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microRNAs and osteocytes

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Abstract

microRNAs, identified in the early 1990s, are believed to regulate approximately 30% of the human genome. The role of microRNA in bone cells was first reported in 2007 in a manuscript showing that microRNA- 223 is essential for osteoclast differentiation *in vitro*, and a few studies reported a role of microRNAs in osteoblasts the same year. The first report of microRNA actions in osteocytes was published in 2010, in which it was demonstrated that the microRNA cluster 23a~27a~24–2 regulates osteocyte differentiation. Since then, few studies have described the role of these 18–25-nucleotide non-coding RNAs on osteocyte biology, reporting osteocytes both as producers and as targets of the actions of microRNAs. We review here the current knowledge on the effects of microRNAs on osteocyte biology.

Keywords

osteocyte; microRNA; bone cell; exosome

Introduction

The first described microRNA (miR) was lin-4, found to be essential for postembryonic development in C. elegans [1]. Since then, miRs have been described in 271 organisms included in the miRbase miR database (http://www.mirbase.org/index.shtml). In particular, bioinformatic analysis revealed 2300 true human mature miRs, approximately 520 high-confidence miR canonical genes, and 120 miR families [2]. It has been estimated that each family of miRs targets more than 400 mRNAs, and 60% of all mRNAs are targeted by miRs.

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Credit author statement

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miRs are small, 18–25-nucleotide non-coding RNAs [3–5] involved in posttranscriptional gene expression regulation, and have been identified as regulators of cell differentiation, proliferation, and survival in physiological and pathological conditions [6, 7]. miRs sequences are located within introns or exons of noncoding RNAs and are transcribed into large primary miRs (pri-miRNAs). In the nucleus, the pri-miRNAs are cleaved into pre-miRNAs, followed by the cleavage by Dicer into small double-stranded RNAs (dsRNAs). These dsRNAs are then loaded into the argonaute protein and assembled in an RNA-induced silencing complex (RISC) where the miR matures. It has been estimated that miRs regulate the expression of one third of the protein-coding genes, in most cases leading to reduced protein expression. A detailed review of miRs genesis and function can be found in a recent manuscript by Saliminehad and colleagues [6]. In bone, the first report of a miR was published by Sugatani and Khruska in a study showing the importance of miR-223 for osteoclast differentiation [8]. The first report of miRs exerting a role in osteocytes was published by Jane Lian's group [9]. As we will describe below, this study showed that the miR-23a~27a~24–2 cluster has a central role in osteocyte differentiation and maintenance of the osteocytic phenotype.

Osteocytes are differentiated osteoblasts surrounded by mineralized bone matrix [10, 11]. Osteocyte differentiation involves changes in morphology, with the appearance of numerous cytoplasmic projections and change in the pattern of miR and gene expression. Through the production of secreted molecules, osteocytes can modulate the function of osteoblasts and osteoclasts on the bone surface and other osteocytes in the matrix, as well as cells in other tissues and organs. In particular, as we will discussed below, it has been proposed that osteocytes can release extracellular vesicles containing miRs that can impact the function of skeletal muscle and adipose tissue [12]. This review describes the current knowledge on the effect of miRs on osteocyte differentiation and function, and the potential role of osteocytic miRs as markers of bone health.

microRNA expression in osteocytes

Long-thought to be inactive bone cells, osteocytes are now recognized as master regulators of the activity of osteoblasts and osteoclasts, essential for the control of bone remodeling and the maintenance of bone mass and strength [11]. Studies performed in osteocytic cell lines as well as in primary osteocytes revealed the expression and role of numerous miRs in these cells (summarized in Table 1). The expression of miRs in osteocytic cells was reported by Sato et al [13] using the murine long bone derived MLO-Y4 osteocytic cell line as a model for osteocytes [14]. These authors compared the miR expression between whole cell preparations and exosomes, and between MLO-Y4 osteocytic cells and the bone marrow-derived stromal cell line ST2 [15] induced to differentiate in the presence of ascorbic acid. Interestingly, this study also includes the levels of miRs present in the circulation of mice in which osteocytes were eliminated by injecting diphtheria toxin to mice expressing the diphtheria toxin receptor under the control of the dentin matrix protein 1 (DMP1) promoter, compared to circulating levels in wild type mice. Deletion of osteocytes led to the upregulation of 30 miRs, whereas another 30 were downregulated in the absence of osteocytes. However, since the measurement in the plasma was made 3 weeks after osteocyte ablation, it is not possible to determine whether the miRs changing in the

circulation are released by osteocytes themselves or from cells other than osteocytes, which are altered following elimination of osteocytes. Nevertheless, some of same miRs downregulated in the serum from osteocyte-deleted mice were enriched in exosomes from MLO-Y4 osteocytic cells compared to whole cell preparations and in exosomes from osteocytic cells compared to those from osteoblastic cells. Taken together, these data suggest that indeed miRs are released by osteocytes *in vivo*.

Another study performed in Ocy454 osteocytic cells (obtained from long bones of mice expressing the green fluorescent protein under the control of the DMP1 promoter and the SV40 antigen [16]) showed that exposure to myostatin results in decreased levels of miR-218 both in whole cell preparations and in Ocy454 cell-derived exosomes [17]. Reduced levels of miR-218 in myostatin-treated cells was associated with increased levels of *Sost* mRNA in Ocy454 osteocytic cells, whereas, conversely, exogenous expression of miR-218 resulted in decreased *Sost* mRNA levels in Ocy454 and IDG-SW3 osteocytic cells (derived from the same mice as the Ocy454 cells [18]).

miR-199a-3p was detected in osteocytic cell preparation from bones of C57BL/6 mice, and its expression was increased 3 months after ovariectomy [19]. Consistent with a direct regulation of miR-199a-3p by estrogen, treatment with 17β-estradiol resulted in decreased levels of the miR in MLO-Y4 osteocytic cells. Reduced miR-199a-3p levels induced by estrogen require ERα, but not ERβ expression. Further, miR-199–3p overexpression abolished the suppression of autophagy induced by estrogen by targeting IGF-1 and mTOR in MLO-Y4 osteocytic cells.

A study performed in primary osteocytes isolated from bone biopsies of patients with adolescent idiopathic scoliosis showed increased expression of miR-145 in the osteocyte fraction, compared to control individuals [20]. The levels of miR-145 positively correlated with Ctnnb1 mRNA levels in bone biopsies from these patients. Further, miR-145 mimic decreased, whereas miR-145 silencing increased sclerostin levels in osteocytic cell preparations from these patients, and the levels of plasma miR-145 negatively correlated with the levels of *Sost*, as well as with osteoprotegerin and osteopontin levels, in the serum from adolescent idiopathic scoliosis patients. Thus, this study suggests a potential role of osteocytic miR-145 on the pathogenesis of this condition.

In a detailed study, Hayashi and colleagues demonstrated the effect of estrogens on miR levels in osteoblastic and osteocytic cells and contrast it with the effect of ovariectomy on the expression of the same miRs in murine bone [21]. These authors found that estrogen addition to osteocytic IDG-SW3 and of calvaria-derived osteoblastic cells led to changes in the levels of miRs and, in particular, to decreases in miR-29b, miR-497, miR-195, and miR30a levels. All of these miRs were identified as potentially targeting Semaphorin3A (Sema3A), and mutations of the miR-497/miR-195 region in the Sema3A gene resulted in loss of regulation by the miRs. Conversely, ovariectomy resulted in increased expression of the four miRs downregulated by estrogen treatment *in vitro*, as well as decreased serum Sema3A levels. Based on this evidence, the authors conclude that estrogen regulates the expression of Sema3A via the inhibition of miR-497 and miR195 in osteoblastic and osteocytic cells. Whereas the results indicate that the regulation of the miRs occurs not only

in osteocytes but also in osteoblasts, the study also showed that the role of Sema3A in osteocytes becomes more relevant as the animals age, suggesting that the osteocytic miRs might be important for osteocyte biology and function in older animals.

More recently, a study analyzed miR levels in exosomes released by MLO-Y4 osteocytic cells subjected to mechanical stimulation by cyclic stretching and found that 121 miRs were increased and 85 were decreased, when compared to exosomes produced by control cells [22]. In particular, the levels of miR-181b-5p were highly upregulated in exosomes from mechanically stimulated osteocytic cells. These authors also found that miR-181b-5p contained in exosomes derived from mechanically stimulated MLO-Y4 osteocytic cells induced osteogenic differentiation of human periodontal ligament stem cells by reducing PTEN levels, thereby activating the Akt signaling pathway.

Regulation of osteocyte differentiation by microRNAs

miRs have been shown to either induce or inhibit osteoblastic cell differentiation from mesenchymal stem cells, as reviewed elsewhere [23]. This bone cell-associated miRs named OsteomiRs are also involved in terminal osteoblast differentiation, regulating the expression of osteocytic genes [24]. In this study by Eguchi and colleagues, the expression of miRs was measured as the mouse mesenchymal stem and bone marrow stromal cell line KUSA-A1 [25], murine calvaria-derived MC3T3 preosteoblasts [26], and human bone marrow stromal cells were induced to differentiate in the presence of β-glycerophosphate, dexamethasone, and either ascorbic acid or KH_2PO_4. As the cells progress in the differentiation pathway, they lose expression of miRs associated with stemness/inhibition of differentiation, while acquiring others that are associated with differentiation or inhibition of stemness. Importantly, these authors propose miRs as osteocyte markers, namely Snord85, whereas others are considered osteocyte negative markers (i.e. miR-101a, miR-10a, let-7 family of miRs, etc.). In addition, these authors proposed that members of the miR-30 family, which are increased during late osteocyte differentiation, might target and repress key osteoblastic genes such as Runx2, Smad1/2, and CCN3.

A miR cluster is defined as a group of two or more miRs transcribed from physically adjacent miR genes, often transcribed as a single unit [27]. The miR-23a~27a~24–22 cluster, also known as the miR-23a cluster, was the first to be identified in osteocytes [9]. In this study, it was demonstrated that the osteoblast transcription factor Runx2 regulates miR-23a~27a~24–22 by direct binding to the promoter in MC3T3 osteoblastic cells. The regulation of the miR-23a cluster by Runx2 is required to increase the levels of Special AT-rich sequence-binding protein 2 (SATB2), which, in turn, is required for the progression of osteoblastogenesis. Further, the expression of the miRs decreased whereas Runx2 increased as osteoblastic cells became more mature. Supporting the notion of osteoblastogenesis regulation by the miRs, overexpression of miR-23a and miR27a resulted in decreased levels of alkaline phosphatase and osteocalcin and reduced mineralization of rat calvaria-derived and of murine MC3T3 osteoblastic cells. After day 21 in culture, osteocytes are formed as Runx2 and SATB2 expression decreases whereas the levels of the components of the miR cluster increase. The mechanism by which the components of the miR-23a cluster promote osteocyte differentiation was further investigated by Brendan Lee’s group[28]. This study

showed that mice overexpressing the components of the miR-23a cluster in osteoblasts exhibit low bone mass and increased osteocyte number, whereas miR-23a or miR-27a loss-of-function results in decreased osteocyte number. The regulation of osteocytogenesis by these miRs result from modulation of the TGF-β signaling pathway and the expression of the *Sost* gene.

Further evidence for a role of miRs on osteocytogenesis was reported by Guo et al [29]. Using insulin-receptor substrate (IRS)-1 deficient mice, this study showed an increase in bone mass associated with higher expression of osteoblastic genes, especially collagen 1a2 (Col1α2). High Col1α2 expression resulted from reduced miR-342 levels, and expression of a miR-342 inhibitor enhanced the transcription of a Col1α2 reported construct. The expression of Col1α2 decreased whereas miR-342 levels increased as bone marrow cells differentiate into osteocytes, as DMP1 expression was being detected. Consistent with a potential role on osteocytogenesis, Col1α2 silencing or expression of a miR-342 mimic inhibits osteocyte differentiation and the expression of DMP1 in bone marrow cells treated with osteoblastogenic media.

Osteocytic connexin43 and the regulation of miRs

Control of osteocytic lifespan is paramount for the maintenance of bone mass and strength [30, 31]. In studies leading to investigation of the mechanisms that control osteocyte viability, we and others found that deletion of connexin43 (Cx43) from osteoblasts and osteocytes results in an increase in osteocyte apoptosis *in vitro* and *in vivo* [32, 33]. In search for the molecular mechanisms for the increased cell death in the absence of Cx43, we examined the levels of apoptosis-associated miRs in MLO-Y4 osteocytic cells in which the expression of Cx43 was silenced using shRNA, compared to scramble-silenced cells [34, 35]. Among the 47 miRs tested using a miR array, the expression of 2 was dysregulated by Cx43 deletion: the levels of the tumor suppressor miR-218 were increased, whereas the levels of the onco-miR miR-21 were decreased [35]. Specifically, expression of miR-218 was increase by 5-fold, compared to scramble-silenced cells. Consistent with a potential regulation of Sost expression by miR-218 in osteocytes described above [17], the levels of sclerostin are decreased in osteocytes present in the cortical bone of mice in which Cx43 has been deleted from cells expressing Cre under the control of the DMP1–8kb promoter [32].

Further studies demonstrated that reduced miR-21 levels are responsible for the increased apoptosis of MLO-Y4 osteocytic cells silenced for Cx43 [35]. Reduced miR-21 level in osteocytic cells is also associated with increased RANKL expression and osteoclastogenic potential of conditioned media from Cx43-silenced MLO-Y4 cells [36]. Consistent with these *in vitro* studies using MLOY4 osteocytic cells derived from a female mouse, deletion of miR-21 in osteocytic cells in female mice expressing the Cre recombinase under the control of the DMP1–8kb promoter (miR-21$^{\Delta Ot}$ mice) led to higher expression of apoptosis-associated genes and the RANKL/OPG ratio in bone. On the other hand, bones from males exhibit a different phenotype, with no changes in RANKL/OPG levels and reduced expression of apoptosis-associated genes. A sex-specific phenotype was also found in the levels of mitochondria-associated genes, cytokine/chemokine production, body weight, bone mineral density, bone formation rate, and osteoblast and osteoclast numbers in bone from

miR-21-deficient mice. On the other hand, the effect of miR-21 deletion on bone strength was similar in both males and females, as detailed below. The molecular basis of the differences in response to miR-21 deletion between males and females remains to be determined.

Circulating markers of bone health: a potential role for osteocytic microRNAs

These is a growing interest in finding serum/plasma markers that faithfully reflect the status of bone health and the activity of osteoblasts and osteoclast under physiological conditions, in pathologies affecting bone mass and strength, and following therapeutic approaches. The potential use of miRs as markers for osteoporosis was reviewed by van der Eerden in 2014 [37]. At that point, there was a limit number of studies (3 references were cited) describing the link between miRs and osteoporosis. A PubMed search using the terms “microRNA-circulation-osteoporosis” now yields 73 manuscripts, showing the growth in this field. However, when “the term “osteocyte” is added to the search, only 2 manuscript plus the one by van der Eerden are retrieved. Given than osteocytes are by far the most abundant bone cell type, it is reasonable to think that osteocytic miRs could be detected in the circulation and serve as indicators of bone health. Consistent with this possibility, deletion of osteocytes leads to a significant change in miR levels in the circulation, as described above [13]. However, no study has been performed directly examining the role of osteocytic miRs on bone metabolism and disease. Nevertheless, comparing the studies in which osteocytes were deleted [13] and those in which osteoporosis-associated miRs were measured [38], one can speculate that miR-133b, which is upregulated in both postmenopausal osteoporotic women and mice with osteocyte deletion, is an osteocytic osteoporosis marker. Although potentially promising, there is no clear evidence that osteocytic miRs can be used as markers of bone health.

miR-21 and osteocytes: regulation of bone strength in a sex-independent manner

We recently reported the consequences of miR-21 deletion from osteocytes on bone mass and strength [36]. We found a sex-dependent effect on bone cell number and activity, leading to increased bone mass in males and no change in female miR-21$^{\Delta Ot}$ mice. On the other hand, both male and female mice exhibit enhanced biomechanical properties.

At the structural level, female miR-21$^{\Delta Ot}$ mice displayed a significant 30–35% increase in both postyield and total displacement which resulted in a 15–20% increase in both postyield and total work. These mechanical benefits came without changes in pre-yield properties including strength, stiffness, or elastic displacement. Male miR-21$^{\Delta Ot}$ mice showed similar postyield structural changes, but the changes were numerically larger (60–90% increase in both postyield and total displacement which resulted in a 55–70% increases in both postyield and total work). In contrast to females, these benefits in males occurred along with decreased elastic displacement resulting in a trend towards decreased work to yield.

Further, male and female miR-21$^{\Delta Ot}$ mice displayed similar structural postyield phenotypes with large and significant enhancements in ductility and energy dissipation but no changes in structural stiffness or strength. These changes were accompanied by a reduction in displacement to yield in males, but this decrease in displacement did not diminish the large

mechanical benefits imparted by improved structural ductility. Since structural level properties can be difficult to interpret given their dependence on the amount (size), distribution, and quality of the tissue, these properties are normalized by size and shape to provide estimated tissue-level properties. In females, the only significant change was a 26% increase in total strain in miR-21$^{\Delta Ot}$ mice. Toughness also trended up (12%) but failed to reach significance. As noted at the structural level, there were no changes in tissue level stiffness or strength indicating that the structural phenotype in females was caused by tissue-level modification that are likely induced by the alteration in osteocytes. In males, total strain was also increased in miR-21$^{\Delta Ot}$ mice and was again numerically larger than the change in females. This increase in strain was accompanied by increased toughness (+28%). Together, these tissue-level enhancements in ductility were accompanied by decreased yield strength which drove a drop in resilience. As noted at the structural level, and similar to females, there were no changes in tissue level stiffness or strength indicating that the structural phenotype in males was also driven by tissue-level changes induced by osteocytes.

Overall, postyield benefits in miR-21$^{\Delta Ot}$ mice displayed as increased ductility and energy dissipation. Although stronger in males, these changes were present in both sexes and were likely an osteocyte-driven impact at the tissue level. Benefits in males were accompanied by negative impacts on the onset of yield at the tissue level, an effect also reflected at the structural level. However, this negative effect in males was minor, and not enough to offset the enhancement in ductility and the beneficial impact on fracture that comes with it. Importantly, these changes appear to be driven by alterations at the tissue level, pointing to the role that miR-21 in osteocytes plays in regulating tissue quality.

Conclusions

The role of miRs on osteocytic cells, including the effect small RNAs have in osteocytogenesis, osteocyte function, and osteocytic regulation of other cells and tissues, is just beginning to be uncovered. To our knowledge, our study deleting miR-21 from osteocytic cells is the only one in which the direct role of a miR on osteocytes have been tested *in vivo*. Thus, more research is needed to determine how miRs modulate osteocyte differentiation and their involvement in the control of bone remodeling by osteocytes.

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Highlights

- microRNAs are small non-coding RNAs that control ~60% of all mRNAs
- osteocytes are both producers and targets of the actions of microRNAs
- miRNAs regulate osteocyte differentiation
- miRNAs released by osteocytes might be useful as markers for bone health

Table 1

microRNAs and osteocyte differentiation and function. The table summarizes the manuscripts reporting microRNAs as modulators of osteocyte differentiation and function.

microRNA	Cell/animal model	Results reported	reference
miR-array	MLO-Y4 osteocytic cells Mice with deleted osteocytes (osteocyte-less)	differential miR expression in exosomes compared to cells, and in the circulation of osteocyte-less mice	[13]
miR-218	Ocy454/IDG-SW3 osteocytic cells	reduced Sost expression	[17]
	MLO-Y4 osteocytic cells	Increased expression in cells lacking Cx43	[35]
miR-199a-3p	Bones from ovariectomized C57BL/6 mice MLO-Y4 osteocytic cells	regulation by estrogen prevention of estrogen-induced autophagy	[19]
miR-145	Primary osteocytes form human bone biopsies	increased levels in cells from patients with adolescent idiopathic scoliosis regulation of sclerostin levels	[20]
miR-29b, miR-497, miR-195, miR30a	IDG-SW3 osteocytic cells calvaria-derived osteoblastic cells	regulation by estrogen regulation of Sema3A expression	[21]
121 miRs increased and 85 miRs decreased	MLO-Y4 osteocytic cells		[22]
miR-181b-5p	MLO-Y4 osteocytic cells	miR-containing exosomes induced osteogenic differentiation of human periodontal ligament stem cells	[22]
Osteomirs: Snord85. miR-101a, miR-10a, and Let-7 and miR-30 miR family	KUSA-A1 and MC3T3 cell lines, and human bone marrow stromal cells	Osteocyte markers and inducers of differentiation	[24]
miR-23a~27a~24–22 cluster	MC3T3 and rat calvaria-derived osteoblastic cells	Osteoblast-osteocyte differentiation	[9]
	Overexpression in osteoblasts Loss of function	Regulation of osteocytogenesis via TGFβ signaling and regulation of Sost expression	[28]
miR-342	Bone marrow cells	Regulation of Col1α2 expression and osteocyte differentiation	[29]
miR-21	MLO-Y4 osteocytic cells Aged wild type mice Mice lacking miR-21 in DMP1-expressing cells	Reduced miR-21 levels are associated with increased osteocytic cell apoptosis Regulation of bone mass and strength	[35, 36]
miR-133b	Circulation	Osteocytic miR potential osteoporosis marker	[13, 38]