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MicroRNAs miR-96, miR-124, and miR-199a Regulate Gene Expression in Human Bone Marrow-Derived Mesenchymal Stem Cells

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ABSTRACT

MicroRNAs are small non-coding RNAs that control gene expression at the post-transcriptional level by binding to 3′-untranslated regions (3′-UTR) of their target mRNAs. They present a promising tool to delineate the molecular mechanisms regulating differentiation of human mesenchymal stromal cells (hMSCs) and to improve the controlled differentiation of hMSCs in therapeutic applications. Here we show that three microRNAs, miR-96, miR-124, and miR-199a, were differentially expressed during osteogenic, adipogenic, and chondrogenic induction of human bone marrow-derived MSCs. miR-96 expression was increased during osteogenesis and adipogenesis, but not during chondrogenesis. miR-124 was exclusively expressed in adipocytes, whereas miR-199a was upregulated in osteoblasts and chondrocytes. Furthermore, functional studies with synthetic miRNA precursors and inhibitors demonstrated that miR-96, miR-124, and miR-199a regulated the expression of genes important for hMSC differentiation, such as *aggrecan*, transcription factor *SOX9*, and fatty acid binding protein 4 (*FABP4*). Modulation of miR-96, miR-124, and miR-199a expression may thus be useful in specific targeting of hMSC differentiation, for e.g., MSC-based therapies. J. Cell. Biochem. 113: 2687–2695, 2012.

KEY WORDS: ADIPOGENESIS; CHONDROGENESIS; MESENCHYMAL STEM CELLS; microRNA; OSTEOGENESIS

Human multipotent mesenchymal stem/stromal cells (hMSCs) present in the bone marrow offer a promise for clinical stem cell therapy. Bone marrow MSCs can be isolated and in vitro differentiated into a variety of cell lineages, including osteoblasts, chondrocytes, and adipocytes [Prockop, 1997]. Cultured hMSCs are hypoimmunogenic and capable of homing, thus having a great potential for various clinical applications [Abdallah and Kassem, 2009], but there are still several open questions related to the control of MSC differentiation. The current endeavors of hMSC-based therapies require a deeper knowledge of the molecular mechanisms controlling the lineage commitment and differentiation of hMSCs.

MicroRNAs (miRNAs) are 20–24 nt, endogenous and evolutionary conserved RNA molecules that negatively regulate the translation of their target mRNAs by binding to their 3′-untranslated regions (3′-UTR) [Bartel, 2004]. Since their discovery in 1993 [Lee et al., 1993], more than a thousand human miRNAs have been annotated but the biological functions of specific miRNAs are still largely unknown. Emerging evidence indicates that miRNAs have a critical role in the self-renewal and differentiation of MSCs [Tomé et al., 2011]. Therefore, regulation of miRNA expression by synthetic oligonucleotides offers a potential tool for the development of MSC-based therapies.

Several miRNAs have been shown to regulate the osteogenic differentiation of human MSCs. For instance, miR-133 inhibits osteogenesis of mesenchymal cells by attenuating the runt-related transcription factor 2 (Runx2) pathway [Li et al., 2008]. Also miR-204 and its homolog miR-211 inhibits osteogenesis and promotes adipogenesis of bone marrow-derived MSCs through negative regulation of Runx2 [Huang et al., 2010]. The miRNA miR-26a negatively regulates the osteogenic differentiation of human

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adipose tissue-derived stem cells (hADSCs) by targeting Smad1 transcription factor [Luzi et al., 2008] while miR-196a is suggested to improve osteogenic differentiation through its predicted target HOXC8 [Kim et al., 2009a]. Moreover, miR-138 suppresses osteogenic differentiation of hMSCs through inhibition of the focal adhesion kinase (FAK) signaling pathway [Eskildsen et al., 2011] whereas miR-20a promotes osteogenic differentiation of hMSC by targeting the negative regulators of BMP signaling [Zhang et al., 2011].

In addition, several miRNAs have been shown to regulate adipogenic differentiation (AD) of hMSCs or hADSCs. For instance, miR-21 enhances AD of hADSCs through modulation of TGF-β signaling [Kim et al., 2009b]. Further studies from the same group recently demonstrated that miR-21 regulates hADSC proliferation through suppression of STAT3 in a high-fat diet induced obesity mouse model [Kim et al., 2012]. In contrast, miR-27b plays a negative role in AD of hADSCs by targeting PPARγ, a transcription factor essential for adipogenesis [Karbiener et al., 2009]. Correspondingly, miR-138 inhibits AD of hADSCs through suppression of adenovirus EID-1 [Yang et al., 2011b]. In addition, miR-369-5p has been suggested to act as a negative regulator of adipogenesis by inhibiting FABP4 expression in hMSCs [Bork et al., 2011]. A recent study with immortalized hMSCs suggested that also miR-155, miR-221, and miR-222 are negative effectors of adipogenesis as their expression levels were decreased during adipogenic induction [Skarn et al., 2011]. In line with this, ectopic expression of these miRNAs was shown to inhibit adipogenesis and to repress adipogenic transcription factors PPARγ and C/EBPα.

In contrast to osteogenic and AD, studies related to the role of miRNAs in chondrogenic differentiation of human MSCs are few. One of the most intensively studied miRNA in chondrocytes is miR-140 due to its specific expression in developing mouse cartilage [Tuddenham et al., 2006]. It has been shown that miR-140 is upregulated during chondrogenic differentiation of hMSCs in parallel with the expression of SOX9 and COL2A1, proteins important for cartilage development [Miyaki et al., 2009]. Furthermore, in vitro and in vivo studies with mice have demonstrated that miR-140 functions as a positive regulator of chondrogenesis through repression of several different targets including HDAC4 [Tuddenham et al., 2006], Smad3 [Pais et al., 2010], and Adamts-5 [Miyaki et al., 2010]. A positive role in chondrogenesis has also been suggested for miR-675, which is abundantly expressed in human articular chondrocytes [Dudek et al., 2010]. Inhibition of miR-675 in chondrocytes resulted in decreased COL2A1 expression whereas overexpression had an opposite effect, suggesting an indirect regulatory role for miR-675 in chondrogenesis. On the contrary, miR-199a* and miR-145 have been identified as negative regulators of chondrogenesis [Lin et al., 2009; Yang et al., 2011a; Martinez-Sanchez et al., 2012]. In vitro studies with mouse C3H10T1/2 multipotent cells found that miR-199a* is a bone morphogenic protein 2-responsive miRNA that suppresses early chondrocyte differentiation via direct targeting of Smad1, the essential transcription factor for chondrogenesis [Lin et al., 2009]. Two recent studies demonstrated that miR-145 is a direct suppressor of SOX9 in murine MSCs and human chondrocytes [Yang et al., 2011a; Martinez-Sanchez et al., 2012].

Our previous studies demonstrated a differential expression profile for miR-96, miR-124, and miR-199a during osteogenic and chondrogenic differentiation of mouse MSCs [Suomi et al., 2008]. The aim of the present study was to further investigate the role of these miRNAs in hMSCs during osteogenic, adipogenic, and chondrogenic differentiation. Here, we show that miR-96, miR-124, and miR-199a regulate the expression of key factors involved in MSC differentiation.

MATERIALS & METHODS

CELL CULTURE AND DIFFERENTIATION

hMSCs were isolated from iliac bone aspirates of four adult females (age 19–38 years) prior to surgical fixation of a fresh lower extremity fracture under spinal anesthesia. The medical histories of the donors were unremarkable without any significant underlying systemic disease or bone-affecting medication. The isolated, plastic-adherent hMSCs have been shown to be multipotent and to express the typical pattern of cell surface markers [Alm et al., 2010]. hMSCs were seeded at 1,000 cells per cm^2 and expanded in plastic tissue culture flasks (Thermo Fisher Scientific, Roskilde, Denmark) in phenol red-free alpha Minimum Essential Media (α-MEM) supplemented with 10% inactivated fetal bovine serum (iFBS) and 100 U/ml penicillin and 100 μg/ml streptomycin (all from Gibco Invitrogen, Carlsbad, California), further referred to as basal medium. Half of the medium was replaced every 3–4 days and cells were passaged when 80% confluence was reached.

hMSCs were cultured in four different conditions: Without induction (MSC) and with osteogenic (OB), adipogenic (AD), or chondrogenic (CC) inductions. Except chondrogenic induction, cells were seeded at 2500 cells/cm^2 in 25-cm^2 culture flasks or 24-well plates and cultured for 4 weeks followed by RNA extraction or fixation in 3% paraformaldehyde. Cultures without induction (MSC) were stained for alkaline phosphatase (ALP) using a histochemical kit (Sigma-Aldrich).

Osteogenic differentiation (OB) was induced by culturing hMSCs in osteogenic medium consisting of phenol red-free α-MEM, 10% iFBS, 10 mM Na-β-glycerophosphate (Fluka BioChemika, Switzerland), 0.25 mM ascorbic acid 2-phosphate (Sigma-Aldrich Corp.), and antibiotics. During the first week, the culture medium was supplemented with 0.1 μM dexamethasone (Sigma-Aldrich). At the end, cultures were stained for ALP.

For AD, hMSCs were cultured in phenol red-free α-MEM, supplemented with 10% FBS, 10 μg/ml insulin (Sigma-Aldrich Corp.), 100 μM indomethacin (Sigma-Aldrich Corp.), 500 μM methyl-isobutylxanthine (Sigma-Aldrich Corp.), 1 μM dexamethasone (Sigma-Aldrich Corp.), and antibiotics. The accumulation of lipid droplets was monitored with a microscope and cells were stained with 0.5% Oil Red O (Sigma-Aldrich Corp.) in methanol.

For chondrogenic differentiation (CC), 200 000 hMSCs were placed in a 15-ml polypropylene tube and centrifuged (8 min, 500 × g) to form a pellet culture. Cells were cultured for 21 days in chondroinductive medium consisting of high-glucose DMEM supplemented with 10 ng/ml TGF-β3 (R&D Systems, UK), 0.1 μM dexamethasone, 0.1 mM ascorbic acid 2-phosphate, 50 mg/ml ITS+ Premix (BD Biosciences). Media were changed every 3–4 days. After

3 weeks, pellets were either lysed for RNA extraction or fixed for 2 h in 3% paraformaldehyde. To evaluate chondrogenic differentiation, pellets were embedded in paraffin, cut into 5 μm sections and stained with toluidine blue for the demonstration of sulphated proteoglycans.

As a baseline reference used for expression analyses, cells were seeded at 1,000 cells/cm^2 and cultured in basal medium for 3–5 days followed by RNA extraction.

GENE EXPRESSION ANALYSIS

Total RNA was extracted from cultured cells using the mirVana miRNA Isolation Kit following the manufacturer's instructions (Ambion). RNA was treated with RQ1 DNase (Promega) and reverse transcribed using M-MLV enzyme (Promega). Expression of selected mRNAs (Table I) was measured by quantitative real-time PCR (qRT-PCR) using TaqMan® Gene Expression Assays (Applied Biosystems), Universal Probe Library probes (Roche, Switzerland, shown in Table I), and TaqMan® Universal PCR Master Mix (Applied Biosystems) following the TaqMan® protocol. Four replicates of each reaction were run in 7900HT Fast Real-Time PCR System (Applied Biosystems). The target transcript levels were normalized against endogenous glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression and compared to the expression level at baseline by $2^{-\Delta\Delta}$Ct method [Schmittgen and Livak, 2008].

miRNA EXPRESSION ANALYSIS

Expression levels of miR-96, miR-124, and miR-199a were detected by qRT-PCR using the TaqMan MicroRNA RT kit (Applied Biosystems) and TaqMan MicroRNA Assays (Applied Biosystems). For RT-reactions, 10 ng of total RNA was used and a no-template reaction was performed for each miRNA separately. Reactions were performed in triplicates in 7900HT Fast Real-Time PCR System. Results were normalized against let-7c expression and compared to the expression level at baseline by $2^{-\Delta\Delta}$Ct method.

TRANSFECTION OF PRE- AND ANTI-miRNA OLIGONUCLEOTIDES

Functional studies were performed with synthetic Pre-miR™ miRNA Precursor Molecules (Ambion) and miRCURY LNA™ microRNA Inhibitors (Exiqon, Denmark) designed for mirR-96, miR-124, and miR-199a. hMSCs from one donor were transfected with 100 nM miRNA precursors, inhibitors, or their negative controls using Nucleofector® (Lonza, Germany) and following the manufacturer's protocol. Transfected cells were seeded at 10 000 cells/cm^2 in 6-well plates or 25-cm^2 culture flasks containing basal medium. After 4 days, cells in 6-well plates were lysed for RNA extraction and the expression levels of mirR-96, miR-124, miR-199a, and genes associated with MSC differentiation (Table I) were detected by qRT-PCR as described above. To evaluate proliferation, cells in 25-cm^2 culture flasks were grown in basal medium for 14 days, then trypsinized and counted.

miRNA TARGET SITE PREDICTION

Target prediction tools TargetScan 5.1 (http://www.targetscan.org) and PicTar (http://pictar.bio.nyu.edu) were applied to search potential target mRNAs for miR-96, miR-124, and miR-199a.

STATISTICAL ANALYSIS

All data are presented as mean ± SD. Data was tested for normal distribution and equal variances using Kolmigorov–Smirnov test and Levene's test, respectively. For analyzing the expression of lineage specific mRNAs and miRNAs during hMSC differentiation, non-parametric analysis of related samples was performed using the Friedman test followed by the Wilcoxon test for paired comparisons. To compare the expression levels of mRNAs in transfected hMSCs, a repeated measures ANOVA was used. Effect of pre-/anti-miRNA transfection on cell proliferation was analyzed as percentage of the negative control and comparisons were done using the paired T-test. P-values less than 0.05 were considered significant. All analyses were performed using SPSS 16.6.

TABLE I. Primer Sequences for qRT-PCR

Gene	Transcript variant	Accession no.	Left primer	Right primer	Probe number
ACAN	1	NM_013227.2	tgcagctgtcactgtagaaactt	atagcaggggatggtgagg	89
	2	NM_001135.2			
ALP	1	NM_000478.3	aacaccacccaggggaac	ggtcacaatgcccacagatt	58
	2	NM_001127501.1			
COMP	–	NM_000095.2	gcaccgacgtcaacgagt	tggtgttgatacagcggact	38
FABP4	–	NM_001442.2	ggatgataaactggtggtgga	cacagaatgttgtagagttcaatgc	85
GAPDH	–	NM_002046.3	acccactcctccaccttga	ttgctgtagccaaattcgttgt	custom
HIF1A	1	NM_001530.2	cagctatttgcgtgtgagga	ttcatctgtgctttcatgtcatc	89
	2	NM_181054.1			
PCK1	–	NM_002591.3	agatggaggaagagggcatc	ggtcagtgagagccaaccag	41
PPARG	1	NM_138712.3	tgacaggaaagacaacagacaaat	gggtgatgtgtttgaacttgatt	7
	2	NM_015869.4			
	3	NM_138711.3			
	4	NM_005037.5			
RUNX2	1	NM_001024630.3	caccatgtcagcaaaacttctt	tcacgtcgctcattttgc	41
	2	NM_001015051.3			
	3	NM_004348.3	gtgcctaggcgcatttca	gctcttcttactgagagtggaagg	29
SOX5	1	NM_006940.4	tctgtcccagcagcgttag	tgacagcatcatggtcatttaag	41
	2	NM_152989.2			
	3	NM_178010.1			
SOX9	–	NM_000346.3	tacccgcacttgcacaac	ggtggtccttcttgtgctg	25

RESULTS

OSTEOGENIC, ADIPOGENIC, AND CHONDROGENIC DIFFERENTIATION OF hMSCs

To study the role of miRNAs in hMSC differentiation, cells were cultured without induction and in osteogenic, adipogenic, or chondrogenic conditions. Cultures without induction stained negative for alkaline phosphatase (ALP) and lacked lipid droplets observed in adipogenic conditions (Fig. 1A). Osteogenesis of hMSCs was demonstrated by histochemical staining for ALP (Fig. 1B) and increased gene expression of *ALP* and *RUNX2* (Fig. 2A). AD was demonstrated by the presence of lipid droplets that stained positive for Oil Red O (Fig. 1C) and the elevated expression of fatty acid binding protein 4 (*FABP4*), phosphoenolpyruvate carboxykinase 1 (*PCK1*) and peroxisome proliferator-activated receptor gamma (*PPARG*) (Fig. 2B). Correspondingly, chondrogenic differentiation was verified by toluidine blue staining (Fig. 1D) and increased expression of type X collagen (*COL10A1*), cartilage oligomeric matrix protein (*COMP*), and SRY (sex determining region Y)-box 5 (*SOX5*) (Fig. 2C).

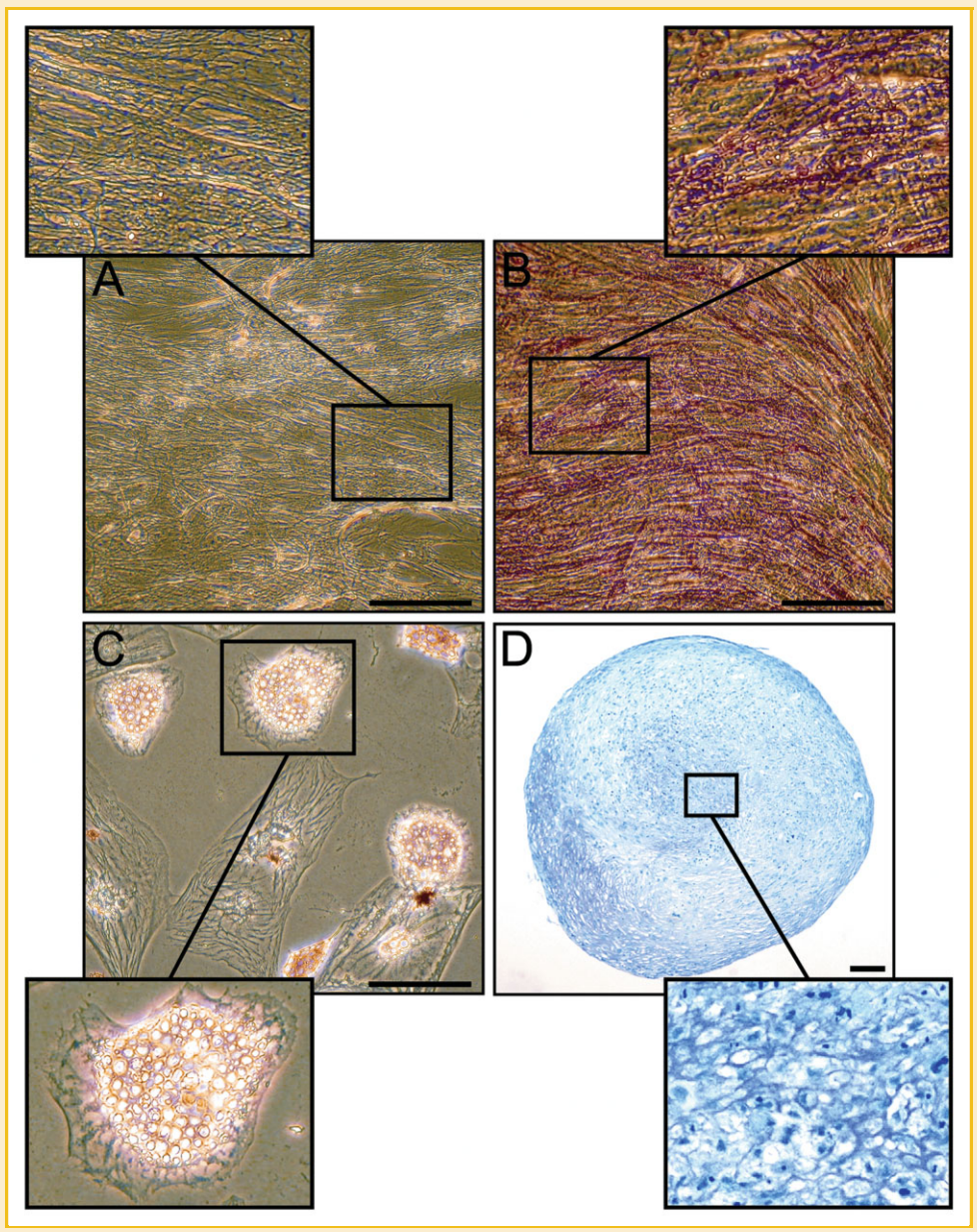

Fig. 1. Differentiation of hMSCs. Cells were cultured without induction (A) and under osteogenic (B), adipogenic (C), and chondrogenic (D) stimuli. ALP staining of hMSCs cultured in basal medium (A) or osteogenic medium (B) for 4 weeks. Oil Red O staining of hMSCs cultured in adipogenic medium for 4 weeks (C), and toluidine staining of cell pellet cultured in chondrogenic medium for 3 weeks (D). Bar = 20 μm in (A–C) and 200 μm in (D) (n = 4). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

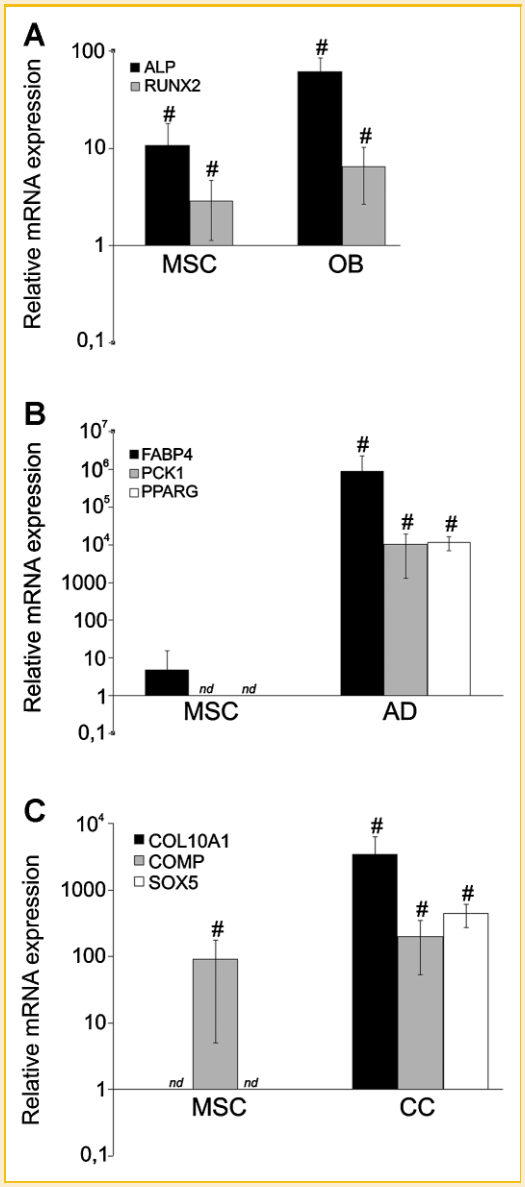

Fig. 2. mRNA expression during hMSC differentiation. The expression of osteogenic marker genes *ALP* and *RUNX2* (A), adipocyte marker genes *FABP4*, *PCK1*, and *PPARG* (B), and chondrocyte marker genes *COL10A1*, *COMP*, and *SOX5* (C) was assessed by qRT-PCR. hMSCs from four patients were cultured without induction (MSC) and under osteogenic (OB), adipogenic (AD), or chondrogenic (CC) stimuli for 3–4 weeks. The data was normalized to *GAPDH* and is presented as mean ± SD of four donors measured in four replicates (nd, not detectable). Undifferentiated hMSCs at days 3–5 served as a reference, in which gene expression values were set to 1. Statistically significant changes in gene expression are marked with #.

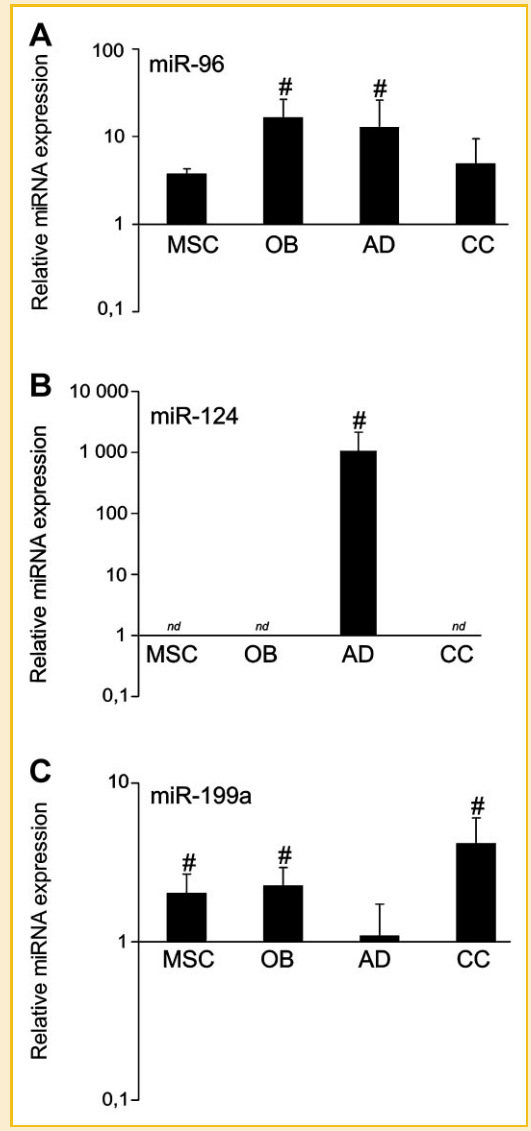

Fig. 3. miRNA expression during hMSC differentiation. hMSCs were cultured in basal medium (MSC) and in osteogenic (OB), adipogenic (AD), and chondrogenic (CC) culture conditions and the expression of miR-96 (A), miR-124 (B), and miR-199a (C) was analyzed by qRT-PCR. The data was normalized to *let-7c* and is presented as mean ± SD of four donors measured in three replicates (nd, not detectable). Undifferentiated hMSCs at days 3–5 served as reference (BL) in which gene expression values were set to 1. Statistically significant changes in miRNA expression are marked with #.

EXPRESSION OF miR-96, miR-124, AND miR-199A DURING hMSC DIFFERENTIATION

Expression of miR-96 was increased in all four culture conditions (Fig. 3). Osteogenic and adipogenic induction increased the expression of miR-96 by 16.3-fold and 12.8-fold ($P = 0.012$), respectively. In basal and chondrogenic conditions, upregulation of miR-96 was statistically insignificant. Interestingly, miR-124 was exclusively expressed during AD ($P = 0.002$) and stayed undetectable in other conditions. Expression of miR-199a was upregulated by ~2-fold in cells without induction ($P = 0.003$) and during OB ($P = 0.002$). Expression stayed at the baseline level during AD, whereas a 4.1-fold upregulation was observed after chondrogenic induction ($P = 0.002$).

FUNCTIONAL ANALYSIS OF miR-96, miR-124, AND miR-199A IN hMSCs

To further characterize the role of miR-96, miR-124, and miR-199a in hMSC differentiation and proliferation, miRNA expression in

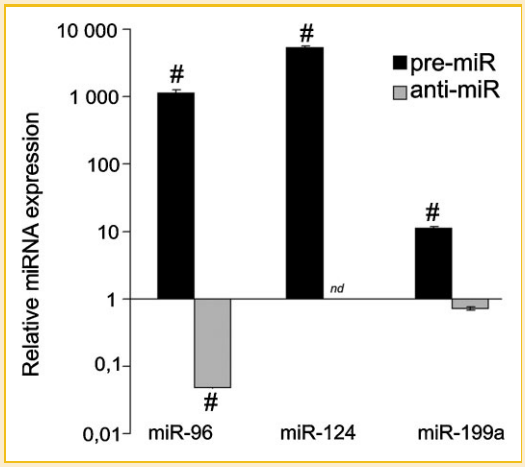

Fig. 4. miRNA expression in transfected hMSCs. Cells were transfected with 100 nM of control RNA, or anti-/pre- miR-96, miR-124, or miR-199a. Expression of the respective miRNAs was analyzed by qRT-PCR 4 days after transfection. Data are presented as mean ± SD from three replicates (nd, not detectable). Statistically significant changes in miRNA expression are marked with #.

hMSCs was modulated by synthetic miRNA precursors and inhibitors. The degree of miRNA overexpression or inhibition was monitored by qRT-PCR four days after transfection and showed satisfactory results. With pre-miRNAs, miR-96, miR-124, and miR-199a levels were elevated by ~1100-fold, ~ 5300-fold, and ~11-fold, respectively (Fig. 4). Transfection with corresponding anti-miRNAs resulted in a decrease in the expression levels by 21-fold for miR-96 and 1.4-fold for miR-199a. As the endogenous miR-124 level in hMSCs was undetectable, the transfection with anti-miR-124 did not show any difference.

To evaluate the biological effects of pre-/anti-miRNA transfections, the expression of selected genes related to hMSC differentiation was measured by qPCR (Table I). An up- or downregulation of ≥2-fold was considered significant and was observed for aggrecan (*ACAN*), *FABP4*, and *SOX9* (Fig. 5).

In general, pre-miR-96 and pre-miR-124 had similar effect on mRNA expression while pre-miR-199a appeared to act oppositely. The most considerable effect was detected with the miR-124 precursor, which upregulated *FABP4* expression by 11.2-fold ($P = 0.045$). Similarly, *FABP4* expression was increased by 3.1-fold after transfection with pre-miR-96 ($P = 0.015$) whereas pre-miR-199a transfection resulted in a 3.1-fold (67.4%) decrease in the *FABP4* mRNA level. Interestingly, pre-miR-124 transfection resulted in a 2.4-fold (57.7%) down-regulation of the *ACAN* expression ($P = 0.005$) while pre-miR-199a upregulated the expression of *ACAN* by 2.5-fold. Inhibition of miR-199a increased the expression of *SOX9* by 2.2-fold, and correspondingly, transfection with pre-miR-199a downregulated the *SOX9* expression by 1.6-fold (37.4%). The results obtained with anti-miR-124 cannot be considered physiologically reliable, as endogenous miR-124 expression remained undetectable in hMSCs.

To determine whether miR-96, miR-124, and miR-199a have an effect on hMSC proliferation, cells transfected with miRNA precursors or inhibitors were counted after 14 days of culture. In

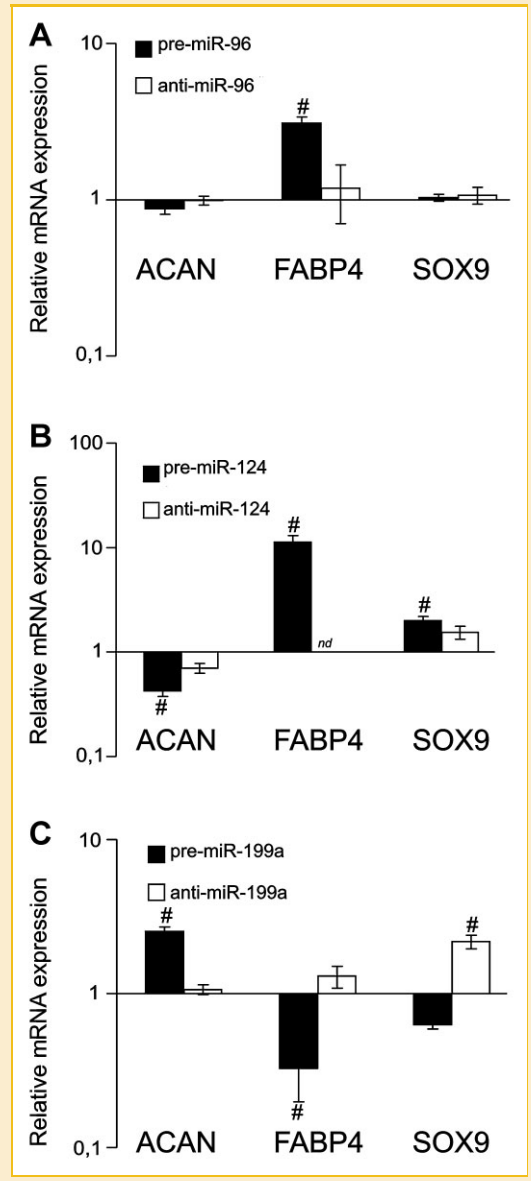

Fig. 5. mRNA expression in transfected hMSCs. Cells were transfected with 100 nM control RNA or pre-/anti- miR-96 (A), miR-124 (B), or miR-199a (C). At day 4, expression of genes related to hMSC differentiation was determined by qRT-PCR. The data was normalized to *GAPDH* and is presented as mean ± SD of four replicates (nd, not detectable). Statistically significant changes in gene expression are marked with #.

general, cells transfected with pre-miRNAs showed reduced proliferation whereas transfection with anti-miRNAs had no effect or slightly increased the proliferation (Fig. 6). Most significant effects were observed with pre-miR-124 and pre-miR-199a, which reduced the proliferation of hMSCs by 74% ($P = 0.002$) and 50% ($P = 0.007$), respectively, as compared to the control.

DISCUSSION

Differentiation of MSCs into various lineages involves complex signaling pathways that are controlled both at the transcriptional

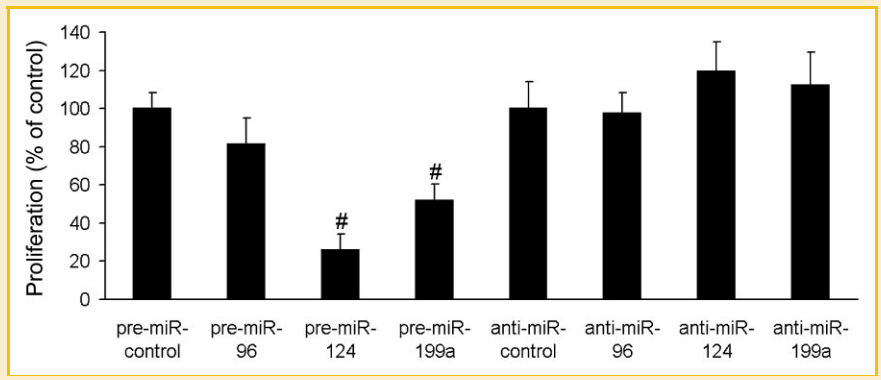

Fig. 6. Proliferation of transfected hMSCs. Cells were transfected with 100 nM control RNA or pre-/anti-miR for miR-96, miR-124, and miR-199a. At day 14, cell proliferation was measured. The results were compared to control and the data is presented as mean ± SD of four replicates. Statistically significant changes in proliferation are marked with #.

and the posttranscriptional levels. In this study, we investigated the expression and function of miR-96, miR-124, and miR-199a in hMSCs during osteogenic, adipogenic, and chondrogenic differentiation. Our results support the emerging concept that miRNAs play important roles in the posttranscriptional regulation of MSC differentiation [Schoolmeesters et al., 2009]. The overall miRNA expression pattern during osteogenic and chondrogenic differentiation of hMSCs was found to be rather similar to our previous observations with mMSCs [Suomi et al., 2008]. However, the fold changes observed in rodent MSCs were somewhat higher than in human MSCs, as shown here. It is widely accepted that the in vitro capacities of rodent and human MSCs are different. Especially the differentiation capacity seems to be much higher in rodent MSCs, as compared to human MSCs [Reilly et al., 2007]. It is therefore not too surprising that this difference is also reflected in miRNA expression levels, considering that they have a role in the regulation of stem cell fate. We collected hMSCs from four volunteers, and observed a considerable inter-individual variation of miRNA expression between the donors. Large inter-individual variation is an unavoidable, well-known problem associated with human MSCs [Hung et al., 2006; Wagner and Ho, 2007]. Furthermore, it has been reported that miRNA expression may vary even between different passages of the same donor as a consequence of replicative senescence [Goff et al., 2008; Wagner et al., 2008]. To minimize such variation in the current experiments, we chose only young, healthy female donors and all inductions were initiated using the same cell passages.

In this study, miR-96 expression was increased in all culture conditions for hMSC differentiation although the increase was not statistically significant during basal or chondrogenic condition. Based on searches with the TargetScan 5.1 target prediction tool, potential miR-96 targets include *SOX5* and *SOX6*, both genes that are critically associated with chondrogenic differentiation [Akiyama and Lefebvre, 2011]. Since the baseline expression levels of *SOX5* and *SOX6* mRNA in hMSCs were too low to be detected, the potential downregulation by pre-miR-96 could not be studied. However, transfection with pre-miR-96 increased the expression of *FABP4* by 3.1-fold. FABP4, mainly expressed by adipocytes and macrophages, belongs to the family of lipid binding proteins that are involved in the uptake and transport of long-chain fatty acids [Zimmerman and Veerkamp, 2002]. FABP4 is a marker for AD, and its upregulation by miR-96 suggests a potential positive effect on in vitro adipogenesis.

miR-124 expression was drastically upregulated during adipogenesis, whereas its expression was low or undetectable in all other culture conditions. Transfection of hMSCs with pre-miR-124 resulted in an 11-fold increase in *FABP4* mRNA level, suggesting a positive role for miR-124 in adipogenesis. In addition, pre-miR-124 decreased *ACAN* expression by 2.4-fold (57.7%) and increased *SOX9* level by 2.0-fold. Aggrecan and SOX9 are both early chondrogenic markers. Aggrecan is the major proteoglycan in the articular cartilage and is responsible for the load-bearing properties of the cartilage through its interaction with hyaluronan [Kiani et al., 2002]. During development, aggrecan is also needed for chondroskeletal morphogenesis [Kiani et al., 2002]. Sox9 is a positive regulator of chondrogenesis and it is required for the commitment of osteo-chondroprogenitors during mesenchymal condensation [Akiyama, 2008]. In addition to the effects on *ACAN* and *SOX9* expression, pre-miR-124 transfection reduced the proliferation of hMSCs by 74%. This finding correlates with a previous study where miR-124 was described to regulate proliferation through suppression of cyclin-dependent kinase 2 in synoviocytes isolated from patients with rheumatoid arthritis [Nakamachi et al., 2009]. Unexpectedly, transfection with anti-miR-124 had similar effects on *ACAN* and *SOX9* expression as pre-miR-124. Since endogenous miR-124 expression in undifferentiated hMSCs remained below the detection limit, the results obtained from anti-miR-124 transfections are not physiologically relevant. The expression and diverse functions of miR-124 in embryonic and adult brain are well established. Moreover, recent studies have suggested that miR-124 regulates Sox9 expression in the developing central nervous system [Cheng et al., 2009; Grandjean et al., 2009; Farrell et al., 2011]. However, to our knowledge, there is no previous data concerning the expression and potential function of miR-124 in hMSCs or adipocytes.

When miR-199a expression was studied in hMSCs, a 2.2-fold upregulation was observed during osteoblastic differentiation and

a 4.1-fold increase was observed during chondrogenesis. The results are analogous to our previous observation in mMSCs, where miR-199a expression was increased twofold during osteogenic and 12-fold during chondrogenic differentiation [Suomi et al., 2008]. Based on target predictions and pathway analyses, we hypothesized that miR-199a is related to hypoxia. Indeed, it was later shown that miR-199a is a master regulator of a hypoxia-triggered pathway at least in cardiac myocytes [Rane et al., 2009]. Moreover, it has been shown that hypoxia promotes the differentiation of mouse mesenchymal cells along the chondrocyte pathway in part by HIF1A-mediated transactivation of Sox9 [Robins et al., 2005]. In our experiments, *HIF1A* expression was increased by 1.8-fold during chondrogenic differentiation of hMSCs and transfection with anti-miR-199a increased the expression of *HIF1A* by 1.3-fold (data not shown). Inhibition of miR-199a resulted in a 2.2-fold up-regulation of *SOX9* expression, whereas pre-miR-199a decreased the expression of *SOX9* by 1.6-fold (37.4%) suggesting a negative role for miR-199a in chondrogenesis. This is supported by a previous study where miR-199a* excised from the 3' arm of miR-199a-1, and miR-199a-2 precursor molecules was shown to negatively regulate early chondrocyte differentiation in mouse C3H10T1/2 cells by targeting Smad1 [Lin et al., 2009]. For unknown reasons, as with miR-124 transfections, *ACAN* and *SOX9* were observed to behave oppositely. This is most probably explained by the totally different physiological functions of aggrecan and SOX9 in chondrocytes. As described before, Sox9 regulates chondrocyte commitment and differentiation [Akiyama, 2008] while aggrecan is the major cartilage matrix protein that maintains tissue mechanoresistance [Kiani et al., 2002].

In summary, we found increased expression of miR-96, miR-124, and miR-199a in hMSCs during osteogenic, chondrogenic, or AD. Furthermore, our results show that overexpression or functional inhibition of these miRNAs altered the proliferation of hMSCs and modulated the expression of genes related to hMSC differentiation. This information may be useful for the development of enhanced in vitro culture techniques required for MSC-based therapies in regenerative medicine.

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