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Overexpression of MicroRNA-122 Enhances In Vitro Hepatic Differentiation of Fetal Liver-Derived Stem/Progenitor Cells

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ABSTRACT

MicroRNAs (miRNAs) are a versatile class of tiny non-coding RNAs involved in regulation of various biological processes. miRNA-122 (miR-122) is specifically and abundantly expressed in human liver. However, the role of miR-122 in differentiation of fetal liver stem/progenitor cells into hepatocytes remains unclear. In this study, dual positive CD34+/CD117+ expressing human fetal liver stem/progenitor cells was enriched by magnetic cell sorting and cultured in vitro. The level of miR-122 was found to be increased at specific time intervals. Interestingly, during the differentiation process of hepatocyte-like cells, the increase in expression of miR-122 was positively correlated with expression of hepatocyte-specific genes. The status of differentiation process was improved by transfection of miR-122 into enriched stem/progenitor cells. The expression level of hepatic-specific genes as well as liver-enriched transcription factors (LETFs) was significantly increased by overexpression of miR-122 in fetal liver stem/progenitor cells. Thus, the study delineated the role of hepato-specific miR-122 in differentiation of fetal liver stem/progenitor cells into hepatocyte-like cells which could be used as a therapeutic target molecule to generate abundant hepatocytes. J. Cell. Biochem. 114: 1575–1583, 2013.

KEY WORDS: FETAL LIVER; STEM/PROGENITOR CELLS; microRNA-122; HEPATOCYTE-LIKE CELLS

MicroRNAs (miRNAs), as a class of endogenous biomolecules regulate the expression of target genes by binding to complementary regions of transcripts to repress their translation or mRNA degradation. MicroRNA 122 (miR-122) is the most abundant miRNA in human liver [Lagos-Quintana et al., 2002]. Hepato-specific miR-122 has been described as a critical facilitator of various homeostatic functions like fatty acid and cholesterol metabolism as well as hepatitis C virus (HCV) replication and hepatocellular carcinoma [Jopling et al., 2005; Krützfeldt et al., 2005; Esau et al., 2006]. Recently, miRNAs have gained significant attention as regulators of a variety of biological processes including maintaining stemness and guiding differentiation of stem/progenitor cells [Rana, 2007; Gangaraju and Lin, 2009].

Liver diseases include a wide spectrum of acute and chronic conditions which are associated with significant morbidity and mortality worldwide [Adam and Hoti, 2009]. Orthotopic liver transplantation is the only effective treatment for patients with end stage liver failure. However, there is a shortage of donor organs, thus the need for development of alternative methods for treating life-threatening liver diseases becoming increasingly important. While cell transplantation and other cell-based therapies have great potential for improving the lives of patients with liver failure [Dhawan et al., 2010; Khan et al., 2010], the lack of available donors for use in such experimental therapies has severely limited their development. An unlimited supply of stem cell-derived hepatocytes would be an invaluable for development of novel cell-based

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therapies. Much interest has developed in recent years in using discarded fetal liver tissues from aborted fetuses. The mammalian fetal liver contains cells with a high proliferative potential consisting of pluripotent stem cells [Piscaglia et al., 2008; Shafritz and Oertel, 2010]. One of the hallmarks traits attributed to human fetal stem cells is a potential for extensive self-renewal. The fetal liver is the main site of hematopoiesis in the fetus until development of the bone marrow. The indefinite cell division capacity of fetal stem cells is an asset as an unlimited source of pluripotent cells for use in tissue replacement.

In this study, for the first time we have examined the role of hepato-specific miR-122 in differentiation of fetal liver derived stem/progenitor cells into hepatocyte-like cells which could be a plausible therapeutic target molecule for treating chronic liver diseases.

MATERIALS AND METHODS

ISOLATION AND ENRICHMENT OF STEM/PROGENITOR CELLS

Human fetal liver tissues were obtained from aborted fetuses at 12–20 weeks' gestation from Nehru hospital following informed consent according to the guidelines set by the Ethical Review Committee (IEC PGI 7442/PG/1Trg/07) of Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India. Single cell suspensions were prepared from each fetal liver at various gestational weeks 12th–20th. Cells were isolated using the human primitive stem/progenitor cell enrichment isolation kit (Miltenyi Biotech., Bergisch-Gladbach, Germany) as described by the manufacturer's instructions and enriched by magnetic activated cell sorting (MACS) positive selection using antibodies to CD34 and CD117.

PRIMARY CELL CULTURE AND HEPATOCYTE DIFFERENTIATION

The stem/progenitor cells recovered at the end of the procedure were tested for viability, after which the cells were seeded in collagen type I-coated petriplates (Biocoat, BD Biosciences) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B. Tissue culture plates (4×10^4 cells/cm^2) were incubated with DMEM containing 10% fetal bovine serum in 5% CO_2. DMEM supplemented with hepatocyte growth factor (HGF, 50 ng/ml), epidermal growth factor (EGF, 20 ng/ml), and basic fibroblast growth factor (bFGF, 10 ng/ml) (Sigma-Aldrich Co, USA) were added and the medium was replaced every third day. The cells were cultured for 3 weeks using this medium; in between the cells were stained for hepatocyte-specific markers. Immunocytochemistry and flow cytometry were used to phenotype the stem/progenitor cells.

IMMUNOCYTOCHEMISTRY

The differentiation status of in vitro cultured cells was analyzed by immunocytochemistry using fluorescence microscope (Olympus, CKx41, Japan). To examine the morphological changes in differentiation process, cells were seeded on glass cover slips in cell culture plates. The cell were fixed for 10 min with ice-cold paraformaldehyde and permiabilized with 0.5% saponin for 5 min, finally blocked with 5% normal bovine serum albumin in PBS for 1 h at room temperature. The cells were then incubated with 1:100 mouse anti-albumin (Anti-ALB) antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and 1:200 rabbit anti-alpha fetoprotein (anti-AFP) antibody (Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. After washing three times with PBS, the cells were incubated with secondary antibodies (goat anti-mouse FITC IgG) for 1 h at room temperature and stained cells were visualized by a confocal microscope and photographed (Carl Zeiss 510 META Confocal Microscope).

FLOW CYTOMETRY

Flow cytometry was performed using antibodies to hepatocyte-specific markers. The cells were incubated with FITC labeled ALB and TRITC labeled AFP antibodies for 30 min at 4°C. For ALB and AFP, intracellular staining was performed after cell permeabilized by using 0.1% saponin in 1% BSA-PBS for 15 min at 4°C. Corresponding controls were used for evaluation of non-specific binding of monoclonal antibodies. Analysis was done on a FACS Calibur flow cytometer (Becton Dickinson, CA) equipped with 15 MW, 488 nm air cooled argon laser using the cell quest software program. The results were expressed in view of the mean fluorescence intensity of the labeled cells in the quadrant region, which could be directly correlated to the percentage of positive cells. Two independent sets of assays were run in duplicate.

GENE EXPRESSION ANALYSIS BY QUANTITATIVE REAL-TIME PCR (qRT-PCR)

Total RNA was extracted from cell samples harvested at different time points (at day 1, day 7, day 14, and day 21) using the TRIzol® reagent (Invitrogen, Carlsbad, CA). Total cellular RNA (2.0 μg) was reverse transcribed following manufacturer's instructions (Fermentas). Parallel-grown stem/progenitor cells without growth factors were used as a control. The expression of targeted mRNAs in cultured stem/progenitor cells was quantified using Light Cycler®, 480 software real-time PCR system (Roche, USA). PCR amplification was performed in a 20 μl volume containing 1× SYBR Green PCR Master mix, 0.2 μM of forward and reverse primer and 2 μl product of reverse transcription. Each PCR reaction was performed in triplex tubes with GAPDH as an endogenous control to standardize the amount of sample RNA. The following primers were used for qRT-PCR: human ALB, 5′-TTG GAA AAA TCC CAC TGC AT-3′ and 5′-CTC CAA GCT GCT CAA AAA GC-3′; AFP, 5′-TGC CAA CTC AGT GAG GAC AA-3′ and 5′-TCC AAC AGG CCT GAG AAA TC-3′; hepatocyte nuclear factor 4α (HNF4α), 5′-CTG CTC GGA GCC ACA AAG AGA TC-3′ and 5′-ATC ATC TGC CAC GTG ATG CTC TG-3′; and CCAAT/enhancer binding protein (C/EBP)α 5′-CAA GAA GTC GGT GGA CAA GAA C-3′ and 5′-CCT CAT CTT AGA CGC ACC AAG T-3′; glyceraldehydes 3-phosphate dehydrogenase (GAPDH), 5′-GTC TTC TCC ACC ATG GAG AAG GCT-3′ and 5′-CAT GCC AGT GAG CTT CCC GTT CA-3′. HepG2 cells were used as a positive control. Reaction conditions were: 95°C for 5 min to denature DNA templates, followed by 40 cycles of 95°C for 15 s denaturation, 62°C for 15 s annealing, and 72°C for 30 s extension. The Ct (number of cycles at which the fluorescence crosses the threshold) value obtained for the mRNA of interest was normalized against

endogenous GAPDH expression to obtain the ΔC_t values. The $\Delta\Delta C_t$ values were then obtained by subtracting the ΔC_t values for each gene of interest against ΔC_t values for the control sample. Fold difference in mRNA expression of the sample from the reference undifferentiated cells was calculated by using $2^{-\Delta\Delta C_t}$ method [Schmittgen and Livak, 2008].

TRANSFECTION OF STEM/PROGENITOR CELLS WITH miR-122 VECTOR

To improve the efficiency of hepatocyte differentiation, the miR-122 expression was induced in stem/progenitor cells by transfecting with miR-122 containing expression vector pEGP-mmu-mir-122 (Cell Biolabs, Inc., USA). Initially cells were enriched from fetal liver tissues by MACS as mentioned earlier. Briefly, the cells were seeded into 24-well culture plates and then transfected with miR-122 construct after 2 days of culture using lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. The cells transfected with vector without miR-122 construct (pIRES-internal ribosome entry site) were used as mock control.

ANALYSIS OF miR-122 EXPRESSION

(i) Polyadenylation reaction
qRT-PCR analysis for miR-122 expression was carried out using 2 μg of total RNA. Polyadenylation reaction was prepared by adding the following components in RNA free 0.5-ml microcentrifuge tubes: 5× poly A polymerase buffer, rATP (10 μM), *E. coli* poly A polymerase, RNase free H_2O and total RNA. The reaction mixture was incubated at 37°C for 30 min and then again incubated at 95°C for 5 min to terminate adenylation, and the reaction tubes were immediately transferred to ice to proceed for first-strand cDNA synthesis.

(ii) cDNA synthesis of poly A tailed miRNA
For the quantification of mature miRNA levels, reverse transcription was performed using the miRNA first-strand cDNA synthesis kit (high-specificity miRNA QPCR core reagent kit, Stratagene, USA) according to the manufacturer's protocol. The completed first-strand cDNA synthesis reaction was placed on ice for immediate use.

(iii) Real-time quantification of miRNA-122
The cDNA synthesized was used as template for real-time PCR amplification for the expression of miR-122 using specific oligonucleotide primers. The reaction mixture was prepared according to the manufacturer's instructions (high-specificity miRNA QPCR core reagent kit, Stratagene). PCR reaction was conducted in 20 μl total volume containing a final concentration of 0.5 μM of each primer, 4 μl ddH_2O, 10 μl of 2× SYBR Green PCR Master Mix and 5 μl of cDNA sample (1:20 diluted) corresponding to 2 μg of total RNA. The following primers were used for amplification of the miRNA-122: forward 5′-TGGAGTGTGACAATGGTGT TTGT-3′, a universal reverse primer miRNA-R 5′-CTC AAC TGG TGT CGT GGA-3′, U6 SnRNA forward: 5-CGCTTCGGCAGCACATATACTAA-3′ and reverse: 5′-TATGGAACGCTTCACGAATTTGC-3′. U6 SnRNA was used as an internal control. Reaction conditions were: 95°C for 5 min to denature DNA templates, followed by 40 cycles of 95°C for 10 s denaturation, 58°C for 15 s annealing, and 72°C for 25 s extension. The target transcript levels were normalized against U6 SnRNA expression and compared expression level to the control samples by $2^{-\Delta\Delta Ct}$. qRT-PCR data are expressed as the mean ± standard deviation (SD) from three separate experiments performed in triplicate.

STATISTICAL ANALYSIS

Real-time PCR data are expressed as mean ± SD. The difference between the groups at the indicated time points were analyzed using one-way analysis of variance (ANOVA) with bonferonni corrections and post hoc for inter group analysis. The differences were considered significant if the $P \leq 0.05$. All statistical calculations were performed using Statistical Package for the Social Sciences (SPSS) for Windows, Version 16 (SPSS, Inc., Chicago, IL).

RESULTS

PRIMARY CULTURE AND DIFFERENTIATION OF FETAL LIVER STEM/PROGENITOR CELLS

Human fetal liver tissues (n = 10) from gestational weeks 12–20 were used for isolation of stem/progenitor cells. As a starting point of our study while collecting the fetal liver tissues, we sought to determine the factors which influencing the number of stem/progenitor cells. The observations indicate that the viability and number of cells from each tissue was dependent on the time interval between the induction of the abortion, the expulsion of the fetus (between 4 and 18 h) and the time period for the collection of the fetal liver samples after expulsion of fetus (varied from 15 min to 6 h). Fetal liver stem/progenitor cell population was enriched by positive selection using antibodies to CD34 and CD117 (Table I). These cells when cultured in presence of growth factors showed typical morphological changes associated with hepatocyte-like cells. The enriched dual positive CD34+/CD117+ cells were seeded

TABLE I. Number of Fetal Liver Samples (n = 10) Collected at Various Weeks of Gestation

Number of samples (n) collected (n = 10) at different gestational weeks	12 (n = 1)	13 (n = 1)	14 (n = 2)	15 (n = 1)	16 (n = 1)	17 (n = 1)	18 (n = 1)	19 (n = 1)	20 (n = 1)
No. of cells isolated	12×10^6	12×10^6	14×10^6	14×10^6	16×10^6	19×10^6	25×10^6	24×10^6	33×10^6
EnrichedCD34+/CD117+ cells by MACS	4×10^5	5×10^5	5×10^5	6×10^5	8×10^5	8.5×10^5	12×10^5	11×10^5	15×10^5
Viability (% mean)	93	85	92	92	89	88	84	86	89

By collagenase digestion method, cells isolated from liver tissues and enriched dual positive CD34+/CD117+ cells by magnetic activated cell sorter.

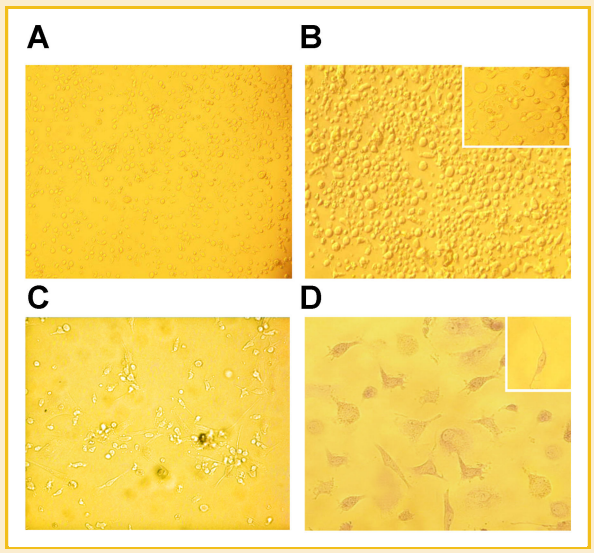

Fig. 1. Morphological analysis of cultured fetal liver stem/progenitor cells at different time intervals during differentiation. Enriched stem/progenitor cells were cultured in presence of growth factors HGF, EGF and bFGF and analyzed by light microscopy (40×). A: At Day 1 (B) Day 7 (3D image) (C) Day 14 (D) day 21. Representative images were taken from three independent experiments.

on collagen type I coated extracellular matrix plates under sterile culture conditions. The sequential morphological changes were observed over a period of 3 weeks (Fig. 1). At the beginning of differentiation, the liver stem/progenitor cells appear to be small and round (Fig. 1A). After first week, the cells begin to change their morphology from round to oval shape with distinctly rounded nuclei during the second week of culture (3D image, Fig. 1B). However, after second week, the cells transformed from smaller compact cells to an elongated spindle shaped cells (Fig. 1C). At later stages of differentiation, that is, after third week, the cells became enlarged and more flattened with characteristic morphological features of hepatocyte-like cells (Fig. 1D).

EXPRESSION OF HEPATOCYTE-SPECIFIC GENES

The enriched dual positive cells were maintained for more than 21 days under optimized culture conditions for differentiation and further quantified by flow cytometry (Fig. 2). On first day of culture, the cells did not show any expression of both hepatocyte-specific markers like AFP or ALB. However, these cells started expressing hepatocyte-specific markers on day 7, while the number of AFP positive cells was relatively low (2.21%) as compared to ALB positive cells (26.06%). However, 11.32% of the cells were found to be positive for both AFP and ALB markers (Fig. 2A). On day 14, we

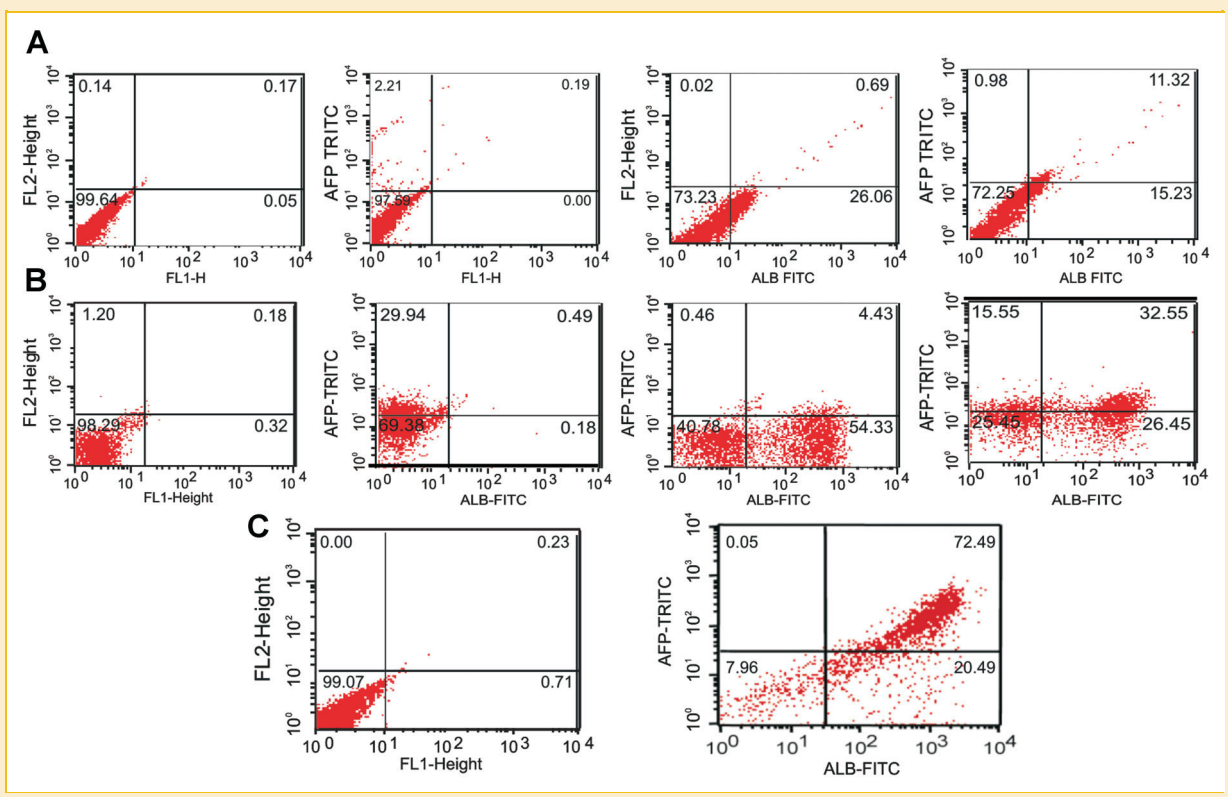

Fig. 2. Differentiation of fetal liver stem/progenitor cells into hepatocyte-like cells analyzed by flow cytometer. In vitro cultured cells were incubated with TRITC labeled AFP antibody and FITC labeled ALB antibody analyzed by flow cytometer and found to be most of the cells were positivity for AFP and ALB. (A) at day 7, (B) day 14, (C) day 21. Unstained cells were taken as negative control.

observed increased population of AFP+ and ALB+ cells which were 29.94% and 54.33%, respectively. Also, the number of dual positive cells co-expressing the hepatic markers (AFP and ALB) significantly increased from 11.32% to 32.55% (Fig. 2B). Interestingly at day 21, a significant increase in dual positive cell population co-expressing AFP+/ALB+ from 32.55% to 72.49% was observed (Fig. 2C).

To confirm the hepatic differentiation potential, in vitro cultured cells were further analyzed by immunocytochemistry as shown in Figure 3. Undifferentiated cells did not express any hepatocyte-specific markers while substantial number of cells expressing both the hepatocyte markers, that is, AFP and ALB were detected during the mid differentiation process (Fig. 3A,B). To further substantiate these results, we performed confocal microscopy which also confirmed the expression of hepatocyte-specific markers (Fig. 3C,D).

Thus, these findings confirmed that more than 70% of fetal liver stem/progenitor cells enriched from fetal liver have the potential to differentiate into hepatocyte-like cells in vitro and depicted higher expression of hepatocyte-specific markers.

EXPRESSION OF HEPATOCYTE AND STAGE-SPECIFIC DIFFERENTIATION GENES BY QUANTITATIVE REAL-TIME PCR

To further corroborate our findings, we quantified the expression of hepatocyte specific genes at indicated time points (at day 1, day 7, day 14, and day 21) of differentiation by quantitative real-time PCR (qRT-PCR) (Fig. 3E). We observed up-regulation in AFP expression (~2-fold only) after 7 days of culture which further increased to 4-fold after 14 days as compared to control. In contrast, after 21 days, the AFP expression was decreased by 2.5-fold as compared to

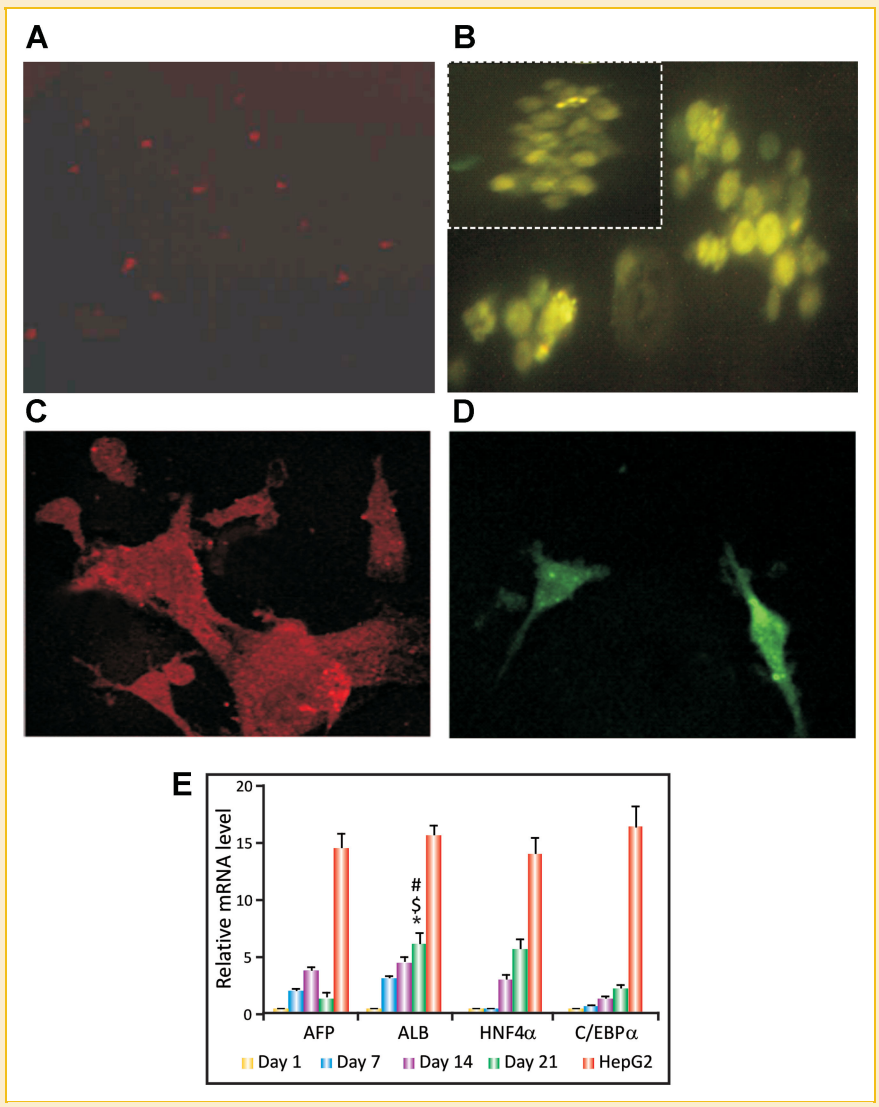

Fig. 3. Differentiation of fetal liver stem/progenitor cells into hepatocyte-like cells by microscopy and real-time PCR. A–D: In vitro cultured fetal liver derived stem/progenitor cells were incubated with AFP and ALB antibodies and analyzed by Fluorescence microscopy (A,B) (40×) and confocal microscopy (C,D) (scale bar 20 μm). A,C: AFP and (B,D) ALB. E: Expression of hepatocyte- and stage-specific genes during in vitro differentiation by real-time PCR. Real-time PCR amplification of the hepatocyte- and stage-specific differentiation genes was performed in triplicate and expression level of each gene was normalized by GAPDH and then compared to control samples. The means of the normalized gene expression values for each time point were calculated and expressed as relative fold changes (mean ± SD).

expression at day 14. The expression level of ALB increased with time from 3.2-, 4.6-, and 6.4-fold at day 7, 14, and 21, respectively. These results were statistically significant when compared the quantitative expression of ALB between 7 days versus 21 days ($P = 0.013$) and 14 days versus 21 days ($P = 0.023$). Over all, these findings were in consistent with the FACS data.

Besides, the hepatocyte-specific genes, we also focused on stage-specific hepatocyte differentiation genes, known as liver-enriched transcription factors (LETFs), like, HNF4α and C/EBPα. Similar to the above data, we did not observe the expression of LETFs during initial days of differentiation. However, the expression of HNF4α and C/EBPα was up-regulated during second week of culture, that is, after 14 days (Fig. 3E). Analyzing our data, we observed a linear increase in expression (3.1- and 5.8-fold) of HNF4α with increase in time period (14 and 21 days) as compared to undifferentiated cells. Similarly, the expression of C/EBPα was found to be gradually up-regulated after 14 days of culture.

Taken together, these results showed expression of hepatocyte-specific genes (AFP and ALB) and stage-specific hepatocyte differentiation genes (HNF4α and C/EBPα) at desired time points reflecting the potential of enriched dual positive (CD34+/CD117+) cells to differentiate into hepatocyte-like cells under optimal culture conditions.

OVEREXPRESSION OF miR-122 IN FETAL LIVER STEM/PROGENITOR CELLS

The study was further focused to gain additional insights into the mechanisms/crucial factors regulating differentiation of stem/progenitor cells. Initially, we also examined the expression pattern of miR-122 in fetal liver tissues. A gradual up-regulation of miR-122 expression was observed from fetal liver tissues of different gestational weeks (data not shown). The expression of hepatocyte-specific genes is known to be induced in enriched stem/progenitor cells when cultured in presence of growth factors in vitro. As our primary data, the miR-122 was up-regulated in stem/progenitor cells as compared with cells maintained monolayer culture. Thus, these findings espouse the role of miR-122 in liver development as well as in differentiation process.

We further performed a transfection studies using miR-122 vector to evaluate whether miR-122 enhances differentiation process of stem/progenitor cells. To confirm the role of miR-122 in differentiation, the same fetal liver derived stem/progenitor cells were transfected with GFP containing pEGP-mmu-mir-122 expression vector (Fig. 4). The cells transfected with pIRES mammalian expression vector were used as a mock control (Fig. 4B). As shown in Figure 4C, GFP expression was detected after 48 h of transfection in the miR-122 transfected cells. To confirm the percentage of miR-122 transfected cells, we performed flow cytometry which showed approximately 17.8% of cells were positive for pEGP-mmu-miR122 (Fig. 4D).

MICRO-RNA STIMULATES THE EXPRESSION OF HEPATOCYTE-SPECIFIC GENES DURING HEPATOCYTE DIFFERENTIATION IN VITRO

To determine whether miR-122 is capable of enhancing the expression of hepatocyte-specific genes, we first validated the miR-122 expression using qRT-PCR. As shown in Figure 5A, at 48 h

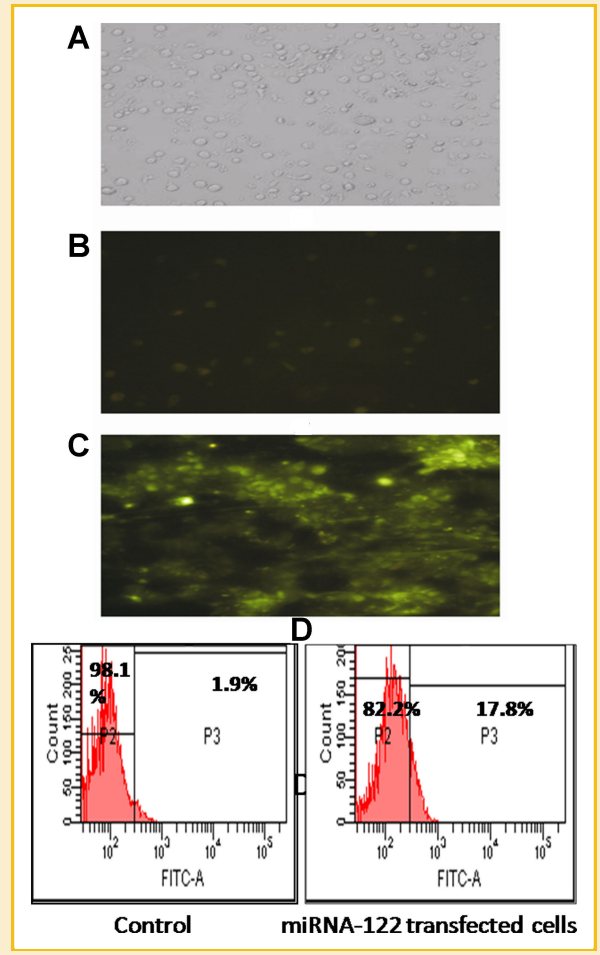

Fig. 4. Overexpression of microRNA-122 into enriched stem/progenitor cells and confirmed by fluorescence microscopy and flow cytometry. Enriched stem/progenitor cells transfected with microRNA-122 expression vector. A: Control cells (B) mock control cells (C) microRNA-122 expression vector transfected cells expressing GFP at 48 h post-transfection. (D) Flow cytometric analysis of microRNA-122 expression vector transfected stem/progenitor cells showed population of GFP positive cells.

post-transfection, the expression of miR-122 was found to be increased as compared to control and mock control. The representative graph showing the expression of hepatocyte-specific genes in mock control cells (Fig. 5B). Further, we performed qRT-PCR assays to detect mRNA levels of hepatocyte-specific genes in transfected stem/progenitor cells. We found that stem/progenitor cells that were transfected with miR-122 resulted in a gradual up-regulation of AFP, ALB, HNF4α and C/EBPα expression levels with time (Fig. 5C), indicating that they might be regulated by miR-122. On day 7, expression of both the markers was up-regulated up to fivefold. At day 14, ~16- and 13-fold increased in expression of AFP and ALB was detected respectively. However, at day 21, we observed remarkable increase in expression of AFP and ALB with 20- and 31-fold respectively. The expression of AFP and ALB from Day 7 to 21 was found to be significant ($P < 0.05$) confirming stimulation of hepatocyte-specific genes by miR-122.

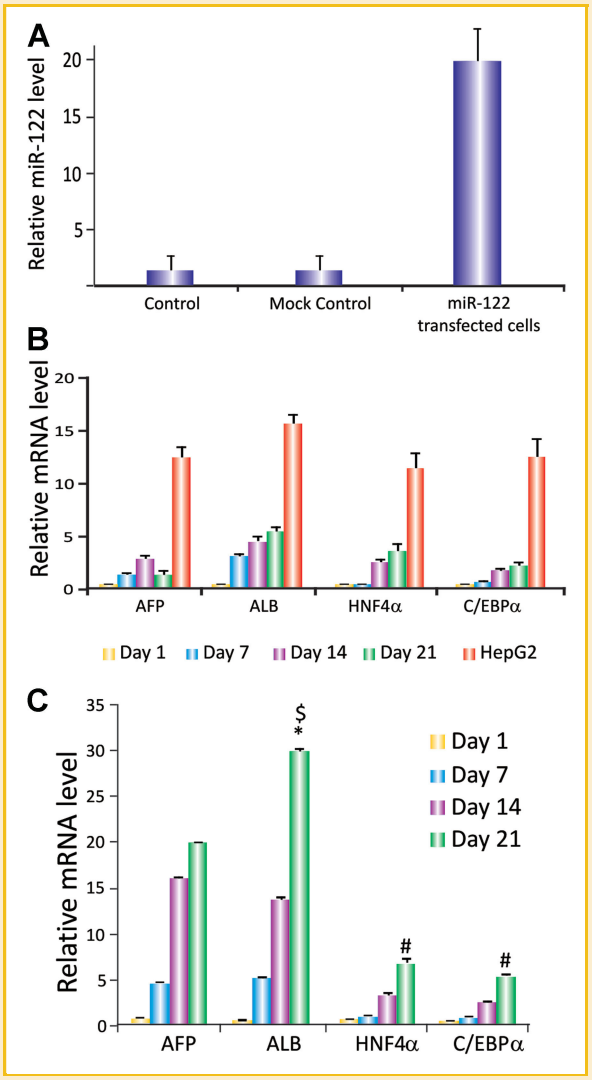

Fig. 5. Expression of microRNA-122 and hepatocyte-specific genes analyzed at specific time intervals after overexpression of microRNA-122 into stem/progenitor cells by quantitative real-time PCR. A: Quantification of mcroRNA-122 in transfected stem/progenitor cells. B: Expression of hepatocyte- and stage-specific differentiation genes in mock control cells. C: MicroRNA-122 stimulates the expression of hepatocyte-specific genes during hepatocyte-like differentiation in vitro. Real-time PCR amplification of the hepatocyte- and stage-specific differentiation genes was performed in triplicate and expression level of each gene was normalized by GAPDH and then compared to control cells. The means of the normalized gene expression values were calculated for each time point and expressed as relative fold changes (mean ± SD).

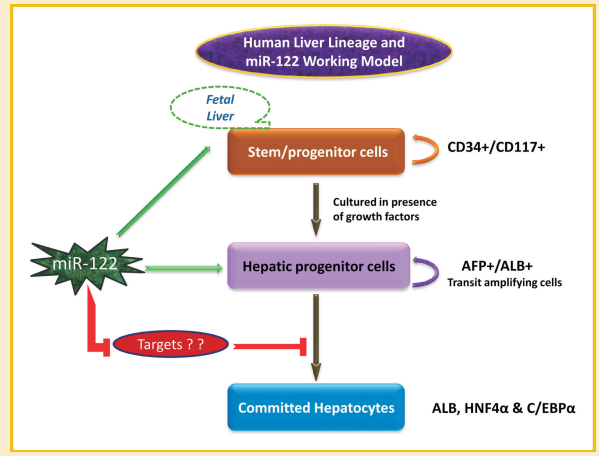

Fig. 6. Model for the role of miR-122 in differentiation of fetal liver stem/progenitor cells into hepatocyte-like cells. Stem/progenitor cells were enriched from fetal livers of different gestational weeks using CD34 and CD117 markers. In presence of growth factors, the enriched cells were differentiated into hepatocyte-like cells. After transfection of miR-122 vector, stem/progenitor cells showed the enhanced expression of hepatocyte-specific markers indicating that miR-122 is playing a crucial role in differentiation process. miR-122 may be gradually activated by liver enriched transcription factors during differentiation process. With the increase of miR-122 expression, the miR-122 targets (?) may be gradually silenced, therefore, increased the expression of hepatocyte- and stage-specific differentiation genes in miR-122 transfected stem/progenitor cells.

Because of the expression of hepatocyte-specific genes, the expression profile of stage-specific hepatocyte differentiation genes was also analyzed at different time points of differentiation. The expression of HNF4α was significantly up-regulated by 3.5- and 6.9-fold after 14 and 21 days of culture respectively ($P < 0.013$). Furthermore, the expression of C/EBPα demonstrated a gradual up-regulation by 2.5- and 5.5-fold after 14 and 21 days of culture respectively (Fig. 5C). Hence, these results suggest that over-expression of miR-122 in stem/progenitor cells was pervasively up-regulate the expression of genes coding for hepatocyte-specific functions during differentiation of fetal liver stem/progenitor cells toward hepatic lineage specification.

DISCUSSION

Stem cells have generated a great deal of interest in recent years because of their potential therapeutic uses. Under the influence of environmental factors (cytokines and extracellular matrix components), they can proliferate, differentiate, and replace damaged cells from adult tissues. To reproduce this process in vitro, it is important to know the ushering factors involved in organ development and differentiation. The multifaceted role of miR-122 in liver makes it an attractive molecule for study, as it likely to have important role in liver development and differentiation. The present work has provided evidence to suggest the role of hepato-specific miR-122 could act as a therapeutic target molecule for differentiation of stem/progenitor cells into hepatocytes (Fig. 6).

In order to analyze the miR-122 expression in differentiation of stem/progenitor cells, fetal liver tissues were digested with collagenase to get the single cell suspension. We have observed the increased expression of miR-122 during fetal liver development (12–20th week of gestation). Similarly, Chang et al. [2004] reported the miR-122 expression is developmentally regulated, increasing in the liver over the course of embryonic development. To determine further the role of miR-122 in differentiation, stem/progenitor cells enriched by MACS were cultured in presence of growth factors in

vitro. In presence of growth factors, fetal liver stem/progenitor cells (CD34+/CD117+ cells) showed typical morphological characteristics of hepatocyte-like cells which were similar to other studies [Nava et al., 2005; Nowak et al., 2005]. Signals from the liver environment are crucial for this shift or differentiation of these cells. Such signals can be derived from the extracellular matrix, soluble factors secreted by the neighboring cells or by direct cell–cell interactions [Erker and Grompe, 2007].

To verify whether mRNA was translated into specific proteins, immunocytochemical analysis was performed. After first week of differentiation, only few cells showed positivity for mature hepatocyte marker (ALB) by immunocytochemistry, however at Day 21 of culture, the number of ALB-positive cells was greater. Besides the characteristics of mature hepatocytes, the fetal liver-derived hepatocytes also appear to retain some immature characteristics, such as persistent AFP expression, a marker of fetal rather than adult hepatocytes. Wauthier et al. [2008] reported reduced expression of mature hepatocytes of AFP as they have acquired the well-known adult-specific expression profile that includes high levels of ALB. Similarly, we also observed the decreased expression of stem/progenitor markers and increased hepatocyte-specific markers, which can be correlated with a process of liver development. High expression level of ALB in our culture demonstrated that the cells are differentiating towards the hepatic lineage. Similar to our results, Liu et al. [2008] also reported the expression of hepatocyte markers in cells isolated from 20 to 24 weeks of gestation.

To investigate whether our in vitro cultured cells could induce the transcription of some factors that are essential for hepatocyte differentiation, we examined the expression of specific genes including ALB, AFP, HNF4α, and C/EBPα at mRNA level. Maturation of cells was further corroborated when a gradual up-regulation of hepatocyte specific genes at later stages of hepatic differentiation was seen. Furthermore, we observed that AFP expression increased initially but the expression decreased on day 21, indicating that these cells gained properties of mature hepatic cells. Therefore, our findings strongly espouse a gradual differentiation into hepatocyte-like cells in vitro.

A network of LETFs controls differentiation and maturation of hepatic cells [Kyrmizi et al., 2006]. The expression of HNF4α and C/EBPα were analyzed in this study because they are found to be highly abundant in both human and mouse liver [Schrem et al., 2004]. Over all, we observed an increased expression of stage-specific differentiation genes HNF4α and C/EBPα during the culture. Recently reported that miR-122 may be controlled by transcription factors enriched in the liver such as HNF4α and C/EBPα, which play pivotal roles in regulating the expression of liver specific genes [Laurent, 2008; Coulouarn et al., 2009; Xu et al., 2010]. This raises the possibility that LETFs positively feedback on miR-122 expression to control hepatocyte differentiation.

In addition to the many homeostatic functions that have been ascribed to miR-122 in the liver, this miRNA may also function as an enhancing differentiation process of stem/progenitor cells. To clarify the potential role of miR-122 in differentiation, transfection studies were performed. As observed a small increase in miR-122 (1.7-fold) is sufficient to perturb hepatic cell differentiation [Laudadio et al., 2012]. Overexpression of miR-122 in fetal liver derived stem/progenitor cells resulted in significantly up-regulated expression of hepatocyte-and stage-specific differentiation genes facilitating hepatic differentiation of stem/progenitor cells derived from human fetal liver. miR-122 can stimulate gene expression by binding to promoters or to mRNAs on their 5′-untranslated region, 3′-untranslated region or coding sequence [He and Hannon, 2004]. At Day 21 of differentiation timeline when a large fraction of stem/progenitor cells have differentiated into mature hepatocyte-like cells, a low level of AFP expression was observed. However, this level was still slightly higher than the level that was observed in stem/progenitor cells before miR-122 transfection. We suspect that this may reflect a heterogenous population of cells in the culture condition, composed of hepatocyte-like cells as well as some hepatic and progenitor stem cells. In a direct or indirect way, most of the miR-122 stimulated genes code for hepatocyte-specific proteins. In our study, under optimized culture conditions, coordinated differentiation of the cells exhibited hepatic stage-specific gene expression (Figs. 4 and 6). Algorithm-based studies have predicted that for hematopoiesis, interactions between the set of miRNAs expressed in CD34+ hematopoietic stem/progenitor cells and mRNAs are critical [Georgantas et al., 2007].

A better knowledge of stem cell basic biology and differentiation pathways may facilitate the translation of their unique biologic properties into novel cellular therapies for tissue regeneration. In conclusion, the hepatocyte-like cells generated by our study have a tremendous potential in development of therapeutics. We also envisaged that miR-122 drives proper hepatocyte-specific gene expression in cultured stem/progenitor cells. Furthermore, studies like this would be helpful for exploring additional avenues through which to study the molecular mechanisms regulating the differentiation of fetal liver stem/progenitor cells and facilitate the development of cell-based therapies for treatment of chronic liver diseases.

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