

## Research article

**HEPATIC DIFFERENTIATION FROM HUMAN MESENCHYMAL STEM CELLS ON A NOVEL NANOFIBER SCAFFOLD**MAHBOOBE GHAEDI<sup>1,2,3</sup>, MASOUD SOLEIMANI<sup>4</sup>, IMAN SHABANI<sup>5</sup>,  
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**Abstract:** The emerging fields of tissue engineering and biomaterials have begun to provide potential treatment options for liver failure. The goal of the present study is to investigate the ability of a poly L-lactic acid (PLLA) nanofiber scaffold to support and enhance hepatic differentiation of human bone marrow-derived mesenchymal stem cells (hMSCs). A scaffold composed of poly L-lactic acid and collagen was fabricated by the electrospinning technique. After characterizing isolated hMSCs, they were seeded onto PLLA nanofiber scaffolds and induced to differentiate into a hepatocyte lineage. The mRNA levels and protein expression of several important hepatic genes were determined using RT-PCR, immunocytochemistry and ELISA. Flow cytometry revealed that the isolated bone marrow-derived stem cells were positive for hMSC-specific markers CD73, CD44, CD105 and CD166 and negative for hematopoietic markers CD34 and CD45. The differentiation of these stem cells into adipocytes and osteoblasts demonstrated their multipotency. Scanning electron microscopy showed adherence of cells in the nanofiber scaffold during differentiation towards hepatocytes. Our results showed that expression levels of liver-specific

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Abbreviations used: AAT –  $\alpha$ -1 antitrypsin;  $\alpha$ FP –  $\alpha$ -fetoprotein; CK8 – cytokeratin-8; CK18 – cytokeratin-18; hMSCs – human mesenchymal stem cells; PLLA – poly L-lactic acid

markers such as albumin,  $\alpha$ -fetoprotein, and cytokeratins 8 and 18 were higher in differentiated cells on the nanofibers than when cultured on plates. Importantly, liver functioning serum proteins, albumin and  $\alpha$ -1 antitrypsin were secreted into the culture medium at higher levels by the differentiated cells on the nanofibers than on the plates, demonstrating that our nanofibrous scaffolds promoted and enhanced hepatic differentiation under our culture conditions. Our results show that the engineered PLLA nanofibrous scaffold is a conducive matrix for the differentiation of MSCs into functional hepatocyte-like cells. This represents the first step for the use of this nanofibrous scaffold for culture and differentiation of stem cells that may be employed for tissue engineering and cell-based therapy applications.

**Key words:** Poly L-lactic acid, Nanofiber, Hepatic differentiation, Mesenchymal stem cells, Electrospinning

## INTRODUCTION

Liver dysfunction is a major health problem in the world. Liver transplantation, while being the only established successful treatment of end-stage liver diseases, is limited by the availability of donor livers, and as a result, many patients die each year while on a waiting list [1, 2]. Hence, researchers have focused on hepatocyte transplantation, which would offer a safer and readily available alternative to whole organ transplantation [3-5]. However, the major limitations of human primary hepatocytes as a source for hepatocyte transplantation include the requirement of large numbers of cells and the low proliferative capacity [6]. Recently, researchers have been working on the potential of using human stem cells to differentiate towards hepatocytes as an alternative to hepatocyte transplantation.

Stem cells are capable of self-renewal and have the capacity to differentiate into specific cell types. These properties make them very promising in tissue regeneration and repair [6, 7]. Many studies have shown the potential of stem cells to differentiate toward hepatocytes. Human mesenchymal stem cells (hMSCs) [8] as well as human embryonic stem cells (hESC) [9], liver stem cells/oval cells [10], cord blood cells [11], bone marrow stem cells [12] and fetal hepatocytes [13] are stem cell types that display potential to develop into hepatocytes. Bone marrow represents an abundant and accessible source of adult stem cells that can differentiate along multiple lineage pathways, including the hepatic lineage [14-17].

Both hematopoietic stem cells (HSCs) and MSCs have the capacity to trans-differentiate into hepatocytes *in vivo* [14, 15]; it appears that MSCs are the most potent component of bone marrow cells in hepatic differentiation [15]. Thus, hMSC-derived hepatocyte transplantation could be a potential treatment for liver disease [8, 15]. Other advantages of using mesenchymal stem cells in tissue regenerative medicine include their ease of isolation, proliferative capacity,

efficiency of *in vitro* transfection and the potential use of autologous cells [7]. Many studies have shown the potential of stem cells to differentiate toward hepatocytes [8-13].

The traditional concept of stem cell-based therapy consists of the isolation of stem cells from patients, expansion and differentiation *in vitro*, and subsequent re-transplantation of autologous cells into the patient. There are many problems associated with this paradigm, particularly during the *in vitro* manipulation process and the delivery and local engraftment of re-transplantation cells [16]. Moreover, stem cell fate is influenced by a number of factors including extracellular matrices (ECMs) and interactions that require robust control for safe and effective regeneration of functional tissue [19].

Appearance of a novel field of science and technology called tissue engineering raises hope for the solution of the problems mentioned above [17]. The ultimate goal of tissue engineering is to design and fabricate natural and functional human tissues and organs suitable for regeneration, repair and replacement of injured or lost human organs [18]. Developing advanced technologies for encouraging the *ex vivo* assembly of functional hepatic tissue for implantation into the human body is one of the challenging tasks facing tissue engineers [20]. Since hepatocytes are anchorage-dependent cells, there has been much effort to maintain hepatocyte functions *in vitro* by adopting different culture configurations [21, 22]. For stem cell differentiation into hepatocytes, there is a need to develop an environment including extracellular matrix to better mimic the *in vivo* liver microenvironment. Three-dimensional (3D) nanofiber scaffolds formed by electrospinning, by virtue of structural similarity to natural ECM, may represent promising structures for tissue engineering applications. They have been shown previously to share morphological similarities with collagen fibrils, and are capable of promoting favorable biological responses to seeded cells by providing a proper matrix for cell attachment, proliferation and differentiation [19, 22, 24].

When choosing a material for a tissue engineering application it must meet a number of requirements including biocompatibility and low toxicity [25, 26]. The most common synthetic biodegradable polymers employed in the tissue engineering field is FDA-approved poly L-lactic acid (PLLA). This biocompatible polymer is widely used as a biomaterial for tissue reconstruction because of its biodegradability, mechanical properties and nontoxic nature [26-29].

We have previously described differentiation of hMSCs to the hepatocyte lineage on a PCL/collagen/PES nanofiber scaffold [30]. In this study, an electrospun PLLA nanofiber scaffold with modified surface properties was produced and its effects on hepatocyte functions were then investigated. The results suggest that such a functional nanofiber scaffold can mimic the *in vivo* microenvironment of hepatocytes and promote hepatocyte-scaffold interaction. Compared with collagen as an extracellular matrix protein and PCL/collagen/PES nanofiber scaffold, the modified PLLA nanofiber scaffold fabricated in this

study demonstrated superior cell adhesion capabilities and biocompatibility, thereby greatly improving the hepatic differentiation and function.

## **MATERIALS AND METHODS**

### **Chemicals and reagents**

Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and trypsin-EDTA solution were obtained from Gibco BioCult (Paisley, UK). Mesencult and Stem Span media were purchased from Stem Cell Technology (USA). Mouse anti-human monoclonal antibodies for albumin and  $\alpha$ -fetoprotein ( $\alpha$ FP) and the goat anti-mouse FITC-conjugated IgG were obtained from DAKO (Denmark) and Oxford Biomedical Research, Inc (UK). The kits for the RNA extraction and cDNA synthesis were purchased from Qiagen (Valencia, CA, USA) and SYBR Green master mix was purchased from Applied Biosystems (Foster, CA, USA). Hepatocyte growth factor (HGF), dexamethasone (DEX), oncostatin M (OSM), Alizarin red staining and Oil red staining kits, PLLA ( $80,000 \text{ g mol}^{-1}$ ), chloroform, EDC/NHS and N,N-dimethylformamide (DMF) were purchased from Sigma Aldrich Co. (USA). Human HepG2 hepatoma cells were obtained from the National Cell Bank of Iran, Pasteur Institute (Tehran, Iran).

### **Preparation of the scaffold**

PLLA was dissolved in chloroform at room temperature and DMF was added to the chloroform just before the electrospinning process. Experiments were conducted at a chloroform/DMF ratio between 100/0 and 80/20 (v/v), while the overall concentration of PLLA in the solution was maintained at 12 wt%. PLLA nanofiber mats were produced by an electrospinning method. Briefly, prepared PLLA solution (25%) in DMF was fed into a blunted needle by using a syringe pump. The collector was a rotating cylindrical drum which was placed at a distance of 15 cm from the needle.

### **Surface modification**

In order to increase hydrophilicity, oxygen plasma treatment was performed. A microwave plasma generator of 2.45 GHz frequency with a cylindrical quartz reactor (Diener Electronics, Germany) was used. Pure oxygen was introduced into the reaction chamber at 0.4 mbar pressure and then the glow discharge was ignited for 10 min. Plasma-treated sheets were cut into 1 cm diameter punches and were immersed in EDC/NHS solution (5 mg/ml) for 6 h for grafting. Then a collagen solution (1 mg/ml) was used to immerse scaffolds overnight. The scaffolds were then rinsed with distilled water and used for cell seeding and surface characterization.

### **Scanning electron microscopy observation**

Morphology of the electrospun PLLA nonwoven mat was observed with scanning electron microscopy (SEM, Philips XL30, Netherlands) after sputter coating with gold. The diameter and the distribution of the diameter of

electrospun PLLA nonwoven mats were measured using image analyzing Image J software (National Institute of Health, USA).

#### **hMSCs isolation and culture**

Human MSCs were obtained from bone marrow aspirates of several healthy donors ranging in age from 19 to 32 years at the Bone Marrow Transplantation Center, Shariati Hospital, Tehran, Iran. Samples were only taken after obtaining informed consent from individuals under an approved institutional review board protocol according to guidelines of the Medical Ethics Committee, Ministry of Health, I.R. Iran. Isolation of human MSCs was performed as previously described [30, 31]. Briefly, the mononuclear cell fraction was separated by centrifugation over a Ficoll-Paque gradient, and resuspended in proliferation medium consisting of low glucose DMEM. Monolayers of adherent cells were cultured at an initial seeding density of  $5 \times 10^5 \text{ cm}^{-2}$  in hMSC medium consisting of Mesencult medium until they reached 70%-90% confluence. Cells were passaged 4 times prior to further analysis so as to ensure removal of contaminating hematopoietic cells.

#### **Flow cytometry analysis**

To characterize the surface markers of hMSCs, approximately  $20 \times 10^5$  cells were detached from the tissue culture flasks after 14 days and were stained with the following anti-human antibodies: FITC-conjugated mouse anti-human CD44, CD73, CD105, CD45; phycoerythrin (PE)-conjugated CD166 and CD45 (leukocyte common antigen). Isotype-matched irrelevant monoclonal antibodies (mAbs) were used as negative controls. Afterwards, cells were analyzed using a FACSCalibur flow cytometer (Beckman Coulter, Fullerton, CA, USA), and the results were analyzed with the Win MDI 2.8 software (Scripps Institute, La Jolla, CA, USA).

#### **Adipogenic and osteogenic differentiation of hMSCs**

$2 \times 10^3$  hMSCs cells were plated in 24-well culture plates. The cultured cells were incubated in the adipogenic medium containing DMEM supplemented with  $1.7 \mu\text{M}$  insulin,  $1 \mu\text{M}$  dexamethasone,  $200 \mu\text{M}$  indomethacin and  $500 \mu\text{M}$  isobutylmethylxanthine for 14 days [31, 32]. Fat droplets within differentiated adipocytes derived from hMSCs were observed using the oil red O staining method.

The potential of hMSCs to differentiate into osteogenic lineages was assessed as previously described [32, 33]. hMSCs were incubated at  $3 \times 10^3 \text{ cells/cm}^2$  in an osteogenic medium containing modified minimum essential medium with  $50 \mu\text{M}$  ascorbate-phosphate,  $1 \mu\text{M}$  dexamethasone and  $10 \mu\text{M}$   $\beta$ -glycerophosphate for 2 weeks. To assess osteogenic differentiation, the cells were stained by specific histochemical staining for calcium using the Alizarin red staining kit. The stained cells were examined with phase contrast microscopy (Nikon, TE-2000, Tokyo, Japan).

**Cell seeding and hepatic differentiation protocol**

$2 \times 10^4$  cells/cm<sup>2</sup> hMSCs at the fourth passage were transferred directly onto the scaffolds in 6-well culture plates, and the same numbers of hMSCs were seeded into collagen I-coated 6-well plates as parallel experiments under the same culture conditions. The hMSC-seeded scaffolds and hMSCs on collagen were incubated in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air at 37°C.

The differentiation of hMSCs was initiated under the previous protocol [34]. Briefly, in the first step, which lasted for seven days, the cells were cultured in medium consisting of  $\alpha$ -MEM supplemented with 15% FBS, 20 ng/ml of HGF and  $10^{-7}$  mol/l of DEX followed by 20 ng/ml of OSM for 2 weeks. The culture medium was changed twice a week and hepatic differentiation was assessed as described below. To minimize variability, only cells of the fourth passage were used for the hepatic differentiation studies.

**Cell morphology**

Cell morphology on the scaffold was investigated by SEM. The cell-loaded scaffolds were rinsed with PBS after 21 days of differentiation and fixed in 2.5% glutaraldehyde for 1 h. After rinsing and dehydrating in sequentially increasing ethanol solutions from 70% to 100% ethanol, the dehydrated samples were dried in a critical point drier (Christ, GAMMA 2-16 LSc) then sputter coating with gold was done followed by observation with SEM.

**Fluorescent immunohistochemistry analysis**

After 3 weeks of differentiation, the cultured cells on the scaffold were harvested with 0.25% trypsin-EDTA solution and mounted on a chambered coverglass. The attached cells on the chamber glass were fixed for 30 min in a 4% paraformaldehyde solution at room temperature and then permeabilized with 0.4% Triton X-100 for 20 min. Corresponding primary antibodies including mouse anti-human albumin (1:1000) and mouse anti-human  $\alpha$ FP (1:500) were used to stain the cells overnight at 4°C. The next day the cells were incubated with FITC-labeled goat anti-mouse IgG at 37°C for 3 h in the dark. Afterwards the cells were incubated with 4,6-diamidino-2-phenylindole (DAPI) (1:1000) for the purpose of nuclear staining. Then they were visualized using a fluorescence microscope. HepG2 cells were stained for albumin and  $\alpha$ FP as a positive control and undifferentiated hMSCs were used as a negative control.

**Real-time RT-PCR reaction**

Real-time quantitative RT-PCR (qRT-PCR) was used to determine the expression of albumin, CK18, CK8 and  $\alpha$ FP in hMSCs cultured on the nanofiber scaffold and collagen-coated plate. Adult human primary hepatocytes isolated from donor livers were provided by Dr. Stephen Strom (University of Pittsburg). These fresh cells were used to prepare cDNA and were used in real-time quantitative RT-PCR as calibrators. Briefly, total cellular RNA was prepared using the RNeasy Mini Kit following the manufacturer's instructions. Single-stranded cDNA was synthesized using the reverse transcription-PCR protocol of

the First Strand cDNA Synthesis Kit. Quantitative real-time PCR was performed using SYBR Green PCR Master Mix. Primers were selected from a database (<http://medgen.ugent.be/rtprimerdb>) and listed in Tab. 1. Concentrations of all primers were optimized before use. All PCR reactions were done in duplicate. The comparative Ct value method using GAPDH as a housekeeping gene for an internal standard was employed to determine relative levels of gene expression.

Tab. 1. List of primers used for quantitative RT-PCR.

Gene	NCBI access number	Length (bp)	Primer sequence
ALB	NM_134326	153	F 5'-AATTGGCAACAGACCTCACC-3' R 5'-GCACTGGCTTATCACAGCAA-3'
AFP	BC097344	155	F 5'-ACCTGACAGGGAAGATGGTG-3' R 5'-GCAGTGGTTGATAACCGGAGT-3'
CK8	BC000654	101	F 5'-CTGGGATGCAGAACATGAGTATTC-3' R 5'-TGTTCCGGCCCCAGTCGATG-3'
CK18	BC000180	86	F 5'-GAGACGTACAGTCCAGTCCTTGG-3' R 5'-TTGTCGGACTCCCTCCACC-3'
GABDH	NM_017008	129	F 5'-TGCCACTCAGAAGACTGTGG-3' R 5'-TTCAGCTCTGGGATGACCTT-3'

### ELISA analysis

Cell culture media were collected at days 0, 7, 14 and 21 after differentiation and used to analyze for secreted albumin and  $\alpha$ -1 antitrypsin (AAT) values using ELISA standard kits from Bethyl Laboratories and Geneway according to the manufacturer's instructions. For each time point after collecting the supernatant, the cells on the nanofiber were trypsinized and counted using a hemocytometer, and albumin and AAT values were normalized to the total number of cells cultured.

### Statistics

All data were measured as means  $\pm$  SEM from at least three independent experiments. An unpaired Student's *t* test was used to analyze the data.  $p < 0.05$  was considered statistically significant.

## RESULTS

### Morphology and average fiber diameter distribution of electrospun PLLA scaffolds

SEM images of the plasma-treated and collagen-grafted PLLA are shown in Fig. 1A. Scanning electron micrographs obtained prior to cell seeding revealed that the 3D scaffold contains randomly oriented, uniformly sized fibers of an average diameter of 300 nm. Oxygen plasma treatment decreased the water contact angle of materials (data not shown), since cells typically adhere better to more hydrophilic materials. The plasma treatment has no significant influence on fiber morphology (data not shown).

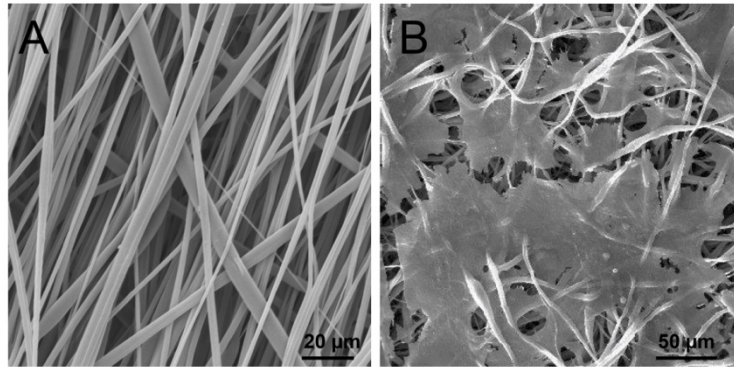


Fig. 1. Scanning electron microscope (SEM) images of PLLA/collagen nanofibers before and after cell seeding. A – SEM image before cell seeding. B – SEM image after differentiation of hMSCs on the nanofibrous scaffold.

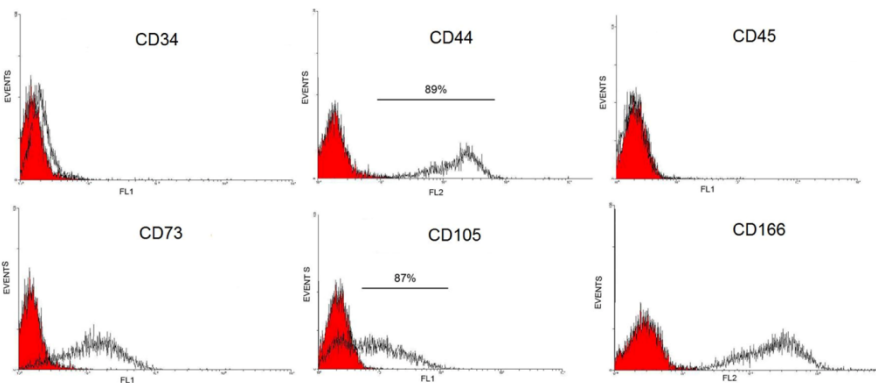


Fig. 2. Flow cytometric analysis of the hMSCs. Flow cytometric analysis was performed for the specific markers of the hMSCs and hematopoietic markers. hMSCs were positive for CD44, CD73, CD105 and CD166 but showed no expression of CD34 and CD45.

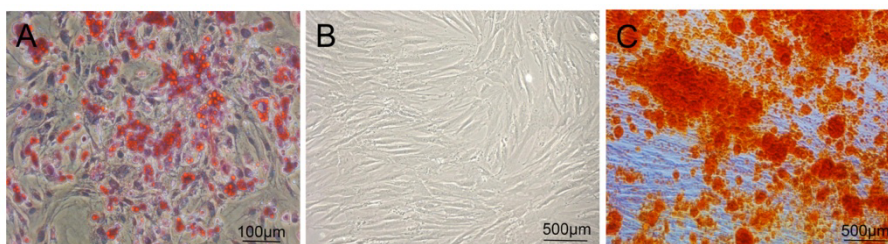


Fig. 3. Transdifferentiation of human bone marrow derived mesenchymal stem cells (MSCs) into adipocytes and osteoblasts. Differentiated MSCs showed positive for (A) oil red staining for adipogenic differentiation and (C) alizarin red staining for osteogenic differentiation. B – Undifferentiated hMSCs as a control group were negative for both staining (alizarin red staining and oil red O staining).



### **Characterization of hMSCs**

Isolated hMSCs were characterized by flow cytometric analysis and mesodermal differentiation potential upon their exposure to mesenchymal supportive conditions containing adipogenic and osteogenic-specific agents. Flow cytometry revealed that the hMSCs were positive for CD73, CD44, CD105 and CD166 and negative for CD34 and CD45 (Fig. 2). The cells also differentiated into adipocytes and osteoblasts, indicating their multipotency. Alizarin red staining confirmed the presence of calcium deposits, characteristic of osteogenic cells, in differentiated cells after 2 weeks of culturing in osteogenic medium (Fig. 3C). The hMSCs' differentiation into adipogenic cells was confirmed by oil red O staining after 4 weeks of culturing in adipogenic medium (Fig. 3A). hMSCs cultured in normal medium served as a control and were negative for both stainings (Fig. 3B). These results demonstrated that hMSCs were successfully isolated and characterized (Figs. 2 and 3).

### **Attachment and proliferation of cells on PLLA scaffold**

A typical SEM micrograph of cells attached to the scaffold on day 21 of differentiation is presented in Fig. 1B. As shown in this figure, the cells adhered well to the surfaces of the nanofibrous scaffolds. They remained attached, proliferated and differentiated on the surface of the PLLA scaffold during three weeks of culture.

### **Evaluation of hepatic differentiation by immunohistochemistry analysis**

To determine *in vitro* hepatic differentiation of hMSCs toward hepatocytes on the scaffold, the expressions of albumin and  $\alpha$ FP were evaluated by immunohistochemistry analysis. Differentiated cells on the nanofiber scaffold were intensely stained for albumin (Fig. 4A) and  $\alpha$ FP (Fig. 5A). These markers were not expressed in undifferentiated hMSCs used as the control (Fig. 4B and 5B).

### **Hepatic gene expression**

We also evaluated the expression of human liver cell markers, namely albumin, CK8, CK18 and  $\alpha$ FP, by real time qRT-PCR in the differentiated cells on nanofibrous scaffolds and collagen-coated plate. qRT-PCR revealed that the levels of albumin, CK8, CK18 and  $\alpha$ FP were significantly higher in hepatocyte-like cells differentiated from hMSCs on the nanofiber scaffold, compared to differentiated cells on the culture plate (Fig. 6). Expression of the genes was not detected in undifferentiated hMSCs (data not shown). These results indicated that the cells differentiated from hMSCs on the nanofiber scaffold show phenotypic properties of human adult hepatocytes.

### **Functional analysis of hMSC-derived hepatocytes on nanofiber scaffold**

hMSCs cultured on the nanofiber scaffold and collagen-coated tissue culture plate were analyzed for synthesis of albumin and AAT using ELISA on day 7, 14 and 21. As shown in Fig. 7, stem cells cultured on the nanofiber scaffold secreted more serum proteins than hMSCs residing on collagen. Looking at the

dynamics of protein secretion over the course of 21 days, hMSCs differentiated on scaffolds produced higher levels of albumin and AAT when compared to cells differentiated in the 2D culture system. Moreover, the secretion of albumin and AAT in the culture media significantly increased on day 21 of differentiation when compared to secretion at day 7 ( $P < 0.05$ ).

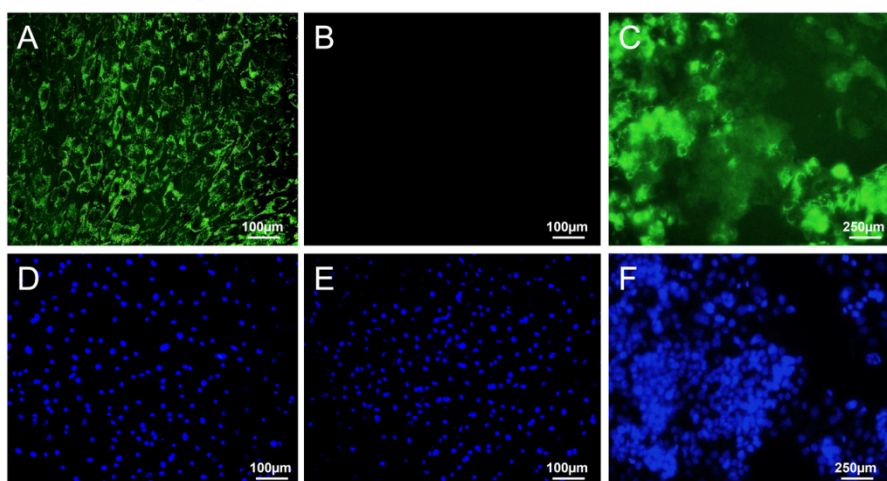


Fig. 4. Immunofluorescent staining of albumin. A, B – albumin staining in differentiated cells on nanofiber scaffold (A) and on undifferentiated cells (B). C – albumin staining in HepG2 cells as a positive control. Nuclei were stained with DAPI for differentiated cells on nanofiber scaffold (D), undifferentiated cells (E), and HepG2 cell line (F).

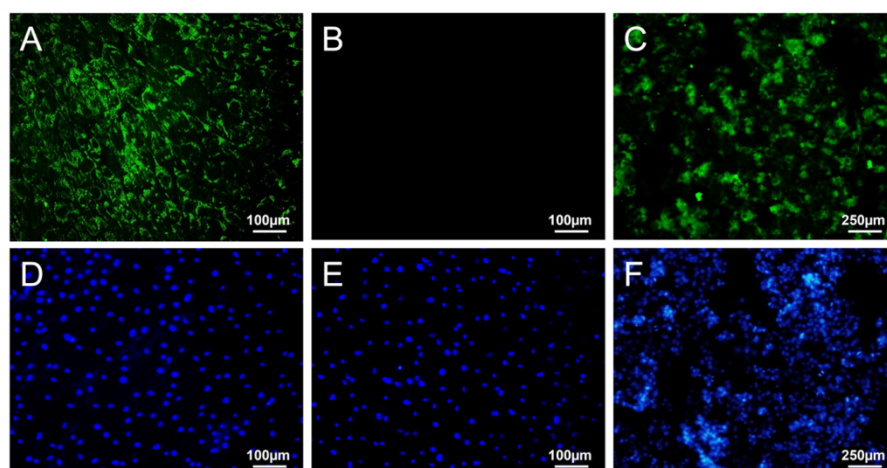


Fig. 5. Immunofluorescent staining of  $\alpha$ -fetoprotein. A, B –  $\alpha$ -fetoprotein staining in differentiated cells on nanofiber scaffold (A), and on undifferentiated cells (B). C –  $\alpha$ -fetoprotein staining in HepG2 cells as a positive control. Nuclei were stained with DAPI for differentiated cells on nanofiber scaffold (D), undifferentiated cells (E), and HepG2 cell line (F).

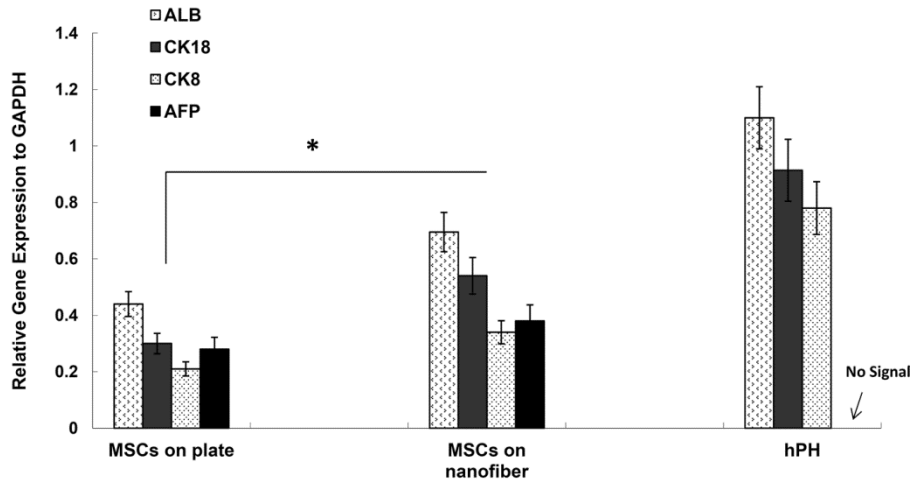


Fig. 6. qRT-PCR analysis of expression of liver-specific genes in hMSC-derived cells on scaffolds. The mRNA expression of albumin,  $\alpha$ -fetoprotein ( $\alpha$ FP), cytoke- ratin-18 (CK-18), and cytoke- ratin-8 (CK-8) was determined by real time quantitative RT-PCR at day 21 after differentiation on both scaffolds and the culture plates. Human GAPDH was used as a housekeeping gene for an internal control, and relative levels of each gene were normalized before cell differentiation. Bars indicate SEM. \* indicates  $p < 0.05$  compared to hMSCs on collagen.

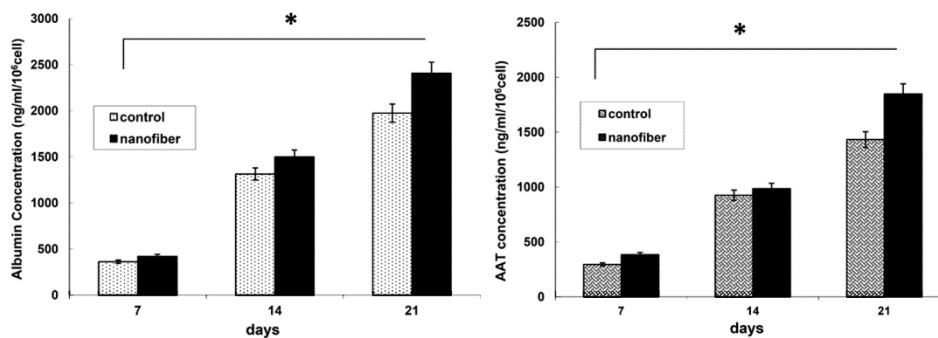


Fig. 7. ELISA analysis of albumin and  $\alpha$ -1 antitrypsin secreted into the medium during differentiation. The supernatants were collected at the different time points during the time course of differentiation of hMSCs on nanofiber scaffolds and on the culture plates, the values of secreted albumin (left panel) and  $\alpha$ -1 antitrypsin (right panel) were measured by ELISA, and the total secreted albumin and  $\alpha$ -1 antitrypsin were normalized to the cell numbers used at each time point. Bars indicate SEM. \* indicates  $p < 0.05$  compared to hMSCs on collagen.

## DISCUSSION

Tissue engineering requires the use of a cell source to allow for the generation and maintenance of tissue-specific biological functions as well as the use of matrix materials [35]. A variety of biodegradable synthetic polymers such as

PLLA, polyglycolic acid (PGA), polyethylene glycol (PEG), polycaprolactones, polyorthoesters, polyanhydrides and polycarbonates have been used for scaffolds [36, 37] because of their ease of synthesis [38].

To obtain three-dimensional tissue constructs, stem or progenitor cells must be combined with a highly porous three-dimensional scaffold, but many of the structures proposed for tissue engineering do not meet all the criteria required by an adequate scaffold because of a lack of mechanical strength and interconnectivity or poor surface characteristics [39]. Fiber-based structures represent a wide range of possibilities that can be tailored for each specific tissue-engineering application [39]. The electrospinning process is a simple, economical means to produce scaffolds of ultrafine fibers derived from a variety of biodegradable polymers. They thus produce an interconnected porous matrix capable of encouraging cell proliferation and cell-cell interactions [24, 25]. The natural scaffold of the cells in human tissues, ECM, is nano-structured and is composed of protein fibers which produce a 3D matrix for cells to attach, differentiate and proliferate. An ideal scaffold should mimic the natural properties of ECM such as porosity and nanosized structure [40].

With respect to the applications of nanofibers for tissue engineering, our aim was to design an artificial matrix that can mimic ECM, in order to support the attachment and differentiation of hMSCs into hepatocyte-like cells. SEM images of the plasma-treated and collagen-grafted PLLA produced by the electrospinning process were obtained prior to cell seeding. As shown in Fig. 1A, nanofibrous PLLA scaffolds contain randomly oriented, uniformly sized fibers and a highly porous structure. The diameters were distributed in the range of 280-320 nm, with an average of 300 nm.

After fabrication and preparation of PLLA, and in order to increase hydrophilicity, oxygen plasma treatment was performed on the nanofibrous scaffold. It is possible to manipulate the regions to which cells will adhere and grow by modifying the surface chemistry of scaffolds with charged gas plasma polymerization deposition [25, 39, 40]. The ability to change the adherent properties of the cells to the PLLA scaffold allows the manipulation of the important cell intrusion phase and subsequent tissue development [40, 41]. Many polymers do not have the desired surface properties to be used as biomaterials. To overcome this problem, surface treatment and modification are employed to improve surface characteristics of the polymers to promote cell attachment, expansion, proliferation and infiltration [42-46]. Plasma treatment is the best way to increase surface hydrophilicity, and protein grafting also improves surface properties of biomaterials [42, 47]. In addition to the fiber size and charged gas plasma effect, collagen was used to improve cell function on the PLLA nanofibrous mats. Collagen is one of the natural polymers that has been used for surface modification as it has many amino acid sequences which are very important in cell-scaffold interactions [41-43, 47].

To test the ability of collagen-grafted PLLA to promote hepatic differentiation, hMSCs were seeded onto the PLLA scaffold and hMSCs on the culture plate

were used as controls. Fig. 1B demonstrates the attachment of the hMSCs onto the collagen-grafted nanofibers. Surface modification by plasma treatment or collagen grafting will aid attachment and improve the morphology of the cells on the treated surface [42, 48].

To determine the extent of hepatic differentiation of hMSCs, intracellular albumin (liver specific protein) and  $\alpha$ FP (a protein indicative of immature hepatocytes) were evaluated by immunohistochemical analysis. As shown in Fig. 4 and 5, a large fraction of hMSCs cultured on the PLLA scaffold expressed  $\alpha$ FP and albumin. But the expression of  $\alpha$ FP points to an immature phenotype of a fraction of cells differentiated from hMSCs. It should be noted that a lower  $\alpha$ FP and albumin signal was observed in hMSCs cultured on Col only. Given that identical differentiation medium was used in both cases, presence of the scaffold on the culture surface and its 3D structure by virtue of structural similarity to natural ECM appears to enhance hepatic differentiation in these experiments.

To assess the differentiation of hMSCs into hepatic lineages, we examined the expression of liver-specific markers such as albumin,  $\alpha$ FP, CK-18 and CK8 by qRT-PCR. qRT-PCR analysis revealed that the levels of  $\alpha$ FP, albumin, CK-18 and CK8 expression were significantly higher in hMSCs cultured on the nanofiber scaffold than on the collagen-coated plate (Fig. 6). Using the PLLA nanofiber scaffold for hepatic differentiation of hMSCs, we successfully converted these cells into hepatic progenitors with 42% and 70% of the cells positive for  $\alpha$ FP and albumin within 21 days, as determined by qRT-PCR (Fig. 6) when compared to hMSCs on Col only (30% and 41% of hMSCs were positive for  $\alpha$ FP and albumin within 21 days).

Secretion of proteins into the blood stream is an important function of the liver. Therefore, production of liver proteins such as albumin and AAT is commonly used to assess the phenotype of hepatocytes cultured in vitro. hMSCs cultured on the nanofiber scaffold and collagen-coated plate were analyzed for synthesis of albumin and AAT using ELISA. As shown in Fig. 7, stem cells cultured on the nanofiber scaffold secreted more serum proteins than hMSCs residing on Col only. Looking at protein secretion over the course of 21 days, one notices only a gradual increase in stem cells on the collagen-coated plate vs. a dynamic change in production of albumin and AAT in stem cells cultured on the nanofiber scaffold. This suggests faster and more pronounced hepatic differentiation of hMSCs cultured on the PLLA scaffold.

Based on the experimental evidence, we demonstrated that the PLLA/collagen nanofibers not only allow the hMSCs to differentiate into hepatocytes but also enhance hMSCs' development into functional hepatocyte-like cells when compared to the conventional culture system. Moreover, compared to the PCL/collagen/PES nanofiber scaffold in our previous study [30], these results suggest that collagen-grafted PLLA nanofibers may be an excellent candidate to form 3D structures in tissue engineering and underscore the potential use of the PLLA scaffold for differentiation of adult stem cells.

## CONCLUSION

Detailed information on the mechanism by which the scaffold architecture enhances stem cell differentiation towards hepatocytes requires further investigation. The presence of biological signals from the biomimetic nanofibers provides a nanoenvironment resembling a 3D natural ECM [19, 22, 24], which in turn enhances the biological activity of growth factors and cytokines for inducing differentiation. Based on our present study, the highly porous collagen-grafted PLLA architecture provides an ECM-like nanoenvironment that is conducive to normal hepatic differentiation. Thus, PLLA nanofiber can potentially be employed as a scaffold for relevant hepatocyte-based applications in liver tissue engineering.

**Authors' contributions.** M.G. and A.S.L. designed the research; M.G. performed the research, collected and analyzed data, and wrote the paper; M.S. and I.S. assisted in doing the research and collecting data; Y.D. assisted in data analysis, partially wrote the paper and revised the paper; A.S.L. financially supported the research and finally approved the paper.

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