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MiR-29a is an enhancer of mineral deposition in bone-derived systems

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ABSTRACT

MicroRNAs (miRNAs) provide a mechanism for fine-tuning of intricate cellular processes through post-transcriptional regulation. Emerging evidences indicate that miRNAs play key roles in regulation of osteogenesis. The miR-29 family was previously implicated in mammalian osteoblast differentiation by targeting extracellular matrix molecules and modulating Wnt signaling. Nevertheless, the function of miR-29 in bone formation and homeostasis is not completely understood. Here, we provide novel insights into the biological effect of miR-29a overexpression in a mineralogenic cell system (ABSa15). MiR-29a gain-of-function resulted in significant increase of extracellular matrix mineralization, probably due to accelerated differentiation. We also demonstrated for the first time that miR-29a induced β-catenin protein levels, implying a stimulation of canonical Wnt signaling. Our data also suggests that SPARC is a conserved target of miR-29a, and may contribute to the phenotype observed in ABSa15 cells. Finally, we provide evidences for miR-29a conservation throughout evolution based on sequence homology, synteny analysis and expression patterns. Concluding, miR-29a is a key player in osteogenic differentiation, leading to increased mineralization *in vitro*, and this function seems to be conserved throughout vertebrate evolution by interaction with canonical Wnt signaling and conservation of targets.

Introduction

Skeletogenesis is a tightly regulated process orchestrated by numerous molecular determinants and cellular activities [1,2]. Although in recent years this process has been greatly investigated, its post-transcriptional regulators are generally unknown. MicroRNAs (miRNAs)[1], an abundant class of small noncoding RNAs, provide a mechanism for fine-tuning of complex cellular processes through binding to the 3′-untranslated region (3′-UTR) of mRNA transcripts to attenuate protein synthesis [3]. MiRNAs are known to control numerous biological processes [4], and were recently implicated in skeletogenesis, where in combination with key transcription factors and signaling molecules they were shown to control the complex program of bone formation [5]. This was evidenced by studies where conditional deletion of Dicer, an enzyme crucial for miRNA biogenesis, in osteoprogenitor cells and in chondrocytes resulted in abnormal formation of bone and cartilage of mouse [6,7]. Other studies have identified a panel of miRNAs that act as negative regulators of bone formation. For instance, miR-206 targeting of connexin43 (Cx43) was shown to impede osteoblast differentiation both *in vitro* and *in vivo* [8]. A set of 11 miRNAs was found to target the master regulator of osteogenesis, Runx2, in both osteoblasts and chondrocytes, and to inhibit osteoblast differentiation [9]. Furthermore, miR-214 was shown to repress ATF4, a transcription factor that orchestrates osteoblast differentiation and function, which consequently inhibited osteoblast activity and matrix mineralization *in vitro* and bone formation *in vivo* [10]. The opposite effect of miRNAs in bone, i.e. positive regulation of osteoblast differentiation, was also demonstrated. For instance, miR-2861 overexpression enhanced osteoblastogenesis in mouse bone marrow stromal cells, whereas its silencing *in vivo* decreased Runx2 protein levels and inhibited bone formation, by targeting HDAC5 [11]. Zhang and co-workers demonstrated that miR-335-5p down-regulates DKK1, thus activating Wnt signaling and promoting osteogenic differentiation [12]. Among this group of miRNAs is the miR-29 family, which expression was shown to increase during mouse osteoblast differentiation [13,14]. This family is composed by miR-29a, miR-29b and miR-29c, which share the same seed sequence and collectively target several genes associated to extracellular matrix (ECM) in bone, including several collagens, matrix metalloproteinase 2 (MMP2), MMP9 and SPARC

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[1] *Abbreviations used:* BMP, bone morphogenetic protein; COL1A1, collagen type I alpha 1; ECM, extracellular matrix; MGP, matrix gla protein; miRNAs, microRNAs; OC2, osteocalcin 2; qPCR, quantitative real-time PCR; SPARC, secreted protein acidic and rich in cysteine; TNAP, alkaline phosphatase; 3′-UTR, 3′-untranslated region.

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(secreted protein acidic and rich in cysteine, also known as osteonectin) [13–15]. In this regard, miR-29b was also found to target known inhibitors of osteoblast differentiation, i.e. HDAC4, TGF3, ACVR2A, CTNNBIP1, and DUSP2 [14], and to promote osteogenesis in mouse. More recently, miR-29b was shown to target osteo-inhibitory genes CDK6, HDAC4 and CTNNBIP1, in human somatic stem cells and to accelerate osteogenic differentiation [16]. MiR-29a was suggested to positively regulate osteoblast differentiation by repression of SPARC, an important protein for ECM assembly and deposition [13]. Furthermore, in mammalian osteoblasts, the transcription of miR-29a was shown to be induced by a key pathway of bone formation, the Wnt signaling, and in turn, miR-29a was shown to repress three antagonists of Wnt, thus potentiating its signaling cascade and contributing for differentiation [17]. Despite all evidences concerning miR-29 effect on mammalian osteogenic differentiation, characterization of its function and regulatory mechanisms in other organisms is far from being understood, which could help to elucidate the intricate and extensive role of this miRNA family. In this regard, teleost fish not only present several anatomic, physiologic and genetic similarities with mammals, but also present several experimental advantages, such as transparency of larvae (crucial for developmental characterization of systems), large progeny or easy manipulation (transgenic preparation), which make them suitable models to investigate vertebrate development, including skeletogenesis [18]. In fact, several miRNAs have been investigated in fish models, both *in vivo* and *in vitro* [19,20], and contributed to elucidate their roles in skeletal formation, demonstrating the suitability of these models.

In this work, we investigated the biological effects of miR-29a overexpression in a fish bone-derived cell line, the ABSa15, capable of *in vitro* mineralization and suitable for miRNA studies [20,21]. We bring novel data regarding miR-29a effect on *in vitro* mineral deposition and osteogenic differentiation as determined by the expression of specific marker genes. We also provide information regarding conservation of miR-29a mechanisms of action and regulation in vertebrates.

Materials and methods

Cell culture maintenance

The ABSa15 cell line was recently deposited in the European Collection of Cell Cultures (Ref. 13112201). This cell line was previously developed in our laboratory from calcified branchial arches of the gilthead seabream (*Sparus aurata*, Linnaeus, 1758) and is capable of *in vitro* mineralization [21]. Also, ABSa15 cells express several genes known as markers for differentiation and mineralization [20]. ABSa15 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (SIGMA), 1% penicillin/streptomycin, 0.2% Fungizone and 2 mM L-Glutamine (all from GIBCO BRL, Gaithersburg, MD) at 33 °C in 10% CO_2. Cells were sub-cultured every 2–3 days through trypsinization.

Extracellular matrix (ECM) mineralization

ASBa15 cells were seeded at a density of 2×10^4 cells/well in 24-well plates. ECM mineralization was induced in confluent cultures through the addition of L-ascorbic acid (50 μg/ml), β-glycerophosphate (10 mM) and $CaCl_2$ (4 mM) to the growth medium. Differentiation medium was renewed every 3–4 days. At appropriate times, mineral deposition was revealed through von Kossa staining and quantified by densitometry analysis or through alizarin red S staining and spectrophotometric quantification as described elsewhere [20,22,23].

RNA extraction and quantitative real-time PCR (qPCR) analysis

Total RNA was extracted as previously described [24], quantified by UV spectrophotometry (NanoDrop ND-1000) and its quality analyzed in agarose gel electrophoresis. Zebrafish adult tissues RNA was obtained from a pool of 5 specimens whereas mouse adult tissues RNA was isolated from a pool of 4 specimens. RNA from cells was extracted from confluent cultures. Quantitative real-time PCR (qPCR) analysis of miRNAs and mRNAs was performed using the StepOnePlus system (Applied Biosystems). For qPCR analysis of miRNAs, total RNA (1 μg) was treated with RQ1 RNase-free DNase (Promega), then polyadenylated, reverse-transcribed and amplified using miRNA-specific primers (Sup. Table 1) and the NCode miRNA First-Strand cDNA Synthesis and NCode SYBR miRNA qRT-PCR kits (Invitrogen), according to manufacturer's instructions. For qPCR analysis of mRNAs, 1 μg of total RNA was treated with RQ1 RNase-free DNase, then reverse-transcribed using MMLV-RT (Invitrogen) and oligo-d(T)-adapter primer (Sup. Table 1), according to manufacturer's instructions. For analysis of miRNAs, PCR amplifications were performed using 1.6 ng of cDNA, gene-specific primers (Sup. Table 1) and Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), according to manufacturer's instructions. For analysis of mRNAs, PCR amplifications were performed using 10 ng of cDNA, gene-specific primers (Sup. Table 1) and SsoFast EvaGreen Supermix (Bio-Rad), according to manufacturer's instructions. Relative mRNA and miRNA expression was calculated using the ΔΔCt method [25] and normalized using expression levels of ribosomal protein L27a (RPL27a) and U6 small nuclear RNA (U6), respectively.

Establishment of stable fish cell clones overexpressing miR-29a

For miR-29a overexpression, oligonucleotides containing forward and reverse sequences of zebrafish pre-miR-29a were annealed and inserted into pcDNA6.2-GW/EmGFP-miR vector downstream of GFP coding sequence using the BLOCK-iT Pol II miR RNAi Expression Vector kit (Invitrogen), according to manufacturer's instructions. Identity and integrity of inserted fragments were confirmed through sequencing.

ABSa15 cells were seeded in 6-well plates at 2×10^5 cells/well, cultured for 14–16 h and transfected with 2.4 μg of pcDNA6.2/EmGFP-miR29a construct and FuGene HD (Roche), according to manufacturer's instructions. After 24 h, cells were sub-cultured into a 10-cm culture dish and blasticidin selection antibiotic was added to cell culture medium (optimal concentration of 2 μg/ml of medium). The amount of antibiotic to be used was determined as described in the manual of BLOCKiT Pol II miR RNAi Expression Vector kit. After 30 days in selective medium (renewed twice a week), cell colonies expressing GFP were identified using Olympus IX-81 fluorescence microscope, and then sequentially sub-cultured into 48-well, 24-well, 6-well and 10-cm culture dishes. Positive clones were assessed for miR-29a expression by qPCR analysis and further characterized for ECM mineralization, as described above.

Transient overexpression of miR-29a during ECM mineralization

miRIDIAN microRNA mimic for dre-miR-29a (denominated MmiR-29 from now on) or negative control 1 (NC) (both obtained from Dharmacon) were delivered in triplicates to ASBa15 cells at a final concentration of 50 nM using EzWay (Koma Biotech) transfection reagent, according to manufacturer's instructions. ABSa15 cells were seeded at a density of 2×10^4 cells/well in 24-well plates and transfected 16 h later. In a parallel experiment (control experiment), cells were transfected with fluorescent oligos to control both transfection efficiency and oligos abundance