In vitro Differentiation of Human Cord Blood-Derived Unrestricted Somatic Stem Cells into Hepatocyte-Like Cells on Poly(ε-Caprolactone) Nanofiber Scaffolds

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Key Words
Cord blood \cdot Stem cell, unrestricted somatic \cdot Poly(ε-caprolactone) \cdot Nanofiber scaffold \cdot Hepatocyte differentiation

Abstract
Tissue engineering of implantable cellular constructs is an emerging cellular therapy for hepatic disease. In this study, we tested the ability of poly(ε-caprolactone) (PCL) nanofiber scaffold to support and maintain hepatic differentiation of human cord blood-derived unrestricted somatic stem cells (USSCs) in vitro. USSCs, self-renewing pluripotent cells, were isolated from human cord blood. The electrospun PCL nanofiber porous scaffold was constructed of uniform, randomly oriented nanofibers. USSCs were seeded onto PCL nanofiber scaffolds, and were induced to differentiate into hepatogenic lineages by culturing with differentiation factors for 6 weeks. RT-PCR analysis of endoderm and hepatic-specific gene expression, immunohistochemical detection of cyto-
keratin 18 (CK-18), α-fetoprotein, albumin, glycogen storage and indocyanine green uptake confirmed the differentiation of USSCs into endoderm and hepatocyte-like cells. In the present study, we show that hepatocyte-like cells differentiated from USSCs on the PCL nanofiber scaffold can be candidate for tissue engineering and cell therapy of hepatic tissues.

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Introduction

A variety of diseases including toxic injury, viral infections, and autoimmune or genetic disorders may cause hepatic dysfunction resulting in chronic liver disease and/or acute liver failure. Liver transplantation which is still the only therapeutic option for end-stage liver disease is limited by the availability of donor organs [Sgodda et al., 2007]. Therefore, it would be greatly beneficial if an unlimited supply of functional hepatocytes from other sources such as stem cells could be generated. The liver is one of the target organs for which stem cell-based therapeutics is very promising.

Many types of stem cells from different sources have been investigated for their hepatic differentiation ability; mostly mouse, but also monkey and human embryonic stem (ES) cells have been used [Banas et al., 2007]. Adult human stem cells are promising candidates for liver regeneration. Currently, the focus is on mesenchymal stem cells (MSCs) found in bone marrow, adipose tissue, scalp tissue, placenta, and umbilical cord blood, as well as in various fetal tissues. These stem cells can differentiate in vitro into multiple types of lineages such as chondrogenic, osteogenic, adipogenic, myogenic and neurogenic [Banas et al., 2007]. It has been reported that MSCs, isolated from bone marrow, adipose tissue, and umbilical cord blood, can differentiate into hepatocytes in vitro and/or in vivo [Jiang et al., 2002; Schwartz et al., 2002; Kakinuma et al., 2003; Kollet et al., 2003; Wang et al., 2003; Anjos-Afonso et al., 2004; Lee et al., 2004; Sato et al., 2005; Talens-Visconti et al., 2006; Aurich et al., 2007; Banas et al., 2007; Sgodda et al., 2007].

Human unrestricted somatic stem cells (USSCs) are self-renewing pluripotent cells isolated from human cord blood by Jäger et al. [2003]. They reported that the culture media and other in vitro conditions influence the osteogenic differentiation potency of USSCs. In addition, USSCs have been differentiated in vitro into osteoblasts, chondrocytes, adipocytes, and neural progenitors as well as in vivo into bone, cartilage, neural and heart tissue [Jäger et al., 2003, 2004; Kögler et al., 2004, 2005, 2006; Fallahi-Sichani et al., 2007]. Moreover, a very recent report has shown differentiation of USSCs into endoderm and hepatic lineages [Sensken et al., 2007].

Traditional culturing methods on plastic surfaces do not accurately represent the in vivo environment, and a paradigm shift from two-dimensional (2-D) to three-dimensional (3-D) experimental techniques is underway. To enable this change, a variety of natural, synthetic and semisynthetic extracellular matrix (ECM) equivalents have been developed to provide an appropriate cellular microenvironment [Serban et al., 2008].

ECM is secreted by cells to form interstitial matrix and basement membrane, which constitutes the framework to which cells are anchored. The basement membrane provides the special orientation and stability required for the organization and development of the characteristic histology of specific tissues. Since ECM plays an important role in the regulation of cell growth and differentiation in addition to its structural function [Rosso et al., 2004], it has been used for tissue engineering in generating substrates for the growth and differentiation of many cell types, including hepatocytes [Semler et al., 2000; Okumoto et al., 2005]. In recent years, tissue engineering has supplied new instruments for the in vitro reconstruction of a natural cellular environment [Zavan et al., 2005].

Currently, there are a number of methods available for manufacturing tissue scaffolds, which include electrospinning, self-assembly, phase separation, solvent casting and particulate leaching, freeze drying, melt molding, template synthesis, drawing, gas foaming, and solid-free forming [Murugan and Ramakrishna, 2007], but only a few of them are capable of producing nanofibrous scaffolds. Among them, electrospinning is considered as an effectual practical method in designing nanofibrous scaffolds with fiber orientation [Yang et al., 2005; Murugan and Ramakrishna, 2007]. Electrospun fibers have been investigated as promising tissue engineering scaffolds since they mimic the nanoscale properties of native ECM [Pham et al., 2006a].

So far, a lot of research efforts have been made to study the efficacy of electrospun scaffolds for tissue engineering applications [Nair et al., 2004; Shields et al., 2004; Khil et al., 2005; Kidoaki et al., 2005; Li et al., 2005a; Sun et al., 2005; Boudriot et al., 2006; Pham et al., 2006a, b]. The spectrum of materials used to prepare nanofiber scaffolds for tissue engineering is extremely broad. Biocompatible and biodegradable polymers of natural and synthetic origin such as poly(glycolic acid),...
poly(\(L\)-lactic acid), poly(\(e\)-caprolactone) (PCL), as well as copolymers from the corresponding monomers in various compositions, segmented polyurethanes, collagen, gelatin, and chitosan are examples in case. Furthermore, synthetic polymers such as poly(\(L\)-lactic acid) or PCL were mixed with or coated with natural polymers such as gelatin or chitosan, in order to provide surfaces with enhanced adhesion and proliferation capability for cells.

A broad spectrum of cells has been seeded on such electrospun scaffolds [Boudriot et al., 2006]. An ideal scaffold for tissue engineering would be highly porous and contains an interconnected pore network through which nutrients and metabolic waste can flow. In addition, it would be biodegradable via a readily controllable mechanism and have suitable surface properties (chemical and physical) for initial cell attachment [Kim and Kim, 2007]. PCL has many advantages because of its biocompatibility, low cost, easy processability and slow hydrolytic degradation rate [Chen et al., 2007a]. It can be fabricated into conduits with the required dimensions, optimum porosity, and viscoelasticity. PCL degrades by hydrolysis and the body resorbs the hydrolyzed products with minimal reaction to the tissues. PCL has one of the slowest degradation rates of all biodegradable polymers and is a good candidate for fabricating scaffolds to be used in engineering tissues that require a long scaffold degradation time, and its degradation products are less detrimental to growing tissues [Pankajakshan et al., 2007]. Li et al. [2005a] tested a 3-D nanofiber scaffold fabricated from PCL for its ability to support and maintain multilineage differentiation of human MSCs (hMSCs) in vitro. They demonstrated that adult hMSCs obtained from a single patient and cultured on a biocompatible and biodegradable PCL-based nanofiber scaffold can be used to engineer adipose, cartilage, and bone in vitro, when provided with the appropriate inductive agents [Li et al., 2005a]. Moreover, they reported that the PCL scaffolds effectively supported TGF-\(\beta\)_1-induced chondrogenesis of adult hMSCs [Li et al., 2005b].

To our knowledge, only one recent report has shown the hepatogenic differentiation potential of USSCs [Sensen et al., 2007]. Moreover, USSCs were seeded onto a collagen/tricalcium phosphate scaffold and xenotransplanted into critical size femoral defects of nude rats, and a survival of human cells within the scaffold and surrounding bone/bone marrow and an increased bone healing rate were shown [Jäger et al., 2007]. However, no one has reported the hepatic differentiation of USSCs or other stem cells on a nanofibrous scaffold fabricated from PCL. In this study, we report the differentiation potential of USSCs cultured in a nanofiber PCL scaffold into a hepatocyte-like cell.

**Materials and Methods**

**Preparation of the Scaffold**

PCL was obtained from Sigma Aldrich (Milwaukee, Wisc., USA). The average molecular weight of PCL was 80,000 g mol\(^{-1}\). Chloroform and N,N-dimethylformamide (DMF) were purchased from Sigma (St. Louis, Mo., USA). These materials were used as received without any further purification. PCL was dissolved in chloroform at room temperature and DMF was added to the chloroform just before the electrospinning process. DMF is not a solvent for PCL but it has a high dielectric constant and could enhance electrospinning. Many studies showed that DMF help during the electrospinning and the formation of electrospun fibers [Lee et al., 2003; Hsu and Shivkumar, 2004].

Experiments were conducted at a chloroform/DMF ratio between 100/0 and 80/20 (v/v), while the overall concentration of PCL in the solution was maintained at 12 wt%.

The experimental setup used for the electrospinning process is shown in figure 1. A variable voltage power supply was used for the electrospinning. The polymer solution was poured into a 10-ml syringe. The PCL solution was delivered with a syringe pump (SP-500, JMS, Tokyo, Japan) at a flow rate of 1 ml min\(^{-1}\) to a 20-gauge syringe nozzle through PE extension tubing. The needle tip could move in restricted distance along the direction of the deposition area and thus gives the capability to have a uniform mat. A voltage range between 12 and 18 was applied to the solution and the jet emerging from the needle to the drum collector. The collector was positioned at a fixed distance of 17 cm from the needle to give the solvent enough time to evaporate from the jet. The rotating drum speed can be controlled ranging from 0 to 10 m min\(^{-1}\). The process time for samples considered for morphological investigations was 1 min, while for in vitro assessment the scaffold was produced in about 2 h. The samples were...
then washed 3 or 4 times in sterile water and immersed in 70% ethanol overnight for elimination of bioburden.

In order to increase hydrophilicity, oxygen plasma treatment was performed. The bare materials were exposed to oxygen plasma at 13.6 MHz for 5 min using a diener electronic plasma device.

**Scanning Electron Microscopy Observation**

Morphology of the electrospun PCL nonwoven mat was observed with scanning electron microscopy (SEM, Vega® Tescan, Cranberry Twp., Pa., USA) after sputter coating with platinum. The diameter and the distribution of the diameter of electrospun PCL nonwoven mats were measured from ImageJ (National Institute of Health, USA) image analyzing software.

Nonwoven fabric samples with proliferated cells were fixed in a 2.5% glutaraldehyde solution in phosphate-buffered saline (PBS). After rinsing and dehydrating in sequentially increasing ethanol solutions to 100% ethanol, the dehydrated samples were dried in a critical point drier (Christ®, GAMMA 2-16 LSc) and after sputter coating with platinum was observed by scanning electron microscopy.

**USSC Isolation and Culture**

Processing of human full-term umbilical cord blood samples and subsequent isolation of USSCs from umbilical cord blood were accomplished as previously described [Kögler et al., 2004]. Briefly, the mononuclear cell fraction was obtained by a Ficoll (Pharmacia-Amersham) gradient separation followed by ammonium chloride lysis of red blood cells. After two washes in PBS, the collected mononuclear cells were resuspended in low-glucose Dulbecco’s modified Eagle medium (DMEM, GIBCO BRL) supplemented with 30% fetal calf serum (GIBCO BRL). After rinsing and dehydrating in sequentially increasing ethanol solutions to 100% ethanol, the dehydrated samples were fixed in 4% paraformaldehyde (Sigma) for 20 min. The cells were permeabilized with 2% Triton X-100 in PBS, when required. The cells were then fixed with 4% paraformaldehyde (Sigma), 0.2% ascorbic acid 2-phosphate, and 10 mM β-glycerophosphate (all from Sigma). Medium was changed every 3–4 days. After 3 weeks of induction, cells were stained with alizarin red S (Sigma) to assess mineralization. For adipogenic differentiation, medium containing 0.5 mM hydrocortisone, 0.5 mM isobutylmethylxanthine, and 60 mM indomethacin (all from Sigma) was incubated with cells for 3 weeks. Medium was changed every 3 days. Cells were rewarshed with PBS, fixed in 10% formalin for 10 min, and stained with oil red O (Sigma) solution.

**Cell Seeding Methods**

USSCs were seeded onto bare PCL scaffolds placed in 24-well tissue culture plates at a plating density of 5 x 10^4 cells/cm² area. The USSC-seeded scaffolds were incubated in a humidified atmosphere of 5% CO₂ and 95% air, at 37°C.

**DAPI Nuclear Staining**

PCL scaffolds were fixed for 10 min in 4% paraformaldehyde solution. After washing with PBS, the disks were stained for 10 min with a 0.6 mg/l solution of 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma) prepared in PBS. The scaffolds were washed 3 times in PBS and were placed on a microscope slide and observed under the microscope (Nikon, Tokyo, Japan).

**Hepatic Differentiation**

To induce hepatic differentiation, a two-step differentiation protocol was performed: step-one differentiation medium, consisting of DMEM supplemented with 50 ng/ml hepatocyte growth factor (HGF, PeproTech EC) and 10 ng/ml fibroblast growth factor 4 (FGF-4, PeproTech EC) for 2 weeks followed by treatment with step-two maturation medium, consisting of DMEM (GIBCO BRL) supplemented with 20% fetal calf serum (GIBCO BRL), 20 ng/ml oncostatin M (OSM, PeproTech EC), 10⁻⁷ M dexamethasone (Sigma), 5 mg/ml insulin, 5 mg/ml transferrin, and 5 mg/ml selenium (ITS, GIBCO BRL). Medium change was performed twice weekly.

**Total RNA Isolation and RT-PCR Analysis**

For RT-PCR analysis, total cellular RNA was extracted using TRI reagent (Sigma). Synthesis of cDNA was carried out with M-MuLV RT and random hexamer as primer, according to the manufacturer’s instructions (Fermentas). PCR amplification was performed using a standard procedure with Taq DNA polymerase (Fermentas) with denaturation at 94°C for 15 s, annealing temperature from 55°C to 68°C for 45 s according to the primers, and extension at 72°C for 45 s. The number of cycles varied between 30 and 40, depending on the abundance of particular mRNA. The primers and product lengths are listed in table 1.

**Immunocytochemistry**

The cells were rinsed twice with PBS-Tween 20 (0.05%) and fixed with 4% paraformaldehyde (Sigma) for 20 min. The cells were permeabilized with 2% Triton X-100 in PBS, when required. The fixed cells were blocked for 30 min at 37°C with 10% goat serum/PBS-Tween 20. Cells were incubated for overnight at 4°C in a humidity chamber with the respective primary antibodies: mouse monoclonal anti-α fetoprotein (AFP, 1:200, R&D Systems), mouse monoclonal anti-albumin (ALB, 1:200, R&D Systems) and mouse monoclonal anti-cytokeratin 18 (CK-18, 1:200, Chemicon). At the end of the incubation time, the cells were rinsed 3 times with PBS-Tween 20 (0.05%) and incubated with the fluorescein isothiocyanate-conjugated secondary antibody, goat anti-mouse (1:100; Sigma), for 30 min at 37°C. After rinsing with PBS, the nuclei were counterstained with DAPI (Sigma), and the cells were then analyzed with a fluorescent microscope (Nikon).
PAS Staining for Glycogen
Glycogen storage was measured using PAS staining. The USSC-derived cells at week 6 were fixed with 4% paraformaldehyde for 15 min at room temperature and then washed 3 times with PBS. Fixed cells were oxidized in 1% periodic acid (Sigma) for 5 min and rinsed 3 times in deionized water. The cells were then treated with Schiff’s reagent (Sigma) for 15 min, and rinsed in deionized water for 5–10 min.

Cellular Uptake of Indocyanine Green
To determine cellular uptake, 1 mg/ml of indocyanine green (ICG; Sigma) in DMEM containing 10% FBS was added to cell cultures on day 28 and incubated at 37°C for 15 min. Cells were then rinsed 3 times with PBS and the cellular uptake of ICG was examined. Cell dishes were replated with fresh DMEM containing 10% FBS. The ICG was undetected in cells 6 h poststaining [Yamada et al., 2002].

Results

Morphology and Average Fiber Diameter Distribution of Electrospun PCL Scaffolds
With increasing DMF content, the bimodal distribution was slightly transferred to a unimodal distribution, but it did not disappear completely (fig. 2a–h). The 12%
Fig. 2. a–h Transition from micro- to nanofiber from a 12% PCL/solvent. a, b Pure chloroform. c, d Chloroform/DMF ratio of 97/3. e, f Chloroform/DMF ratio of 92/8. g, h Chloroform/DMF ratio of 87/13. Tip to target distance = 17 cm, flow rate = 1 ml·min⁻¹, voltage = 12 kV. i–l The role of voltage in reducing the fiber diameter in 12% PCL/(chloroform/DMF ratio of 92/8). i, j 15 kV. k, l 18 kV.
PCL solution was very viscous and the extent of splitting of fiber was too low. In figure 2, a series of samples with chloroform/DMF ratio of 92/8 at different process parameters is shown (fig. 2i–l). With increasing voltage, the fiber diameter decreases significantly. In addition, unimodal fiber distribution was observed. It seems that increasing voltage favored the splitting and splaying of the jet and improved the morphology of electrospun fibers. A chloroform/DMF ratio of 92/8 and 18 kV voltage give the smallest fibers of about 300 nm and with the narrow-

Fig. 3. Characterization and differentiation potential of human USSCs. Flow cytometry analysis of the surface markers of isolated USSCs (a). Data displayed are representative for 3 independent experiments. The cells were stained with alizarin red S for calcium phosphate precipitates (the black arrow shows mineralized bone nodule) after incubation with osteogenic differentiation medium (b) and with oil red for lipid depositions after incubation with adipogenic differentiation medium (c). Scale bars represent 100 µm.
est fiber diameter distribution; thus, these parameters were selected for in vitro assessment.

With increasing the voltage, the 1st second of fiber depositing encountered with some kind of jet instability resulted in a very thick fiber or even unshaped pieces of a solvent-free polymer. Obviously, these phenomena had some unwanted influence on fiber distribution, which is uncontrollable. However, after passing the first stage, stable jet again began the normal deposition.

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 changes in fiber morphology (data not shown).

**Characterization of USSCs**

The morphology of the USSCs was similar to that of hMSCs: spindle-shaped with fibroblast-like colonies adhering to the plastic surface (fig. 4a). The expression of surface markers of hMSCs was analyzed after isolation of USSCs. Representative histograms for USSCs are shown in figure 3a.

The undifferentiated USSCs were positive for CD10, CD44, CD166, HLA-ABC, CD90, CD54, and CD105, but were negative for CD34, CD45, CD117, CD106, and CD31. The expression of CD133 was variable in different experiments (not shown). In order to test their adipogenic and osteogenic differentiation abilities, differentiation induction medium was added to treat cells. Then differentiation was confirmed using alizarin red S and oil red O staining for osteogenesis and adipogenesis, respectively (fig. 3b, c). The results showed that placenta-derived multipotent cells have adipogenic and osteogenic differentiation abilities.

**Morphologic Changes in Cultured USSCs**

Cell morphology of the differentiated USSCs on tissue culture plate did not change greatly by step one of differentiation (weeks 1–3), though the fibroblastic morphology was lost and cells developed a broadened flattened shape. A round shape and a cuboidal morphology developed by step two of differentiation (weeks 4–6).

DAPI nuclear staining was employed to visualize USSCs over the surface of the polymer. Because PCL scaf-
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Expression of Hepatic-Specific Genes by Differentiated USSCs

RNA samples were obtained from undifferentiated USSCs, differentiated USSCs on nanofiber scaffold after 6 weeks of culture, differentiated USSCs on tissue culture plate after 6 weeks of culture, and HepG2 cell line as a positive control. mRNA expression of endodermal and hepatocyte-specific genes, such as CCAAT/enhancer binding protein (C/EBP), alpha (CEBPA), Forkhead box A2 (FOXA2), cytokeratin 8 (CK-8), cytokeratin 18 (CK-18), transthyretin (TTR), AFP, apolipoprotein B (APOB), ALB, tryptophan 2,3-dioxygenase (TDO2), phosphoenolpyruvate carboxykinase 1 (PCK1), cytochrome P450,
family 7, subfamily a, polypeptide 1 (Cyp7a1), tyrosine aminotransferase (TAT), and glucose-6-phosphatase (G6P) were analyzed by RT-PCR (fig. 6). mRNA expression of these endodermal and hepatocyte-specific genes was detected in differentiated USSCs on nanofiber scaffold, differentiated USSCs on a tissue culture plate and HepG2 cell line. In our results, undifferentiated USSCs expressed only mRNA of CEBPA, CK-8, CK-18, and APOB genes.

**Confirmation of Hepatic Differentiation by Immunofluorescence Staining**

Further, confirmation of the hepatic protein expression was done by immunofluorescence staining. On approximately week 6 the hepatocyte-like cells expressed several hepatic proteins such as AFP, ALB, and CK-18 (fig. 7a–f). The 3-D structures of differentiated cells on nanofiber scaffold were intensely stained for AFP, ALB, and CK-18, although the immunoreactivity was observed in the surrounding cells (fig. 7a–c). Undifferentiated USSCs and technical controls without primary antibody showed no immunoreactivity (data not shown).

**Glycogen Storage**

Glycogen storage was determined by PAS staining in USSCs cultured on nanofiber scaffold after 6 weeks of differentiation. Positively stained glycogen granules were detected in the cytoplasm of differentiated USSCs on nanofiber scaffold (fig. 8a). Undifferentiated cells were negative for PAS staining (data not shown). HepG2 cells were used as a positive control (fig. 8b).

**Cellular Uptake of ICG**

ICG is an organic anion that is clinically used as a test substance to evaluate liver function. ICG uptake was observed in the cytoplasm of differentiated USSCs on nanofiber scaffold and the positive cells showed green staining (fig. 8c); there was no ICG uptake in undifferentiated USSCs when stained with ICG (data not shown), indicating that ICG uptake only occurred after differentiation. HepG2 cells were used as a positive control (fig. 8d).

**Discussion**

In the present study, we show that hepatocyte-like cells differentiated from USSCs on nanofiber scaffold remained adherent to the PCL scaffold, and expressed hepatic marker genes and proteins. To assess the differentiation of USSCs into hepatic lineages, we examined mRNA and protein expressions of liver-specific markers. The results showed mRNA expression of endodermal makers, such as CEBPA, FOXA2, CK-8, CK-18, TTR, and AFP, FOXA2 (HNF3β) and CEBPA are required for hepatic specification and are important mediators of hepatocyte differentiation [Duan et al., 2007]. APOB, ALB, TDO2, PCK1, Cyp7a1, TAT and G6P are indicators of more mature hepatocytes and play important functions in the liver metabolism (fig. 6). Differentiated USSCs also expressed several endoderm and hepatic proteins such as AFP, ALB, and CK-18 (fig. 7). To determine whether cells with morphological and phenotypic characteristics of hepatocytes also acquired functional properties, functional assays such as glycogen storage and ICG uptake (fig. 8) were performed.
Fig. 7. Expression of endoderm and liver-specific proteins by differentiated USSCs on nanofiber scaffold after 6 weeks of culture (a–c). Hepatocyte-like cells expressed several hepatic proteins such as CK-18 (a), AFP (b), and ALB (c). d–f DAPI nuclear staining. Undifferentiated USSCs were negative (not shown). Representative data from one of three experiments are shown. Scale bars represent 100 μm.

Fig. 8. Glycogen storage determined by PAS staining (a, b) and cellular uptake of ICG (c, d). Positively stained glycogen granules (a) and cellular uptake of ICG (c) were detected in the cytoplasm of differentiated USSCs on nanofiber scaffold after 6 weeks of differentiation. HepG2 cells were used as a positive control (b, d). Undifferentiated USSCs were negative for PAS staining and uptake of ICG (not shown). Representative data from one of three experiments are shown. Scale bars represent 100 μm.
Positively stained glycogen granules detected in the cytoplasm of differentiated cells demonstrated a functional production and storage of glycogen by differentiated USSCs. In addition, we examined ICG uptake, which is one of the liver-specific functions used for the identification of differentiated hepatocytes in vitro [Duan et al., 2007]. ICG is an organic anion that is clinically used as a test substance to evaluate liver function because it is nontoxic and eliminated exclusively by hepatocytes [Yamada et al., 2002].

In differentiating toward the endoderm lineage, stem cells in the presence of growth factors might theoretically also enter the differentiation pathway of endodermal cells other than hepatocytes, such as pancreatic or intestinal cells but we can decrease or limit their undesired differentiation pattern by using specific growth factors and suitable procedures so that they cannot interfere with our results. According to this idea and with the knowledge that, unlike the embryonic stem cells, USSCs have no spontaneous differentiation into other lineages, we did not study other endodermal contaminations. We even consider that the dominant population of mature and functional hepatocytes can overcome the small population of undesired cells and create the desired results.

During embryonic development, the production of growth factors such as HGF and FGFs has been associated with endodermal specification. In addition, OSM, glucocorticoids and insulin are involved in the late maturation stage leading to an increase in liver-specific gene expressions [Hamazaki et al., 2001]. Moreover, DMSO has been shown to maintain hepatic morphology [Banas et al., 2007]. Hence, we designed a two-step protocol for hepatic differentiation from USSCs. aFGF, FGF-4 and HGF were added at an early stage (weeks 1–3) in order to induce the endoderm and hepatic differentiation. OSM, ITS, dexamethasone and DMSO were added as late-stage factors from week 3 until week 6.

Engineering tissues essentially requires an appropriate cell source. The appropriate use of specific cells is therefore one of the defining factors for the success of tissue engineering [Murugan and Ramakrishna, 2007].

USSCs have characteristics similar to bone marrow-derived MSCs and are capable of differentiating in vitro and in vivo into several cell lineages [Jäger et al., 2003; Kögler et al., 2004, 2005, 2006; Fallahi-Sichani et al., 2007]. The advantages of using USSCs are that they can be easily harvested and cultured and differentiated into a spectrum of tissue-specific phenotypes of mesenchymal origin, such as bone, cartilage, muscle and tendon, as well as hepatocytes. As published previously [Kögler et al., 2004], in various animal models USSCs differentiated in vitro into osteoblasts, chondrocytes, adipocytes, and neural progenitors as well as in vivo into bone, cartilage, hematopoietic cells, and neural, liver and heart tissues. In addition, USSCs compared with MSCs from other sources seem to be an immature mesodermal progenitor for MSC and are an easily accessible source of cells with a high proliferation capacity which can maintain a normal karyotype [Kögler et al., 2004]. In contrast to MSC from BM, USSCs have longer telomeres, which may explain the high expansion capacity. They have a much broader differentiation potential [Kögler et al., 2004, 2005]; therefore, they may be an ideal source of stem cells for tissue engineering. Recently, Sensken et al. [2007] described the in vitro differentiation of USSCs towards an endodermal pathway using different matrices, growth factors and organic substances. They reported that USSCs cultured in the presence of growth factors, retinoic acid, Matrigel™ matrix and different cocultures can differentiate in vitro into an endodermal-like cell with a phenotype similar to hepatic cells [Sensken et al., 2007].

Cell-cell or cell-matrix interaction is important for the maintenance of hepatocyte function and maturation. The maintenance of hepatocyte functions in vitro is useful for cellular therapies using primary hepatocytes, such as bioartificial liver assist devices or hepatocyte transplantation. Since hepatocytes are anchorage-dependent cells, there have been many efforts to maintain hepatocyte functions in vitro by adopting different culture configurations [Ng et al., 2005]. These efforts include culturing the hepatocytes or hepatocyte-like cells differentiated from stem cells on a 2-D and 3-D matrix made of synthetic polymers or from natural ECM components such as Matrigel and collagen [Schwartz et al., 2002; Imamura et al., 2004; Ng et al., 2005; Okumoto et al., 2005; Baharvand et al., 2006]. The control of the physicochemical properties of the matrix may be useful for optimizing the functional maintenance of hepatocytes in vitro [Ng et al., 2005]. Since ECM plays a major role in the regulation of cell growth and differentiation, by virtue of its capacity for the storage and presentation of growth factors, it was considered that culturing stem cells on a thin layer of ECM in the presence of growth factors might provide a superior environment to encourage their differentiation into hepatocyte-like cells [Okumoto et al., 2005]. In addition, the interactions of hepatocytes with the ECM environment are important for hepatic polarity and differentiated function maintenance [Du et al., 2008]. Control-
ling cell-microenvironment interactions such as cell-cell contact and the presentation of ECM and soluble ligands are important for the development of tissue engineering constructs and in vitro cultures that mimic the organization and complexity of normal tissue architecture [Langer and Vacanti, 1993; Khademhosseini et al., 2004]. Ultrafine nanofiber scaffolds improved mechanical strength and more extensive substrate for cellular attachment as compared to larger fibers, as a result of a higher surface area to volume ratio [Li et al., 2005a]. The electrospinning process was originally developed to produce ultrafine polymer fibers and has recently been used as a novel technique to synthesize scaffolds that mimic the architecture and mechanical properties of the ECM of native tissues [Li et al., 2005a].

The electrospinning process is a simple, economical means to produce scaffolds of ultrafine fibers derived from a variety of biodegradable polymers, and these electrospun polymers produce an interconnected porous matrix capable of encouraging significant cell proliferation and cell-cell interactions in both MSCs and mouse embryonic stem cells [Li et al., 2002; Dawson et al., 2008].

Properties of biocompatible scaffolds, synthetic or natural, that must be taken into careful consideration include optimal fluid transport, delivery of bioactive molecules, material degradation, cell-recognizable surface chemistries, mechanical integrity and the ability to induce signal transduction [Dawson et al., 2008]. Among various types of biomaterials, PCL has many advantages because of its biocompatibility, low cost, easy processability, and slow hydrolytic degradation rate. It has been used for the reconstruction of various tissues such as bone, skin, nerve and retina [Chen et al., 2007a]. However, PCL is known for its poor cellular adhesion and surface modifications are needed for any tissue engineering applications. To increase hydrophilicity, sodium hydroxide and plasma treatment, NaOH treatment was used and reported to achieve better cell adhesion. Researchers have also tried to coat ECM proteins, such as collagen [Chen et al., 2007a].

In this study, our goal was to obtain PCL scaffolds with a reduced average diameter and morphologically similar fibers with minimal variation in pore size. This was obtained by three important process parameters: concentration (viscosity), voltage, and collector screen distance. The exact effects of each of these parameters on fiber morphology are described in a separate article [Chen et al., 2006]. We observed that there is a range of electrospinning process parameters that allow us to obtain uniform fibrous scaffolds with a single modal distribution. Our studies have shown that PCL solution concentration plays a dominant role in determining these morphologies. Others have also reported similar features [Deitzel et al., 2001; Chen et al., 2006; Chen et al., 2007b].

The disadvantage of the chloroform only system is the large fiber diameter. This disadvantage was reported by Lee et al. [2003] who used the MC and DMF solvent system. MC alone could not produce PCL nanofibers. With increasing DMF volume fraction in solvent mixture, spinning was enhanced, jet splay and splitting had begun and the average diameter of the fibers was reduced (fig. 2). Lee et al. reported that as DMF volume fraction increased, surface tension and viscosity decreased while conductivity and dielectric constant increased. When PCL was electrospun without any DMF, a bimodal fiber diameter distribution was observed. These results may be explained with data of Demir et al. [2002] which suggest that repeated splaying of the fiber jets is the main reason for the development of bimodal distribution.

Nanofer scaffolds formed by electrospinning, by virtue of structural similarity to natural ECM, may represent promising structures for tissue engineering applications. Li et al. [2002, 2005a] have previously shown that electrospun 3-D nanofibrous structures share morphological similarities with collagen fibrils, and are capable of promoting favorable biological responses from seeded cells. In another study, they have demonstrated that electrospun nanofibrous scaffolds fabricated from PCL, effectively supported TGF-β1-induced chondrogenesis of adult hMSCs [Li et al., 2005b]. It has been found that fibroblasts proliferated better on a less stiff PCL membrane. Tan and Teoh [2007] investigated the effect of stiffness of the PCL membrane on fibroblast cell proliferation. Their results indicated that 3T3 fibroblasts, belonging to soft tissues, prefer to proliferate in a lower stiffness environment, which is closer to their native environment. In another study, the nanofibrous scaffold fabricated from PCL has been tested for its ability to support and maintain multilineage differentiation of hMSCs in vitro. It has been demonstrated that adult hMSCs cultured on a biocompatible and biodegradable PCL-based nanofibrous scaffold can be used to engineer adipose, cartilage, and bone, in vitro, when provided with the appropriate inductive agents [Li et al., 2005a]. However, these nanofibrous scaffolds have not been previously evaluated for their ability to support the differentiation of stem cells into hepatocytes.
In conclusion, differentiation of USSCs cultured in a nanofiber PCL scaffold demonstrates that this culture system can potentially be used as an alternative to the ECM-based culture for relevant hepatocyte-based applications in liver tissue engineering and drug discovery.

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References


