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Hepatocyte nuclear factor Foxa-2 effectively differentiates mesenchymal stem cells to functional hepatocytes

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Overexpression of hepatocyte nuclear factor (Foxa-2), a master regulator significantly enhances the hepatic differentiation by triggering targeted liver-specific gene expression for liver development. We investigated the function of key regulators that facilitate hepatic differentiation. The functional hepatocyte was determined by observing morphological changes, expression of liver specific markers through immunocytochemical analysis, qRT-PCR, Western Blot and specific liver function assays. Our study confirmed that induction of functional hepatocyte shows typical epithelial morphology, express hepatic genes and acquire hepatocyte functions. Notably, these cells expressed the markers of mature hepatocytes, including albumin, tyrosine aminotransferase, cytokeratin, Cyp7A1 and hepatic transcription factors. Furthermore, these cells exhibited hepatic functions *in vitro*, including glycogen storage, albumin production, urea secretion and functional assessment of hepatocytes. We have developed a robust and efficient method to differentiate mesenchymal stem cells into induced functional hepatocytes, which exhibits characteristics of mouse hepatocytes. Apparently, induction of functional hepatocytes will pave a better cellular therapy or effective cell source within a short timeline to facilitate the development of hepatocytes for future clinical applications of regenerative medicine.

Keywords: Cytochrome P450, Hepatocyte nuclear factor,, Liver development differentiation, Nuclear transcription factor

Fulminant hepatic failure (FHF) is the major outcome from of a primary liver insult, which leads to failure of multiple organs with innumerable complications. The death from advanced FHF is high and the accessibility of donor livers for transplantation is an alternative way to rescue FHF patients. However, the limited availability of donors resulted in the burgeoning interest in developing stem cell therapy as alternatives to liver regeneration[1,2]. Stem cells presented in all organs play a very critical role in maintaining the tissue homeostasis. Normal physiological surroundings, hepatic progenitors reveal a low proliferative potential and location of stem cell niche inside the intrahepatic biliary tree maintaining the liver specific stem cells under quiescent state. Upon liver injury, the stimulated stem cell niche augments bi-potential transient cells to differentiate into mature bile duct cells and hepatocytes[3]. Bone marrow and or blood derived mesenchymal stem cells have also been considered as an alternative cell source apart from tissue derived stem cells that are able to differentiate into functional hepatocytes[4]. More reports are available suggesting embryonic stem cells (ESCs) and umbilical cord stem cells (UCSTs) have the potential to differentiate into mature hepatocytes due to its pluripotent nature. Moreover, its therapeutic practice is limited due to responses to recipient's immune system and formation of teratomas upon transplantation[5].

The two winged helix transcription factors belongs to the Foxa family members includes HNF 3α, β, and γ (hepatocyte nuclear factor) are known as Foxa 1,

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Abbreviations: AAT, Alpha-1 antitrypsin; Alb, Albumin; α-FP, alpha-fetoprotein; β-actin, beta-actin; C/EBP CCAAT-enhancer-binding proteins; CK, cytokeratin; CYP3a11, cytochrome P450 3a11; bEGF, epidermal growth factor-4b; bFGF, fibroblast growth factor; FHF, Fulminant hepatic failure; HNF, hepatocyte nuclear transcription factor; ICG; indocyanine green, mMSCs; mouse mesenchymal stem cellsPAS, periodic acid Schiff's staining; PEPCK, Phosphoenolpyruvate carboxykinase; PXMP1-L, peroxisomal membrane protein 1-like protein; TAT, tyrosine-aminotransferase; and TBS-T, Tris-buffered saline with 0.2% Tween 20

Foxa 2, and Foxa 3, respectively, considered as key transcription factors in regulating the proper formation of foregut and hindgut during embryonic development[6]. Regulation of hepatogenesis and maintenance of hepatic homeostasis, a Foxa family member plays a leading role right from embryogenesis to adult liver functioning. Foxa proteins were also shown to regulate chromatin remodeling *via* nucleosome relaxation which is very crucial in priming hepatogenesis before the actual transcription of hepatic genes begins[7]. Mouse embryonic studies indicated the potential role of Foxa proteins as pioneer factors in determining definitive endoderm in nascent liver[8].

Foxa-2 is a liver-enriched nuclear transcription factor that plays a key function in the early development of the endoderm which is the precursor to form the liver. During embryonic liver development process, the establishment of defined foregut endoderm from cardiac mesoderm signals and stimulation of liver-specific gene expression were the two major events that regulate hepatogenesis. These transcription factors showed a significant role in creating the endodermal domain that finally contributes for liver development. HNF-4α is necessary for Foxa-2 expression and endoderm development that responds to the fibroblast growth factors (FGF) signal, via foregut endoderm commitment upon receiving the signal. Also, FGFs from the cardiac mesoderm were found to stimulate liver markers in the ventral foregut endoderm, resulting in outgrowth of the endoderm and hepatic differentiation[9]. It has been demonstrated that Foxa 1 and Foxa 2 deficient endoderm failed to react to FGF2 signal that made it further inefficient in activating the hepatic program in *in vitro* explant culture system[10].

The chromatin structure of the albumin enhancer region during hepatogenesis, showed GATA and Foxa factors bound to the enhancer of gut endoderm prior to the albumin gene initiation[11-13]. These findings suggested that GATA and FOXA as genetic activators of hepatic differentiation[14,15]. Several data demonstrated that binding of Foxa-2 and GATA protein could facilitate the interaction with CBP (CREB binding protein) which enhances transcriptional activation[16]. It is also known that Foxa proteins binds DNA-nucleosome core region and resulted in the decompacting of the chromatin, which further facilitates the binding of the other transcription factors[17,18].

In vitro hepatic differentiation can also be stimulated from stem cells by means of inducers including chemical compounds, growth factors and cytokines, but limited to low differentiation efficiencies that hampers isolating functional hepatocytes. Overexpression of nuclear factors can directly differentiate the stem cells phenotypically, including trans-differentiation, de-differentiation and differentiation[19].

In the present study, we tried to evaluate the effectiveness of overexpression of Foxa-2 on hepatic trans-differentiation potential of bone marrow MSCs.

Materials and Methods

Animals

Normal inbred 6 week old BALB/c mice were procured from King's Institute, Chennai, an enlisted supplier of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animal), India. The animals were provided with pellet diet and water *ad libitum* and were sacrificed on the same day of purchase to isolate bone marrow Mesenchymal Stem Cells (MSC's). All procedures used in the study were done by following the guidelines of the CPCSEA, (Government of India) and approved by Indian Institute of Technology Madras, Institutional Ethics Committee (Ref. No. IEC/2010/08/RSV-4/03).

MSC isolation

Bone marrow derived MSCs (BM-MSCs) were isolated according to the method followed by Soleimani & Nadri[20]. The isolated population were cultured in DMEM/F12 medium supplemented with 20% FBS, 100 µg/mL penicillin and streptomycin at 37°C in humidified incubator with 5% CO_2. After 2 days, BM-MSCs were washed with medium to remove the non-adherent floating cells. The culture was maintained until it reaches confluence of 80% by changing the medium every 3 days. Briefly, on reaching confluence the populations were trypsinized and maintained till 4 to 5 passages. Further, all assays and studies were done between 3 to 15 passages.

Characterization of BM-MSCs and its multilineage differentiation

Characterization of BM-MSCs at passage 3 was done by immunocytochemistry. Cells were trypsinized and washed twice with Dulbecco's phosphate buffered saline (DPBSA). Further, the pellet was then fixed with 4% paraformaldehyde for 20 min and washed with DPBSA. Then the cells were permeabilized with 0.25% Triton X for 20 min and washed with DPBSA twice. The cells were blocked

with 5% FBS for 90 min and washed with DPBSA twice. Cells were stained for the following monoclonal antibodies CD29, CD44, CD90, CD 105, Sca-I, CD 34 and CD45 for 16 h at 4°C. After incubation, the cells were washed with DPBSA thrice and the appropriate secondary antibody conjugated with PE and FITC was added along with the nuclear stain Hoechst for 2 h in RT (28°C) in dark. The cells were washed with DPBSA twice and observed under Fluorescence microscope Nikon Ti Eclipse, Japan.

Construction of PCDNA3.1/Foxa-2 recombinant clone selection

The CMV-Foxa2 (HNF-3β) expression vector containing the full-length rat cDNA (1.6-kb EcoRI fragment) and two truncated Foxa2 plasmids expressed from the cytomegalovirus promoter. The upstream primer was 5′-CGGAATTCTACTCTTCCG TGAGCAAC-3′, and the downstream primer was 5′-CGCGGATCCGGACGAGTTCATAATAG-3′. The PCR product was cloned into pcDNA 3.1 Myc (procured from Prof. Andrew Russo, University of Iowa), resulting in the generation of PCDNA3.1/FOXa-2. The Foxa-2 fragment was confirmed by digesting with Xho I and Hind III restriction sites and sequenced. After passage 4, the BM-MSCs were transfected with the recombinant plasmid using Lipofectamine 2000 (Invitrogen). The target gene overexpression was confirmed by RT-PCR and immunofluorescence. The transfected BM-MSCs were cultured in medium with G418 (neomycin) (with increasing concentration) for the selection of resistant clones. Further, the selected clones were used for the differentiation studies.

***In vitro* differentiation of MSCs**

Osteogenic lineage

BM-MSCs after 4th to 15th passages were harvested for assessing osteogenic lineage differentiation and mineralization as per the method of von Kossa and alizarin red S staining[21].

Adipogenic lineage

BM-MSCs after 4th to 15th passages were harvested, adipogenic lineage differentiation and lipid accumulation was assessed biochemically by Oil Red-O and alizarin red S and staining[21].

Hepatogenic differentiation

BM-MSCs of passage 3 were cultured on type-I collagen coated 6-well plates till 80% confluence in DMEM/F12 (20% FBS + antibiotics). Induction of functional hepatic transdifferentiation was carried out according to Lee *et al*.[22] with minor changes. Briefly, cultured BM-MSCs (2×10^4 cells/cm^2) were subjected to serum deprived condition with 10 ng/mL fibroblast growth factor (bFGF) and 20 ng/mL epidermal growth factor-4b (bEGF) for 2 days in DMDM medium. The medium was replenished after 48 h, by hepatocyte differentiation medium (DMEM containing well defined growth factor cocktail of 10 ng/mL bFGF + 20 ng/mL HGF + 0.61 g/L nicotinamide) for six days. The medium was replaced with maturation medium [DMEM containing well defined growth factor cocktail of dexamethasone (1.0 μM/l) + oncostatin M (20 ng/mL) + ITS (Insulin, Transferrin, selenium, linoleic acid) (50 mg/mL)]. Cultures were maintained for 28 days, during which maturation medium was changed once every four days[7,22]. Induction of functional hepatic differentiation was carried out by considering BM-MSCs with the following groups: Gr. I: Normal BM-MSCs maintained in DMEM/F12 (20% FBS) throughout the studies, Normal BM-MSCs as a negative control group (BM-MSCs); Gr. II: Normal BM-MSCs maintained in hepatogenic differentiation systems (D-MSCs); and Gr. III: Foxa-2 transfected BM-MSCs maintained in hepatogenic differentiation systems (Foxa-2-MSCs).

Characterization of Foxa-2 Induced BM-MSC-derived functional hepatocytes

Examining the morphological changes

During hepatogenic differentiation of **BM-**MSCs differences in morphology of cell differentiation in all groups were examined at different time intervals by phase contrast microscopy (Olympus, CX-41).

Evaluating the functional characteristics of hepatocyte

The expression of functional specific markers of hepatocytes (Albumin (Alb), Cytokeratin 18 (CK18), Cytokeratin 19 (CK19) and Foxa-2) was investigated for all groups (induced and uninduced) after 3 weeks of induction according to Dhanasekaran *et al*.[3] and observed by fluorescence microscope (Nikon Tie, Japan).

Expression of functional specific hepatic genes

Total RNA was extracted by Trizol reagent (Sigma Aldrich, USA) from all the induced and uninduced groups (BM-MSCs, D-MSCs and Foxa-2-MSCs) according to the manufacturer's instructions The cDNA templates were attained by reverse transcription of total RNA using MMLV-RT (Thermo scientific, USA) and oligo-dT primers (NEB, USA). RT- PCR and quantitative real-time PCR (qRT-PCR) with primers designed specific for functional

hepatocytes listed in Table 1 using Applied Biosystems SYBR Select Master mix (Applied Biosystems 7500 Real-Time PCR Systems, Life Technologies, USA). Relative mRNA quantification by normalization to β-actin housekeeping gene and the fold change was then calculated by C_T ($2^{-\Delta\Delta CT}$) method. The analysis was performed in triplicate for statistical validation.

Functional Assessment of Foxa-2 Induced BM-MSC-derived hepatocytes

Liver functional activities were determined to validate the functionality of Foxa-2 induced BM-MSC-derived hepatocytes.

Assessment of glycogen storage by PAS

The insoluble stored glycogen was assessed in hepatogenic differentiated cells by periodic acid Schiff's staining (PAS) for all groups (induced and uninduced) after 3 weeks of induction according to Dhanasekaran *et al*.[7] and visualized under phase contrast microscope.

Albumin and Urea Secretion

The albumin secretion was determined in the supernatant of the induced and un-induced (BM-MSCs, D- MSCs and Foxa-2-MSCs) cells at different time periods (0, 6, 12, 24 and 28 days) using a mouse ALB ELISA quantitation kit (Bethyl, USA) according to the manufacturer's instructions. Urea concentration was assessed in induced and uninduced (BM-MSCs, D-MSCs and Foxa-2-MSCs) cells at various time intervals using urea assay kit (Sigma) were measured as per manufacturer's instruction. The data were normalized based on cells (10^5 live cells) which is collected from the conditioned media for the assays.

Cellular uptake and release of ICG

Assessment of liver function was performed by examining the uptake and release of indocyanine green (ICG) dye assay was performed in induced and un-induced (BM-MSCs, D-MSCs and Foxa-2-MSCs) as per Lee *et al*.[22]. The cells were photographed to ensure the release of ICG under inverted light microscope.

Evaluating the functional hepatic protein expression

Expressions of the functional specific hepatic markers (Albumin, CK18, CK19 and Foxa-2) were investigated for all groups (induced and uninduced) after 3 weeks of induction. The hepatic protein expression was detected using enriched chemiluminescence Pierce ECL Plus western blotting detection reagents (Pierce, USA) according to the manufacturer's instruction.

Table 1 — Primers used for RT-PCR and qRT-PCR

Genes	Primer Sequence (5′-3′)	Temp. (°C)	Length (bp)
β –Actin	FP:5′-TTCCTTCTTGGGTATGGAAT-3′ RP:5′-GAGCAATGATCTTGATCTTC-3′	55	206
Albumin	FP:5′-TGAACTGGCTGACTGCTGTG-3′ RP:5′-CATCCTTGGCCTCAGCATAG-3′	57	718
Trans-thyretin	FP:5′-AGTCCTGGATGCTGTCCGAG-3′ RP:5′-TTCCTGAGCTGCTAACACGG-3′	62	440
αFP	FP:5′-TCGTATTCCAACAGGAGG-3′ RP:5′-AGGCTTTTGCTTCACCAG-3′	55	174
AAT	FP:5′-AATGGAAGAAGCCATTCGAT-3′ RP:5′-AAGACTGTAACTGCTGCAGC-3′	57.2	484
HNF-1α	FP:5′-CGAAGATGGTCAAGTCGTAC-3′ RP:5′-GGCAAACCAGTTGTAGACAC-3′	55	500
HNF-1β	FP:5′-TTCAGTCAACAGAACCAGGG-3′ RP:5′-CTCTGTGCAATGGCCATGAC-3′	57.2	721
HNF-3α	FP:5′-CATGAGAGCAACGACTGGAA-3′ RP:5′-TTGGCGTAGGACATGTTGAA-3′	55.2	182
HNF-3β	FP:5′-AGAGGACTGAGGTAACTGAC-3′ RP:5′-GACTCGGACTCAGGTGAGGT-3′	60.2	415
HNF4	FP:5′-GATTGACAACCTGCTGCAGG-3′ RP:5′-CCTGCAGCAGGTTGTCAATC-3′	55	769
HNF-6	FP:5′-CAGCGTATCACCACCGAGCT-5′ RP:5′-CTCTGTCCTTCCCATGTTCT-3′	55	250
CK8	FP:5′-ATCGAGATCACCACCTACCG-3′ RP:5′-TGAAGCCAGGGCTAGTGAGT-3′	55	127
CK18	FP:5′-CGAGGCACTCAAGGAAGAAC-3′ RP:5′-GCTGAGGTCCTGAGATTTGG-3′	57	130
CK19	FP:5′-ACCCTCCCGAGATTACAACC-3′ RP:5′-CAAGGCGTGTTCTGTCTCAA-3′	58	160b
C/EBP/α	FP:5′-TGGACAAGAACAGCAACGAG-3′ RP:5′-TCACTGGTCAACTCCAGCAC-3′	56	126
C/EBP/β	FP:5′-GAGCGACGAGTACAAGATGCGG-3′ RP:5′-TTGTGCTGCGTCTCCAGGTTG-3′	61	95
P450 CYP3a11	FP:5′-TGAGGCAGAAGGCAAAGAAA-3′ RP:5′-GGTATTCCATCTCCATCACA-3′	55	590
PXMP1-L	FP:5′-CTTCAGACCCAGAGAGAGCTG-3′ RP:5′-CCCGTGTTGCCTGTGATGAGC-3′	62	475
AAT	FP:5′-AATGGAAGAAGCCATTCGAT-3′ RP:5′-AAGACTGTAACTGCTGCAGC-3′	55	240
PEPCK	FP:5′-CTTGTCTACGAAGCTTCTCAG-3′ RP:5′-AGTGACCTCGAAGTGGAACC-3′	59	280
TAT	FP:5′-TGAGCAGTCTGTCCACTGCCT-3′ RP:5′-ATGTGAATGAGGAGGATCTGAG-3′	62	358

Statistical analysis

All data presented in this study as the mean ± SD (standard deviation). One-way repeated measures analysis of variance (ANOVA) was used to analyze group differences. P <0.05 was considered as statistically significant between the groups.

Results and Discussion

Isolation and Characterization of BM-MSCs

The heterogeneous populations were found to be plastic adherent and showed a typical fibroblastic morphology under phase contrast microscope. Immuno-cytochemistry was done to address the surface marker profile of isolated cells. MSCs were found to express markers like CD29, CD90, CD44 and Sca I [Fig 1A(i-iv)],

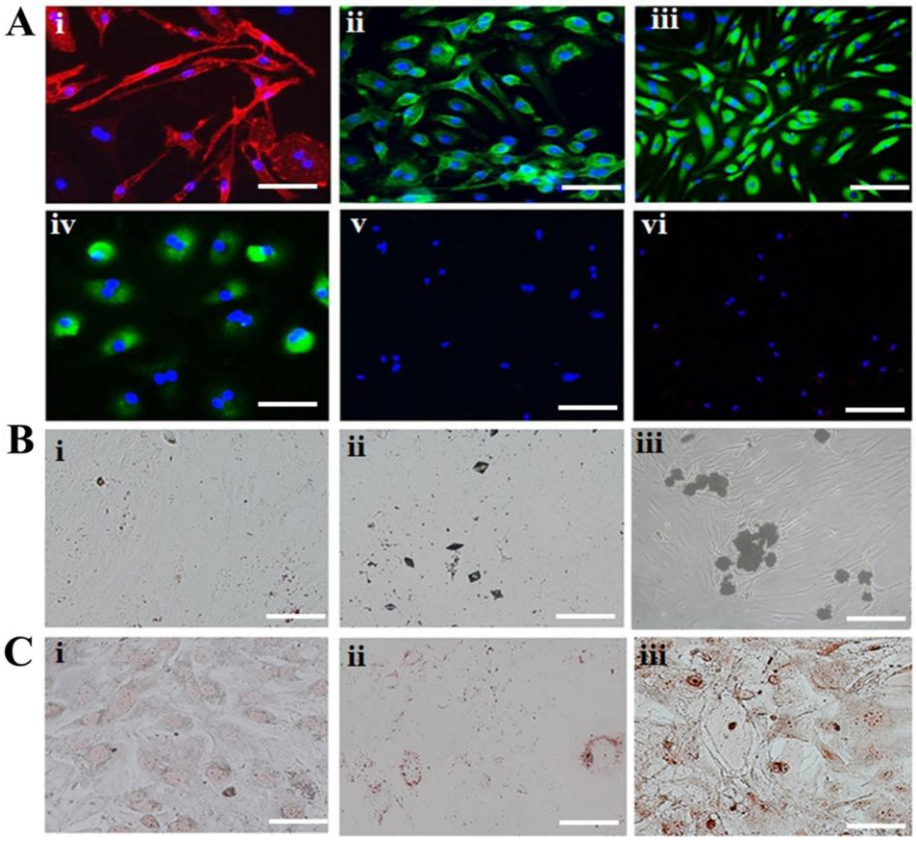

Fig. 1 — Characterization of bone marrow derived MSCs. (BM-MSCs) (A) Immunocytochemistry shows that BM-MSCs is positive for the expression of (i) CD29, (ii) CD44, (iii) CD90 and (iv) Sca 1, but negative for the expression of (v) CD34 and (vi) CD45; (B) MSCs are induced to differentiate into osteoblasts, (i) Control BM-MSCs (un-induced) with their normal morphology stained negative, (ii) positive stain for mineralization by Oil Red O stain (iii) and positive stain for mineralized matrices by von Kossa assay; and (C) Under adipogenic conditions, MSCs accumulate lipid vacuoles, (i) Control BM-MSCs (un-induced) with normal morphology stained negative with alizarin red , (ii) positive strain for oil droplets by Oil Red O assay (iii) and positive stain for oil droplets by alizarin red. [Scale bars, 100 µm. (Original magnification, X100)]

and was negative for CD34 and CD45 [Fig.1 (v and vi)]. BM-MSCs were subjected to adipogenic and osteogenic differentiation using defined chemical cues. Alizarin-red staining used to confirm osteogenic lineage showed calcium mineralization in differentiated BM-MSCs. Fig. 1B(i) shows un-induced control BM-MSCs with their normal morphology stained negative, Fig. 1B(ii) is stained positive for mineralization by Oil Red O stain and Fig. 1B(iii) and depicts the mineralized matrices by von Kossa assay. Accumulation of intracellular lipid droplets was observed that indicates the adipogenic lineage as seen by the oil aggregation in differentiated BM-MSCs. Fig. 1C(i) shows uninduced control BM-MSCs with normal morphology stained negatively with alizarin red, Fig. 1C(ii) indicates BM-MSCs with oil droplets stained positively by Oil Red O assay and Fig. 1C(iii) stained positive for alizarin red. Scale bars, 100 µm. (Original magnification, X100).

Stage-specific Foxa-2-MSCs selectively promotes hepatic differentiation

D-MSCs and Foxa-2-MSCs were induced for hepatogenic differentiation. Upon differentiation, the phenotype of mesenchymal stem cell changes from its inherent fibroblastic spindle to polygonal shape drastically within 28 days of hepatogenic induction, as observed under phase contrast microscope (Fig. 2). In the absence of growth factors, no changes in the cellular morphology of BM-MSCs (Group I) [Fig. 2A (i-iv)] was observed, whereas the addition of growth factors produced morphological changes resembling mature cells depends upon the time period. Compared with control, morphological changes were more prominent and homogenous in Foxa-2 transfected cells that were hepatocyte-like cells. In group II D-MSCs with growth factors showed a large number of a heterogeneous population consisting of hepatoblasts (day 12), hepatocytes, (day 20) and mature

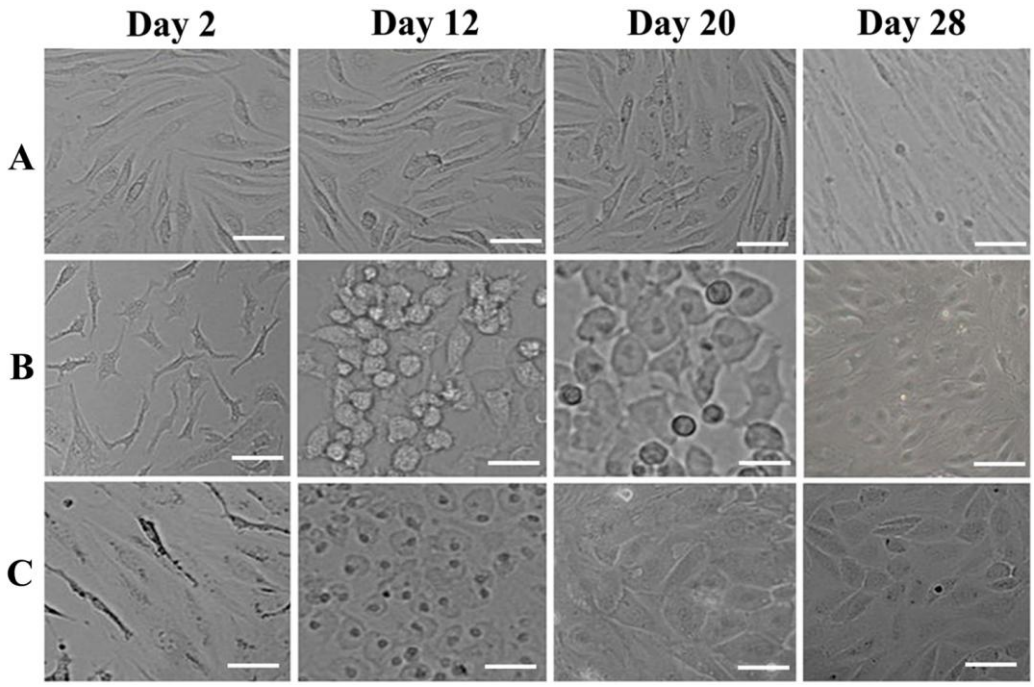

Fig. 2 — Phase contrast micrographs showing *in vitro* morphological changes observed during hepatic trans-differentiation of BM-MSCs with and without foxa-2 transfection for different days (2, 12, 20 and 28 days). (A) Control BM-MSCs as a negative control shows the normal morphology; (B) MSCs derived hepatocytes generated by hepatogenic induction without Foxa-2 transfection (D-MSCs) (group II)) with different days; and (C) Foxa-2 transfected MSCs induced functional hepatocytes generated by hepatogenic induction for different days. [Scale bars, 100 µm. (Original magnification, X100)]

hepatocyte-like cells [day 28, Fig 2B (i-iv)]. When compared, these cells with Foxa-2-MSCs showed morphological changes that highly resemble heterogeneous population consisting of hepatoblasts (day 12) and mature hepatocyte-like cells (day 20, 28) were observed [Fig 2C (i-iv)]. This enhanced effect of hepatocyte differentiation might be due Foxa-2 over expression which selectively promotes induction of functional hepatic differentiation and represses biliary differentiation.

Induction of functional hepatocyte markers expression by Foxa-2

To confirm induction of functional hepatocyte differentiation, we evaluated the functional efficacy of hepatocyte via immunocytochemical staining for liver markers that includes; Alb, CK18, CK19 and Foxa-2 (Fig. 3A). Our analyses for albumin secretion indicated rather than D-MSCs, the Foxa-2-MSCs had higher efficiency to secrete albumin at day 28 while other markers were stained diffusely in cytoplasm; CK19 was barely detectable (Fig. 3A), but the expression of CK18 was increased in Foxa-2-MSCs than D-MSCs. Foxa-2 expression was observed more in Foxa-2-MSCs and localized in the nucleus (Fig. 3A). However, hepatic marker expression in D-MSCs was negligible. BM-MSCs cells were negative for the hepatocyte marker profiles.

Liver Function Activity of Foxa-2-MSC Induced Functional Hepatocytes

Albumin secretion was negligible at day 6 and 12, which continuously increased significantly (P <0.05) and reached its maximal values at day 28 in Foxa-2-MSCs induction group compared to D-MSCs induction group and control BM-MSCs (Fig. 3B). Urea production also reveals that Foxa-2-MSCs induced hepatocytes had higher levels on day 28 compared to D-MSCs. In comparison with Foxa-2-MSCs, urea production was significantly decreased (P <0.05) in D-MSCs. Urea production in BM-MSCs was absent (Fig.3C). Urea secretion and albumin production were normalized for 10^5 cells per well in all groups (Fig. 3 C and B). We measured the ability of stored glycogen as the functional activity of differentiated hepatocytes, a specific activity of hepatic cells by PAS staining. Microscopic analysis showed that the Foxa-2 induced functional hepatic cells revealed profound levels of glycogen storage (pink) in comparison with D-MSCs and negative controls (BM-MSCs) (Fig. 4). The PAS staining was distributed evenly over the cells which indicated that

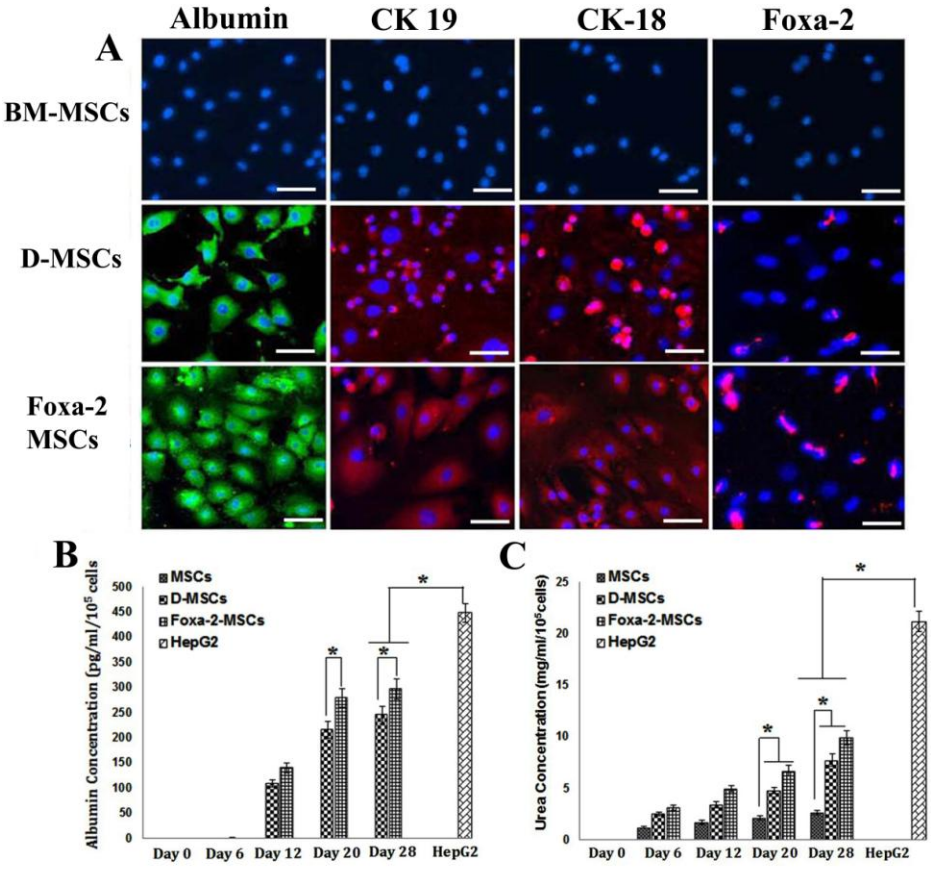

Fig. 3 — Immunofluorescence analysis, albumin and urea secretion of specific hepatic markers. (A) Immunofluorescence micrographs (merged) showing positive expression of hepatocyte specific markers, albumin (Alexafluor 488: green colour), CK-18 (Alexa Fluor 594: red colour), CK-19 (Alexa Fluor 594: red colour) and Foxa2 (Alexa Fluor 594: red colour) in BM-MSCs derived hepatocyte-like colonies with and without Foxa-2 transfection after 28 days of differentiation and negative expression in undifferentiated BM-MSCs. [Nuclei were stained with DAPI (blue colour). (Original magnifications × 100) (Scale bars =100 μM)]; (B) Secretion of albumin in a time-dependent manner. [* denotes the significant increase (*P <0.05) in albumin secretion in Foxa-2 transfected MSCs compared to D-MSCs (group II) cells. # denotes the significant increase (*P <0.05) in albumin secretion in group II and group III cells compared to normal MSCs]; and (C) Secretion of urea in a time-dependent manner. [*denotes the significant increase (*P <0.05) in urea secretion in Foxa-2-MSCs transfected cells compared to D-MSCs treated cells. # denotes the significant increase (*P <0.05) in urea secretion in both groups compared to normal BM-MSCs untreated]

stored glycogen was measured only in the cytoplasm. This finding suggested that the stored glycogen detected in the Foxa-2-MSCs were highly positive for glycogen than in D-MSCs. The cytoplasmic glycogen storage, cuboidal morphology, prominent nucleoli, and nuclear-to-cytoplasmic ratio highly specific to functional hepatocytes were acquired more prominently in Foxa-2 overexpressed group (Foxa-2-MSCs) group III compared with other groups. Similarly, the microscopic observation of induced functional hepatocytes from Foxa-2-MSCs showed higher levels of ICG uptake and rapid release of ICG when compared to D-MSCs cultures, while control BM-MSCs cultures completely excluded the anionic solution (Fig. 4 ICG uptake and release).

RT-qPCR and RT-PCR analysis of functional hepatic markers expression by Foxa-2

Relative quantification of functional hepatic specific marker genes (Albumin, CK8, CK18, CK19, p450 (Cyp)3a11, Transthyretin, AAT, TAT, AFP, PXMP1-L, PEPCK, and γ-GT) by qRT-PCR revealed their upregulation in Foxa-2-MSCs induced hepatocytes group as well as D-MSCs group (Fig 5 A-F), with no expression observed in untreated BM-MSCs group. Compared to D-MSCs, the Foxa-2-MSCs exhibited considerably enhanced expression of Foxa 1 and HNF1α (Fig. 5D), integral members of the combinatorial hepatic regulatory network that regulates hepatic transcription as well as upregulated expression of HNF-4α and HNF-6 (Fig. 5D). There

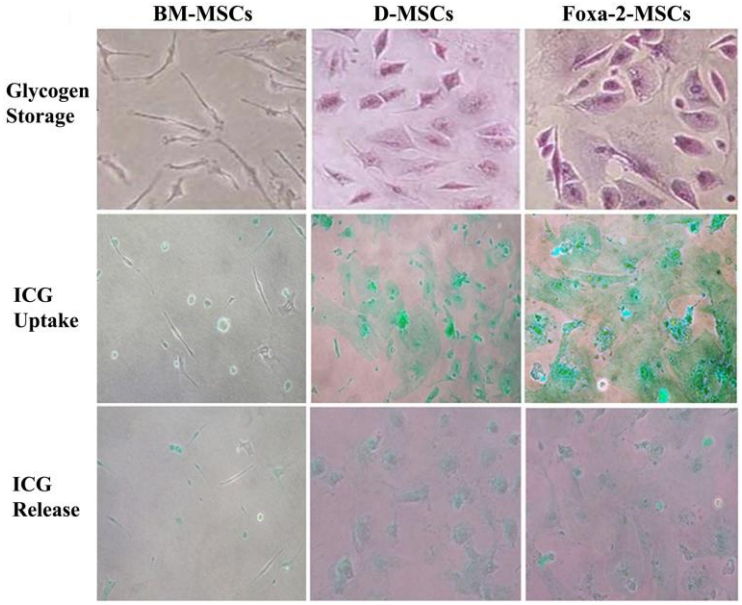

Fig. 4 — Light micrographs showing glycogen storage and ICG uptake and release during hepatic trans-differentiation of MSCs with and without foxa-2 transfection. (A) PAS staining for glycogen storage showed differentiated and undifferentiated cells; and (B) Uptake and release of ICG depicts the function of differentiated hepatocytes. [ICG = indocyanine green. Scale bars, 100 µm. (Original magnification, X100)]

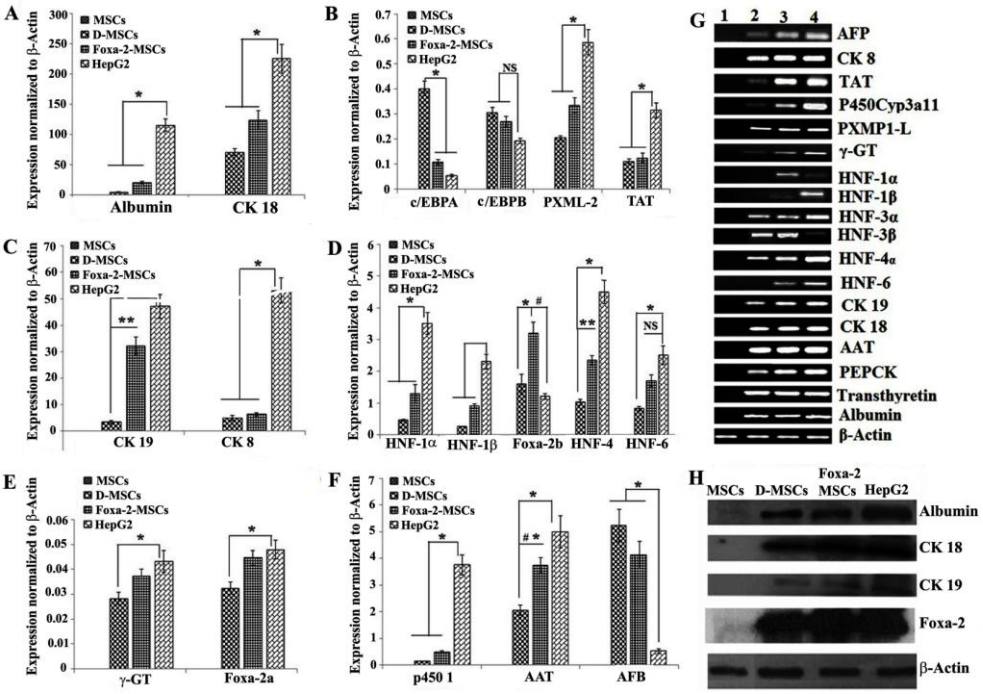

Fig. 5 — Quantitative real time PCR, RT-PCR and Western Blot expression of hepatocyte specific genes. (A-F) shows qRT-PCR of hepatocyte specific genes. [Positive expression of ALB, AFP, CK19, CK18, CK 8, HNF-1α, HNF-1β, Foxa-2a,, HNF-4α, HNF-6, p450 1, Foxa -2b, γ-GT and AAT were observed in both D-MSCs and Foxa-2-MSCs induction group, compared to untreated BM-MSCs. There was a significant difference in expression between D-MSCs and Foxa-2-MSCs induction group (***P* <0.05), but it was significantly lesser compared to HepG2 (**P* <0.05). Negative expression of AFP, c/EBPA and c/EBPB were observed in both (D-MSCs and Foxa-2-MSCs), but it was significantly higher compared to HepG2 (**P* <0.05). $^{\#}P$ <0.05 denotes the significant difference between Foxa-2-MSCs and HepG2. The expressions of genes were normalized to β-actin and mRNA of untreated BM-MSCs was used as calibrator]; (G) Gel picture showing of hepatic specific genes which is compared with HepG2 as positive control. [Lane-1: Normal BM-MSCs (Control), Lane-2: D-MSCs, Lane-3: Foxa-2-MSCs and Lane-4: HepG2]; and (H) Expression of hepatocyte-specific proteins such as Albumin, CK18, CK19, HNF-3β with β-actin (positive control) by Western Blot analysis. [Normal BM-MSCs, D-MSCs induced hepatocytes, Foxa-2-MSCs induced functional hepatocytes and HepG2]

was a significant difference in expression between the two D-MSCs and Foxa-2-MSCs induction group (**P <0.05), but it was significantly lesser compared to HepG2 (*P <0.05). Negative expression of AFP, c/EBPA and c/EBPB were observed in both (D-MSCs and Foxa-2-MSCs), but it was significantly higher compared to HepG2 (*P <0.05). $^{\#}P$ <0.05 denotes the significant difference between Foxa-2-MSCs and HepG2. The expression of genes was normalized to β-actin and mRNA of untreated BM-MSCs was used as calibrator.

Induced functional hepatocyte mRNA expression of Albumin, CK8, CK18, CK19, p450 (Cyp)3a11, Transthyretin, AAT, TAT, AFP, PXMP1-L, PEPCK, and γ-GT were augmented in Foxa-2-MSCs when compared with D-MSCs which reflects the progression of mature functional hepatocytes. Hepatic transcription factor mRNA expression (HNF-1α, HNF-1β, HNF-3α, HNF-3β, HNF-4α, HNF-6) were increased in Foxa-2-MSCs induced hepatocytes when compared with D-MSCs hepatocyte which efficiently promotes hepatic maturation.

Additionally, functional hepatocyte specific markers includes albumin, CK18, CK19 and Foxa-2 level was found to be upregulated in Foxa-2-MSCs induced hepatocytes when compared to D-MSCs hepatocyte in immunoblot assay with HepG2 cell line as positive control (Fig. 5H).

In recent research study using rat models of transplantation of primary hepatocyte revealed efficacious donor chimerism that rescued animals from severe hepatic failure[23]. Thus, hepatocyte transplantation has gained major attention in liver regeneration therapies and also emerged as a potential remedy to solve the problem of treating end stage liver diseases. We endeavored to analyze the role of liver specific transcription factor Foxa-2 in inducing or enhancing hepatogenesis in BM-MSCs. Many reports were available regarding the multipotential differentiation ability of BM-MSCs, hence they are considered as the better source for transplantation without the risk of immune rejection[24,25]. Currently, the protocol uses various growth factors and cytokines for inducing hepatogenesis in MSCs. However, the efficacy of MSCs differentiating into hepatocytes needs improvement to derive the functional cell type of clinical importance. It has been reported that epidermal growth factor (EGF) induced hepatogenesis by triggering proliferation in hepatocytes which resulted in re-establishing the liver homeostasis following liver injury[26]. Early investigation demonstrated that bFGFs play a key function in endoderm development of liver in embryogenesis which is secreted by cardiac mesoderm. It also demonstrated that the Foxa-2 expression is a major key factor for the development of a definitive endoderm to respond to bFGF signals for hepatogenesis[22]. HGF possesses multipotential functions reported as enhancing the hepatocyte proliferation and morphogenesis. Oncostastin M (OSM) is a cytokine for promoting hepatic maturation. Finally OSM and HGF are involved in later stages of hepatogenesis along with insulin and dexamethasone at the latter stage of *in vitro* differentiation[27]. However, still optimizing the culture conditions for deriving functional hepatocyte from the adult source is still not fully established. For achieving success in optimizing the cultural protocol for deriving functional hepatocytes, it is necessary to understand the specific signaling biology in hepatogenesis.

Earlier studies suggested that, the establishment of defined foregut endoderm and induction of functional hepatic gene expression were the two important events during embryonic hepatogenesis. Foxa-2 and Gata-4 bind with hepatic enhancers and alter them as possessing effective potential to be expressed following induction. These transcription factors have a capacity to identify their specific binding sites in the chromatin[27,28]. Thus, among the hepatic transcription factors presented in the definitive endoderm, Foxa-2 and Gata 4 act as mediators of competence in the foregut endoderm [14,15], and both of these factors are important for the induction and maintenance of hepatic gene expression[29]. Henceforth, in all the earlier reports of developmental embryogenesis it was clearly proposed that Foxa-2 plays an important function in inducing hepatogenesis and maintaining the expression of functional hepatocyte genes to carry over its function. Current study focused to optimize the method of deriving functional hepatocytes via overexpressing the liver enriched transcription factor Foxa-2 (HNF3-β). This study utilizes various liver specific metabolic function activities to critically evaluate the degree of differentiation.

In our study, we overexpressed Foxa-2 followed by culturing in growth factor defined media for four weeks and the results were compared with D-MSCs.

It was observed the cellular phenotype derived from Foxa-2-MSCs induced hepatocytes were homogenously polygonal in shape when compared with D-MSCs. However, the efficacy of hepatogenesis was further assessed by screening for the stable expression of liver specific genes. Hepatic and endodermal specific markers includes HNF-3β, HNF-4α, HNF-1α, HNF-1β, HNF-3α, HNF-6, cytokeratins (CK-8, CK-18, and CK-19), transthyretin (TTR), α-feto protein (AFP), α-1-antitrypsin (AAT), albumin, tyrosine aminotransferase (TAT), PXMP1-L, phosphorenol pyruvate carboxy kinase (PEPCK) and cytochrome P450 subunit 3a11 (Cyp3a11) have been determined by semi-quantitative RT-PCR analysis in D-MSCs and Foxa-2-MSCs (Fig. 5A-G). qRT PCR data clearly validated the expression profiles of liver specific genes between two groups. As expected there was two to five fold increase in the expression of genes such as PEPCK, Cyp P450 3a11, PXMP-1L, Foxa-2, Albumin, alpha fetoprotein, TAT, CK-19, CK-8, γGT, Fox2b respectively. Very little fold change of 0.5 was observed in the case of α-1 antitrypsin, CK 18, HNF-1α, HNF4 and HNF6. These HNFs transcription factors were considered as major transcription factors in regulating hepatogenesis and in our study, we showed significant changes in Foxa-2-MSCs when compared with D-MSCs differentiation[30]. The functional expression of CK19 and CK8 was significantly enhanced in Foxa-2-MSCs induced group compared with the D-MSCs group. CK8, CK18, and CK19 are specific cyto-skeletal proteins of hepatocytes[31,32] which maintain the morphology of hepatocytes while being expressed in fetal liver differentiation. AAT and TTR representing endodermal differentiation have been reported to be expressed in functional hepatocytes[33].

Our study of over expressing Foxa-2 has significantly increased the expression of AFP which is a known endodermal early fetal hepatic differentiation marker and ALB is a functional hepatocyte marker most abundantly synthesized by mature hepatocytes[34,35]. TAT and PEPCK were considered as hepatocyte specific enzyme marker for pre-natal or post-natal functional hepatocyte differentiations and represent a major function of the liver[36-38]. Detectable urea secretions were found throughout the 28 day culture period and were higher in Foxa-2-MSCs induced group. Functionally, the Foxa-2-MSCs induced group was found to store glycogen mimicking the *in vivo* characteristics. ICG uptake and release assay suggested that Foxa-2-MSCs induced groups were able to retain more concentration of ICG followed by its release after culturing in the normal media conditions. Cytochrome P450 (Cyp) 3a11 protein encoding CYP3A11 gene expressed in the liver which is the main detoxifying protein reported to be expressed in embryoid bodies *in vitro* differentiation of ESCs into hepatocytes[39,40]. In our study, considerable expression of Cytochrome P450 3a11 were found in significant levels as compared to D-MSCs.

Conclusion

It has been correlated widely that Foxa proteins play a significant role in *in vivo* hepatogenesis. By over-expressing lineage specific transcription factors in non-hepatic source we could achieve functional hepatocytes and this study endeavoured to interpret the role of Foxa-2 in *in vitro* hepatogenesis. Findings of this study suggest that BM-MSCs can trans-differentiate towards functional hepatocytes *in vitro* using overexpression of foxa-2. We found Foxa-2 over-expression system induces better hepatocyte differentiation resulted in the significant increase in expression levels of functional hepatocyte genes and proteins to carry over liver specific functions like production of albumin, urea secretion and storage of glycogen. These foxa-2-MSCs derived hepatogenesis, might be clinically relevant for autologous cellular therapy and also for *in vitro* preclinical toxicity studies.

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Conflict of interest

Authors declare no competing interests.

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