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Age-associated changes in microRNAs affect the differentiation potential of human mesenchymal stem cells: Novel role of miR-29b-1-5p expression

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bone.2021.116154.

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Abstract

Age-associated osteoporosis is widely accepted as involving the disruption of osteogenic stem cell populations and their functioning. Maintenance of the local bone marrow (BM) microenvironment is critical for regulating proliferation and differentiation of the multipotent BM mesenchymal stromal/stem cell (BMSC) population with age. The potential role of microRNAs (miRNAs) in modulating BMSCs and the BM microenvironment has recently gained attention. However, miRNAs expressed in rapidly isolated BMSCs that are naïve to the non-physiologic standard tissue culture conditions and reflect a more accurate *in vivo* profile have not yet been reported. Here we directly isolated CD271 positive (+) BMSCs within hours from human surgical BM aspirates without culturing and performed microarray analysis to identify the age-associated changes in BMSC miRNA expression. One hundred and two miRNAs showed differential expression with aging. Target prediction and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses revealed that the up-regulated miRNAs targeting genes in bone development pathways were considerably enriched. Among the differentially up-regulated miRNAs the novel passenger strand miR-29b-1-5p was abundantly expressed as a mature functional miRNA with aging. This suggests a critical arm-switching mechanism regulates the expression of the miR-29b-1-5p/3p pair shifting the normally degraded arm, miR-29b-1-5p, to be the dominantly expressed miRNA of the pair in aging. The normal guide strand miR-29b-1-3p is known to act as a pro-osteogenic miRNA. On the other hand, overexpression of the passenger strand miR-29b-1-5p in culture-expanded CD271+ BMSCs significantly down-regulated the expression of stromal cell-derived factor 1 (CXCL12)/ C-X-C chemokine receptor type 4 (SDF-1 (CXCL12)/ CXCR4) axis and other osteogenic genes including bone morphogenetic protein-2 (BMP-2) and runt-related transcription factor 2 (RUNX2). In contrast, blocking of miR-29b-1-5p function using an antagomir inhibitor up-regulated expression of BMP-2 and RUNX2 genes. Functional assays confirmed that miR-29b-1-5p negatively regulates BMSC osteogenesis *in vitro*. These novel findings provide evidence of a pathogenic anti-osteogenic role for miR-29b-1-5p and other miRNAs in age-related defects in osteogenesis and bone regeneration.

Keywords

Human mesenchymal stem cells; Aging; microRNA; Osteoporosis; SDF-1; CXCL12

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1. Introduction

Aging, in addition to being inevitable, appears to in part involve an evolutionary conserved, genetically programmed decline in function that affects us all [1]. Age-related bone loss, including osteoporosis, is characterized by a progressive decline in bone mass and mineral density [2]. As with many age-associated disorders a number of emerging changes in epigenetic regulation of gene expression has been observed with osteoporosis [3–6].

Bone is a primary structural connective tissue, as well as a component of several key physiological systems regulating the immune system [7,8], hematopoiesis [9,10], peripheral wound repair [11,12], kidney function [13], ionic and energy balance [14]. As might be expected this critical organ is regulated by multiple cross-linked pathways. Bone remodeling involves two stem cell populations; hematopoietic stem cells giving rise to osteoclasts and mesenchymal stromal/stem cells (BMSCs) giving rise to osteoblasts. BMSCs are involved in both peripheral injury repair and locally within the bone marrow (and periosteum) differentiating into adipocytes or osteoblasts [15–17]. In age-related osteoporosis, increased bone fragility is derived from an imbalance in bone resorption relative to bone formation [18–20]. Accumulating evidence suggests that age-related osteoporosis is a stem cell disease. The aged bone and BM microenvironment suppresses the function of adult stem cells in bone formation [21]. This includes changes in expression of inflammatory mediators in aging that are linked to bone mass [22–25]. One of the inflammatory factors we have been focused on is the osteogenic cytokine/chemokine stromal cell-derived factor 1 (SDF-1), also called CXC-motif-ligand-12 (CXCL12). In addition to its chemotactic role in cell mobilization and migration, SDF-1 is a critical regulator of BMP-2 activity, a key osteogenic pathway [26–30]. SDF-1 levels and bioactivity decrease with age in the BM microenvironment and in BMSCs [26,31–35].

Recent studies suggest microRNAs (miRNAs) play a major role in the regulation of physiological bone development and BMSC differentiation decisions during aging [36–42]. Until now, research has focused on the role of miRNAs in extensively cultured BMSCs from a variety of species [43–45]. Significant progress has been made in identifying numerous tissue specific miRNAs, which are selectively expressed at particular stages in bone and joint tissue development [41,46]. However, no studies have reported the age-related miRNA changes in rapidly isolated uncultured BMSCs that possess a relatively uncompromised memory of their resident miRNA gene expression signature. We used a rapid non-culturing approach to isolate BMSCs in less than 3 h to avoid artifacts in assessing the profiles of miRNAs expressed in BMSCs *in vivo* [47,48]. Non-physiological tissue culture generated artifacts result in differences in gene expression, cell differentiation, function, and frequently exposure to factors that induce senescence.

Currently available studies on miR-29b and related family members miR-29a, b2, & c have focused on the nominal 3p guide strands including miR-29b-1-3p (previously miR-29b-1-1). The strands processed from the miR-29 family 3p arms have been widely considered as the functional mature miRNA arms [49–51]. In contrast, miR-29b-1-5p (previously miR-29b-1-1*), as well as the other 5p arm miRNAs from miR-29a, b2 & c have largely been ignored owing to their suggested status as non-functional passenger strand by-

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products of the biogenesis process. Recently and importantly, our research group found that kynurenine, a tryptophan metabolite that accumulates with age is a driver of osteoporosis [26]. Importantly, we have shown that Kynurenine upregulates miR-29b-1-5p expression while suppressing the 3p guide strand in human and murine BMSCs. MiR-29b-1-5p directly targets HDAC3 and SDF-1 (CXCL12) and impairs the osteogenic differentiation ability of BMSCs [26]. Here we identify novel age-associated changes in human CD271+ BMSC miRNA gene expression that are involved or potentially involved in the molecular regulation of the SDF-1 axis, as well as multiple other osteogenic genes, their regulatory pathways, and ultimately bone homeostasis.

2. Methods

2.1. Mesenchymal stem cell direct isolation and expansion

Bone marrow was obtained under sterile conditions from orthopaedic surgery patients as per the Institutional Review Board (IRB) of Augusta University. Briefly, bone marrow was aspirated from the iliac crest of orthopeadic surgical patients for use in their bone repair surgery, and 2–10 mL of the surgical waste bone marrow was collected in EDTA tubes for our BMSC isolation. In some cases, bone marrow was aspirated from vertebral bodies of spinal orthopaedic surgery patients as per the Institutional Review Board (IRB) of the Medical University of South Carolina (Fig. 6). Bone marrow was filtered through 100 μm filter to remove bone fragments and cell clumps. Cells were layered in Ficoll-Paque in an appropriate conical tube and centrifuged for 35 min at 445 ×*g*. Bone marrow mononuclear cells (BM MNC) were carefully transferred to a new canonical tube, washed and centrifuged again at 200 ×*g* for 10–15 min to remove platelets. The CD271 positive (+) BMSCs were isolated according to the manufacturer's protocol using a CD271 isolation kit (Miltenyi Biotec Inc., 130–092–283). Briefly, cells were magnetically labeled by mixing 5×10^7 cells in 2 ml of Bone Marrow with 50 μl of FcR blocking reagent and 50 μL of CD271-APC and then incubated for 10 min at 4 °C in the dark. Cells were washed, centrifuged and resuspended in 50 μl of FcR blocking reagent and 100 μl of Anti-APC MicroBeads. The mixture was incubated for 15 min at 4 °C, then washed and centrifuged. For magnetic separation, the cell mixture was loaded onto a MS column placed in the magnetic field of a MACS Separator. The unlabeled fraction of cells was collected for other uses, and the column was washed 3 times. The column was removed from magnetic separator and the CD271 labeled cells were collected by flushing the column into a collection tube. The cells were then either immediately treated to isolate RNA or placed in BMSC cell culture for rapid expansion as described below. From surgical aspiration to RNA collection takes approximately 2–3 h *versus* 3 weeks minimum in culture with multiple passages for standard human BMSC isolations prior to use of the cells as BMSCs.

For the microarray study, the direct-isolation procedure was used to quickly capture CD271+ BMSCs from bone marrow without culturing or standard plastic adherence. For other studies, CD271+ BMSCs were isolated directly from bone marrow, washed with standard culture medium composed of DMEM medium (Corning, 10–013-CM), 1% antibiotics-antimycotics (AA; Invitrogen, 15,240–062) and 15% FBS, transferred to 100 mm culture dish and incubated at 37 °C in humidified atmosphere at 5% carbon dioxide (CO_2). After 24

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h, the medium with non-adherent cells was removed, and the adherent cells were carefully washed in DPBS, further expanded in fresh standard culture medium. Culture-expanded CD271+ BMSCs of passage 1 were used for quantitative real-time polymerase chain reaction (qPCR), while passages 2–4 were used for studies described below.

2.2. microRNA Array and miRNA target prediction

Microarrays were performed using an Affymetrix GeneChip® miRNA 2.0 Array at the Integrated Genomics Core, Georgia Regents University, GA. Directly captured CD271+ BMSCs from 3 "young" patients (29–41 years of age) and 4 "old" patients (64–73 years of age) were lysed with TRIzol (Invitrogen, 15,596–018). RNA was isolated as described earlier [52] and further purified using the RNeasy MinElute Cleanup Kit (Qiagen, 74,204). FlashTag™ Biotin HSR RNA Labeling Kit (Affymetrix®, 901,910) was used to prepare biotin labeled cRNA probes from 1 microgram (μg) of purified total RNA. Briefly, in the first step, single stranded cDNA was synthesized by reverse transcription. Single stranded cDNA was further converted into double stranded cDNA which was extracted with phenol/chloroform and then precipitated with ethanol. An *in vitro* transcription (IVT) reaction was then carried out in the presence of biotinylated UTP and CTP to produce biotin-labeled cRNA from the double stranded cDNA. The resulting cRNA was then fragmented in the presence of heat and Mg++ and were hybridized in GeneChip Hybridization Oven 640 (Affymetrix®) at 60 °C for 16 h. The miRNA array was then washed and stained with streptavidin-phycoerythrin using the GeneChip Fluidics Station 450 (Affymetrix®) and then scanned using GeneChip Scanner 3000 (Affymetrix®). Data was extracted from the images, quantile-normalized, summarized (median polish), and $\log^2$-transformed with the miRNA QC software from Affymetrix®. The results were assessed by 2-way ANOVA analysis of young *vs* old CD271+ BMSCs (with sex and age categories as the variables) using the Partek® Genomics Suite™ (PGS7.19.1125) [53]. The miRNA profile was analyzed for hierarchic clustering of miRNA to generate heat maps. The results were normalized using robust multichip average (RMA).

In addition to validated targets for miRNA from reported studies, pathway analysis of significantly differentially expressed miRNAs and their associated putative genes were performed using built-in TargetScan5.0, Partek® Pathway and KEGG databases (https://www.genome.jp/kegg/pathway.html) within PGS7.19.1125.

2.3. miRNA mimic and inhibitor transfection

Transient transfections of miRNA mimics and inhibitors were performed using HiPerFect transfection reagent (Qiagen, 301,704) according to manufacturer's protocol. In brief, shortly before transfection, 2×10^4 cells/cm^2 of culture-expanded CD271+ BMSCs were seeded per well of a 24-well plate in 0.5 ml of complete media. For the short time until transfection, the cells were incubated under 37 °C and 5% CO2. The miRNA mimic or inhibitor was diluted to final concentration of 5 nM or 50 nM in 100 μl culture medium without serum. 3 μl of HiPerFect Transfection Reagent was added to the diluted miRNA and was mixed by vortexing. Samples were incubated for 5–10 min at room temperature to allow the formation of transfection complexes. Formed complexes were added dropwise onto the cells. Uniform distribution of the transfection complexes was achieved with gentle

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swirling of the plate. Cells with the transfection complexes were kept under their normal growth conditions and monitored. Syn-hsa-miR-29b-1-5p mimic and anti-hsa-miR-29b-1-5p inhibitor were purchased from Qiagen (see Supplementary Table. 5A). AllStars Negative Control siRNA (cat# SI03650318, Qiagen) was used as a control for Syn-hsa-miR-29b-1-5p mimic and miScript Inhibitor Neg. Control (cat# 1027271, Qiagen) was used as a control for anti-hsa-miR-29b-1-5p inhibitor. The cell lysates and the conditioned medium were harvested at different experimental conditions and used for miRNA, mRNA qPCR assays, ELISA, differentiation assays and migration assays.

2.4. Mature microRNA qPCR

Directly captured or culture-expanded CD271+ BMSCs were lysed in TRIzol and the RNA was isolated as described earlier [54]. The cDNA was prepared in a reverse transcription reaction using miScript II RT kit (Qiagen, 218,160). Three nanograms of cDNA served as the template for real-time PCR iCycler™ (Bio-Rad) using a miRNA-specific miScript Primer assay (forward primer; see Supplementary Table. 5B) and the miScript SYBR Green PCR kit (Qiagen, 218,073), which contains miScript Universal Primer (reverse primer) and the Quantitect SYBR Green PCR Master Mix. The expression levels of miRNAs were normalized to RNU6B and SNORD61. Unless otherwise stated, experimental groups were compared to control groups (Younger CD271+ BMSCs, Control miRNA, Control inhibitor).

2.5. mRNA qPCR

Culture-expanded CD271+ BMSCs were lysed by TRIzol. RNA isolation and subsequent cDNA synthesis (Bio-Rad, 170–8891) were performed as previously described [54]. cDNA was amplified in duplicates in each 40-cycle reaction using an iCycler™ (Bio-Rad) with annealing temperature set at 60 °C, ABsolute™ QPCR SYBR® Green Fluorescein Mix (ABgene, Thermo Fisher Scientific), and custom-designed qPCR primers (Supplementary Table 5C) (Thermo Fisher Scientific). A melt curve was generated to analyze the purity of amplification products. The expression levels of mRNA were normalized to β-actin and 18 s. Relative expression of mRNA was evaluated by using the comparative CT method (Ct) [55]. Unless otherwise stated, experimental groups were compared to control groups (younger CD271+ BMSCs, control miRNA, control inhibitor).

2.6. Western blotting

Whole cell lysates of BMSCs were prepared in RIPA lysis and extraction buffer (#89901 ThermoFisher Scientific) containing protease and phosphatase inhibitor cocktail (Millipore Sigma). Protein concentration was determined using Pierce BCA Protein Assay Kit (#23225 ThermoFisher Scientific) and equal amounts (30 μg) of protein lysates were subjected to SDS-PAGE using gradient 4–12% NuPAGE Bis-Tris gels (#NP0321 Invitrogen) and transferred to 0.2 mm nitrocellulose membranes using Power Blotter Select Transfer Stacks (#PB3310 ThermoFisher Scientific).

Membranes were blocked with 5% Bovine Serum Albumin (#A2153 Sigma-Aldrich) in TBST. Specific primary antibodies were used to detect osteogenic markers Collagen 1A1 (mouse, #sc-293,182, Santa Cruz), Osteocalcin (rabbit, #sc-630,045, Santa Cruz), RUNX2 (mouse, #ab76956, Abcam), BMP2 (rabbit, #MBP1–19751, Novus Biological), and

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Beta Actin (mouse, #A5441, Sigma). Bound antibodies were visualized with Pierce ECL detection system (#32106 ThermoFisher Scientific) on Amersham Imager 680 (GE Healthcare, Pittsburgh, PA). The intensity of immunoreactive bands was quantified using Image Lab (Bio-Rad, Hercules, CA).

2.7. Osteogenic differentiation assay

The ability of culture-expanded CD271+ BMSCs to differentiate into osteogenic cells was validated according to earlier described methods [54,56]. In brief, cells were plated in 24-well plates at 5000 cells/cm^2 and cultured in DMEM for 24 h. Culture medium was then aspirated and replaced with differentiation-specific medium. For osteogenesis, the cultures were incubated in DMEM that was supplemented with 5% FBS, 1% AA, 0.25 mM ascorbic acid (Sigma-Aldrich, A4544), 0.1 mM dexamethasone (Sigma-Aldrich, D4902), and 10 mM β-glycerophosphate (Sigma-Aldrich, G9891). The freshly prepared medium was replaced 2 times per week for 3 weeks. Osteogenic differentiation was assessed by staining for bone mineralization with Alizarin Red (AR; Sigma-Aldrich, A5533). The cells were fixed with 10% formalin for 20 min at room temperature (RT) and stained with 40 mM AR, pH 4.1 for 20 min at RT. Stained monolayers were visualized by phase-contrast microscopy using an inverted microscope (Nikon, Melville, NY). Differentiation was quantified as previously described [26,57]. In brief, cells were destained by using 10% cetylpyridinium chloride (Sigma-Aldrich, C0732). Collected samples were then analyzed by using a microplate reader at 590 nm.

2.8. Transwell migration assay

In vitro chemotaxis was assayed using the HTS Transwell® 96-well system (8 μm pore size; Corning®, 3374 and 3583). Bone marrow aspirate supernatants or medium conditioned by culture-expanded CD271+ BMSCs at different experimental conditions were collected and loaded in the bottom chamber of the black receiver plate as a source of chemoattractants. The inserts (upper compartment) of the transwell plate were placed to expose to the lower chamber containing the samples. BMSCs derived as previously described [58] from 18-month-old male C57BL/6 J mice were starved overnight in a Phenol Red Free DMEM supplemented with 1% FBS. After the incubation, the cells were dissociated with trypsin/ EDTA and samples each containing 2×10^3 cells in 50 μl of migration buffer (Phenol Red Free DMEM/1% FBS) were added to the transwell inserts and incubated for 6 h at 37 °C in 5% CO_2 to allow migration across the porous membrane. SDF-1 (100 ng/mL) diluted in a migration buffer was used as a positive control and fresh migration buffer was used as a negative control. At the end of the incubation, cells that had migrated into the lower chamber and attached to the lower surface of the filter were detached by trypsin/EDTA, lysed and stained with Cyquant® dye (Molecular probes, C7026) and counted using a fluorescence plate reader (M1000 Infinite®; Tecan). All experiments were performed in triplicate.

2.9. Luciferase reporter assay

Culture expanded CD271+ BMSCs were seeded at 1.5×10^4 cells/cm^2 in a 96 well plate (Costar®, 3917) and were co-transfected with the LightSwitch 3’-UTR reporter GoClone plasmids (30 ng/μl; SwitchGear Genomics, COL1A1-S809272 or empty vector- S890005) together with *syn*-hsa-29b-5p or Control miRNA (5 nM; Qiagen) using DharmaFECT DUO

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transfection reagent (Fisher Thermo Scientific, 2010–01). Luciferase activity was analyzed 24 h post transfection using LightSwitch Luciferase Assay reagents (SwitchGear Genomics, LS010). The signal was normalized to empty vector and the luciferase signal ratio of the average mimic signal divided by the average signal from the control miRNA was calculated. All transfection experiments were conducted in triplicate.

2.10. Statistical analysis

Statistical significance was assessed by comparing mean values ± standard deviation (± SD) using Student's *t*-test and analysis of variance (ANOVA) followed by the Tukey's *post hoc* test for independent groups. Experiments were performed 3 independent times. Significance was assumed for $p < 0.05$. Data were analyzed using GraphPad Prism version 8.3.0 for Windows (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Aging induces differential expression of more than 100 miRNAs among human miRNA genome

Using flow cytometry analysis, we confirmed that the phenotype of isolated CD271+ BMSCs was consistent with the accepted profile for BMSCs (92.1% CD90; 80.1% CD105; 12.5% CD14/CD20/CD34/CD45; 91.4% CD73) (Supplementary Fig. 1) [59]. To investigate the age-related changes in miRNA expression, we performed miRNA profiling on uncultured CD271+ BMSCs captured from 3 younger (29–41 years of age) and 4 older (57–73 years of age) patient's BM aspirates. Two-way ANOVA analysis identified 102 of the whole human miRNA genome to be significantly differentially expressed with aging ($p < 0.05$ and absolute fold change<1.1). Among the differentially expressed miRNAs, 39 were up-regulated and 63 were down-regulated with age (Supplementary Table. 1 and 2). Mapping of hierarchical cluster analysis using the normalized miRNA expression data confirmed that the expression of miRNAs in younger CD271+ BMSCs is distinctly different from the older CD271+ BMSCs (Fig. 1A and 1B).

To investigate the potential biological impact of differentially expressed miRNAs, we computationally identified putative miRNA targets employing TargetsScan5.0 built inside Partek® Genomics Suite™ (PGS7.19.1125). This was a conservative approach; we combined the 39 up-regulated miRNAs to identify their mRNA targets and found that 29 of these 39 miRNAs had validated gene targets. That is the genes linked to these miRNAs as targets had been identified and independently experimentally validated as target genes, a total of 17,538 putative genes were targeted by this group of miRNAs, of which 5229 were distinct genes. Similarly, we combined the 63 down-regulated miRNAs with their mRNA targets using TargetScan4.0 and found that 28 of these 63 miRNAs target a total of 5621 known putative genes (of which 3672 are distinct). While the other miRNAs have predicted targets they have not been validated as functional targets. Verified Genes identified as miRNA targets were analyzed with Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. Supplementary tables 3A and 3B summarize the multiple predicted pathways regulated by genes targeted by differentially expressed miRNAs. For up-regulated miRNAs, the target gene clusters were associated with 317 different cellular pathways. Out of the

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317 pathways, 105 pathways were significantly enriched by the genes targeted by 29 up-regulated miRNAs (enrichment score greater than 3 and p-value<0.05). Interestingly, the critical osteogenic wnt signaling pathway was the second highest scoring pathway (enrichment score: 24.74, p 0.0001) (Supplementary Fig. 4), signaling pathways regulating pluripotency of stem cells (enrichment score: 18.81, p 0.0001) (Supplementary Fig. 5), longevity regulating pathway (enrichment score: 10.05, p 0.0001) and cellular senescence pathway (enrichment score: 9.33, p 0.0001) were among the highest enriched pathways by genes targeted by up-regulated miRNAs with aging. For down-regulated miRNAs, the target gene clusters were associated with 324 different cellular pathways, 117 pathways were significantly enriched by the genes targeted by 28 down-regulated miRNAs (enrichment score greater than 3 and p-value<0.05). As more predicted gene targets are validated the pathway analysis will potentially change to reflect the added gene targets.

In addition to verified targets for miRNA reported in Partek® Genomics Suite™, predicted miRNA targets of interest relevant to the SDF-1 signaling axis and osteogenesis were selected according to other public database information. Three databases were considered; miRDB (http://mirdb.org), TargetScan ((http://www.targetscan.org/vert_72/) and TargetMiner (https://www.isical.ac.in/~bioinfo_miu/targetminer20.htm). Supplementary tables 4A and 4B summarize the multiple predicted genes that might be targeted by differentially expressed miRNAs. Osteogenic genes including bone morphogenetic protein-2 (BMP2), β-arrestin, stromal derived factor-1 (SDF-1/CXCL12), insulin-like growth factor 1 (IGF-1), leptin receptor and peroxisome proliferator-activated receptor gamma 2 (PPAR-γ2) that were putatively targeted by the differentially expressed miRNAs. This was a focused discovery and does not show all potential gene targets or rank potential interactions. Predicted genes of interest targeted by miR-29b-1-5p were experimentally assessed as describe below.

To confirm the data obtained from microarray analysis we isolated miRNAs from an additional set of patient BM aspirates using the same rapid CD271+ BMSCs isolation approach. However, to have enough miRNA for multiple by quantitative real-time PCR (qPCR) experiments we culture-expanded 14 sets of isolated CD271+ BMSCs once (approximately 2–3 days to reach 70% confluency) then isolated the miRNA ($n = 7$; young 18–48 years of age; and n = 7 old 71–80 years of age). We then performed qPCR with primers for 2 of the identified up-regulated and 2 down-regulated miRNAs. In addition to miR-29b-1-5p, which we were interested in further assessing, this included three randomly selected miRNAs chosen for validation by qPCR. The qPCR analysis revealed comparable patterns of miRNA up- or downregulation as seen with the microarray analysis supporting our results (Fig. 2). Next, rapidly isolated CD271+ BMSCs from additional sets of young and old BM aspirates were further culture-expanded up to 3–4 cell passages to attain sufficient cell numbers for additional experiments looking at the gene expression and functional assays described in Sections 3.2 and 3.3 below. This suggests that short-term culture-expanded directly-isolated CD271+ hBMSCs retain a stable miRNA and mRNA expression profile within early passages.

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3.2. Aging downregulates SDF-1/CXCR4 signaling axis genes and osteogenic genes

Computational predictions indicated that the differentially expressed miRNAs with age might play a role in modulating several gene regulatory networks associated with skeletal development and homeostasis. To that end, we performed qPCR analysis of genes relevant to the SDF-1/CXCR4 signaling axis, as well as selected osteogenic genes in culture-expanded CD271+ BMSCs ($n = 6$ young 13–45 years of age; n = 6 old 69–80 years of age). The results showed that all osteogenic and SDF-1 axis gene expression levels assessed were significantly down-regulated with age except for the adipogenic transcription factor PPAR-γ2 (Fig. 3). This supports the finding we recently reported on SDF-1α protein expression in the supernatant of the human BM aspirates (AKA BM interstitial fluid, BMISF) by ELISA and the BMISF's ability to mediate migration of BMSCs in a standard transwell assay. We showed that SDF-1α secretion in older BMISF was significantly reduced compared to younger BMISF and had a reduced ability to drive cell migration [60].

3.3. The passenger strand miR-29b-1-5p is up-regulated with aging and regulates SDF-1/CXCR4 signaling axis genes, osteogenic genes and osteogenic differentiation in CD271+ BMSCs

According to miRBase (http://www.mirbase.org/), hsa-miR-29b-1 encodes minor miRNA hsa-miR-29b-1-5p at the 5p arm, ranging from nt.10 to 33 of pre-miRNA with 623 read counts, and major (mature) miRNA hsa-miR-29b-1-3p at the 3p arm, ranging from nt.51 to 73 with 39,647 read counts. However, among the significantly up-regulated miRNAs, the miRNA expression profiling detected miR-29b-1-5p as the dominant mature miRNA of the duplex miRNA pair (Fig. 1, Supplementary Table 1). We validated this initial microarray observation by qPCR in additional independently isolated hBMSCs populations, this again showed that miR-29b-1-5p expression was up-regulated with aging compared to miR-29b-1-3p (Supplementary Fig. 2). Therefore, we selected 2 culture-expanded CD271+ BMSCs isolated from an 18-year-old female and a 72-year-old female. Based on the rationale that the level of the 5p arm was lower in young hBMSCs, but high in the older hBMSCs we transiently up-regulated miR-29b-1-5p levels using a synthetic miRNA mimic in the younger culture-expanded CD271+ BMSCs, and down-regulated miR-29b-1-5p levels employing a synthetic miRNA inhibitor in the older culture-expanded CD271+ BMSCs. The transfected cells showed no gross morphological changes compared to control transfected cells. As expected, following transfection with the mimetic qPCR showed that the expression of miR-29b-1-5p was increased by ~1000-fold in the younger CD271+ BMSCs 24 h after transfection compared to controls (Fig. 4A). Expression levels remained high even after 48 h (data not shown). We also confirmed that overexpressing miR-29b-1-5p did not alter the expression of miR-29b-1-3p (Fig. 4B). To evaluate the biological effects of the *syn*-hsa-miR-29b-1-5p mimic the expression of the same selected genes related to the SDF-1/CXCR4 signaling axis and BMSC differentiation that were changed with age in hBMSCs (Fig. 3) were measured by qPCR. Similar to aging overexpressing miR-29b-1-5p in young hBMSCs significantly decreased the transcript levels of all assessed genes relative to controls except PPAR-γ2 (Fig. 4C). To verify that the observed miR-29b-1-5p-mediated decrease in SDF-1α mRNA causes a direct impairment in functional protein, we investigated secreted levels of SDF-1α protein from younger CD271+ BMSCs cultured in standard proliferation medium following transient transfection with syn-hsa-miR-29b-1-5p

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mimic. ELISA analysis revealed that miR-29b-1-5p significantly decreased SDF-1α levels in cell culture media compared to controls (Supplementary Fig. 3A). Functional transwell migration analysis showed that miR-29b-1-5p significantly attenuated the chemotactic potential of the conditioned media relative to controls as a result of decreased SDF-1α (Supplementary Fig. 3C). Pretreatment of migratory BMSCs with AMD3100 significantly reduced CXCR4 mediated SDF-1 chemotaxis. Overall, this suggests that overexpression of miR-29b-1-5p decreases SDF-1α levels and chemotactic activity in younger CD271+ BMSCs.

To further confirm the functional activity, syn-miR-29b-1-5p mimic transfected younger CD271+ BMSCs were cultured in osteogenic medium to induce osteogenic differentiation. Results indicated that overexpression of miR-29b-1-5p significantly attenuated the osteogenic differentiation as indicated by Alizarin red staining of the mineralized matrix and the mRNA expression of COL1A1 (Fig. 4D and E). We further verified these results using luciferase reporter assays that contained putative 3'UTR sequence for COL1A1 binding (miR-29b family 'seed sequence). Consistent with bioinformatical prediction, when compared to control, overexpression of miR-29b-1-5p significantly suppressed the luciferase activity of COL1A1 Goclone reporter plasmids (Fig. 4F). Although, without a mutated miR29b-1-5p binding site 3'UTR reporter construct we can't clearly demonstrate direct *versus* indirect targeting of COL1A1 by miR-29b-1-5p.

Transfection with the anti-hsa-miR-29b-1-5p antagomir inhibitor resulted in a decrease in miR-29b-1-5p expression levels by ~19-fold relative to controls in the older CD271+ BMSCs after 24 h (Fig. 5A). Similar to treatment with mimics, the transfected cells showed no gross morphological changes compared to control cells. Again, similar to young cells being treated with the miR-29b-1-5p mimetic, treatment with the antagomir inhibitor did not alter the expression level of miR-29b-1-3p (Fig. 5B). This suggests that the 2 arms do not directly interfere with each other's expression or show complementary pairing. qPCR analysis showed that the transcript levels of SDF-1α and β, CXCR4, BMP-2, runt-related transcription factor 2 (Runx2), and COL1A1 were augmented while the expression of PPAR-γ2 was reduced in miR-29b-1-5p inhibitor transfected cells compared to controls (Fig. 5C), moving in the opposite direction from mimetic effects on young BMSCs (Fig. 4). We also examined SDF-1α protein levels in the conditioned medium collected from older CD271+ BMSCs following transient transfection with anti-hsa-miR-29b-1-5p inhibitor and its effects on migratory BMSCs (Supplementary Fig. 3B and D). While the inhibitor did not show increased SDF-1α protein levels by the ELISA, it did demonstrate significantly enhanced chemotactic migration of inhibitor treated BMSCs relative to controls. Pretreatment of migratory BMSCs with AMD3100 significantly reduced chemotaxis towards the younger BM supernatant, suggesting potentially increased SDF-1α mediated CXCR4 based signaling.

Next, we transiently transfected anti-miR-29b-1-5p inhibitor in the older CD271+ BMSCs prior to inducing *in vitro* osteogenesis. Inhibiting miR-29b-1-5p expression increased osteogenesis as assessed by Alizarin red staining at three weeks (Fig. 5D). However, it did not appear to significantly augment expression of COL1A1 mRNA levels in the older cells at three weeks (Fig. 5E), although by that time point collagen synthesis may have ended.

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We also assessed the protein level expression of a subset of osteogenic markers on three separate rapidly-isolated low passage (2–3) human BMSC lines transfected with either the miR29b-1-5p mimetic or inhibitor (Fig. 6A–D). This showed the 5p mimetic suppressed the protein expression of Runx2, osteocalcin and Col1A1, while the 5p inhibitor increased their expression relative to controls 48 h following transfection. Additionally, the 5p mimetic reduced Alkaline Phosphatase (ALP) activity, an early osteogenic marker, while the 5p inhibitor increased ALP activity (Fig. 6E). Interestingly, neither showed significant effects on BMP2 although there was a trend for the inhibitor to increase BMP2 expression at this time point, assessing at a later time point such as 72 h is needed to see if BMP2 protein levels are affected since we showed changes in the gene expression for BMP2 at 24 h (Figs. 4C and 5C).

In order to see if extensive passaging increases the expression of miR29b-1-5p in rapidly isolated human BMSCs we compared the miRNA expression from four human cell lines at low passage (2–3) then again at high passage (12–13). By passage 8 the cell proliferation rate started to significantly decline, eventually going from a doubling about every 7 days at the lower passages to 10–14 days by passage 12 (Fig. 6F). Of interest there was a biphasic effect with the levels of miR29b-1-5p appearing to increase with high passages (12–13) of older human BMSCs (67 & 70 year old), while there was an apparent decrease in younger (18 & 36 year old) BMSCs. This increase in miR29b-1-5p levels with extensive passaging of older human BMSCs, but not younger BMSCs, echoed the original observations of the higher expression of miR29b-1-5p from the directly isolated non-passaged microarray data (Fig. 1) and the two independent experiments (Fig. 2 and Supplementary Fig. 2) with directly isolated low passage (1) human BMSCs showing higher miR29b-1-5p with age (18–45 *vs* 71–80 year old). Taken together, the results suggest that miR-29b-1-5p is an age-associated negative regulator of osteoblast differentiation characterized by discrete anti-osteogenic properties.

4. Discussion

To date, few studies have investigated the miRNA expression patterns in BMSCs with age and these have used extensively culture expanded BMSCs that likely do not accurately reflect the *in vivo* profiles of miRNA expression [61–63]. A close relationship between the dysregulation of miRNAs and changes in bone formation and loss has been described [64,65]. Here we examined the age-related changes of the human miRNA expression profile in native uncultured CD271+ BMSCs using microarray analysis and identified 102 miRNAs that were differentially expressed with age. Although the gene targets of a number of the differentially expressed miRNAs from our microarray are not well defined in the Partek® Genomics Suite™ verified target database we used, many of the predicted biological functions had a direct association with musculoskeletal system and its development. Overall, these data suggested that the differentially expressed miRNAs might affect stem cell renewal, differentiation, and chemotaxis by changing the fate of hBMSCs in their niche.

Among up-regulated miRNAs, miR-29b-1-5p has been shown to increase with age [26,66–68]. No previous studies have reported the effect of aging on the expression or regulation of miR-1244, a second miRNA we confirmed was up-regulated with age in hBMSCs. However,

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its role in sensitizing non-small lung cancer cells to cisplatin therapy has been reported, and we saw it targeted SDF-1 axis genes reducing their expression when its mimetic was transfected in young hBMSCs (data not shown) [69,70]. Among the down-regulated miRNAs we confirmed miR-1231 was reduced with age. Little is known about it but a lower expression of miR-1231 with an increased expression of miR-29b was described in human colon cancer cells [71]. The biological functions of the remaining down-regulated miRNAs were validated to link with skeletal system development, cancer and neurodevelopmental disorders [72–77].

miRNAs are short non-coding RNA species which are initially transcribed as primary-miRNA molecules, cleaved into precursor miRNAs with 5p/3p arms and a terminal loop, then further processed into a miRNA duplex [64]. The miRNA duplex is unwound into 3p arm/strand and 5p arm/strand. One strand is then selected as the mature guide strand (miRNA) and the other becomes the complementary passenger strand (miRNA* or miRNA star sequence) through a selection process that is not fully understood. Mature miRNA downregulates target mRNA gene expression; either by mRNA degradation or by blocking its translation [78,79]. In contrast, the passenger strand miRNA* was thought to be typically degraded. However, recent studies found that mature functional gene targeting miRNAs can be generated from both the 3p and 5p arm of many duplexes. This has complicated the earlier narrative and it is now understood that this can result in two mature miRNAs from one duplex that can undergo arm-switching where the different arms can each be dominantly expressed and functional in different tissues, developmental stages, pathologies or species, and now it is emerging in cells or tissues during aging as well [80–85]. microRNA dysregulation is coming to be understood as a common inductive feature found in many diseases and in aging [86–88].

The principles governing miRNA expression and activity are starting to be well elucidated, however; the regulation of arm-selection and arm-switching is still poorly understood [89,90]. Genes for the 4 members of the miR-29 family are arranged in pairs at 2 genomic sites [91] and their role in osteogenesis has been well described, with the caveat it is assumed that it is the 3p guide strand being discussed since the bone literature is silent on the 5p passenger strand, unless there is some unrealized confusion as to the strand responsible for described actions [92–95]. miR-29b facilitates osteogenic differentiation of human BMSCs [67,96] and somatic stem cells [97]. Remarkably, at the early stages of osteogenesis, miR-29b was shown to target inhibitors of *in vivo* bone formation including histone deacetylase 4 (HDAC4), transforming growth factor beta-3 (TGFβ3) and dual specific phosphatase. miR-29b was found to control the osteogenic function of Runx2 and directly attenuate collagen gene expression allowing for efficient collagen fibril maturation and mineral deposition [68,98].

As such, available data in the literature strongly support the notion that miR-29 family members exert pro-osteogenic effects. In contrast, our recent study by Elmansi et al., demonstrated that the passenger strand, miR-29b-1-5p, is an anti-osteogenic miRNA that down regulates SDF-1 and HDAC3 levels in murine BMSCs, directly targeting their 3’UTR binding sites [26]. Here, we show that miR-29b-1-5p expression in rapidly directly isolated CD271+ BMSCs suppresses osteogenic genes and osteogenic differentiation, and that

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its inhibition can rescue these effects. Most studies on miR-29b used its mature guide sequence processed from the 3p arm, miR-29b-1-3p, while miR-29b-1-5p was largely considered a non-functional by-product of the biogenesis process. The miR-29 family member miR-29b-1-3p has been well characterized. Using the miR-29b-1-3p sequence, it was shown that the expression of miR-29b is low during the early matrix deposition phases of osteoblastogenesis and increases as the matrix matures and the osteoblasts achieve terminal maturation [99]. In contrast, little is known about miR-29b-1-5p, the other mature miRNA expressed from the same precursor. Xu et al., [100] reported the role of miR-29b-1-5p on cell growth in bladder urothelial cancer. We are the first to demonstrate that CD271+ BMSCs from older patients show age-related arm-switching and produce the passenger strand miR-29b-1-5p as the dominant mature miRNA with little effect on miR-29b-1-3p levels. We have previously reported that treatment of BMSCs with the age-associated tryptophan metabolite kynurenine signals *via* the aryl hydrocarbon receptor (AhR) transcription factor [26]. AhR mediated kynurenine signaling increases miR-29b-1-5p levels and induces an aging-like inhibition of osteogenesis in murine BMSCs *via* the targeting the CXCL12 axis, HDAC3, and other osteogenic genes [26]. Since the sequences of mature miRNA products from 5p and 3p arms are different (Supplementary Table 5B), they are predicted to target primarily distinct sets of mRNAs, although they have some common target genes. The latter may be the result of these genes having separate binding sites for both miRNA strands in their 3'UTRs (data not shown).

Together with a number of other labs we have demonstrated that SDF-1/BMP-2 signaling is critical for regulating the differentiation and survival of BMSCs [54,101,102]. BMP-2 stimulates the master transcription factor, Runx2, and SPARC, which in turn independently or cooperatively stimulate osteoblast genes like COL1A1 at early stages, and others like osteocalcin at later stages of differentiation [103,104]. Furthermore, aged BMSCs are linked to poor wound healing, angiogenesis, proliferation and anti-apoptosis capabilities show reduced Sirtuin 1 (Sirt1) and SDF-1 gene expression [35]. Based on bioinformatics analysis, miR-29b-1-5p was identified to target several genes including SDF-1, mothers against decapentaplegic homolog 4 (SMAD4), calcitonin receptor and Sirt1. We altered the individual miR-29b-1 3p/5p arm levels using mimics and antagomir inhibitors to recapitulate or rescue age-related changes in culture expanded CD271+ BMSCs. We investigated the possibility that miR-29b-1-5p can post-transcriptionally regulate SDF-1α expression. The SDF-1α secretion was significantly lower with reduced chemotaxis activity in the conditioned medium collected from the miR-29b-1-5p mimic expressing CD271+ BMSCs. Additionally, using luciferase reporter assays, we show that miR-29b-1-5p targets directly or indirectly the COL1A1 mRNA 3'UTR. Our group has shown that SDF-1 enhances osteogenesis by regulating BMP-2 signaling *in vitro* [105] and *in vivo* [106]. Here we show that among the many miRNAs whose expression changes with age miR-29b-1-5p by itself is a potent anti-osteogenic factor, that overexpressing miR-29b-1-5p significantly reduces the osteogenic potential of younger CD271+ BMSCs by downregulating SDF-1 expression along with other osteogenic genes. In contrast, inhibiting miR-29b-1-5p expression in the older CD271+ BMSCs can rescue osteogenesis, supporting the idea that targeting miR-29b-1-5p might be a potential therapeutic strategy to address age-related bone loss or dysfunctional bone repair.

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5. Conclusion

Aging alters the miRNA profile in hBMSCs and potentially this change results in a shift in the targeting of mRNAs representing many different genes related to BMSC function and cell fate. Each individual miRNA may have many target genes and the sum of the shift up or down in gene expression across these miRNAs consequently decreases osteogenic activity in aged BMSCs. While there are at least 39 up-regulated and 63 down-regulated miRNAs, we looked at changes related to only a small set focusing on the novel arm-switching increase in the passenger strand of a well characterized and critical osteogenic regulatory miRNA, miR-29b-1. This work suggests that miR-29b-1-5p might act as a potential therapeutic target to rescue osteogenic function in aged BMSCs. However, there are still a large number of potentially important miRNAs to confirm and validate, as well as to clarify their interacting roles in age-driven changes in osteogenic and other BMSC functions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviation

AhR	Aryl hydrocarbon receptor
BM	Bone marrow
BM MNC	Bone marrow mononuclear cells
BMISF	BM interstitial fluid
BMP-2	Bone morphogenetic protein-2
BMSCs	Bone marrow mesenchymal stromal/stem cells
CXCL12	CXC-motif-ligand-12
CXCR4	C-X-C chemokine receptor type 4
IGF-1	Insulin-like growth factor 1
KEGG	Kyoto Encyclopedia of Genes and Genomes

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miRNAs	microRNAs
PPAR-γ2	Peroxisome proliferator-activated receptor gamma 2
qPCR	Quantitative real-time PCR
RUNX2	Runt-related transcription factor 2
SDF-1	Stromal cell-derived factor 1
Sirt1	Sirtuin 1

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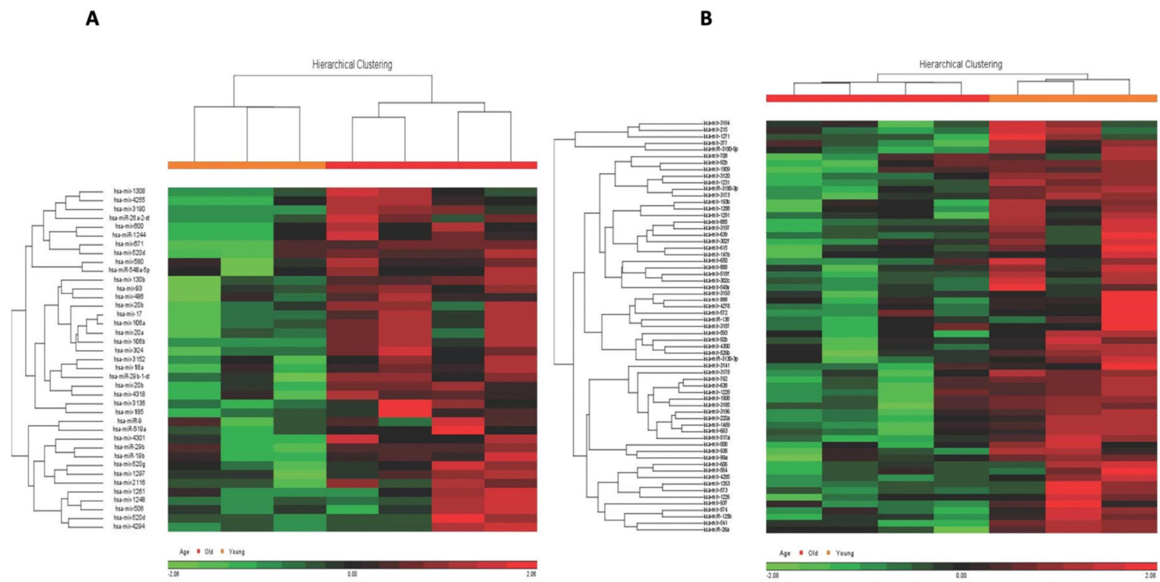

Fig. 1.

Differentially expressed miRNAs in CD271+ BMSCs with age. The figure shows the hierarchical cluster analysis of differentially expressed ($P < 0.05$) miRNAs between younger (29–41 years of age-orange colour) and older (57–73 years of age-red colour) CD271+ BMSCs using Partek® Genomics Suite™ software. (A) is showing 39 up-regulated miRNAs while (B) is showing 63 down-regulated miRNAs with aging. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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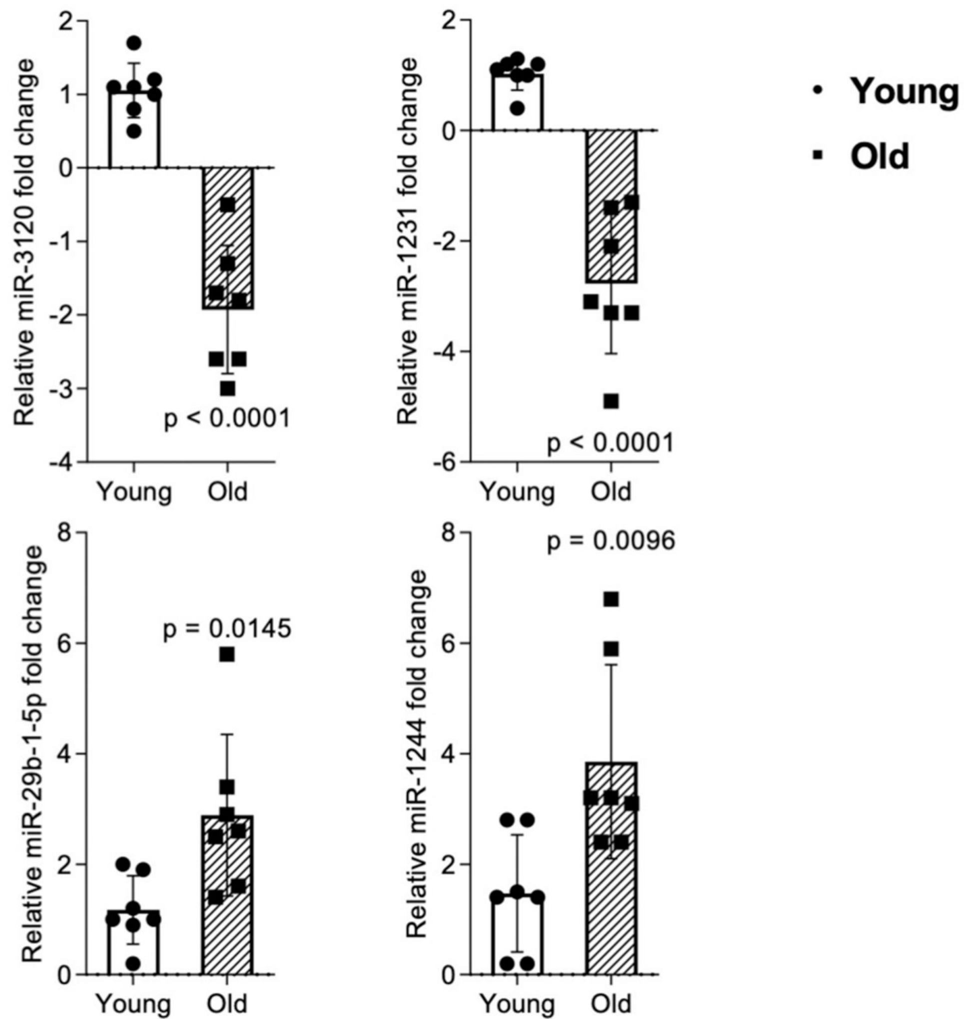

Fig. 2.

Validation of some miRNAs expression levels in CD271+ BMSCs. qPCR analysis for culture expanded CD271+ BMSCs (passage 1) verified down-regulation of miR- 3120 and miR- 1231 (upper panel) and upregulation of miR-29b-1-5p and miR-1244 (lower panel) in older (71–80 years of age) CD271+ relative to younger (18–48 years of age) CD271+ BMSCs. miRNA expression levels were normalized to the average of SNORD1 and RNU-6 expression. Data are shown as means ± SD, n = 7 young and n = 7 old groups.

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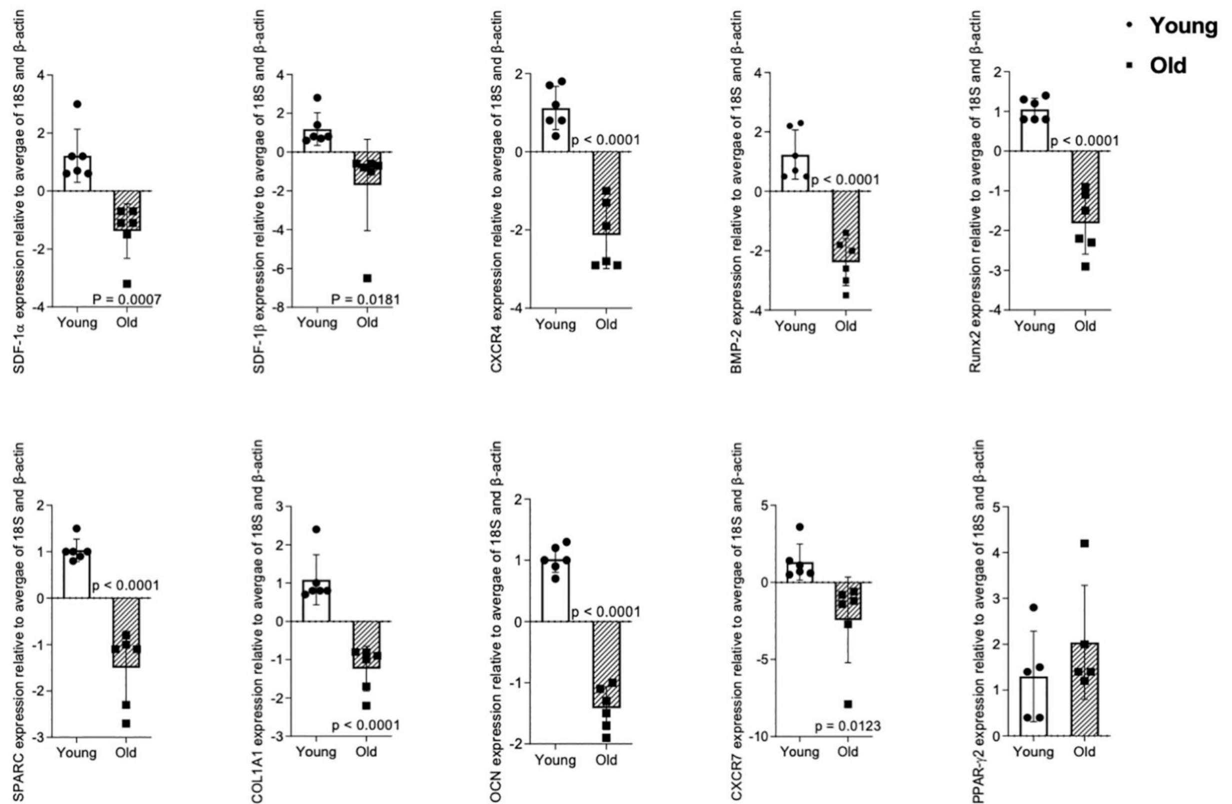

Fig. 3.

Aging regulation of SDF-1/CXCR4 signaling in CD271+ BMSCs. Analysis of gene expression by qPCR showed that except PPAR-γ2 other selected gene transcript levels were significantly down-regulated in older (69–80 years of age) when compared to younger (13–45 years of age) CD271+ BMSCs. Gene expression levels were normalized to average of β-actin and 18 s expression, and the fold change was calculated in relation to younger CD271+ BMSCs. Data are shown as means ± SD, n = 6 young and n = 6 old groups.

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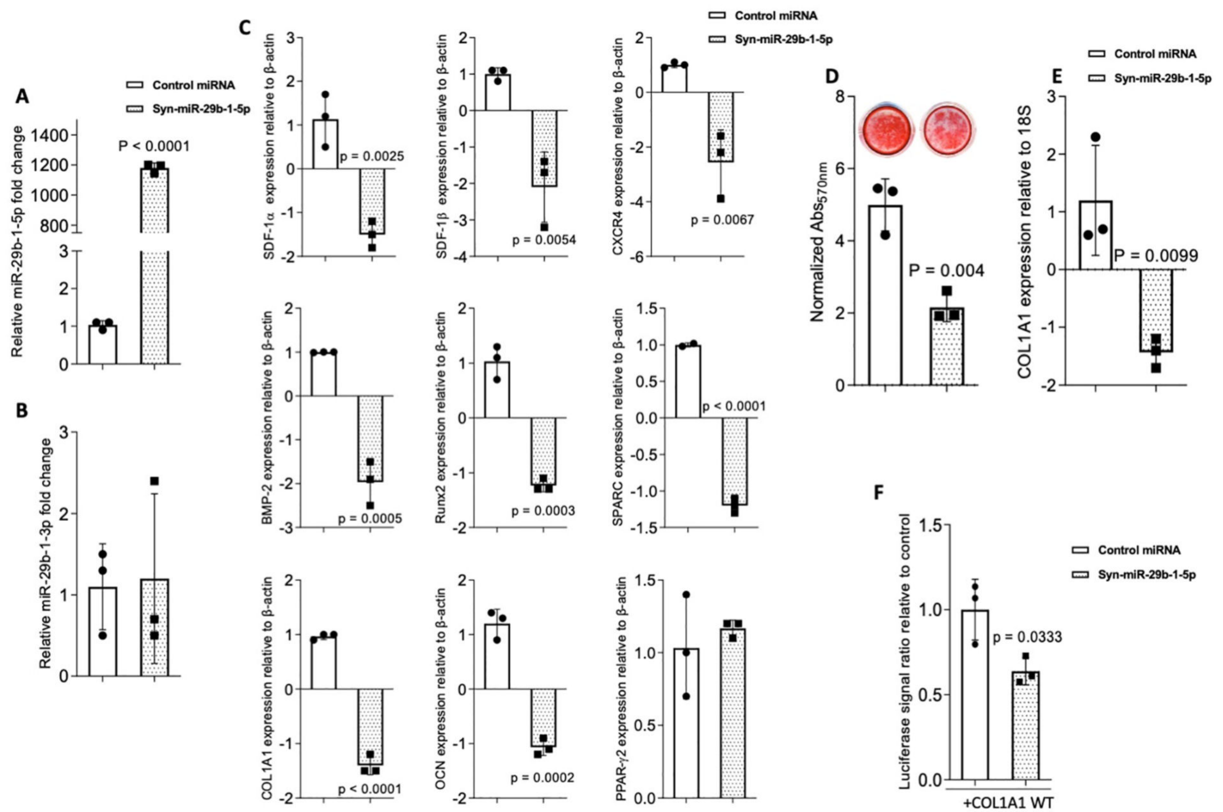

Fig. 4.

Effect of overexpressing *syn*-miR-29b-1-5p mimic on regulating SDF-1/CXCR4 signaling axis genes, osteogenic genes and osteogenic differentiation in younger CD271+ BMSCs. Syn-miR-29b-1-5p mimic and control miRNA were transfected into a younger culture-expanded CD271+ BMSCs (Passage 3). **(A)** After 24 h, the transfection of efficiency was verified using RT qPCR and was presented as fold change relative to control miRNA. miRNA expression levels were normalized to the average of SNORD1 and RNU-6 expression. **(B)** The figure showing the expression level of miR-29b-1-3p. **(C)** Analysis of gene expression by qPCR showed that except PPAR-γ2 other selected gene transcript levels were significantly down-regulated in syn-miR-29b-1-5p mimic transfected cells. Gene expression levels were normalized to β-actin expression, and the fold change was calculated in relation to control miRNA. Data are shown as means ± SD, $n = 3$. **(D)** Mimic and control miRNA were transfected into a younger CD271+ BMSCs. After 24 h, CD271+ BMSCs were induced with osteogenic medium for 21 days. Alizarin red staining was performed and quantified. The results show that overexpression of miR-29b-1-5p significantly inhibits osteogenic differentiation of CD271+ BMSCs. **(E)** qPCR analysis showed that overexpressing miR-29b-1-5p significantly suppressed the COL1A1 transcription level in CD271+ BMSCs at day 21 during osteogenesis. Gene expression levels were normalized to 18 s expression and the fold change was calculated in relation to control miRNA. Data are shown as means ± SD, n = 3. **(F)** Signals from 3'UTR reporters for the COL1A1 was significantly knocked down in the presence of the miR-29b-1-5p mimic when compared to the signals for control miRNA. Data are shown as means ± SD, n = 3. (For interpretation of

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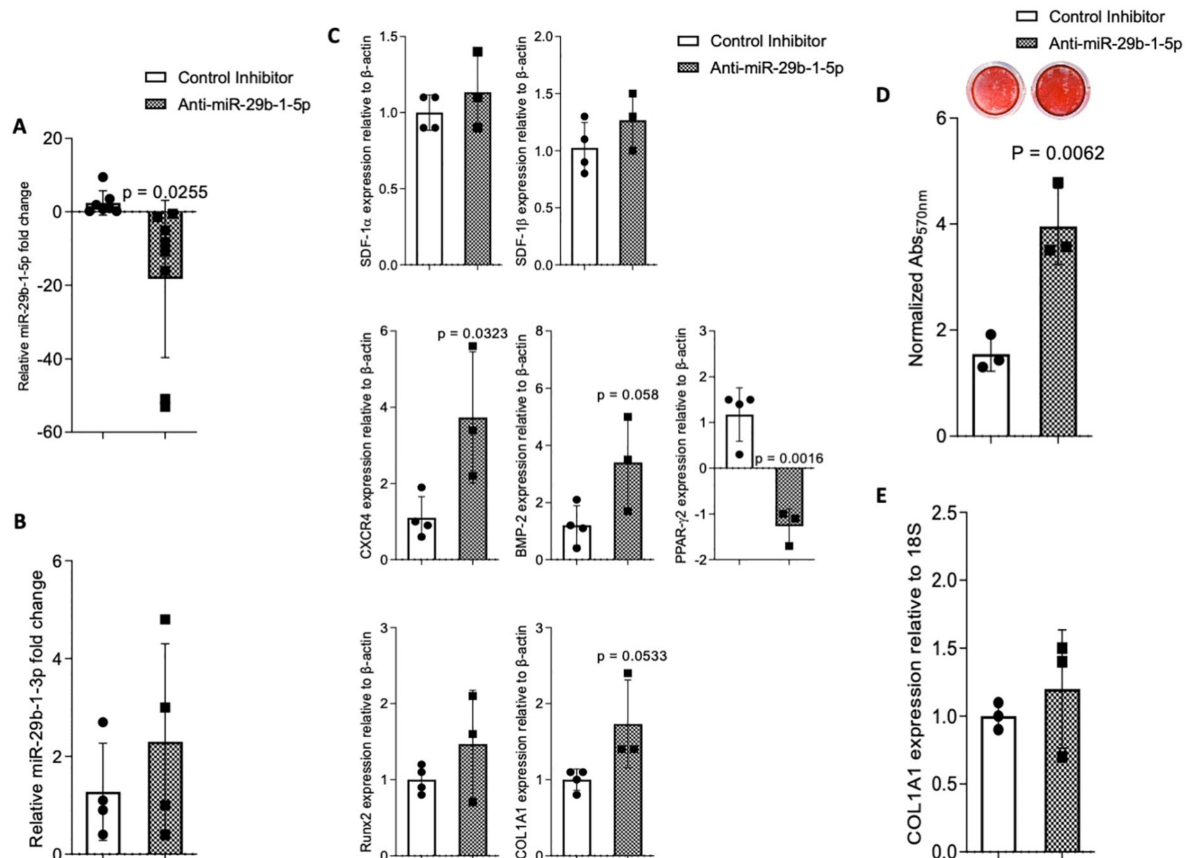

Fig. 5.

Effects of expressing anti-miR-29b-1-5p inhibitor on regulating SDF-1/CXCR4 signaling axis genes, osteogenic genes and osteogenic differentiation in CD271+ BMSCs from older patients. Anti-miR-29b-1-5p inhibitor and control inhibitor were transfected into an older culture-expanded CD271+ BMSCs. **(A)** After 24 h, the transfection of efficiency was verified using miScript qPCR. miRNA expression levels were normalized to the average of SNORD1 and RNU-6 expression and the fold change was calculated relative to control inhibitor. $n = 6$ or 8/group **(B)** The figure showing the expression level of miR-29b-1-3p. $n = 4$/group **(C)** Analysis of gene expression by qPCR showed that except PPAR-γ2, other selected gene transcript levels were up-regulated in CD271+ BMSCs expressing low levels of miR-29b-1-5p. Gene expression levels were normalized to f β-actin expression and the fold change was calculated in relation to control inhibitor. Data are shown as means ± SD, $n = 3$ or 4/group. **(D)** Inhibitors to miR-29b-1-5p and control inhibitor were transfected into an older CD271+ BMSCs. After 24 h, transfected CD271+ BMSCs were induced with osteogenic medium for 21 days. Alizarin red staining for mineralization indicated that the downregulation of miR-29b-1-5p stimulated osteogenic differentiation in CD271+ BMSCs. $N = 3$/group **(E)** Gene expression level of COL1A1 was analyzed by qPCR and was shown as fold change relative to control inhibitor. Gene expression levels were normalized to 18 s expression. Data are shown as means ± SD, n = 3/group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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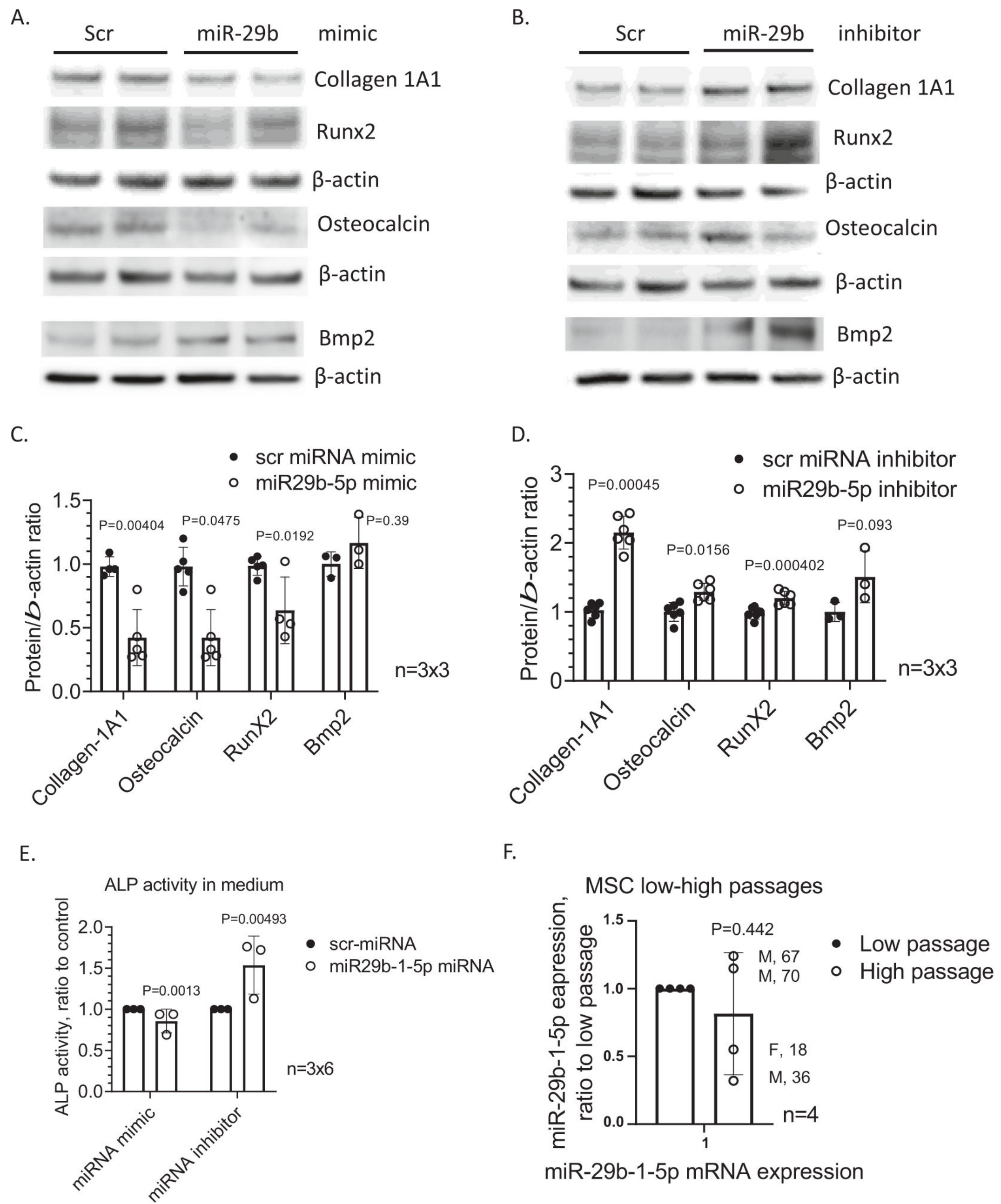

Fig. 6.

miR29b-1-5p inhibits osteogenic protein expression and ALP activity. **(A&C)** miR29b-1-5p mimic suppress expression of osteogenic markers, Collagen-1A1, osteocalcin, and Runx2. **(B&D)** miR-29b-1-5p inhibitor upregulates osteogenic markers in human BMSC. **(A&B) -** representative images of protein expression, (**C&D**) - protein expression changes ratio, data expressed as protein to beta-actin ratio, adjusted to scrambled miRNA controls. **(C&D)** results are a combination of triplicated experiments from 3 separate low (2–3) passage human BMSC lines transfected with the miR29b-1-5p mimetic or inhibitor with 3 biological replicates and 3 technical replicates in each independent experiment (1-way ANOVA). **(E)** Alkaline Phosphatase activity assay with the same 3 separate human cell lines with

6 technical replicates for each experiment (1-way ANOVA). **(F)** Four independent rapid directly isolated human BMSC lines were assessed for the expression of miR29b-1-5p at either passage 2 or 3 then again at either passage 12 or 13. BMSCs from older patient showed increased expression of the miRNA with extensive passaging compared to low passage (when set to 1).

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