ARTICLE
Journal of Cellular Biochemistry 115:1582–1593 (2014)

Journal of Cellular Biochemistry

MicroRNA-122 Overexpression Promotes Hepatic Differentiation of Human Adipose Tissue-Derived Stem Cells

Nahid Davoodian,[1] Abbas S. Lotfi,[1]* Masoud Soleimani,[2] and Seyed Javad Mowla[3]

[1]*Department of Clinical Biochemistry, Faculty of Medical Science, Tarbiat Modares University, Tehran, Iran*
[2]*Department of Hematology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran*
[3]*Department of Genetics, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran*

ABSTRACT

MicroRNAs are the regulatory molecules in post-transcriptional regulation of gene expression, which affect diverse biological processes and have been found to play important roles in regulating stem cell character in plants and animals. The aim of this study was to identify the role of miR-122 during hepatic differentiation of human adipose tissue-derived stem cells (hADSCs), and also to investigate whether overexpression of miR-122 could enhance differentiation of hADSCs toward functional hepatocyte-like cells without any extrinsic factor. To investigate this, the level of miR-122 was monitored by quantitative real-time PCR (qRT-PCR) at specific time intervals following hepatic differentiation of hADSCs using growth factors. For the next step, lentiviral transduction was applied to overexpress miR-122 in hADSCs for up to 21 days. Hepatic functionality was evaluated by analyzing specific hepatocyte genes and biochemical markers at different time points of differentiation induction. The qRT-PCR results revealed that miR-122 was upregulated during hepatic differentiation of hADSCs. Additionally, the stable miR-122 overexpression in hADSCs resulted in increased expression of specific hepatocyte markers such as ALB, AFP, CK18, CK19, and HNF4a compared with the negative control cells. Moreover, urea and albumin production as well as glycogen deposits were observed in the treated cells. Therefore, our findings demonstrate that the hepatic differentiation process could be improved by the overexpression of miR-122 in hADSCs, making it a potential therapeutic resource for liver disorders. J. Cell. Biochem. 115: 1582–1593, 2014.

KEY WORDS: MicroRNA; miR-122; HEPATIC DIFFERENTIATION; HUMAN ADIPOSE TISSUE-DERIVED STEM CELLS

The liver is the largest organ in the adult body and plays an important role in detoxification, metabolism, and protein synthesis (Ishibashi et al., 2009), making it an important organ for survival. Currently, millions of patients worldwide suffer from end-stage liver disease, and the only available therapy since 1982 has been liver transplantation (LT). Development in surgical techniques, clinical immunosuppressants, and post-surgical care has increased the survival of these patients. However, problems such as donor organ shortage, high cost, and life-long use of common immunosuppressants, which in itself are responsible for complications such as renal dysfunction, malignancy, and cardio vascular disease, have limited the success of this procedure (Fiegel et al., 2006; Locke et al., 2009; Åberg et al., 2011).

Due to the problem of organ donor limitations, life-long use of immunosuppressants, and rejection risk, cell-based therapy has been proposed as an alternative strategy for hepatic disease. Therefore, various sources of stem cells have been examined in different laboratories to evaluate their regenerative and therapeutic potential (Lodi et al., 2011).

Mesenchymal stem cells (MSCs) are multipotent stromal cells that can be isolated from bone marrow, adipose tissue, placenta, umbilical cord blood, peripheral blood, liver, and others. MSCs have numerous advantages compared to other stem cells, including their immunomodulatory properties, multidirectional differentiation potential, fewer clinical concerns and faster growth, making them the better choice for stem cell therapy (Lin et al., 2011).

Recently, some studies have shown that adipose tissue-derived stem cells (ADSCs) can be differentiated toward osteogenic, adipogenic, neurogenic, myogenic, chondrogenic, and hepatogenic lineages (Zuk et al., 2002; Safford et al., 2002; Miranville et al., 2004; Planat-Benard et al., 2004; Romanov et al., 2005). Additionally, due to the expression of hepatocyte-specific markers (Zemel et al., 2009), long culture period, and higher proliferation capacity (Visconti

*Correspondence to: Prof. Abbas S. Lotfi, Ph.D., Department of Clinical Biochemistry, Faculty of Medical Science, Tarbiat Modares University, Tehran, Iran. E-mail: lotfi_ab@modares.ac.ir
Manuscript Received: 27 January 2014; Manuscript Accepted: 11 April 2014
Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 15 April 2014
DOI 10.1002/jcb.24822 • © 2014 Wiley Periodicals, Inc.

et al., 2006), great interest has been generated in the use of ADSCs as an ideal source for regenerative therapy of liver disease.

MicroRNAs (miRNAs) are small, non-coding RNAs that act as key regulators of gene expression in a sequence-specific manner. Some reports estimate that each animal miRNA regulates hundreds of different mRNAs, making a large proportion of the transcriptome (about 50% in humans) a target of miRNA regulation (Voinnet et al., 2009; Huntzinger et al., 2011). Furthermore, miRNAs have gained considerable attention as regulators of a variety of cellular process, including animal and plant development, cell proliferation and differentiation, apoptosis, and metabolism (Bartel et al., 2004; Lewis et al., 2005; Dykxhoorn et al., 2010). Additionally, it has recently been shown that certain miRNAs play an important role during the differentiation of MSCs into specialized cell types, including osteocyes, chondrocytes, neurons, adipocytes, and hepatocytes (Ling et al., 2011). Furthermore, several studies have demonstrated that miRNAs are specifically expressed in certain stages of development, tissues, and cell types (Lagos et al., 2002; Wienholds et al., 2005). Of the liver-specific miRNAs, miR-122 has most frequently been shown to be important, through expression and function, in lipid metabolism (Esau et al., 2006), cholesterol biosynthesis (Krutzfeldt et al., 2005), liver development (Chang et al., 2004; Xu et al., 2010), and some liver diseases (Kutay et al., 2006; Girard et al., 2008; Padgett et al., 2009).

Based on studies showing the importance of miR-122 in liver development and function, the present study seeks to determine the role of miR-122 overexpression during differentiation of human adipose tissue-derived stem cells to hepatocyte-like cells. Furthermore, the study investigates whether overexpression of miR-122 could enhance differentiation of hADSCs toward functional hepatocyte-like cells without additional extrinsic factors.

MATERIAL AND METHODS

ISOLATION AND CULTURE OF hADSCs FROM HUMAN ADIPOSE TISSUE

Human adipose tissue was obtained from discarded lipoaspirate during liposuction procedures, according to the guidelines of the Institutional Medical Ethics Committee.

hADSCs were isolated as described by Zuck et al. (2001). Briefly, after extensive washing of the lipoaspirate with sterile phosphate buffered saline (PBS), digestion was performed using 0.075% (w/v) collagenase type I (Sigma-Aldrich, USA) in PBS at 37°C for 30 ;min, with vigorous shaking. Following digestion, Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) was added to inactivate the enzyme. Then, centrifugation was carried out at 1200 x g for 10 ;min and erythrocytes were removed from the cell pellet through resuspension in 160 ;mM NH_4CL. Finally, the cells were collected by centrifugation as before (10 min at 1200 x g) and resuspended in complete medium (DMEM, 10% FBS and 1% penicillin-streptomycin). The extracted cells were plated in 25 cm^2 cell culture flasks (Nunc, Denmark) and incubated at 37°C and 5% CO_2. 24 hours after isolation, medium was changed to eliminate non-adherent cells. After reaching 80–90% confluence, the cells were harvested using 0.025% trypsin-EDTA and used for the following analyses and differentiation assays.

FLOW CYTOMETRY ANALYSIS

hADSCs were evaluated for cell surface antigen expression by flow cytometry analysis. After detaching cells using trypsin-EDTA, approximately 2×10^5 cells were labeled with FITC- or PE-conjugated anti-CD90, anti-CD105, anti-CD45, anti-CD44, anti-CD34 or mouse IgG isotype control antibodies (Abcam, USA). Analysis was performed using fluorescence-activated cell sorter (FACS) caliber software.

OSTEOGENIC AND ADIPOGENIC DIFFERENTIATION OF hADSCs

hADSCs were plated at a concentration of 3×10^3 cells/cm^2. Cells were then treated with osteogenic differentiation medium (Bioidea, Iran). The medium was changed every 3 days over the course of 14 days. Then cells were fixed with 4% paraformaldehyde and stained using Alizarin red S (Sigma-Aldrich, USA). Adipogenic medium (Bioidea, Iran) was used for 14 days to induce differentiation into adipocytes, and subsequently fixed and stained using Oil Red O (Sigma-Aldrich, USA).

HEPATIC DIFFERENTIATION

Induction of hADSCs into hepatocyte-like cells was performed using a two-step differentiation process. Between passage 3 and 5, cells were seeded at a concentration of 1000 cells/cm^2. First, cells were treated for 1 week with DMEM supplemented with 10% FBS, 20 ng/mL hepatocyte growth factor (HGF), and 10^{-7} mol/L dexamethasone. For the following 2 weeks, cells were cultured in DMEM medium containing 10% FBS, 20 ng/mL HGF, 10^{-7} mol/L dexamethasone, and 10 ng/mL oncostatin M (OSM). The medium was changed twice a week, and after 21 days cells were used for hepatic differentiation assays.

RNA ISOLATION AND QUANTITATIVE REAL-TIME PCR

In order to evaluate hepatocyte-specific genes in hADSCs-derived hepatocyte-like cells, qRT-PCR was performed. Primers targeting albumin (ALB), alpha fetoprotein (AFP), cytokeratin 18 (CK18), cytokeratin 19 (CK19), CAT-1 (cationic amino acid transporter 1, a target of miR-122), and HNF4a were used (Table I). Total cellular RNA was isolated on days 1, 7, 14, and 21 using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and 2 μg of total RNA was reverse transcribed into cDNA using the Power cDNA Synthesis Kit (Intron, Kyungki-Do, Korea), following the manufacturer's protocol. PCRs were carried out using 100 ng of cDNA with a three-step procedure: denaturation at 95°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 60 s, for a total of 40 cycles. β-actin was used as a reference gene for normalization. Applied Biosystems (StepOne Real-time PCR system, USA) was used for qRT-PCR, and the comparative CT method (ΔΔCt) served as the analysis method for the relative quantification of gene expression.

IMMUNOCYTOCHEMISTRY

After fixation with 4% paraformaldehyde, cells were permeabilized for 30 min using 0.2% Triton X-100 in PBS. A solution of PBS with 1% BSA and 0.1% Triton X-100 was used for blocking; cells were then incubated with monoclonal anti-human albumin (ALB) and alpha 1 fetoprotein (AFP) diluted in PBS with 1% BSA. Next, cells were washed three times with PBS and treated with Alexa Flour 594 donkey anti-mouse IgG for 1 h. A final wash was carried out with

TABLE I. Primers Which Used for qRT-PCR

Name of Gene	Primer Sequences
Human Albumin	F 5′-GAGACCAGAGGTTGATGTGATG-3′
	R 5′-AGGCAGGCAGCTTTATCAGCA-3′
Human AFP	F 5′-CATGAGCACTGTTGCAGAGGAGA-3′
	R 5′-CGTGGTCAGTTTGCAGCATTCTG-3′
Human CK-18	F 5′-TTGATGACACCAATATCACACGA-3′
	R 5′-TATTGGGCCCGGATGTCTG-3′
Human CK-19	F 5′-GCGGCCAACGGCGAGCTA-3′
	R 5′-GCAGGACAATCCTGGAGTTCTC-3′
Human HNF4a	F 5′-CTTCTTTGACCCAGATGCCAAG-3′
	R 5′-GAGTCATACTGGCGGTCGTTG-3′
Human CAT-1	F 5′-CCCACCCCCATAGCTCC-3′
	R 5′-TCCTGAAGTAGACTCAGTGGAACG-3′
Human β-actin	F 5-CTGGAACGGTGAAGGTGACA-3′
	R 5′-AAGGGACTTCCTGTAACAATGCA-3′

PBS, and then cells were stained with 4′,6-diamidino-2-phenylindole (DAPI, for nuclear staining) for 5 min. Finally, the plates were visualized under an inverted fluorescence microscope (Nikon 200, Japan).

GLYCOGEN STAINING

Cells were first washed with PBS and fixed for 15 min using 4% paraformaldehyde. Next, oxidation was performed with 1% periodic acid for 5 min and cells were then washed again with PBS. Subsequently, cells were treated using Schiff's reagent (Sigma-Aldrich, USA) for 15 min; after rinsing in H_2O, hematoxylin was used for counterstaining. Finally, cells were observed under an inverted microscope.

UREA PRODUCTION

Determination of urea concentration was performed in cell culture media at different times of culture (1, 7, 14, 21 days) using a colorimetric assay kit (Zistchem, Iran). This assay is based on the reduction of ammonia produced via urea hydrolysis.

PRODUCTION OF ALBUMIN

Albumin production was evaluated in cell culture media on days 1, 7, 14, and 21 by enzyme linked immunosorbent assay (ELISA, E80-129; Bethyl Laboratories, Montgomery, Texas) according to the manufacturer's protocol.

VIRUS PRODUCTION AND TRANSDUCTION OF hADSCs

miR-122 lentiviral plasmid and negative control (scramble) vectors were purchased from Applied Biological Materials Inc, USA. Viral production was performed as described previously by Zufferey and Trono (2000). Briefly, 293T cells were cotransfected with lentiviral vector, psPAX2 (packaging plasmid), and pMD2.G (encoding the VSV G envelope protein) using calcium chloride with DNA coprecipitation. Cells were incubated overnight at 37°C and 5% CO_2. Early the next morning, after aspiration of the medium, complete medium was added and incubated for 24 h. The supernatant was collected 24 and 48 h post-transfection and concentrated using ultracentrifugation for 90 min at 72000 × g, at 4°C. The viral particles were divided into aliquots and stored at −80°C, and 15 μl of concentrated virus was taken for titration. 293T cells were used as target cells and the percentage of green fluorescent protein (GFP)-positive cells was determined by FACS.

Between passage 3 and 5, hADSCs were detached using 0.25% trypsin/EDTA and seeded at a density of 1×10^4 cells/well. The following day, cells were transduced with medium supplemented with concentrated virus (at a multiplicity of infection (MOI) of 50) for 24 h. The following day, the medium was replaced and after 7, 14, and 21 days the cells were analyzed using a variety of assays. Negative control scramble vector was used for triggering nonspecific effects, and to thereby demonstrate that lentiviral particles induce no specific changes in the differentiation marker genes.

QUANTITATIVE REAL-TIME-PCR FOR miR-122

The level of miR-122 was measured in transduced cells by qRT-PCR. First, total RNA was extracted with TRI reagent (Sigma, St. Louis, Missouri). After removing genomic DNA by DNase I (Fermentas, Vilnius, Lithuania), RNA was reverse transcribed using a universal cDNA synthesis kit (Exiqon, Woburn, Massachusetts), according to the respective manufacturer's protocol. The cDNA was amplified using the Sybr green master mix (Exiqon, USA) in combination with the miR-122 LNA PCR primer set (Exiqon, USA). The U6 snRNA PCR primer set (Exiqon, USA) was used to amplify U6 as a reference gene for normalization of target transcript levels. Each PCR reaction was assayed in triplicate and the comparative CT method (ΔΔ CT) was applied for data analysis.

STATISTICAL ANALYSIS

The results are expressed as mean ± SEM. Statistical significance was assessed using the Student *t*-test; *P* values below 0.05 were considered significant.

RESULTS

CHARACTERIZATION OF hADSCs

In order to characterize hADSCs, the specific surface markers of MSCs were analyzed using flow cytometry. Furthermore, the mesodermal differentiation potential (multilineage potential) of these cells was examined by treating cells with medium containing osteogenic and/or adipogenic-specific agents. It was shown that these cells express MSC-specific markers, such as CD90 (98.56%), CD105 (98.49%), and CD44 (99.84%) while being negative for the hematopoietic markers CD45 (1.98%) and CD34 (0.83%) (Fig. 1A).

Osteogenic differentiation of hADSCs was confirmed using alizarin red staining that visualizes the presence of calcium matrix production, and thereby serve as a specific indicator of osteogenic differentiation (Fig. 1Bb). As well as this, Oil Red-O staining was performed to determine adipogenic differentiation of hADSCs. This Oil Red-O is used to stain cells containing intracellular lipid droplets, and showed a successful differentiation of hADSCs toward an adipogenic lineage (Fig. 1Ba). Undifferentiated hADSCs were used as a negative control for the staining procedures (Fig. 1Bc and d). Taken together, these results showed the typical features of MSCs in isolated hADSCs.

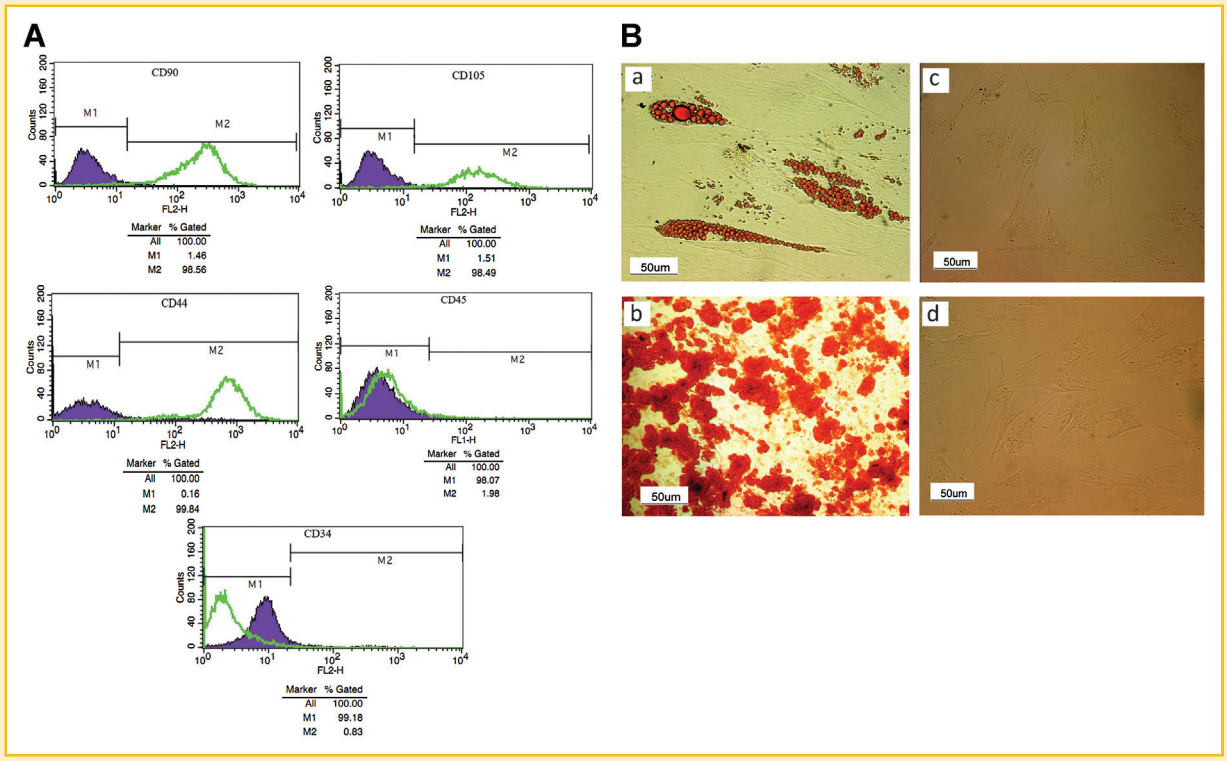

Fig. 1. Characterization of hADSCs. A. Flow cytometry analysis of hADSCs from the first passage. hADSCs expressed MSC specific markers including CD90 (98.56%), CD105 (98.49%), and CD44 (99.84%). These cells were negative for CD45 (1.98%) and CD34 (0.83%). The filled area shows the negative control, the unfilled area represents the respective specific antibody. B. Differentiation of hADSCs into adipocytes and osteoblasts. hADSCs were treated with osteogenic or adipogenic medium. Differentiated cells were stained with alizarin red to detect calcium mineralization and Oil Red to detect lipid droplets. Differentiated cells were positive for Oil Red staining (a) and alizarin red staining (b). No staining was detected for undifferentiated cells using either staining (c, d). Abbreviation: hADSCs, human adipose-derived stem cells.

MORPHOLOGICAL CHANGES AND EXPRESSION OF HEPATOCYTE-SPECIFIC MARKERS IN HEPATOCYTE-LIKE CELLS

Differentiation of hADSCs into hepatocyte-like cells was induced using medium containing growth factors, over the course of 21 days. Hepatic induction in these cells resulted in remarkable changes in hADSCs morphology, which were analyzed at various stages of the differentiation procedure. Initially, cells exhibited a spindle-like shape, which became broad and flattened after 14 days. Especially during the last week, cells exhibited a round and polygonal shape with granule accumulation in the cytoplasm (Fig. 2). In order to confirm the hepatic metabolic functions of hADSCs-derived hepatocyte-like cells, glycogen storage ability was assessed in the differentiated polygonal cells using glycogen staining. A significant fraction of cells stained positive for glycogen deposition, while undifferentiated hADSCs were negative for glycogen staining (Fig. 4A). Additionally, the RNA of hepatocyte-like cells was extracted at different times throughout the differentiation procedure (at days 1, 7, 14, and 21) and the mRNA level of hepatocyte-specific markers such as AFP, ALB, CK18, CK19, and HNF4a were examined by qRT-PCR. The expression level of CK19 was not changed significantly during the differentiation procedure (Fig. 3C). However, induction of differentiation resulted in a significant increase of both CK18 and HNF4a expression by day 21 (Fig. 3C). ALB and AFP levels increased early in the differentiation process, thereby acting as early markers of hepatic differentiation. Following differentiation, the expression of AFP was significantly increased by the seventh day after culturing and continued to be highly expressed until day 21 (Fig. 3C). Changes in the ALB levels were noted later at day 14 after induction of differentiation, increasing up to twofold by day 21 (Fig. 3C). The results were normalized using the level of β-actin, and then compared with results obtained from undifferentiated hADSCs.

In addition, protein expression of ALB, AFP, and HNF4a were observed using immunostaining 21 days after induction of differentiation. Positive staining for the three markers was detected in hADSCs-derived hepatocyte-like cells (Fig. 4B, C, and D). As expected, undifferentiated hADSCs were negative for HNF4a staining, and showed low expression of ALB and AFP, while the staining was more intense in hepotocyte-like cells compared to undifferentiated hADSCs (Fig. 4B, C, and D).

Finally, in order to determine the functionality of hADSC-derived hepatocyte-like cells, albumin and urea production were analyzed. Production was measured in culture media at different times of the differentiation procedure (at days 1, 7, 14, and 21). Upon induction of differentiation, the synthesis of urea was elevated in a time-dependent manner, with the most significant increase at day 14 and 21 (Fig. 3A); the secretion of albumin was detected at significant level at day 21 (Fig. 3B).

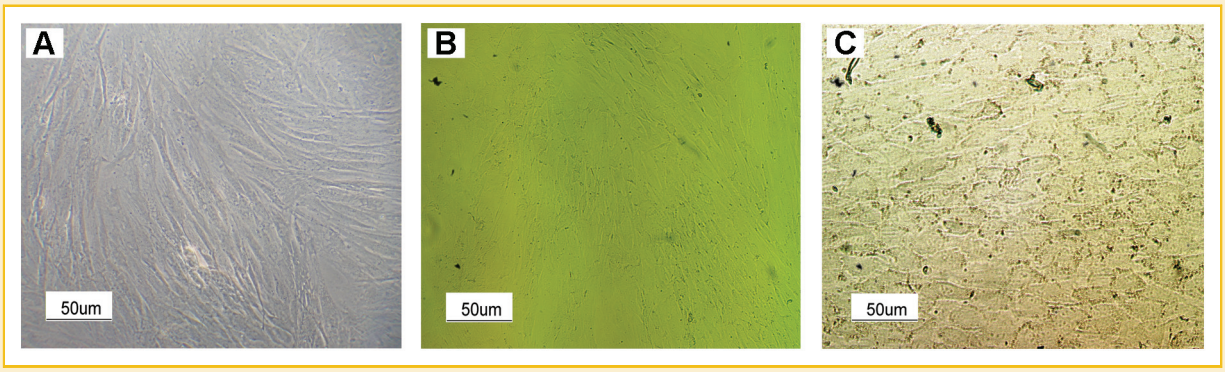

Fig. 2. Morphology of hADSCs at day 0, 14, and 21 after induction of hepatic differentiation. These cells were treated with HGF (20 ng/mL) and dexamethasone (10^{-7} mol/L) for a week and with OSM (10 ng/mL), HGF (20 ng/mL) and dexamethasone (10^{-7} mol/L) for the following 2 weeks. hADSCs represented the spindle shape and fibroblast like morphology (A), hepatic induction in these cells resulted in significant changes toward a round shape at 14 (B) and 21 (C) days after hepatic induction. Abbreviations: hADSCs, human adipose tissue-derived stem cells; HGF, hepatocyte growth factor; OSM Oncaostatin M.

OVEREXPRESSION OF miR-122 IN HUMAN ADIPOSE TISSUE-DERIVED STEM CELLS

We observed that the level of miR-122 was gradually upregulated over time in hADSCs during hepatic differentiation using growth factors (Fig. 5). Therefore, we examined whether the hepatic differentiation process in hADSCs would be affected by the overexpression of miR-122. Overexpression of miR-122 in hADSCs was performed using a lentiviral vector containing GFP; additionally, we transduced the cells with scramble lentiviruses as a negative control. The calculated titer for both lentiviral particles was 10^9 viral particles/mL. After 48 h of transduction, the percentage of GFP-positive cells was about 90% (data not shown).

Analysis by qRT-PCR revealed that miR-122 was highly expressed in transduced hADSCs relative to the negative control (Fig. 6A). Moreover, functionality of miR-122 was confirmed by examination of its ability to repress CAT-1, a known miR-122 target gene. The expression level of CAT-1 was significantly downregulated after 14 and 21 days (Fig. 6B).

THE EFFECT OF miR-122 OVEREXPRESSION ON HEPATIC DIFFERENTIATION OF hADSCs

To determine the impact of miR-122 overexpression on hepatic differentiation of hADSCs, we evaluated the mRNA level of several hepatocyte markers, using qRT-PCR to compare cells transduced with viruses containing miR-122 and cells transduced with the negative control. A gradual upregulation was observed for ALB, AFP, CK18, and CK19 over time, showing the possibility of a correlation of miR-122 overexpression with hepatocyte specific gene expression (Fig. 7C). The expression levels of ALB and AFP significantly ($P < 0.05$) rose at day 14 and 21 (Fig. 7C). Furthermore, after 7 days, a significant increase was observed for CK18 and CK19 which lasted until day 21 (Fig. 7C). HNF4a was also upregulated, although this was only detected at the final stage of the procedure (day 21) (Fig. 7C). We also examined the expression of AFP, ALB, and HNF4a by immunostaining at day 21. The cells which were transduced with viruses containing miR-122 showed positive staining for all three markers, whereas immunostaining was negative for the cells transduced with the negative control (Fig. 8B, C, and D). In addition, the functionality of the transduced cells was assessed. The cells transduced with miR-122 viruses showed glycogen deposition using glycogen staining (Fig. 8A). Moreover, urea secretion was observed over time, with a significant increase at days 14 and 21 (Fig. 7A). Figure 7B shows the albumin production at different time points of the differentiation, showing a significant increase in albumin production at day 21 for the cells transduced with miR-122 viruses.

DISCUSSION

Recent observations have demonstrated that hADSCs possess the ability to differentiate into hepatocyte-like cells. hADSCs have a hepatogenic potential allowing them to easily differentiate toward a hepatocyte-specific phenotype (Zemel et al., 2009). Moreover, these cells can be obtained from patients with a minimally invasive procedure, compared to BMSCs. The abundance, easy accessibility, and high proliferation rate of these cells, as well as the possibility to keep the cells in culture over a long time period, were features making hADSCs the ideal choice for this study.

So far, many studies have attempted to find cheaper and easier methods for generating liver cells or hepatocyte-like cells from different kinds of stem cells. In this context, various hepatic differentiation media containing different growth factors and cytokines have been reported to be capable of inducing the differentiation of hADSCs into hepatocyte-like cells.

Recently, the importance of specific miRNAs during differentiation and development of certain tissues has been shown. The striking role of the liver-specific miRNA, miR-122 in embryonic development of mouse liver (Chang et al., 2004) and differentiation of embryonic stem cells (ESCs) into hepatocyte-liked cells (Chen et al., 2010; Kim et al., 2011) has been reported recently. Additionally, Doddapaneni et al. (2013) reported the role of miR-122 in differentiation of fetal liver stem/progenitor cells into hepatocyte-like cells. Likewise, other reports have demonstrated the regulation of some liver functions by miR-122 (Xu et al., 2010). Furthermore, it has been shown that the expression of miR-122

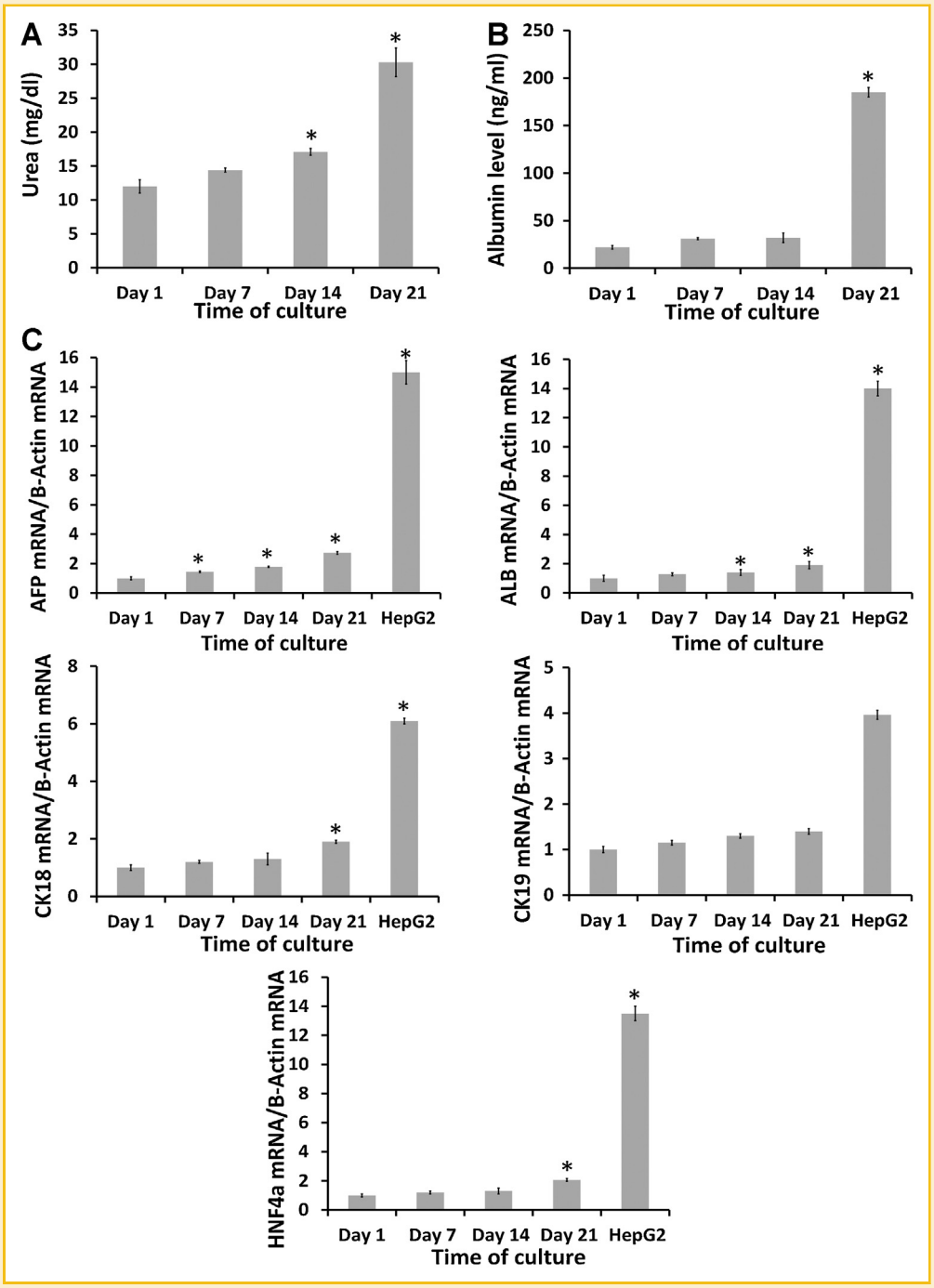

Fig. 3. Differentiation of hADSCs into hepatocyte-like cells using growth factors. Urea production: urea was detected in cell culture medium at different time-points of culture in hepatic differentiation medium (A). Albumin production of hepatocyte-like cells at different time intervals (B). qRT-PCR analysis of selected hepatocyte specific markers during hepatic differentiation of hADSCs. The expression of AFP, ALB, CK18, CK19, and HNF4a were measured by qRT-PCR. Data were normalized by β-actin and are shown relative to undifferentiated hADSCs (day 0). The results are represented as mean ± SEM (C). Abbreviations: ALB, albumin; AFP, alpha fetoprotein; CK18, cytokeratin 18; CK19, cytokeratin 19, HNF4a, hepatocyte nuclear factor; hADSCs, human adipose-derived stem cells; qRT-PCR, quantitative real-time PCR.

increased during hepatic differentiation of some types of stem cells, including ESCs and liver-derived progenitor cells (LDPCs) (Chen et al., 2010; Kim et al., 2011). Based on these results clearly showing the significant role of miR-122 in liver function and differentiation, this study attempts the first analysis of the effect of an over-expression of miR-122 in hADSCs. The study focuses at the effect of miR-122 on the differentiation process, and determines whether miR-122 can be applied as a substitute factor for inducing hepatic differentiation of hADSCs, replacing the expensive and time consuming use of growth factors.

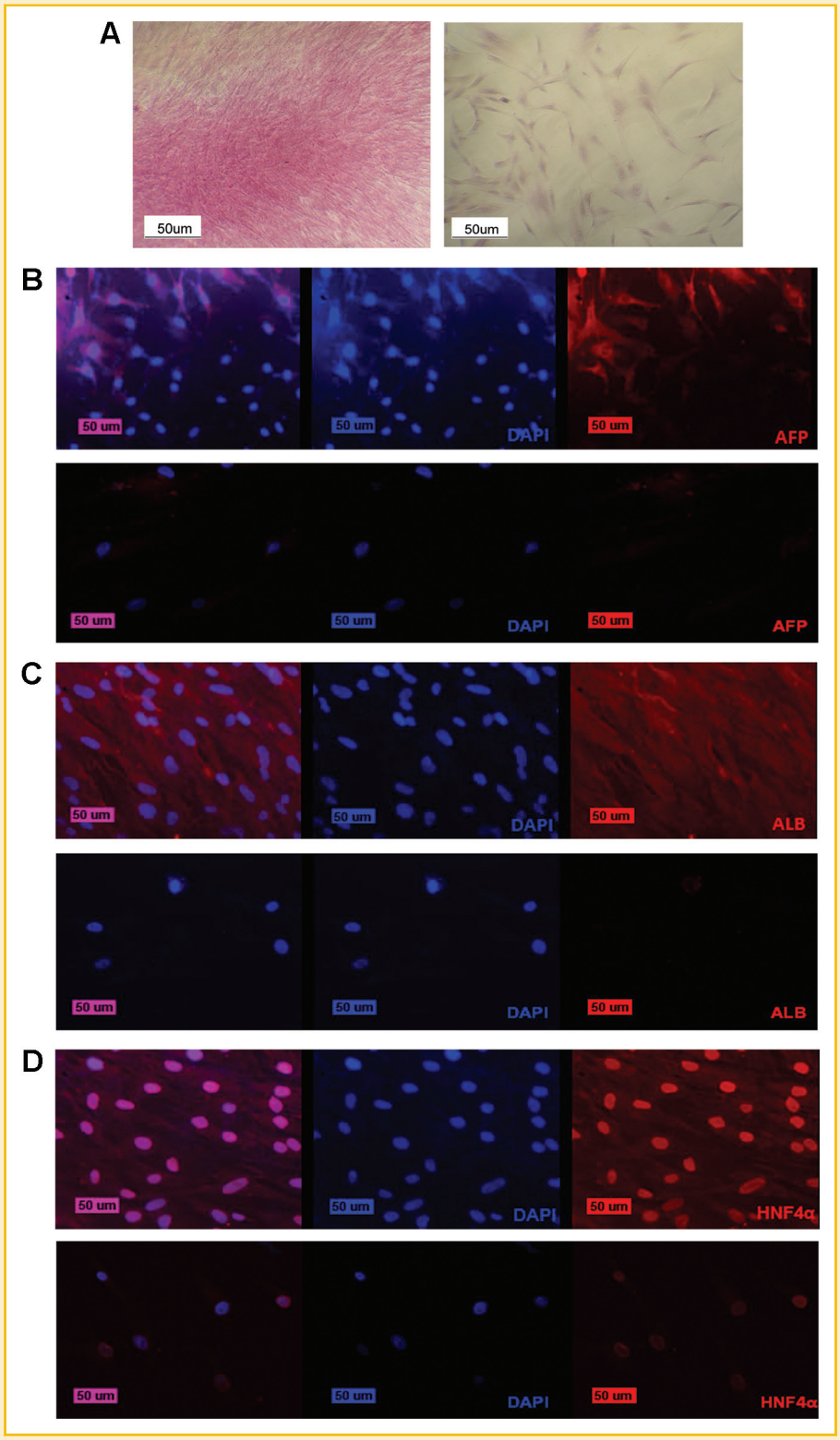

Fig. 4. Differentiation of hADSCs into hepatocyte-like cells using growth factors. Glycogen staining of hepatocyte-like cells derived from hADSCs and undifferentiated hADSCs (A). Immunocytochemistry assay for AFP, ALB, and HNF4a in hepatocyte-like cells, respectively (B, C, and D). Abbreviations: ALB, albumin; AFP, alpha fetoprotein; CK18, cytokeratin 18; CK19, cytokeratin 19, HNF4a, hepatocyte nuclear factor; hADSCs, human adipose-derived stem cells.

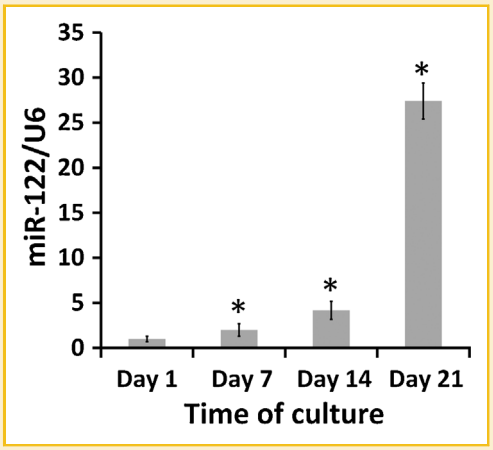

Fig. 5. miR-122 expression in hADSCs during hepatic differentiation with growth factors. qRT-PCR analysis of miR-122 expression levels during the hepatic differentiation of hADSCs at day 1, 7, 14 and 21. Data were normalized by U6 snRNA and expressed relative to undifferentiated hADSCs (day 0). The results are represented as mean ± SEM. Abbreviations: hADSCs, human adipose derived stem cells; qRT-PCR, quantitative real-time PCR.

Initially, we used growth factors to differentiate hADSCs into hepatocyte-like cells. The hepatic properties of hepatocyte-like cells were confirmed by examination of hepatocyte specific markers and biochemical functions. Besides the morphological changes that were observed by inverted microscope, expression of hepatocyte-specific genes such as ALB, AFP, and HNF4a was confirmed using qRT-PCR. Moreover, immunofluorescence for albumin, alpha fetoprotein, and HNF4a showed the expression of these markers on a translation level. Additionally, the observation of glycogen deposits and urea production in differentiated cells suggested that a large proportion of cells successfully differentiated into hepatocyte-like cells. Analysis by qRT-PCR revealed a gradual increase of miR-122 upon hepatic differentiation induction in hADSCs (2-fold, 4-fold, and 27-fold at day 7, 14, and 21, respectively), which confirmed the role of this miRNA during hepatic differentiation.

Furthermore, there is evidence of a potential role of some miRNAs in directing differentiation. Rahimian et al (2011) described the involvement of miR-124 in the maturation of monoblastic U937, whereas overexpression of miR-375 in hESCs induced differentiation into a pancreatic lineage (Lahmi et al., 2013). Interestingly, Doddapaneni et al. (2013) showed that overexpression of miR-122 in fetal liver stem/progenitor cells was able to promote hepatic differentiation. Therefore, in line with these results and our finding that showed the increased expression of miR-122 during differentiation of hADSCs into hepatocyte-like cells, we examined if miR-122 alone could affect hepatic differentiation of hADSCs, without the addition of any extrinsic factors.

For this purpose, we used lentiviral transduction to overexpress miR-122 in hADSCs, a tool known to be effective for ex vivo MSC modification (McGinley at al., 2011). The level of miR-122 overexpression was monitored using qRT-PCR, which revealed a continuous high expression of miR-122 in transduced hADSCs throughout the differentiation process, relative to negative control cells. Additionally, we confirmed the functionality of miR-122 in transduced cells by analyzing its ability to repress expression of CAT-1, a validated target for miR-122.

As expected, the overexpression of miR-122 in hADSCs resulted in a significant upregulation of several genes associated with hepatic functions. Analysis by qRT-PCR revealed that the expression levels of ALB, AFP, CK18, and CK19 were remarkably increased. We also found that hADSCs transduced with miR-122 showed a fourfold upregulation of hepatocyte specific markers when compared to hADSCs treated with growth factors. Moreover, our data demonstrated that the expression of HNF4a was upregulated during the late stage of the differentiation process. HNF4a is an important nuclear transcription factor which controls the expression of several hepatocyte-specific genes (Schrem et al., 2002). Recently Xu et al. (2010) identified HNF4a as an important regulator of miR-122, additionally showing the

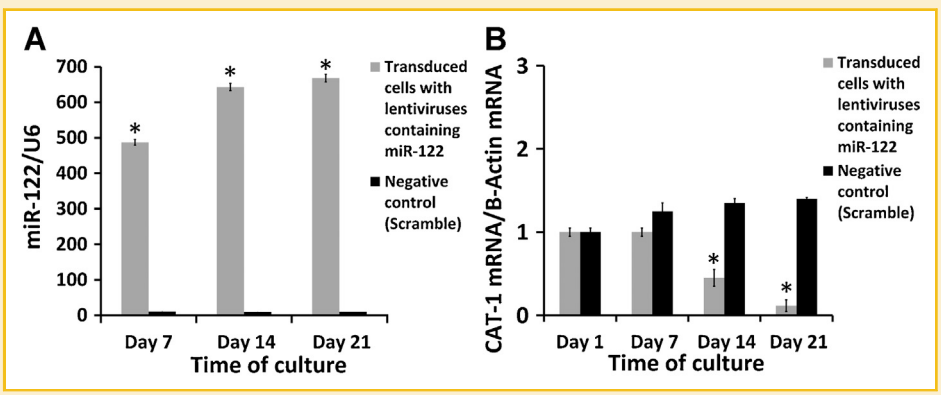

Fig. 6. miR-122 overexpression in hADSCs. qRT-PCR result of miR-122 expression levels in hADSCs transduced with lentiviruses containing either miR-122 or negative control (scramble). Data were normalized to U6 snRNA and expressed relative to undifferentiated hADSCs (day 0). The results are represented as mean ± SEM (A). The expression level of CAT-1, a known miR-122 target gene in hADSCs transduced with lentiviruses containing either miR-122 or negative control (scramble) at different time intervals. Data were normalized with β-actin and compared relative to undifferentiated hADSCs (day 0). The results represented as mean ± SEM (B). Abbreviations: CAT-1, cationic amino acid transporter 1; hADSCs, human adipose-derived stem cells.

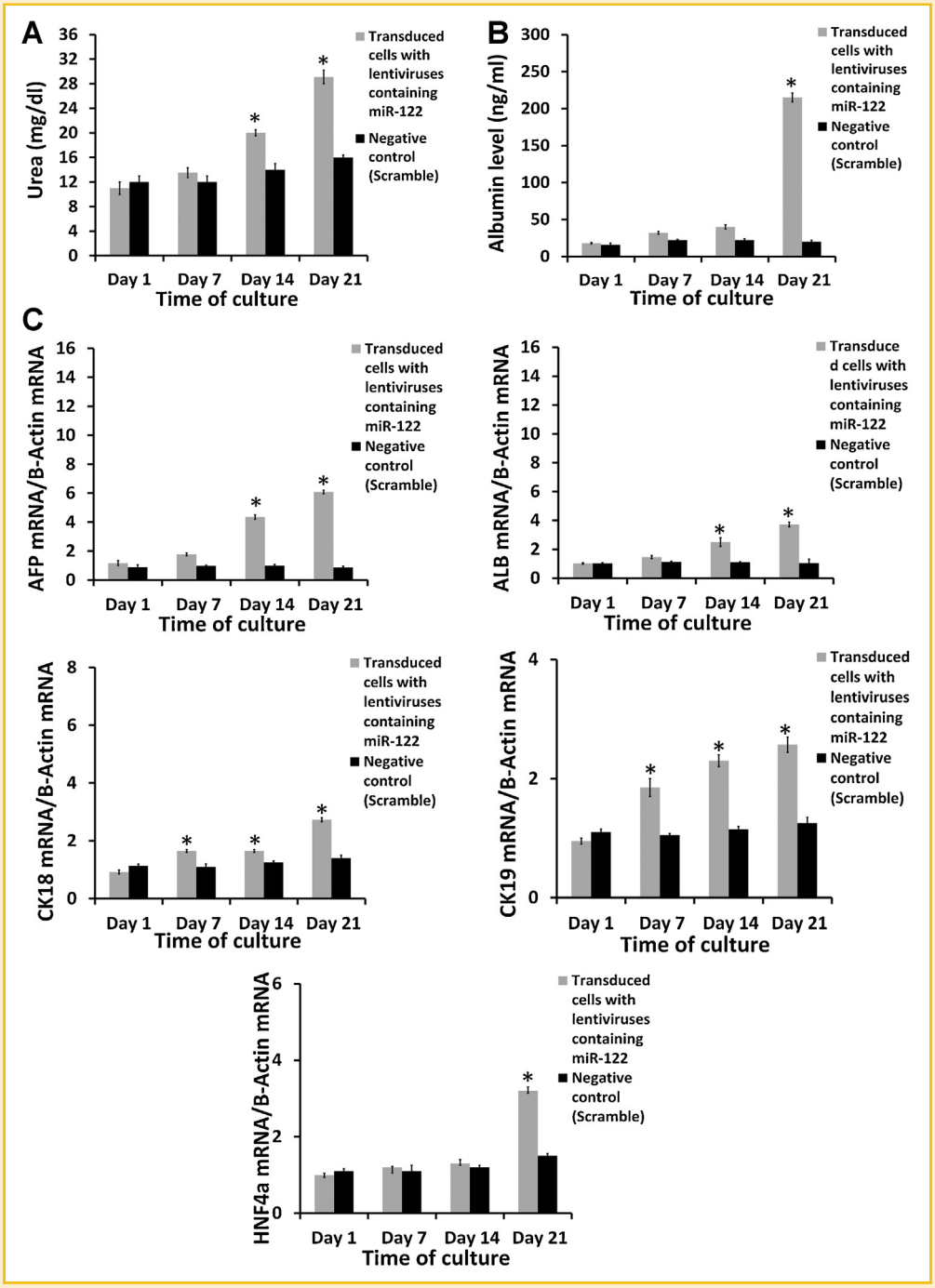

Fig. 7. Effect of miR-122 overexpression on hepotocyte specific markers in hADSCs. Production of urea: urea was detected in cell culture medium at day 1, 7, 14, and 21 in hADSCs transduced with lentiviruses containing either miR-122 or negative control (scramble) (A). Albumin production by hADSCs transduced with lentiviruses containing either miR-122 or negative control (Scramble) (B). Hepatic differentiation was evaluated with qRT-PCR analysis of selected factors, including AFP, ALB, CK18, CK19, and HNF4a at different time points. Data were normalized with β-actin and expressed relative to undifferentiated hADSCs. The results are represented as mean ± SEM (C). Abbreviations: as in Figure 3.

role of miR-122 in controlling the expression of several genes involved in hepatic proliferation and differentiation. Based on these findings it is hypothesized that there is a positive feedback loop between miR-122 and HNF4a to promote the differentiation process.

In this study, the differentiation of hADSCs into hepatocyte-like cells was also supported by immunostaining of ALB, AFP, and HNF4a, proteins present in adult liver cells. At day 21, the secretion of albumin, the protein produced most abundantly by hepatocytes, was detected using ELISA. We were also able to detect the production of urea, as well

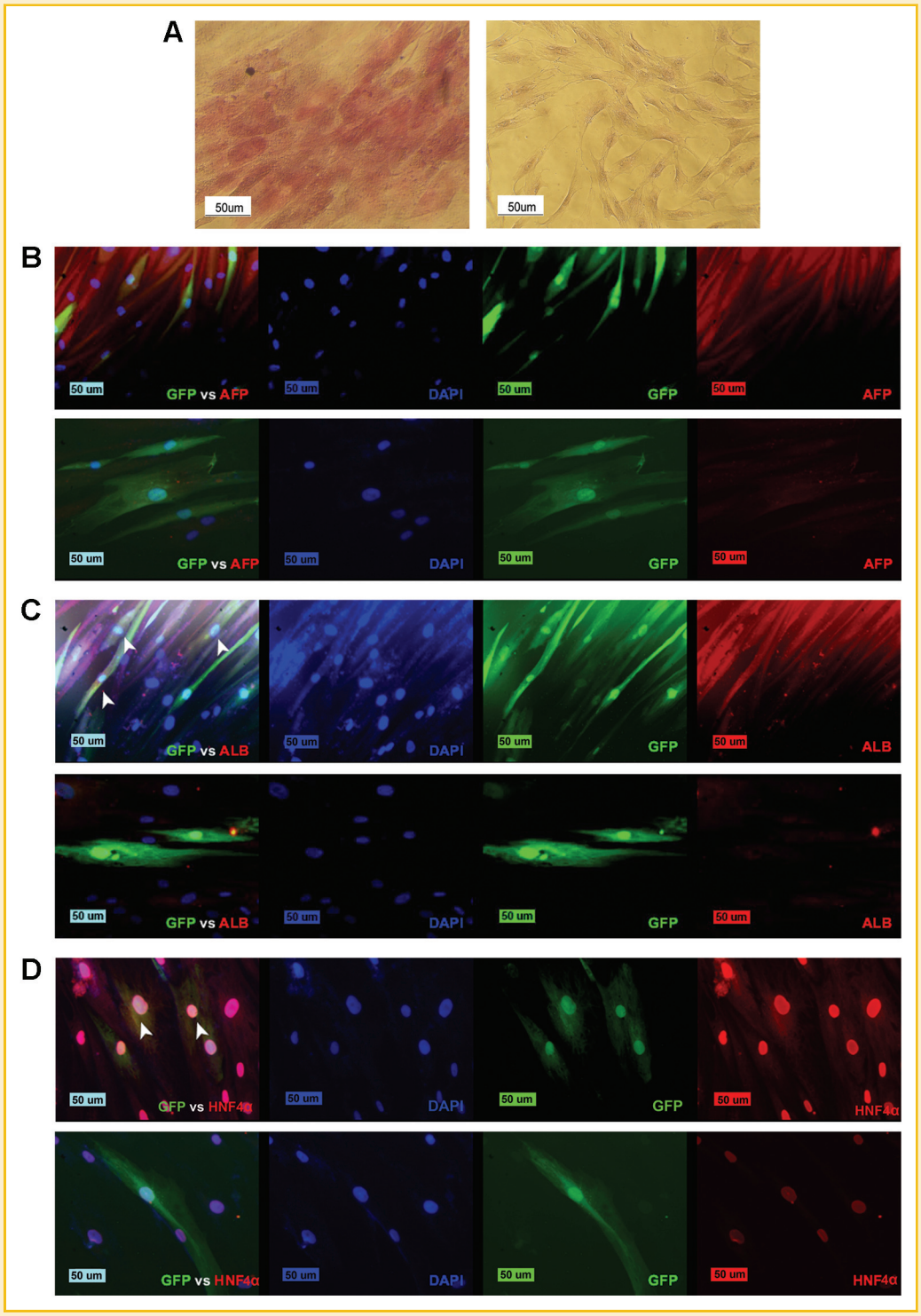

Fig. 8. Effect of miR-122 overexpression on hepotocyte specific markers in hADSCs. Glycogen deposition was detected using Schiff's reagent in hADSCs transduced with lentiviruses containing either miR-122 or negative control (Scramble) (A). Immunocytochemistry assay for AFP, ALB, and HNF4a in hADSCs transduced with lentiviruses containing either miR-122 or negative control (scramble), respectively (B, C, and D). Abbreviations: as in Figure 4.

as glycogen storage ability in the transduced cells. These results clearly demonstrate that hADSCs transduced with miR-122 display the key properties of hepatocyte-like cells and thereby show the ability of miR-122 to direct differentiation toward a hepatogenic lineage.

Recently, it was demonstrated that undifferentiated hADSCs express several hepatocyte markers, revealing the potential of these cells to acquire hepatocyte-like functions (Zemel et al., 2008). In our study, we were also able to detect low levels of some hepatic

differentiation markers in untreated hADSCs; however, the expression of those markers was lower than in treated cells. Therefore, hADSCs could be an attractive candidate for achieving a functional hepatocyte. Furthermore, our results provide evidence that miR-122 overexpression in hADSCs enhances their ability to differentiate into hepatocyte-like cells without the help of external factors. Altogether these findings suggest that overexpression of miR-122 in hADSCs could serve as a valuable tool in the formation of functional and transplantable hepatocyte-like cells.

Overall, our study confirmed that miR-122 can act as a prominent factor in differentiation of hADSCs into hepatocyte-like cells. Therefore, overexpression of miR-122 in hADSCs could prove to be useful in enhancing hepatocyte formation, and thereby in regenerative therapy for liver disease. Further studies are necessary to clarify the molecular mechanisms of single miRNAs during hepatic differentiation.

REFERENCES

Åberg F, Iisoniemi H, Höckerstedt K. 2011. Long-term results of liver transplantation. Scand J Surg 100:14–21.

Bartel DP. 2004. MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell 116:281–297.

Chang J, Nicolas E, Marks D, Sander C, Lerro A, Buendia MA, Xu C, Mason WS, Moloshok T, Bort R, Zaret KS, Taylor JM. 2004. MiR-122, a mammalian liver specific microRNA, is processed from hcr mRNA and may downregulate the high affinity cationic amino acid transporter CAT-1. RNA Biol 1:106–113.

Chen Y, Zhou HO, Sarver AL, Zeng Y, Roy-Chowdhur y J, Steer CJ, Sahin MB. 2010. Hepatic differentiation of liver-derived progenitor cells and their characterization by microRNA analysis. Liver Transolant 16:1086–1097.

Doddapaneni R, Chawla YK, Das A, Kaur J, Ghosh S, Chakraborti A. 2013. Overexpression of microRNA -122 enhances in vitro hepatic differentiation of fetal liver-derived stem/progenitor cells. J Cell Biochem 114:1575–1583.

Dykxhoorn DM. 2010. MicroRNAs and metastasis: Little RNAs go a long way. Cancer Res 70:6401–6406.

Esau C, Davis S, Murray SF, Yu XX, Pandey SK, Micha el Pear, Watts L, Booten SL, Graham M, McKay R, Subramaniam AK, Propp S, Lollo BA, Freier S, Bennett CF, Bhanot S, Moni a BP. 2006. MiR-122 regulation of lipid metabolism revealed by in vivo antisense targeting. Cell Metab 3: 87–98.

Fiegel HC, Lange C, Kneser, Lambrecht W, Zander AR, Rogiers X, Kluth D. 2006. Fetal and adult liver stem cells for liver regeneration and t issue engineering. J Cell Mol Med 10:577–587.

Girard M, Jacquemin E, Munnich A, Lyonnet S, Henrion-Caude A. 2008. MiR-122, a paradigm for the role of microRNAs in the liver. J Hepatol 48(4):648–656.

Huntzinger E, Izaurralde E. 2011. Gene silencing by microRNAs: Contributions of translational repression and mRNA decay. Nature Reviews 12:99.

Ishibashi H, Nakam M, Kom A, Migita K, Shimod S. 2009. Liver architecture, cell function, and disease. Semin Immunopathol 31:399–409.

Kim N, Kim H, Jung I, Kim Y, Kim D, Han YM. 2011. Expression profiles of miRNAs in human embryonic stem cells during hepatocyte differentiation. Hepatol Res 41:170–183.

Krutzfeldt J, Raje wsky N, Braich R, Rajeev KG, Tuschl T, Manoharan M, Stoffel M. 2005. Silencing of microRNAs in vivo with antagomirs. Nature 438:685–689.

Kutay H, Bai S, Datta J, Motiwala T, Pogribny I, Frankel W, Jacob ST, Ghoshal K. 2006. Downregulation of miR-122 in the rodent and human hepatocellular carcinomas. J Cell Biochem 99(3):671–678.

Lahmy R, Soleimani M, Sanati MH, Behmanesh M, Kouhkan F, Mobarra N. 2013. Pancreatic islet differentiation of human embryonic stem cells by microRNA overexpression. J Tissue Eng Regen Med.

Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W, Tuschl T. 2002. Identification of tissue-specific microRNAs from mouse. Curr Biol 12:735–739.

Lewis BP, Burge CB, Bartel DP. 2005. Conserved seed pairing, often flanked by adenosines indicates that thousands of human genes are microRNA targets. Cell 2005(120):15–20.

Lin H, Xu R, Zhang Z, Chen L, Shi M, Wang FS. 2011. Implications of the immunoregulatory functions of mesenchymal stem cells in the treatment of human liver diseases. Cell Mol Immunol 8:19–22.

Ling GA, Robert CH, Zhao B, Yaojiong Wu. 2011. The role of microRNAs in self-renewal and differentiation of mesenchymal stem cells. Exp Hematol 39:608–616.

Locke JE, Shamblott MJ, Cameron AM. 2009. Stem cells and the liver: Clinical applications in transplantation. Adv Surg 43:35–51.

Lodi D, Iannitti TM, Palmieri B. 2011. Stem cells in clinical practice: Applications and warnings. J Exp Clin Cancer Res 30:9.

Miranville A, Heeschen C, Sengenes C, Curat CA, Busse R, Bouloumie A. 2004. Improvement of postnatal neovascularization by human adipose tissue-derived stem cells. Circulation 110:349–355.

McGinley L, McMahon J, Strappe P, Barry F, Murphy M, O'Toole D, O'Brien T. 2011. Lentiviral vector mediated modification of mesenchymal stem cells and enhanced survival in an in vitro model of ischaemia. Stem Cell Res Ther 2:12.

Padgett KA, Lan RY, Leung PC, Lieo A, Dawson K, Pfeiff J, Mao TK, Coppel RL, Ansari AA, Gershwin ME. 2009. Primary biliary cirrhosis is associated with altered hepatic microRNA expression. J Autoimmun 32(3-4):246–253.

Planat-Benard V, Silvestre JS, Cousin B, Andre M, Nibbelink M, Tamarat R, Clergue M, Manneville C, Saillan-Barreau C, Duriez M, Tedgui A, Levy B, Penicaud L, Casteilla L. 2004. Plasticity of human adipose lineage cells toward endothelial cells: Physiological and therapeutic perspectives. Circulation 109:656–663.

Rahimian A, Soleimani M, Kaviani S, Aqaee H, Atashi A, Arefian E, Nikougoftar M. 2011. Bypassing the maturation arrest in myeloid cell line U937 by over-expression of microRNA-424. Hematology 16:5.

Romanov YA, Darevskaya AN, Merzlikina NV, Buravkova LB. 2005. Mesenchymal stem cells from human bone marrow and adipose tissue: Isolation, characterization, and differentiation potentialities. Bull Exp Biol Med 140:138–143.

Safford KM, Hicok KC, Safford SD, Halvorsen YD, Wilkison WO, Gimble JM, Rice HE. 2002. Neurogenic differentiation of murine and human adipose-derived stromal cells. Biochem Biophys Res Commun 294:371–379.

Schrem H, Klempnauer J, Borlak J. 2002. Liver-enriched transcription factors in liver function and development. Part I: The hepatocyte nuclear factor network and liver specific gene expression. Pharmacol Rev 54:129–158.

Visconti RT, Bonora A, Jover R, Mirabet V, Carbonell F, Castell J, Lechón MJ. 2006. Hepatogenic differentiation of human mesenchymal stem cells from adipose tissue in comparison with bone marrow mesenchymal stem cells. World J Gastroenterol 12(36):5834–5845.

Voinnet O. 2009. Origin, biogenesis, and activity of plant microRNA. Cell 136:669–687.

Wienholds E, Kloosterman WP, Miska E, Alvarez-Saavedra E, Berezikov E, Bruijn ED, Horvitz HR, Kauppinen S, Plasterk RH. 2005. MicroRNA expression in zebrafish embryonic development. Science 309:310–311.

Xu H, He JH, Xiao ZD, Zhang QQ, Chen YQ, Zhou H, Qu LH. 2010. Liver-enriched transcription factors regulate microRNA-122 that targets CUTL1 during liver development. Hepatology 1431–1442.

Zemel R, Bachmetov L, Ad-El D, Abraham A, TurKaspa R. 2009. Expressi on of liver specific markers in naive adipose-derived mesenchymal stem cells. Liver Int 1326–1337.

Zufferey R, Trono D. 2000. Production of high-titer lentiviral vectors. Curr Protoc Hum Genet 12(10):1–12.

Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH. 2002. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 13:4279–4295.

Zuk PA, Zhu M, Mizuno H. 2001. Multilineage cells from human adipose tissue: Implications for cell-based therapies. Tissue Eng 7:211–228.