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HEPATOLOGY

Biochemical and molecular characterization of hepatocyte-like cells derived from human bone marrow mesenchymal stem cells on a novel three-dimensional biocompatible nanofibrous scaffold

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Abstract

Background: There is significant interest in using nanofibers in tissue engineering from stem cells. The transdifferentiation of mesenchymal stem cells into the hepatic lineage in a nanofibrous structure has not been reported. In this study, a three dimensional nanofibrous scaffold is introduced for differentiation of human bone marrow derived mesenchymal stem cells (hBMSCs) into hepatocytes.

Methods: A scaffold composed of Poly (ε-caprolactone), collagen and polyethersulfone was fabricated by the electrospinning technique. After characterization of isolated hBMSCs, the performance of the cells on the scaffold was evaluated by Scanning Electron Microscopy (SEM) and MTT assay. Cytological, molecular and biochemical markers were measured to confirm differentiation potential of hBMSCs into hepatocytes.

Results: The isolated cells possessed the basic properties of mesenchymal stem cells (MSCs). Based on scanning electron microscope (SEM) analysis and MTT assay, it was shown that the cells adhere, penetrate and proliferate on the nanofibers. Cultured cells on the nanofibers differentiated into hepatocyte-like cells and expressed hepatocyte specific markers such as albumin, α-fetoprotein, cytokeratin-18, cytokeratin-19 and cytochrome P450 3A4 at mRNA levels. Appearance of a considerable number of albumin-positive cells cultivated on the scaffold ($47 \pm 4\%$) as compared to the two-dimensional culture system ($28 \pm 6\%$) indicates the supporting role of the scaffold. The efficiency of the cells to produce albumin, urea, transferrin, serum glutamic pyruvic transaminase and serum oxaloacetate aminotransferase in hepatocytes on the scaffold further attest to the functionality of the cells.

Conclusion: The data presented in this study show that the engineered nanofibrous scaffold is a conductive matrix which supports and enhances MSC development into functional hepatocyte-like cells.

Introduction

Tissue engineering proves to be a temporary treatment for patients suffering from hepatic failure.[1,2] For successful tissue regeneration, the cells constituting the tissues to be regenerated are necessary. Considering the proliferation activity and differentiation potential of cells, stem cells are practically promising. Human bone marrow derived mesenchymal stem cells (hBMSCs) have great potential for liver tissue engineering because autologous BMSCs can be harvested, expanded extensively *ex vivo*, and differentiated into a hepatic phenotype for transplantation back into the patient.[3] Differentiation of hBMSCs into hepatocyte-like cells in standard monolayer or two-dimensional (2D) cultures is now well established[4–6] but the challenge remains to develop robust protocols to generate functional hepatocytes from hBMSCs suitable for transplantation.

A complementary key ingredient in regenerative medicine and tissue engineering is the use of biologically compatible scaffolds that can be readily adopted by the body system without harm. So far, natural matrices have been used in liver tissue engineering from stem cells.[7–10] These natural polymers are suitable for cell interaction, however, scaffolds fabricated purely from these

molecules exhibit poor mechanical strength and are not easy to handle. Large batch-to-batch variations upon isolation from biological tissues, as well as restricted versatility in designing devices with specific biomechanical properties are other limitations assigned to the natural scaffolds.[11]

Advances in polymer chemistry have facilitated the engineering of synthetic matrices that can be precisely manipulated with regard to physical and mechanical characteristics. The scaffolds should mimic the structure and biological function of the native extracellular matrix (ECM). A well known feature of native ECM structures is the nanoscaled dimensions of their physical structure.[11,12] In recent years, with respect to nanofibers for tissue engineering purposes, a wide variety of nanofibrous scaffolds have been produced.[13–17] Design of nanofibers is an important concern in the effective applications of these nanostructured materials. For example, nanofibers containing drugs have been used for controlled release drug administration.[18–20]

Different techniques have been used for the formation of nanofibrous materials.[21] There is increasing interest towards employing electrospinning for scaffold fabrication because the mechanical, biological, and kinetic properties of the scaffold are easily manipulated by altering the polymer solution composition and processing parameters. It has been shown that electrospun three-dimensional (3D) nanofibrous structures share morphological similarities to ECM, and are capable of promoting favorable biological responses from seeded cells.[22,23]

Poly (ε-caprolactone) (PCL), an aliphatic polyester which is bioresorbable and biocompatible, is generally used in pharmaceutical products.[24] The experimental results have shown that, although synthetic biodegradable PCL supports cell growth, to proliferate more and encourage cell ingrowth for better integration between cells and the scaffold, the biologically inert PCL nanofibers need effective hybridization with bioactive molecules.[25] It has been reported that electrospinning of PCL with collagen gives encouraging results in improving the cell-scaffold interactions.[25,26] Besides, polyethersulfone (PES) has many fascinating properties including favorable mechanical strength, thermal and chemical resistance, and excellent biocompatibility.[27,28] Therefore, the polymer blend of PCL/collagen/PES can overcome the shortcomings of natural and synthetic polymers, resulting in a new biomaterial with good biocompatibility and improved mechanical, physical, and chemical properties.

In this study, the potential of hBMSCs to differentiate into functional hepatocytes within designed PCL/collagen/PES nanofiber has been investigated. Cytochemical, biochemical and molecular features of hepatocyte-like cells differentiated from hBMSCs on the scaffold were used to show the role of nanofibrous structure to support differentiation.

Materials and methods

Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), glutamine, antibiotics and trypsin-EDTA (ethylenediaminetetraacetic acid) solution were obtained from Gibco BioCult (Paisley, UK). Albumin and transferrin ELISA Quantitation kits were purchased from Bethyl Laboratories (Montgomery, TX, USA) and Cygnus Technologies (Southport, NC, USA). Quantitative kits for urea, serum glutamic pyruvic transaminase (SGPT) and serum oxaloacetate aminotransferase (SGOT) were obtained from Olympus (Dublin, Ireland) and Hemagen Diagnostics Inc (San Diego, CA, USA), respectively. Antibodies for flow cytometric assay were obtained from Dako (Glostrup, Denmark) and Oxford Biomedical Research (Oxford, UK). RNA extraction kit, cDNA Synthesis Kit and materials for PCR amplification were purchased from Qiagen (Valencia, CA, USA) and Fermentas (Hanover, MD, USA). Chambered coverglass was obtained from Nalge Nunc International (Rochester, NY, USA). Hepatocyte growth factor (HGF), dexamethasone (DEX), oncostatin M (OSM), PES, mouse anti human antibodies for albumin, and α-fetoprotein, rabbit anti mouse FITC-conjugated immunoglobulin G (IgG), type I collagen, PCL, alizarin red staining kit, oil red O-staining kit, Cell growth determination kit (MTT kit) and other reagents were from Sigma Chemical Co (St Louis, MO, USA). Human hepG2 hepatoma cells were obtained from Pasteur Institute of Iran.

Isolation and culture of human BMSCs

Bone marrow aspirates (10 mL) were obtained from iliac crests of human donors ranging in age from 19 to 32 years at the Bone Marrow Transplantation Center, Shariati Hospital, Tehran, Iran. Bone marrow was obtained after informed consent according to guidelines of the Medical Ethics Committee, Ministry of Health I. R. Iran. Based on the previous reports,[29,30] mesenchymal stem cells were isolated from bone marrow as described below. The aspirates were diluted with phosphate buffer saline (PBS). Cell solution was gently overlaid on the Ficoll-Hypaque (D = 1.077 g/mL) to eliminate unwanted cell types that were present in the marrow aspirate. Mononuclear cells were recovered from the gradient interface and washed with PBS after centrifugation 400 *g* for 30 min at room temperature (RT).

The isolated mononuclear cell layer were then washed in PBS, resuspended in growth medium containing DMEM-low glucose supplemented with 15% FBS, 2 mM glutamine, 100 μg/mL streptomycin, 100 U/mL penicillin and plated in polystyrene plastic 75-cm^2 tissue-culture flasks. The cell cultures were maintained at 37°C in a humidified 5% CO_2 incubator. Following 3 or 4 days incubation, the non-adherent cells were washed away leaving behind the adherent cell population that was growing as fibroblastic cells in clusters. Hematopoietic stem cells and non-adherent cells were removed with every 3–4 days changes in medium. When cells reached 70–90% confluence, cultures were harvested with 0.25% trypsin-EDTA solution and plated in 25-cm^2 plastic cell culture flasks at the density of 10^4 cells/cm^2.

Immunophenotyping of human BMSCs by flow cytometry

hBMSCs were detached from the tissue culture flasks after 14–33 days *in vitro* with trypsin/EDTA and counted. About 2×10^5 cells were divided into aliquots and centrifuged at 1000 rpm for 5 min at RT. The pellet was resuspended in human serum and incubated for 30 min on ice. After recentrifugation at 1000 rpm for 5 min, the pellet was suspended in 3% human serum albumin (HSA)/PBS and incubated with appropriate antibodies including Fluorescent isothiocyanate (FITC)-conjugated mouse anti human CD44

(H-CAM), CD13, CD105 (Endoglin or SH2), CD34 and Phycoerythrin (PE)-conjugated CD166 (ALCAM), CD45 (leukocyte common antigen) for 1 h on ice, washed twice in PBS, and centrifuged for 5 min. The cells were resuspended in 100 μL of PBS and studied by a Coulter Epics-XL flow cytometer (Beckman Coulter, Fullerton, CA, USA). An isotype control with FITC or PE-labeled was included in each experiment, and specific staining was measured from the cross point of the isotype with the specific antibody graph. Creation of histograms was performed with Win MDI 2.8 software (Scripps Institute, La Jolla, CA, USA).

Fabrication and preparation of PCL/collagen/PES nanofiber scaffold

PCL was dissolved in chloroform to obtain a 10 wt% solution and type I collagen dissolved in hexafluoroisopropanol (HFIP) at a concentration of 8%. PES was dissolved in an organic solvent mixture (90:10) of N, N, dimethylformamide and tetrahydrofuran at a final concentration of 15 wt%. The electrospinning setup utilized in this study consisted of three syringes, a ground electrode (stainless steel drum, 3 and 5 mm outer diameter 10 cm length), and a high voltage supply. The distance between the tips and rotating drum was in the range of 10–25 cm and the positive voltage applied to the polymer solutions was in the range of 15–30 kV. From the bulk material of electrospun nanofiber mats, small discs with areas of approximately 0.8 cm^2 were cut out and placed in 24-well plastic cell culture plates. All scaffolds were soaked in 70% filtrated ethanol for 2 h, and then were incubated in DMEM at 37°C in a humidified 5% CO_2 incubator overnight prior to cell seeding to facilitate cell attachment onto the nanofibers.

Growth curve

The hBMSCs (1×10^4 third passage cells/cm^2) were transferred directly onto the scaffolds in 24- well culture plates and incubated for 1 hour. Thereafter, the scaffolds were soaked in DMEM-low glucose supplemented with 15% FBS. The cell proliferation was evaluated during 12 days via 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction based on cell growth determination protocol.[31] Briefly, culture medium was removed and replaced with 0.5 mL of medium without FBS; then 25 μL MTT stock solution (5 mg/mL) was added to each culture being assayed. After 3 h incubation, the medium was removed and the converted dye was solubilized with acidic anhydrous isopropanol (0.1 N HCl in absolute isopropanol). Absorbance of converted dye was measured at a wavelength of 570 nm with background subtraction at 670 nm. Data obtained as the mean ± standard error of mean (SEM) of values of three cell seeded scaffold.

Osteogenic and adipogenic differentiation of hBMSCs

The potential of the isolated cells to differentiate into osteogenic and adipogenic lineages was examined. For osteogenic differentiation, hBMSCs were induced for 2 weeks by α-MEM supplemented with 10% FBS, 0.1 μM dexamethasone, 10 μM β-glycerophosphate, and 50 μM ascorbate-phosphate.[32,33] The culture medium was changed two times per week for up to two weeks. The cells were fixed with methanol for 10 min at RT and identified by specific histochemical staining for calcium with alizarin red staining kit. The staining was examined with a phase contrast microscope (Nikon, Tokyo, Japan).

For adipogenesis, the cultured cells were incubated in adipogenic medium DMEM supplemented with 10% FBS, 1 μM dexamethasone, 200 μM indomethacin, 1.7 μM insulin, 500 μM isobutyl-methylxanthine, 0.05 U/mL penicillin, and 0.05 μg/mL streptomycin for 2 weeks. After 14 days, cultured cells were detected for adipocyte identification, using oil red O-staining. Briefly, cells were fixed in 10% solution of formaldehyde in aqueous phosphate buffer for 1h, washed with 60% isopropanol, and stained with oil red O-solution (in 60% isopropanol) for 10 min, followed by repeated washings with distilled water before being destained in 100% isopropanol for 15 min.[34,35] Control cultures without the differentiation stimuli were maintained in parallel to the differentiation experiments and stained in the same manner.

Hepatic differentiation protocol

Cultured hBMSCs at passage 3 (2×10^4 cells/cm^2) were seeded on control 24-well plastic cell culture plates (as 2D culture system) and the scaffold (3D). Hepatic differentiation was performed using a 2-step protocol employing HGF, DEX and OSM based on the previous reports.[5,7] Briefly, in the first step which lasted for seven days, the cells were cultured in a culture medium consisting of DMEM-low glucose supplemented with 15% FBS, 20 ng/mL HGF, and 10^{-7} mol/L DEX. At the second phase, OSM was added at a concentration of 10 ng/mL and continued until 21 days of differentiation. The culture media was changed twice weekly and hepatic differentiation was assessed by different experiments.

Scanning Electron Microscopy (SEM)

hBMSCs grown on scaffolds were washed with PBS to remove non-adherent cells and then fixed in 3% glutaraldehyde for 3 h at RT, dehydrated through a series of graded alcohol solutions. After drying, the scaffolds were mounted on aluminum stubs, sputter-coated with gold-palladium (AuPd) and viewed using SEM (Philips XL30, Amsterdam, the Netherlands).

Immunocytochemistry

After 3 weeks of differentiation, cultured cells on scaffold were harvested with 0.25% trypsin-EDTA solution and mounted on Chambered coverglass. After 24 h incubation at 37°C in a humidified 5% CO_2 incubator, attached cells on Chambered coverglass and 2D cultured cells were washed twice with PBS and fixed with 4% paraformaldehide for 30–45 min at room temperature, then permeabilized with 0.4% triton X-100 for 20 min. Washed cells were incubated overnight at 4°C with primary antibodies, including mouse anti-human albumin (1:1000), and mouse anti-human AFP (1:500). Subsequently, the cells were washed three times with PBS and incubated with fluorescence labeled second antibody, FITC-labeled goat anti-mouse IgG at 37°C for 3 h in a dark room. After washing with PBS, cells were incubated with DAPI (4′, 6-diamidino-2-phenylindole; 1:1000) for nuclear staining. The cells were visualized and photomicrographed using a fluorescence

Table 1 Primers and annealing temperatures used for Reverse transcription polymerase chain reaction (RT-PCR)

Gene	Sequence	Product size (bp)	Annealing temperature (°C)
Albumin	F 5′-TGC TTG AAT GTG CTG ATG ACA GGG-3′ R 5′-AAG GCA AGT CAG CAG GCA TCT CATC-3′	161	68
AFP	F 5′-TGC AGC CAA AGT GAA GAG GGA AGA-3′ R 5′-CAT AGA CGA GCA GCC CAA AGA AGAA-3′	216	69
CK-18	F 5′-CCC GCT ACG CCC TAC AGAT-3′ R 5′-ACC ACT TTG CCA TCC ACT ATCC-3′	271	56
CK-19	F 5′-TGC GTG ACA TGC GAA GCC AAT-3′ R 5′-ACC TCC CGG TTC AAT TCT TCA-3′	98	53
CYP3A4	F 5′-CCT TAC ATA TAC ACA CCC TTT GGA AGT-3′ R 5′-AGC TCA ATG CAT GTA CAG AAT CCC CGG TTA-3′	382	62
β-actin	F 5′-GTC CTC TCC CAA GTC CAC AC-3′ R 5′-GGG AGA CCA AAA GCC TTC AT-3′	198	52

AFP, alpha-fetoprotein; CK-18, cytokeratin-18; CK-19, cytokeratin 19; CYP3A4, cytochrome P450 3A4.

microscope (Nikon, TE-2000, Tokyo, Japan). The ratio of immunopositive cells to the total number of cell nuclei labeled with DAPI was recorded. Human hepG2 hepatoma cells cultured in 24-well culture plates were simultaneously stained for albumin and AFP considered as positive control.

Reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR was used to evaluate the expression of albumin, AFP, cytokeratin-19 (CK-19), cytokeratin-18 (CK-18), and cytochrome P4503A4 3A4 (CYP3A4) in differentiated cells on scaffold. Briefly, total RNA was isolated from cells using an RNA extraction kit. Prior to reverse transcription (RT) RNA samples were digested with DNase I to remove contaminating genomic DNA. Standard RT was performed using a Reverse RevertAid™ First Strand cDNA Synthesis Kit and 2 μg of total RNA, 0.5 μg oligo (dt_{18}) per reaction, according to the manufacture's instructions. PCRs were conducted using approximately 200 ng cDNA to amplify a number of hepatocyte marker genes listed in Table 1. After initial denaturation at 95°C for 1 min, PCR amplification was continued at 95°C for 40 s, annealing temperature for 40 s, and 72°C for 1 min for a total 30 cycles, and final extension at 72°C for 5 min. Amplified DNA fragments were electrophoresed on 2% agarose gel. The gels were stained with ethidium bromide (10 μg/mL) and photographed on a UV transilluminator (Uvidoc, Cambridge, UK).

Production of Albumin and Transferrin

Conditioned media obtained from differentiated cells (1×10^5 cells per scaffold or every well of 24-well plates) were collected on days 0, 7, 14, 21 and stored at −20°C until assay. The conditioned media were assayed for albumin production using a quantitative enzyme-linked immunosorbent assay kit (ELISA) according to manufacturer's recipes. Finally, albumin concentration was calculated from a standard curve prepared from different dilutions of albumin standard. Furthermore, the transferrin concentration was assayed in culture media collected directly from cultured cells on scaffold and 2D culture system on day 21 of differentiation using the ELISA kit.

Urea production

Differentiated cells on scaffold and 2D cultures were incubated with medium containing 5 mM NH_4Cl for 24 h in 5% CO_2 at 37°C on day 21 of differentiation. Following incubation, obtained supernatant from 1×10^5 cells per scaffold or well was collected and urea concentration was measured by a colorimetric assay kit. This assay is based on reduction of ammonia produced via urea hydrolysis. Undifferentiated hBMSCs were used as negative control.

Determination of SGPT and SGOT

Cultured cells on scaffolds and 2D culture system were harvested with 0.25% trypsin-EDTA solution on day 21 of differentiation. After centrifugation of cells at 1200 rpm for 5 min, the pellet was resuspended in 300 μM lysis buffer containing 50 mM Tris-HCl, 1% Triton X-100, 150 mM NaCl, 1 mM phenylmethylsulfonylfluoride (PMSF) (pH ~7.5) on ice. The cell lysate was then homogenized with a short sonication and centrifugation at 12 000 rpm for 10 min. SGOT and SGPT levels in supernatant media derived from 10^5 cells per scaffold or well were measured with quantitative kits. The methods were based on kinetics UV-test according to the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC).

Statistical analysis

Triplicate samples obtained from the differentiated and undifferentiated cells were analyzed in duplicate for determination of albumin, urea, SGPT, SGOT, and transferrin. The results were given as mean ± SEM. Statistical analysis was performed by using a Student's *t*-test with significance reported when $P < 0.05$.

Results

Characterization of isolated hBMSCs

hBMSCs were cultivated from the mononuclear cell fraction of bone marrow samples. To ensure removal of contamination, the cells were selected based on plastic adherence and passaged 3 times prior to experiments.

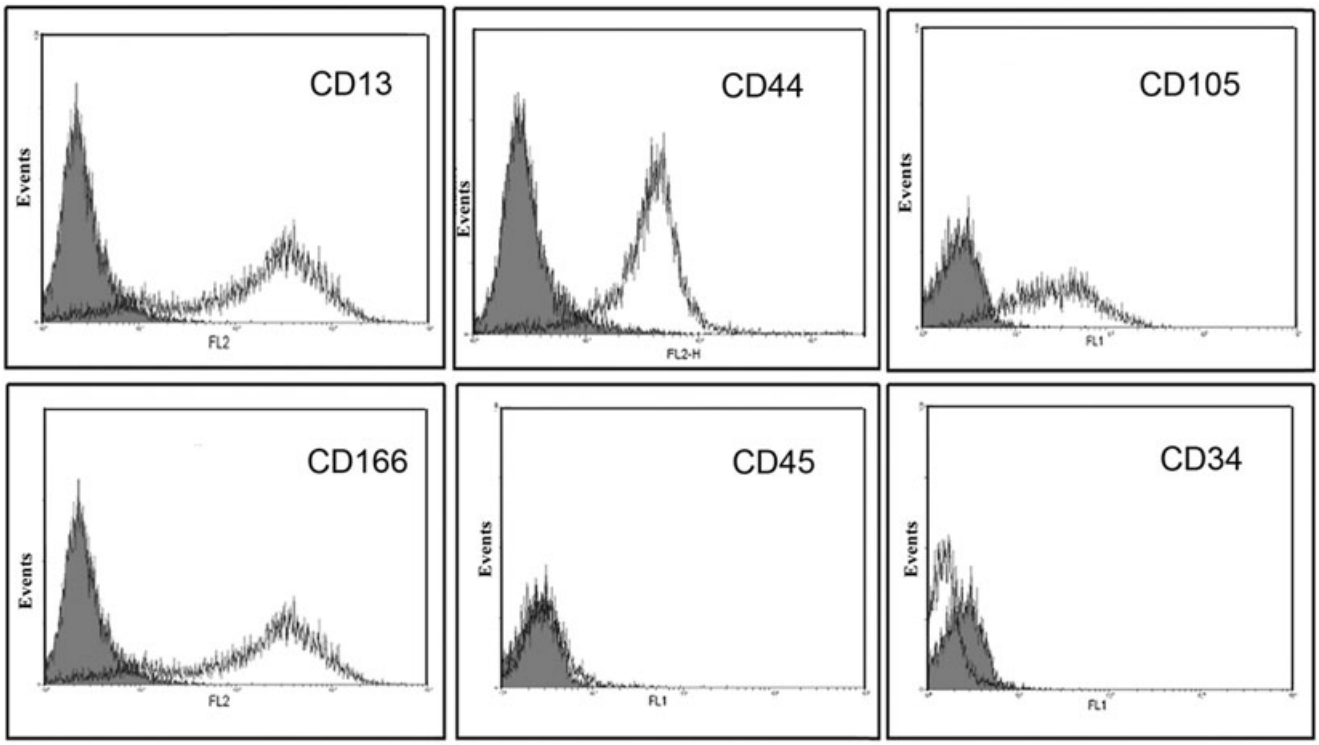

Figure 1 Immunophenotyping of human bone marrow derived mesenchymal stem cells (hBMSCs) using flow cytometry. Mesenchymal stem cells (MSCs) were positive for CD44 (H-CAM), CD166 (ALCAM), CD105 (Endoglin or SH2) and CD13. These cells were negative for CD34, and CD45. The shaded area shows the profile of the negative control. The results are representative of 3 independent experiments.

To ascertain that the culture-expanded cells were genuine MSCs, their immunophenotyping profile and mesodermal differentiation potential in mesenchymal-supportive conditions containing osteogenic and/or adipogenic-specific agents was examined. Flow cytometric analysis showed that the human BMSCs express CD13, CD44, CD105 and CD166, whereas they were negative for CD34 and CD45 (Fig. 1). Alizarin red staining confirmed the presence of calcium deposits, characteristic for osteogenic cells in differentiated osteoblasts. Likewise, lipid droplets in differentiated adipocytes were located by oil red O-staining, whereas undifferentiated hBMSCs were negative in both staining procedures (Fig. 2). These results indicate that the isolated cells have the basic properties of genuine MSCs.

Morphological observations

Figure 3A shows typical SEM micrographs of the hybrid matrix composed of PCL, collagen and PES nanofibers. In this nonwoven, randomly oriented nanofibrous scaffold, PES was distributed uniformly in the PCL nanofibrous structure. The region of distribution of PES and collagen were ranging from 100 to 200 nm. However, the diameter of pure PCL nanofibers was broadly distributed in the range of 200–1500 nm. Furthermore, the hybrid matrix produced fibers with approximately 500 μm thickness. Figures 3B and C show typical SEM micrographs of cells attached to scaffold prior to differentiation and on day 21 of differentiation. As shown in this figure, cells penetrated and adhered well on the surface of the nanofibrous hybrid scaffold. Proliferation profile of the hBMSCs during 12 days of *in vitro* culturing showed a progressive expansion of the attached cells on the scaffold (Fig. 4).

Immunocytochemical staining

Hepatic differentiation from hBMSCs, was confirmed by showing expression of albumin (the most abundant protein synthesized by functional hepatocytes), and AFP (a protein indicative of hepatocyte morphology) expression. Changes in albumin and AFP accumulation in cells cultured on scaffolds and 2D culture systems showed the presence and differentiation of hepatocyte-like cells from the stem cells (Figs 5 and 6). The results of albumin and AFP expression in differentiated cells were controllable to those of human hepG2 hepatoma cell line (positive control). Appearance of binuclear cells indicates possible hepatocyte maturation (Fig. 5A). The percentage of albumin, and AFP positive cells were 28 ± 6% and 15 ± 5% in 2D, 47 ± 4% and 26 ± 5% in 3D cultured cells, and 90 ± 6% and 88 ± 10% in hepG2 respectively. These markers did not express in MSCs as control group (Figs 5C and 6C) whereas nucleus staining with DAPI shows a strong signal in both differentiated and undifferentiated cells.

Hepatic gene expression

The expression of a panel of hepatocyte specific markers such as albumin, CK-19, AFP, CK-18 and CYP3A4 measured at mRNA

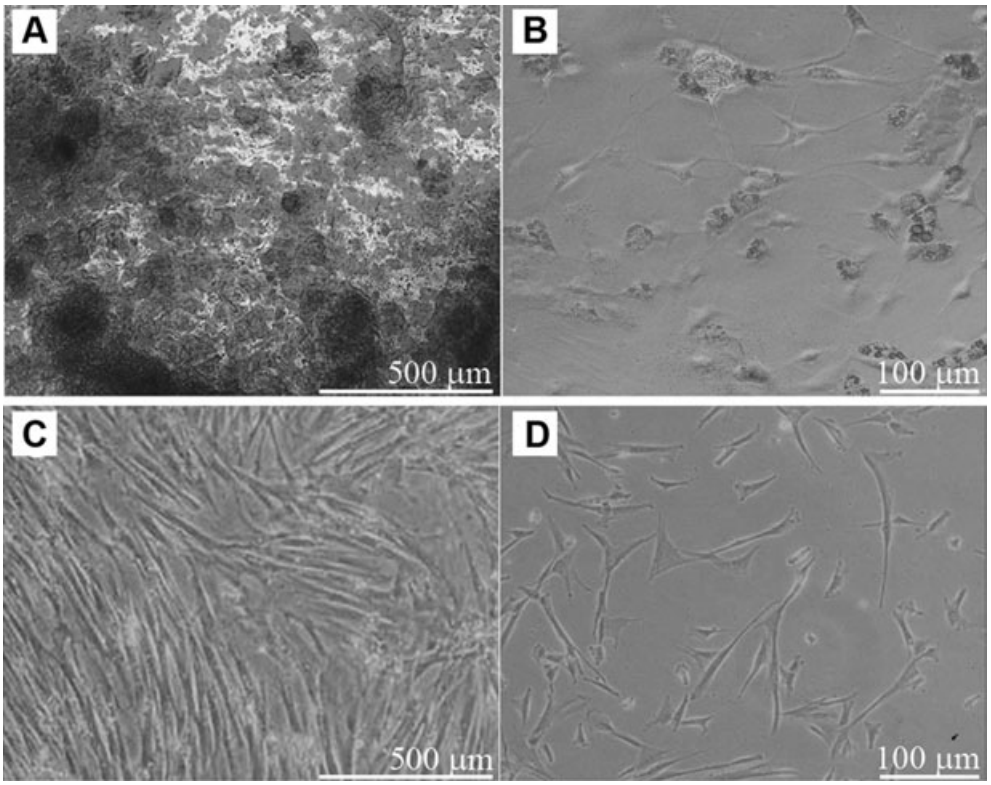

Figure 2 Transdifferentiation of human bone marrow derived mesenchymal stem cells (hBMSCs) into adipocytes and osteoblasts. Differentiated hBMSCs to osteogenic and adipogenic lineages were positive for Alizarin red staining (A) and oil red O-staining (B). Undifferentiated hBMSCs as control group were negative for both staining (C; alizarin red staining and D; oil red O-staining).

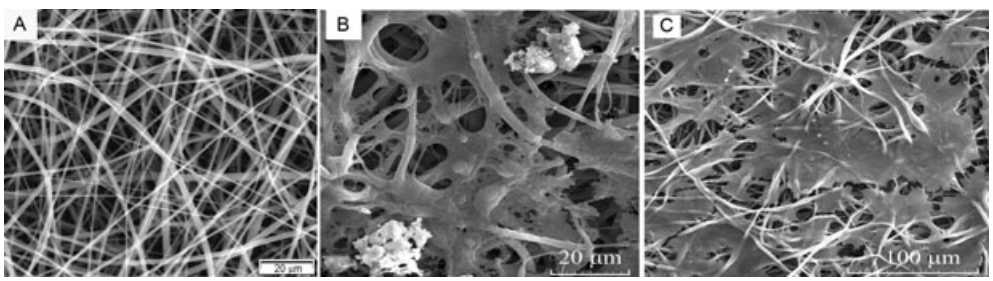

Figure 3 Scanning electron microscope (SEM) images of Poly (ε-caprolactone) (PCL)/collagen/polyethersulfone (PES) nanofibers before and after cell seeding. (A) Before cell seeding, (B) SEM micrographs of the interaction between human bone marrow derived mesenchymal stem cells (hBMSCs) and nanofiber scaffold before differentiation, (C) SEM image after differentiation of hBMSCs on the nanofibrous scaffold.

levels in cells on day 0, 10 and 21 of differentiation clearly shows the development of hBMSCs into hepatocyte-like cells on the scaffold. As shown in Fig. 7, the hBMSCs did not express mRNA of hepatocyte lineage genes, but, following treatment, the mRNA expression of the studied genes was detected in induced cells on day 10 of differentiation and increased on day 21 of differentiation. The expression of beta-actin, used as an internal control, was the same in undifferentiated and differentiated cells.

Functional assay

Typical functional hepatic features such as synthesis and/or secretion of albumin, SGPT, SGOT, transferrin, and urea were measured in hepatocyte-like cells derived from the stem cells.

Albumin synthesis in the cells was detected only in cells subjected to hepatic differentiation. Accordingly, albumin was secreted in the culture media and significantly increased on day 21 of differentiation. hBMSCs differentiated on scaffolds produced

significantly higher levels of albumin when compared to cells differentiated on 2D culture system on day 21 of differentiation ($P < 0.008$). On days 7 and 14 of differentiation, the differences in albumin levels between differentiated cells in 2D and 3D systems were negligible (Fig. 8). The levels of urea and transferrin excreted in culture media from differentiated cells on scaffold were approximately 2-fold greater than that in the 2D culture system on day 21 (urea: $P < 0.04$; transferrin: $P < 0.02$). Moreover, differentiated cells on the scaffold produced higher levels of SGPT as compared to cells differentiated in 2D culture ($P < 0.01$). The levels of urea, transferrin and SGPT in culture media or cell lysate derived from undifferentiated hBMSCs on both 3D and 2D culture systems were negligible. The difference in SGOT levels was not significant in cells differentiated on the scaffold compared to those in the 2D culture system (Table 2).

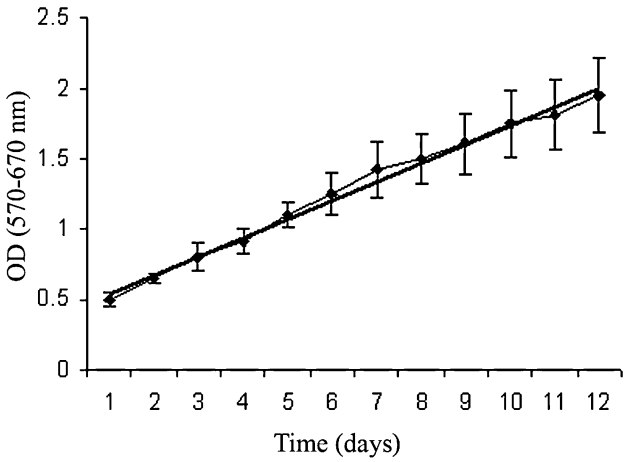

Figure 4 Cell proliferation of human bone marrow derived mesenchymal stem cells (hBMSCs) cultured on the scaffold. At time 0, 1×10^4 third passage cells/cm^2 were seeded on per scaffold; the proliferation of mesenchymal stem cells (MSCs) was examined during 12 days of cultivation by thiazolyl blue (MTT) staining. Absorbance of converted dye was measured at a wavelength of 570 nm with background subtraction at 670 nm. Data are shown as the mean ± SEM of values of three scaffolds seeded with cells.

Discussion

The hepatogenic differentiation of stem cells in natural matrix has been the subject of different reports.[7–10] However, the use of such natural scaffolds has been associated with some limitations. The problem with the control of pore size and porosity, large batch-to-batch variations upon isolation from biological tissues and poor biomechanical strength are the major concerns.

In recent years, different synthetic scaffolds have been fabricated and used for liver tissue engineering.[36–41] Although there is a significant interest in using nanofibers in tissue engineering from stem cells, reports on the transdifferentiation of stem cells into the hepatic lineage in a nanofibrous configuration is scanty. With respect to the applications of nanofibers for tissue engineering, our interest was to design an artificial matrix that can mimic ECM, to support the differentiation of hBMSCs into hepatocyte-like cells. Based on the experimental evidences, we demonstrated that the PCL/collagen/PES nanofibers not only allow the hBMSCs differentiated into hepatocyte but also enhance MSCs development into functional hepatocyte-like cells when compared to the conventional culture system.

As described in the methods section, following characterization of isolated MSCs derived from bone marrow, they were transferred to the ECM-like nanofibers. Expression of the adhesion molecules such as CD44, CD166, CD105, and CD13, the markers for MSCs[42] were used to characterize the cells. The purity of the isolated

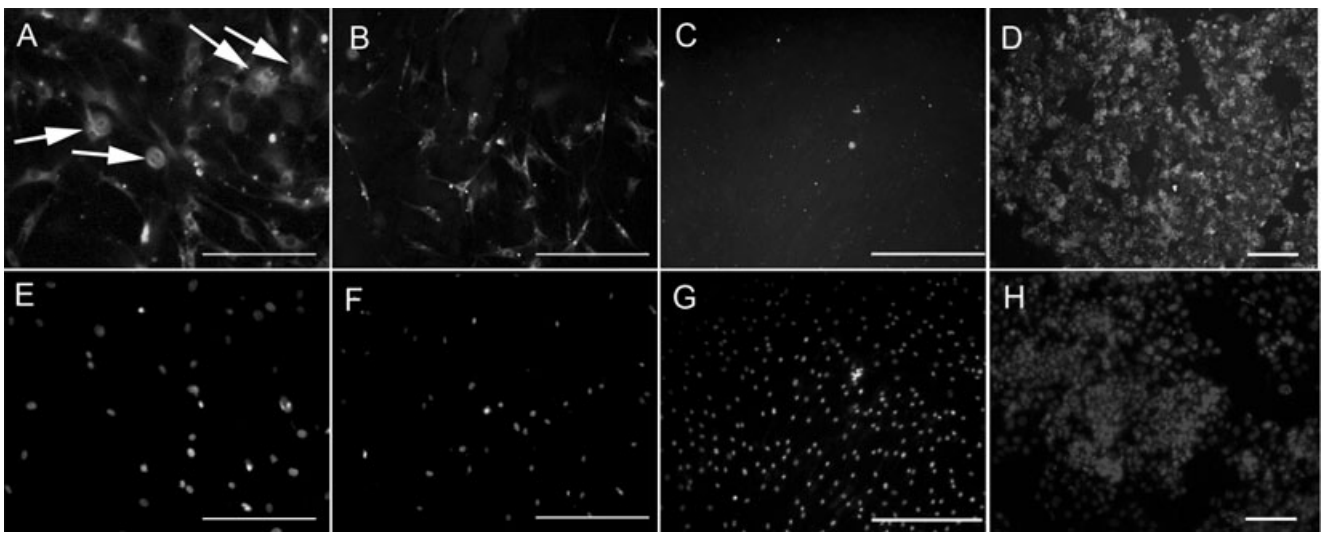

Figure 5 Immunofluorescent staining of albumin. Sections A and B show albumin staining in differentiated cells on scaffold and 2D culture system, respectively. Section C shows negative staining for undifferentiated cells. Section D (positive control) show albumin staining in hepG2 cells. Nuclei were stained with DAPI (4′, 6-diamidino-2-phenylindole; 1:1000) in the same cells (E–H). Some of positive cells were binuclear (arrowhead). Scale bar for figures A–C and E–G: 500 μm; all others: 100 μm.

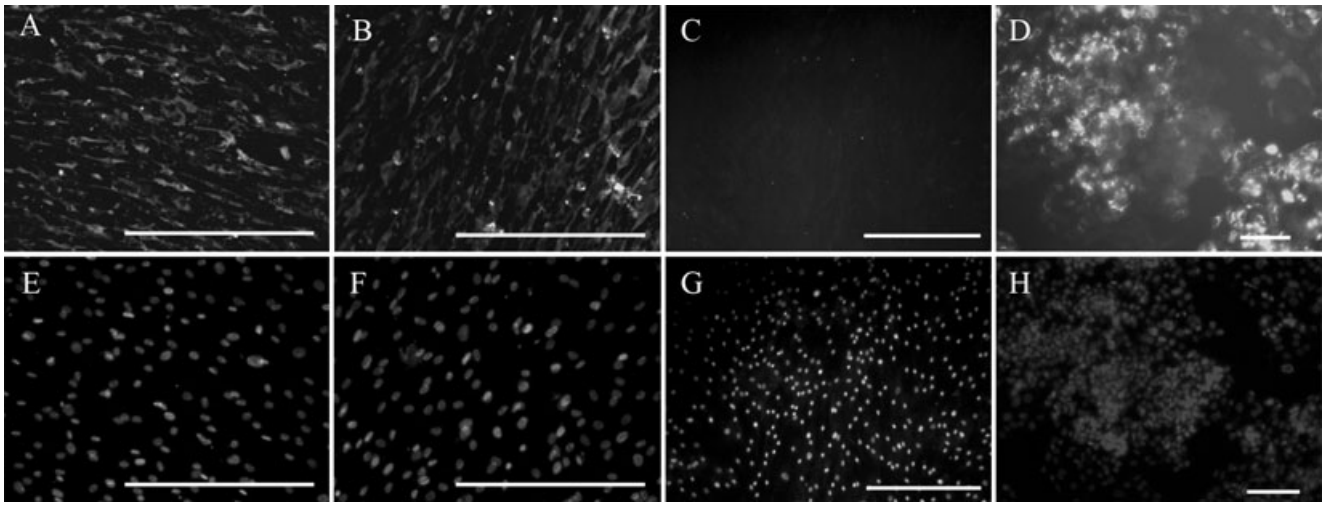

Figure 6 α-fetoprotein staining by immunocytochemistry. (A) Differentiated cells on scaffold, (B) 2D culture system, (C) human bone marrow derived mesenchymal stem cells (hBMSCs) and (D) hepG2. Sections E–H show Nucleus staining by DAPI (4′, 6-diamidino-2-phenylindole; 1:1000). Scale bar for figures A–C and E–G: 500 μm; all others: 100 μm.

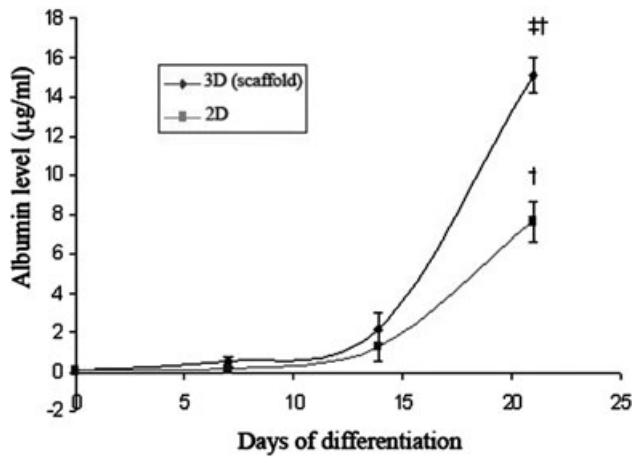

Figure 7 Albumin levels in differentiated cells on scaffold and 2D culture system by ELISA. Albumin levels are expressed as μg/mL on days 0 (before differentiation), 7, 14 and 21 of differentiation. $^{\dagger}P < 0.05$; significant differences between different time schedules of differentiation. $^{\ddagger}P < 0.05$; significant differences between 2D and 3D culture system.

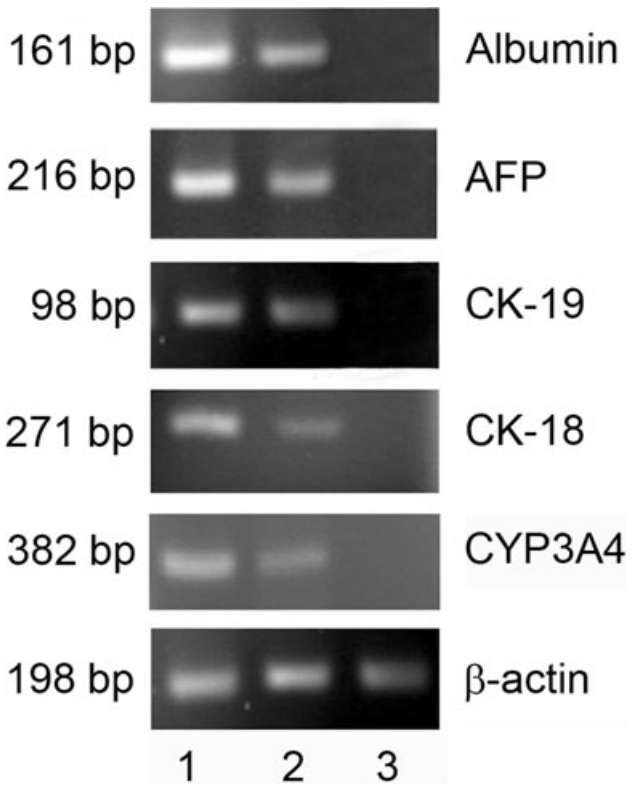

Figure 8 Expression of liver specific genes in human bone marrow derived mesenchymal stem cells (hBMSCs) derived cells on scaffolds. The mRNA expression of albumin, α-fetoprotein (AFP), cytokeratin-18 (CK-18), cytokeratin-19 (CK-19) and cytochrome P450 subunit 3A4 (CYP3A4) using reverse transcription polymerase chain reaction (RT-PCR) is shown on days 10 (lane 2) and 21 (lane 1). β-actin, a house keeping gene was used as internal control. The cells before differentiation were used as control (lane 3).

hBMSCs was confirmed by showing negative expression of certain markers known as hematopoietic lineage markers namely; CD34, and CD45 (Fig. 1). Transdifferentiation ability of isolated cells to osteogenic and adipogenic lineages was another evidence to approve the nature of MSCs (Fig. 2).

Previous studies showed that nanoscaled features of the engineered scaffold influenced cell behaviors.[43] For instance, it has been demonstrated that cell adhesion and proliferation increased with decreasing fiber diameter in the range of 10 μm to 500 nm.[44] The pore size of the hybrid polymer prepared by us was within 200–1500 nm range, which can create a nanofiber environment, allowing slow diffusion of small molecules, metabolites and

Table 2 Synthesis and production of urea, serum glutamic pyruvic transaminase (SGPT), serum oxaloacetate aminotransferase (SGOT) and transferrin

		Urea (mg/dL)	SGPT (IU/L)	SGOT (IU/L)	Transferrin (μg/mL)
Scaffold	Differentiated cells	29 ± 8*,**	9.1 ± 0.8*,**	8.3 ± 1.9*	6.5 ± 0.6*,**
	Undifferentiated cells	0.08 ± 0.05	0.04 ± 0.02	0.02 ± 0.02	0.003 ± 0.001
2D	Differentiated cells	14.2 ± 4*	4.2 ± 1*	6.9 ± 2*	2.2 ± 0.5*
	Undifferentiated cells	0.06 ± 0.04	0.03 ± 0.01	0.06 ± 0.04	0.004 ± 0.002

All the experiments were carried out in triplicate. Data are shown as the mean ± SEM of values of three separate samples. *$P < 0.05$; significant versus undifferentiated cells. **$P < 0.05$; significant against differentiated cells in 2D culture system.

macromolecules such as gases, nutrients, and growth factors (Fig. 3A).

The results of cell attachment and proliferation (Figs 3 and 4) revealed that this highly porous network is adequate for survival of cells for an extended period of time. The artificial scaffolds formed by PCL/collagen/PES blend not only provide a suitable support for proliferation, but also maintain cell stability during differentiation (Fig. 3C). In addition to the fiber size effect, the presence of collagen biomacromolecules as a major natural component in the nanofibers is assumed to be responsible for favorable response of hBMSCs. It has been shown that PCL fibers alone do not sufficiently support proliferation process, probably because PCL lacks appropriate amino groups and hydrophilicity (data not shown), whereas the hybrid PCL/collagen/PES nanofibers possess the amide group enables cell adherence and proliferation (Figs 3 and 4).

The expression of hepatic markers such as albumin, AFP, CK-18, CK-19 is commonly used to show the performance of stem cells derived hepatocytes.[6,45,46] Expression of albumin as a late marker of hepatic differentiation at protein and mRNA levels in the differentiated cells on the scaffold attest to the effective differentiation process (Figs 5A and 7). The percentage of albumin-positive cells was higher when hBMSCs were cultured on the scaffold (47 ± 4%) as compared to that in 2D culture system (28 ± 6%).

A progressive expression of CK-18 at mRNA levels further confirms hepatocyte maturation and commitment. The detoxification capacity of cells derived from hBMSCs on the scaffold was used as an additional marker to show the function of the hepatocyte-like cells. Expression of CYP3A4, a member of superfamily enzyme involved in the metabolism of a majority of drugs[47,48] indicates the functional properties of the differentiated cells (Fig. 7). On the other hand, the expression of AFP and CK-19 (early marker of hepatic differentiation) suggests the presence of certain hepatic progenitors such as oval-shaped cells in the preparation (Fig. 7). A surge in the levels of SGPT, SGOT, transferrin, urea and albumin in the cultured cells 21 days after differentiation was used as evidence for the functionality of the differentiated. In comparison with 2D culture, higher production levels of albumin, urea, SGPT, and transferrin in differentiated cells on the scaffold were obtained ($P < 0.05$) which may suggest the conductive effect of the ECM-like structure for hBMSCs differentiation (Fig. 8 and Table 2).

Detailed knowledge about the part played by the scaffold architecture for enhancing the stem cell differentiation especially into hepatocytes needs further study. It is assumed that the presence of biological signals from the biomimetic nanofibers provide a nanoenvironment resembling a 3D natural ECM, which would enhance the biological activity of growth factors and cytokines for inducing differentiation.

Based on accumulating data, the high porous PCL/collagen/PES architecture provides an ECM-like nanoenvironment that is conducive to normal hepatic differentiation. This biocompatible nanofibrous meshes can act as suitable storage depot and delivery vehicle for transplantation and promising for the development of a bioartificial liver system.

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