lumbar spine or total hip BMD and among these miRNAs, Cao et al. validated only miR-422a, which was significantly up-regulated in patients with low BMD. MiR-422a targets genes related to the inhibition of osteoclastogenesis such as c-CBL and TOB2 [162]. In particular, c-Cbl degrades NFATC1, the principal transcription factor of OC development, and Tob2 interacts with VDR to suppress RANKL expression, and the actions of miR-422 determine then an excessive bone resorption in postmenopausal women [162]. In another clinical study, always conducted analyzing small cohorts of postmenopausal osteoporotic ($n = 6$) and healthy ($n = 6$) women of Mexican Mestizo origin, 2578 miRNAs were screened [146]. Thirty-five miRNAs identified by their significant different regulation between selected cohorts were subsequently validated if markedly up-regulated (miR-548, miR-1270, and miR-8084) or down-regulated (miR-6124, miR-6165, and miR-6824). MiR-1270 was found significantly up-regulated; it targets IRF8 mRNA, which codifies a transcription factor interfering with NFATC1 activity and promoting osteoclastogenesis [146]. Small cohorts of patients were also analyzed by Weiner et al. that identified 6 out of 165 miRNAs that exhibited significant expression differences in serum levels of post-menopausal women with osteoporotic femoral neck fractures ($n = 7$) in comparison to gender- and age-matched controls ($n = 7$) [180]. The cohorts were selected to identify those circulating miRNAs, which might be released from bones after fractures to prime bone healing processes. Three up-regulated miRNAs (miR-10a, miR-10b, miR-22) and three down-regulated miRNAs (miR-133b, miR-328, let-7g) identified in the 'discovery step' were subsequently analyzed in validation cohorts (11 control and 12 fractured patients), confirming significant regulation for only let-7g, miR-22 and miR-328 [180].

In a recent interesting clinical study by Kocijan et al., screening 187 miRNAs in blood of fractured osteoporotic ($n = 36$; 10 pre-menopausal, 10 post-menopausal and 16 men) and in healthy ($n = 39$; 12 pre-menopausal and 11 post-menopausal and 16 men without baseline values of bone turnover markers) patients, 3 up-regulated (miR-152, miR-320a and miR-335) and 16 down-regulated miRNAs (let-7b, miR-7, miR-16, miR-19a, miR-19b, miR-29b, miR-30e, miR-93, miR-140, miR-186, miR-215, miR-324, miR-365, miR-378a, miR-532, miR-550a) were identified in idiopathic and postmenopausal osteoporotic patients [181]. Up-regulated miR-320a and down-regulated miR-378 determine inhibition of OB differentiation through negatively regulation of Wnt signal and Casp levels, respectively, while down-regulated miR-7b, miR-186, miR-365 and miR-532 promote osteoclastogenesis. MiR-29b down-regulates different negative regulators of OB differentiation as well as OC differentiation. However, the up-regulation of miR-152 and miR-335 and down-regulation of let-7b, miR-7, miR-16, miR-30e, miR-93 miR-140, miR-550a, that improve osteogenesis, are in contrast with osteoporotic development. Until now, miR-19a, miR-19b, miR-215, miR-324 have not yet been described to have a validated target that affect bone remodeling. They also identified that: (1) miR-19b, miR-93, miR-324 and miR-532 were significantly correlated with lumbar spine BMD; (2) miR-140 and miR-320a were correlated with body mass index (BMI); (3) miR-29b and miR-365 were correlated with markers of bone formation as Procollagen I Intact N-Terminal (PINP) and OCN; and (4) miR-365 was also correlated with markers of OC activity (PTH and TRAP) [181]. Subsequently, the same authors correlated the 19 identified circulating miRNAs to altered bone morphometry (histomorphometry and microtomography) results achieved in patients with idiopathic osteoporosis ($n = 10$ pre-menopausal women and $n = 16$ men aged < 50) or post-menopausal osteoporosis ($n = 10$) with peripheral or vertebral low traumatic fractures [182]. No differences were found regarding bone histomorphometric and microtomographic parameters among groups, but among the 19 screened miRNAs, miR-29b, miR-324 and miR-550a had highly significant correlations between their levels and morphometric parameters. In particular, miR-29b was found positively correlated to some histomorphometrical parameters, *i.e.* mineral apposition rate (MAR), bone formation rate/bone surface (BFR/BS) and bone surface/bone volume (BS/BV), miR-324 was correlated only with MAR and BFR/BS and miR-550a was correlated with MAR and BS/BV and negatively correlated with trabecular bone volume (BV/TV). Finally, the regulation of miR-29b, miR-324 and miR-550a was investigated in patients undergoing anti-resorptive therapy (ATR), revealing a significant down-regulation of miR-29b and miR-324 [182].

Differently from the above clinical studies, Garmilla-Ezquerra et al. and De-Ugarte et al. identified several miRNAs by considering cohorts of osteoporotic patients with fracture and osteoarthritic patients [183,184]. In detail, Garmilla-Ezquerra et al. identified 13 miRNAs, showing significant differences between aged osteoporotic (all with fractures) and osteoarthritic (without fractures) patients [183,184]. The

analysis of these selected miRNAs in the 'replication step' showed that only 2 miRNAs, miR-518f and miR-187, presented significant differences between the selected cohorts. MiR-518f was present in high concentration in bones of 74% of osteoporotic patients and negatively regulated several genes involved in Wnt pathway inhibiting osteogenesis. Conversely, they found that miR-187 was down-regulated mainly in osteoarthritic patients (90%) and in 74% of osteoporotic ones. However, the role of miR-187 in osteoporosis has not completely understood; the results obtained by this study might be confounding since its regulation of pro-inflammatory cytokine genes (IL6 and TNF), which negatively influences OB and positively regulates OC differentiation and activity, respectively, might be active in both selected cohorts. Finally, the authors were not able to prove evidence for the same expression of miR-187 and miR-518f in OB cultures of about half of the cohort patients, stating that this result might be the consequence of the lack of bone microenvironment [183]. The second study that considered two small cohorts of patients, 7 osteoporotic patients with fracture and 6 osteoarthritic patients (submitted to hip replacement), evaluating miRNAs from discarded bone tissue samples, showed that miR-320a and miR-483 were significantly over-expressed in osteoporotic patients [184]. MiR-320a was extensively studied in both cancer and osteoblastic cell function for its role in targeting Catenin Beta (CTNNB1) and RUNX2. MiR-483 targets Erk1, which is implicated in FGF pathway; its over-expression inhibits osteoblastogenesis as demonstrated by the diminished expression of BMP2, type I collagen, ALP and OCN, indicating the abnormal functionality of osteoporotic bone. The authors concluded that the deregulation of these miRNA suggests a dysfunction in the pathways implicated in OB cell renewal in the osteoporotic bone. However, this study presents a possible bias not stated by the authors and related to the use of Let-7e in the normalization of miRNA analysis [184].

Another interesting study carried out by You et al. discovered circulating miRNA comparing microarray containing 851 human miRNAs of postmenopausal osteoporosis ($n = 5$) and healthy premenopausal ($n = 5$) women, identifying 33 miRNAs significantly regulated. Among them, they validated one of the most strongly down-regulated miRNA, miR-27a, in larger postmenopausal osteoporotic ($n = 81$, aged 51–62 years) and healthy premenopausal women ($n = 74$, aged 40–46 years) cohorts [55]. Their selection was related to the fact that miR-27a is an essential factor on MSCs differentiation and its expression levels are regulated during osteoblastogenesis and adipogenesis. For this purpose, they performed further *in vitro* and *in vivo* loss and gain function investigations on human BMSCs and mouse model, respectively, whose results highlighted for the first time how miR-27a acts on down-regulating Mef2c, which is involved in OB differentiation [55].

Preclinical *in vitro* and *in vivo* (mouse and rat) model were also used by other authors to evaluate and/or identify miRNAs implicated on osteoporosis [111,185,186]. In *in vitro* (loss and gain of function) and *in vivo* (OVX mouse) studies on the role of long non-coding RNA maternally expressed gene 3 (LncRNA MEG3) on osteogenesis, it was highlighted that lncRNA MEG3 down-regulates Zip1, Runx2, Cxcl11 and Cxcr3 *via* elevating miR-133a, and then inhibits the OB differentiation of BMSCs from OVX mice. The authors validated this data also on small cohorts of post-menopausal osteoporotic women ($n = 10$) and pre-menopausal healthy women ($n = 10$) achieving similar results to those obtained in mice [185]. In a subsequent *in vivo* study, always using a mouse model, Liu et al. highlight the role of miR-96 in osteoblastogenesis inhibition by OSX mRNA targeting that leads to bone mass reduction. After this result, they then compared miR-96 levels among different groups of postmenopausal osteoporotic ($n = 20$) and elderly osteoporotic ($n = 20$) women with premenopausal ($n = 20$) and healthy elderly ($n = 20$) women. MiR-96 was markedly up-regulated in elderly patients with osteoporosis, respect to healthy elder controls and confirmed that its levels were also negatively correlated with BALP and BMD [111]. Finally, Chen et al. compared pooled RNA from 2 rat osteoporotic models ($n = 4$ OVX rats and $n = 4$ hindlimb-unloading osteoporotic male rats), respect to healthy controls ($n = 4$ sham female, $n = 4$ male) and then validated miRNAs on osteoporotic ($n = 10$) and osteopenic ($n = 7$) postmenopausal patients respect to healthy controls ($n = 19$). MiR-30b, miR-103, miR-142 and miR-328 were confirmed as down-regulated miRNAs in the serum of postmenopausal osteoporotic patients, while only miR-30b was down-regulated significantly in osteopenic patients, suggesting its early regulation in osteoporosis development. The down-regulation of miR-30b, miR-103 that target OB genes (SMAD1, RUNX2), promotes osteogenesis, while miR-142 and miR-328 inhibit OB differentiation. Furthermore, down-regulated miR-142 also promotes OC differentiation [186].

By analyzing the results obtained by the above-mentioned studies, it could be stated that some miRNAs clearly provide more evidence of their possible use as biomarkers in osteoporosis, compared to those showing partial or contrasting evidences. MiR-124, miR-125b, miR-133 and miR-148a were found up-regulated in serum of osteoporotic patients in three, three, five and three studies, respectively, while miR-29b was found down-regulated in three studies. On the contrary, miR-21 and miR-23a, reported deregulated in seven and three clinical studies, respectively, which provided, nevertheless, contrasting results on their up- or down-regulation, quite possibly due primarily to the small cohorts of patients recruited, and secondly to the selected control patients. Regarding this last aspect, osteoporotic patients were often compared with control ones presenting bone pathologies such as fractures or osteoarthritis. This, however, might have contributed to the emergence of contrasting results, since the presence of these pathologies could have modified systemic expression miRNAs leading to conclusions biased by confounding factors, for instance, the lack of controls of dietary supplements (*e.g.* calcium, vitamin D) of recruited cohorts of patients.

6. Conclusion

Structural bone integrity and bone homeostasis are maintained by the balance between bone modeling and remodeling controlled by osteoblasts and osteoclasts, respectively. During aging, a progressively decrease of osteoblast differentiation abilities is observed, as well as a preferential differentiation in adipocytes of MSCs, determining an increase of fat tissue in bone marrow. Increased fat tissue inhibits further osteoblast differentiation through soluble factors, increasing aging osteoporosis risk [187]. Furthermore, postmenopausal conditions determine a diminution of hormone levels, inducing bone loss and augmented fracture risks.

In this review, we focused our attention on deregulated miRNAs that can destabilize bone homeostasis, through interfering in osteogenesis and osteoclastogenesis, leading to osteoporosis. The clinical studies showed that only some of these miRNAs are deregulated in osteoporosis and although the role of these molecules in bone homeostasis is undoubted, they often lack adequate controls to indicate if these miRNAs may be usable as biomarkers in clinical use.

Abbreviations

3′UTR	3′ Untranslated Region
AP1	Activator Proteins 1
Acvr	Activin Receptor
APC	Adenomatous Polyposis Coli
ADSCs	Adipose Stem Cells
ARF	ADP Ribosylation Factor
ALP	Alkaline Phosphatase
ATR	Anti-Resorptive Treatments
AGO2	Argonaut 2
ATG	Autophagy-Related Gene
BIM	B-Cell Lymphoma 2 Interacting Mediator Of Cell Death
BCL-6	B-Cell Lymphoma 6
BFR	Bone Formation Rate

BGLAP Bone Gamma-Carboxy-Glutamic Acid-Containing Protein
BMMs Bone Marrow Macrophages
BMSCs Bone Marrow Mesenchymal Stem Cells
BMD Bone Mineral Density
BMPs Bone Morphogenetic Proteins
BMP2K Bone Morphogenetic Proteins 2 Kinase
BAMBI Bone Morphogenetic Proteins and Activin Membrane-Bound Inhibitor
BMPR Bone Morphogenetic Proteins Receptor
BS Bone Surface
BV Bone Volume
CALCR Calcitonin receptor
CHIP Carboxy Terminus Of Hsp70-Interacting Protein
c-Cbl Cellular-Casitas B-Lineage Lymphoma
CASP3 Caspase 3
CTNNB Catenin Beta
CTNNBIP1 Catenin Beta Interacting Protein 1
CTSK Cathepsin K
C/EBPα CCAAT-Enhancer-Binding Proteins
CDC25A Cell Division Cycle 25A
CLC7 Chloride Channel 7
CNTF Ciliary neurotrophic factor
COL1A1 Collagen Type I Alpha 1 Chain
COL4A2 Collagen Type IV Alpha 2
COL5A3 Collagen Type V Alpha 3
c-FMS Colony-Stimulating Factor-1 Receptor
CTGF Connective Tissue Growth Factor
Cx43 Connexin 43
CBFA1 Core-Binding Factor α1
CXCL C-X-C Motif Chemokine Ligand
CXCR C-X-C Motif Chemokine Receptor
CDK Cyclin-Dependent Kinase
CRIM1 Cysteine-Rich Motor Neuron 1
DAPK-3 Death Associated Protein Kinase 3
DC-STAMP Dendritic Cell-Specific Transmembrane Protein
DGCR8 Di George Syndrome Critical Region Gene 8
DM Diabetes Mellitus
Dkk Dikkopf
Dlx Distal-Less Homeobox
DUSP-2 Dual Specificity Phosphatase 2
DXA Dual-Energy X-Ray Absorptiometry
E2F3 E2F Transcription Factor 3
EGFR Epidermal Growth Factor Receptor
ERK Extracellular Signal-Regulated Kinase
FIAT Factor inhibiting ATF4-mediated transcription
FGF Fibroblast Growth Factor
FGFR Fibroblast Growth Factor Receptor
FAK Focal Adhesion Kinase
GPR85 G Protein-Coupled Receptor 85
GSK3β Glycogen Synthase Kinase 3β
GEF-1 Guanine Nucleotide Exchange Factor-1
Hh Hedgehog
HBEGF Heparin-Binding EGF-Like Growth Factor
Hmga2 High Mobility Group AT-Hook 2
HDAC Histone Deacetylase
HOXA10 Homeobox A 10
HOXC8 Homeobox C 8
hiPSC Human induced pluripotent stem cell
IKK Inhibitor of Kappa B Kinase
IGF1 Insulin-Like Growth Factor 1
IGF1R Insulin-Like Growth Factor 1 Receptor
IRF Interferon Regulatory Factor
IL Interleukin
JAG1 Jagged 1
JAK Janus Tyrosine Kinases
LEF Lymphoid enhancer-binding factor
Let-7 Lethal 7
LPS Lipopolysaccharides
LRP Low Density Lipoprotein Receptor-Related Protein
Kdm6b Lysine-Specific Demethylase 6b
M-CSF Macrophage Colony-Stimulating Factor
MMP Matrix metalloproteinases
MSCs Mesenchymal Stem Cells
MITF Microphthalmia-Associated Transcription Factor
MiRNAs MicroRNAs
MAR Mineral Apposition Rate
MAFB Musculoaponeurotic Fibrosarcoma Oncogene Homolog B
Mef2c Myocyte Enhancer Factor 2C
NFIA Nuclear Factor I A
NF-kB Nuclear Factor Kappa B
NFATC1 Nuclear Factor of Activated T Cells C1
OB Osteoblast
OCN Osteocalcin
OC Osteoclast
ON Osteonectin
OPN Osteopontin
OPG Osteoprotegerin
OSX Osterix
OVX Ovariectomized
PTH Parathyroid Hormone
PDLSCs Periodontal Ligament Tissue-Derived Mesenchymal Stem Cells
PBMCs Peripheral Blood Mononuclear Cells
PPARG Peroxisome Proliferator Activated Receptor Gamma
PTEN Phosphatase and Tensin Homolog
PI3K Phosphatidylinositol 3 Kinase
PINP Procollagen I Intact N-Terminal
PDCD4 Programmed Cell Death Protein 4
PIAS3 Protein Inhibitor of Activated STAT 3
qRT-PCR Quantitative Real-Time Polymerase Chain Reaction
Rab27a Ras-Related Protein
Erbb Rb-B2 Receptor Tyrosine Kinase
RANK Receptor Activator of Nuclear Factor Kappa-B
RANKL Receptor Activator of Nuclear Factor Kappa-B Ligand
RECK Reversion-Inducing Cysteine-Rich Protein with Kazal Motifs
RUNX2 Runt-Related Transcription Factor 2
SOST Sclerostin
sFRP Secreted Frizzled Related Protein
STAT Signal Transducer and Activator of Transcription
SMAD SMA- And MAD-Related Protein
SMURF1 SMAD Ubiquitination Regulatory Factor 1
SLC39A1 Solute Carrier Family 39 Member 1
SATB2 Special AT-Rich Sequence-Binding Protein
SPRY1 Sprouty RTK Signaling Antagonist
STUB Stress Induced Phosphoprotein 1 Homology And U-Box Containing Protein
SOCS Suppressor of Cytokine Signaling
TRAF Tumor Necrosis Factor Receptor Associated Factor
TLR Toll Like Receptor
TV Trabecular Bone Volume
TCF Transcription Factor
Tob1 Transducer of Rb—B2 Receptor Tyrosine Kinase 1
TGFBI Transforming Growth Factor Beta Induced
TGFBR1 Transforming Growth Factor Beta Receptor 1
TGFBR2 Transforming Growth Factor Beta Receptor 2
TGFβ Transforming Growth Factor-Beta
TGIF2 Transforming Growth Factor B Induced Factor 2
TRAP Tartrate-resistant acid phosphatase
TNFα Tumor Necrosis Factor α
VEGF Vascular Endothelial Growth Factor
VDR Vitamin D Receptor
Wnt Wingless-Type MMTV Integration Site Family

Zip1 Zinc Transporter 1

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Conflict of interest

The authors declare that they have no competing interests. No benefits in any form were received or will be received from a commercial party related directly or indirectly to the subject of this article.

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