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Unrestricted somatic stem cells differentiation into hepatic-like cells in a three dimensional arrangement

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ABSTRACT

Reports describing the human cord blood derived mesenchymal stem cells (hUSSCs) hepatocytic differentiation potential are rare and there is no report describing fibrin base 3D culture of hepatocyte-like cells differentiated from cord blood unrestricted somatic stem cells. Hepatocyte-like differentiated stem cells has the potential to provide compensation for acute liver failure patients. Therefore, we characterized cord blood Unrestricted Somatic Stem Cells (USSC) and their hepatocyte differentiation capabilities for functional liver support in a three dimensional arrangement. Moreover, three-dimensional (3D) network and its distribution to grow and differentiate stem cells in so far as possible mimic to native tissue holds huge potential in liver tissue engineering. In this study, we isolate USSCs from human cord blood and validate them with flow-cytometry. Briefly, we differentiated cells using DMEM containing FGF4, Activin A, HGF and 2% FBS into hepatocyte-like cells (HLCs) in two conditions; 3D state in biocompatible fibrin Hydrogel and or conventional culture (2D) in polystyrene plates for 21 days. Immuncytochemical and gene expression analysis revealed the expression of *KRT18, KRT19, albumin, Sox17, FoxA2* and *Prox1* increased in 3D fibrin culture compared to 2D conventional culture. In combination, these data reveal that using 3D networks can resemble near natural tissue properties for effective generating HLCs which used to cell replacement in the future. As well as, USSC represent a stem cell source with a substantial hepatic differentiation capability which hold the possible for clinical applications.

Keywords: Unrestricted Somatic Stem Cells, Three-dimensional networks; Tissue engineering, hepatocytic differentiation.

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Introduction

Cell-based therapies applications have been discussed for several years as an orthotopic liver transplantation alternative. Although liver cell transplantation is already well-established, limits in appropriate organ availability and after cryoconservation diminished tissue viability impair clinical trial. Since different stem cells collected from various sources reveal a hepatic differentiation potential in vitro and in vivo; hepatocytes and hepatocyte-like cells, derived

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from hepatic or non-hepatic stem cells, are therefore considered as potential alternatives (1). In particular, mesenchymal stem cells (MSC) from cord blood (CB), adipose tissue, or bone marrow are stated as a potential stem cell source for hepatocyte or hepatocyte-like cell generation in both in vivo and in vitro approaches (2-5). Nevertheless, studies illustrating in vitro hepatic features of stem-cell-derived hepatocyte-like cells were essentially confined to a histologic and molecular level rather than a functional approach, characterizing the hepatic-like phenotype solely by a marginal panel of markers and demonstrating simultaneous marker expression in undifferentiated nonhepatic cells. Thus, standardized techniques to characterize hepatocyte functionality from differentiated cells must be considered as a necessary trailing part of researches (6-8).

In addition to hematopoietic stem cells (HSC), human umbilical CB contains, different non-hematopoietic adherently growing cell populations. CB-derived stem cells have higher telomere length and proliferation capacities compared to bone marrow derived MSC which is accompanied by a lower exposure to pathogens until extraction (9-11). CB-MSC and unrestricted somatic stem cells (USSC) share many overlapping features concerning the immunophenotype and differentiation potential. Compared to CB-MSC, USSC have a limited adipogenic differentiation potential associated with a strong expression of delta-like 1/ pre-adipocyte factor 1 (DLK-1/PREF1) (12). DLK1 is referred to as a fetal and immature tissue marker, expressed in multiple embryonic tissues but downregulated in the majority of post-natal cells. Moreover, DLK1 seems to be a highly sensitive and specific marker for hepatoblastomas and is strongly expressed in hepatoblasts of the fetal liver (13, 14) currently being the basis for isolation of hepatoblasts from murine fetal liver (15). The USSC potential to undergo partial hepatic differentiation in vitro has already been reported and was proven in vivo in the pre-immune in utero fetal sheep model (16-18). In cooperation with Ruhparwar, studies have been performed concerning liver regeneration in the mature ovine model after acute ischemia and subsequent injection of USSC into portal vein, resulting in engraftment of USSC into ovine liver parenchyma as functional hepatic-like cells without fusion (18).

With the aim of tissue engineering approaches three main components have become available in regenerative medicine; stem cells, growth factors and biomaterial which act as a scaffold. This would be very efficient to restore cells performance that no longer remain capable to carry out their function or even situated in damaged tissues.

Fibrin is a natural suitable polymer widely applied in tissue engineering applications (19-22). Fibrinogen is converted into fibrin through the reaction of thrombin in the presence of high amounts of calcium ions, during fibrin network formation (22). These reactions make 3-dimensional organized clot resembling classic wound healing which accrue in the body (22, 23). It is to remember that beside of its elasticity and biocompatibility, it provides suitable mechanical support for cell migration and cell entrapment (22).

In this study we use the fibrin complex as a three dimensional setting to mimic mechanical properties of the native liver tissue. By the way, we were used mechanical properties of the fibrin and differentiate USSCs into hepatocyte-like cells in an

aforementioned environment in comparison to conventional cell (2D condition) culture.

Materials and Methods

Isolation and characterization of human USSCs from human Umbilical cord blood

The study was performed at the proteomics Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran. All cord blood samples were negative for HTLV-I, HIV-I, and hepatitis B. We were recognized for the appropriate usage of human cord blood by the ethical and safety committee of the Shahid Beheshti University of Medical Sciences. Isolation and characterization of human USSCs was performed as described in previous studies (10, 11, 17). Briefly with some modifications, human cord blood was collected from 30 informed consent mother's umbilical cord vein. The mean age of donors was 28 years. Red blood cells were lysed, after sample collection using ammonium chloride (NH4Cl) (Sigma Chemical Co, St. Louis) and the isolation procedure was continued via Ficoll-Hypaque (GE healthcare, Hatfield, UK). A cell solution was overlaid onto the Ficoll-Hypaque gently and centrifuged at 400g for 30 minutes at room temperature. The white cell layer between the Ficoll and plasma was collected after centrifugation, and diluted with Hank's balanced salt solution (HBSS). It was then centrifuged at 400g for 10 min at room temperature. The isolated mononuclear cell layer was washed in PBS, then re-suspended in growth medium containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 mg/ml streptomycin, and 100 U/ml penicillin and containing low glucose (all provided from Gibco, Massachusetts, USA). The isolated mononuclear cell layer was then plated onto polystyrene plastic of 75 cm2 tissue-culture flasks (Nunc, Germany). The cells being cultured were maintained at 37_C in a humidified, 5% CO2 incubator (Binder, Germany). Cultures were harvested with 0.25% Trypsin–EDTA solution, when cells reached 80% confluence, (Gibco, Massachusetts, USA). Non-adherent cells were removed after 48 hr. The medium was replaced every 3–4 days. The phenotype of USSC was evaluated using mouse monoclonal antibodies against human CD105, CD90, CD31, CD34, CD44, CD45 and CD73 (all from Miltenyi Biotec, Auburn, Calif., USA). The cells were detached using trypsin-EDTA and incubated with isotype controls or the specific antibodies in 100 µl of 3% bovine serum albumin in PBS for 1 h at 4 ° C. The cells were then fixed with 1% paraformaldehyde-PBS and analyzed with a CyFlow SL machine and flowing software version 2-5-1 (free software, University of Turku).

USSCs 3D culture in fibrin gel

Fibrin gel formation organization previously described by Roozafzoon et al. (22), briefly, 1.5 mg of fibrinogen (Sigma-Aldrich Chemie GM, Steinheim, Germany) was dissolved in 0.5 ml M199 medium (Sigma-Aldrich Chemie GM, Steinheim, Germany) and 2×10^5 cells/ml of USSCs were loaded to 500µl of the prepared fibrinogen solution then added to 24-well culture plate. Then, 15µl of a thrombin solution (120 U/ml in 1 M CaCl2, NaCl, pH: 7.4) (Sigma-Aldrich Chemie GM, Steinheim, Germany) and 50µl of fetal bovine serum (FBS) were added to fibrinogen solution (3 mg/ml). After gelatinization at 25 C, the dish was placed in an incubator at 37C and 98% relative humidity for 1 h to maintain its final three dimensional complex structures.

Hepatocyte in vitro differentiation

For Hepatocyte differentiation, USSC were plated at 5×10^4 cells/cm^2 in DMEM (Gibco, UK) containing 30% fetal bovine serum (FBS; Gibco,

UK). After 72 h, when cells reached a confluency of 80%, plates were washed twice with PBS and incubated with ''pre-induction medium'' (PIM) (Figure 1A) enriched with 100 ng/ml activinA for 24 h. Subsequently, for 4 days, PIM was supplemented with 100 ng/ml activinA, 20 ng/ml FGF4 (0.5% FCS for first 2 days, 2% FCS on days 3 and 4). Additionally, cells were cultured in PIM with 2% FCS, 1_ITS (5mg/ml insulin, 5mg/ml transferrin, 5 ng/ml selenium), 5 ng/ml HGF, 10 ng/ml FGF4, 5 ng/ml BMP2, 10 ng/ml EGF, and 1 mM RA for two more days. After pre-induction, PIM was changed into serum-free ''hepatocyte differentiation medium'' (HDM) enriched with 10 ng/ml FGF4, 20 ng/ml HGF, 10 ng/ml EGF, and 1mM RA for 7 days of hepatic induction. For subsequent hepatic maturation, HDM was supplemented with 10 ng/ml FGF4, 20 ng/ml HGF, 30 ng/ml OSM, and 10^{-7}M dexamethasone (Fig. 1A). Cytokines, growth factors with cell culture additives were purchased from sigma company (Sigma-Aldrich Chemie GM, Steinheim, Germany) (activinA/FGF4/BMP2/OSM), Sigma–Aldrich (Sigma-Aldrich Chemie GM, Steinheim, Germany) (EGF/dexamethasone), and Peprotech (Hamburg, Germany) (HGF). Culture of human hepatocytes (Primacyt Cell Culture Technology, Schwerin, Germany).

Gene expression analysis by real time PCR

Real time-PCR was performed to evaluate the expression of Sox17, FoxA2, Prox1, KRT18 and KRT19 genes. Total RNA was isolated from the cells using an RNA extraction kit (Fermentas, Ontario, Canada). To remove contamination of genomic DNA, RNA samples were treated with DNaseI (Fermentas, Ontario, Canada). The primer sequences used are shown in Table 1. Reverse transcription was performed using the RevertAid first strand cDNA synthesis kit (Fermentas, Ontario, Canada) using 2 mg of total RNA per reaction, according to the manufacturer's instructions. The PCR thermal cycling program was as follows: initial denaturation at 95$\mathring{C}$ for 1 minute, followed by 40 cycles of 95$\mathring{C}$ for 40 seconds, annealing temperature for 40 seconds, and extension at 72$\mathring{C}$ for 1 minute, with a final extension at 72$\mathring{C}$for 5 minutes. Levels of mRNA for tested genes were quantified using $^{\Delta\Delta}$CT

Table 1. Primer Sequence for evaluation of hepatocyte-like cell

Primers	Sequence (5'>3')	Tm
KRT-18	F: TTGGCCACCCCGTTTCTGG	60
289bp	R: TCGTTCAGGCTTTGCATGGT	
KRT-19	F: AGAATTGAACCGGGAGGTCG	59
342bp	R: GCTGTAGGAAGTCATGGCGA	
Sox17	F: GGGTACGCTGTAGACCAGAC	61
317bp	R: TCGCCCTTCACCTTCATGTC	
GAPDH	F: GATGCCCCCATGTTCGTCATG	60
	R: GGGTGTCGCTGTTGAAGTCAG	
FoxA2	F: TGATTGCTGGTCGTTTGTTGT	60
155bp	R: TCATGTTGCTCACGGAGGAG	
Prox1	F: GAGGGTGGGAAAGGGGTTTT	59
204bp	R: TCAAACGGCACTGAGCTTGT	

method and normalized against human GAPDH as a housekeeping gene.

Immunocytochemistry Analysis

To confirm results from real time PCR, Immunocytochemistry (ICC) test were performed for candidate markers (KRT18, KRT19). The hepatocyte like cell from umbilical cord blood USSCs were used for ICC. The cells were fixed in 4% paraformaldehyde, incubated at 4$\mathring{C}$ for 30 minutes, and then at room temperature for 30 minutes. After two times washing with cold PBS, 0.4% Triton X-100(Sigma-Aldrich Chemie GM, Steinheim, Germany) was added to the cells. Following incubation at room temperature for 40 minutes, the cells were rinsed with 0.1% PBS-Tween and placed in a blocking solution containing 1% human serum albumin in PBS for 30 minutes. After washing two times with PBS, the primary antibodies Anti-KRT18, Anti-KRT19 were added to the cells and then incubated at 4 $\mathring{C}$ for 12 hours in a dark room. Following three times washing with 1% PBS-Tween solution, the secondary IgG PE-conjugated antibody (Sigma-Aldrich Chemie GM, Steinheim, Germany) was added to the cells at a 1:30 dilution. After 3 hours, the cells were washed with PBS-Tween solution. DAPI 0.5 µg/ml (Sigma-Aldrich Chemie GM, Steinheim, Germany) was used to counter stain the nuclei.

Periodic Acid–Schiff Staining (PAS) for glycogen

The functional assay of the hepatocyte-like cells was performed by PAS staining for glycogen storage detection. The control and experiment cultures were fixed with 4% paraformaldehyde. The samples then were washed in distilled water and incubated in 0.5 % periodic acid for 5 min rinsed three times in de-ionized water (dH2O). Consequently, the cultures were washed and bathed with Schiff's reagent for 15 min. Samples were counterstained with Mayer's hematoxylin for 1 min, rinsed in dH2O, and examined by light microscopy.

Results

USSC were Isolated and Characterized *in vitro*:

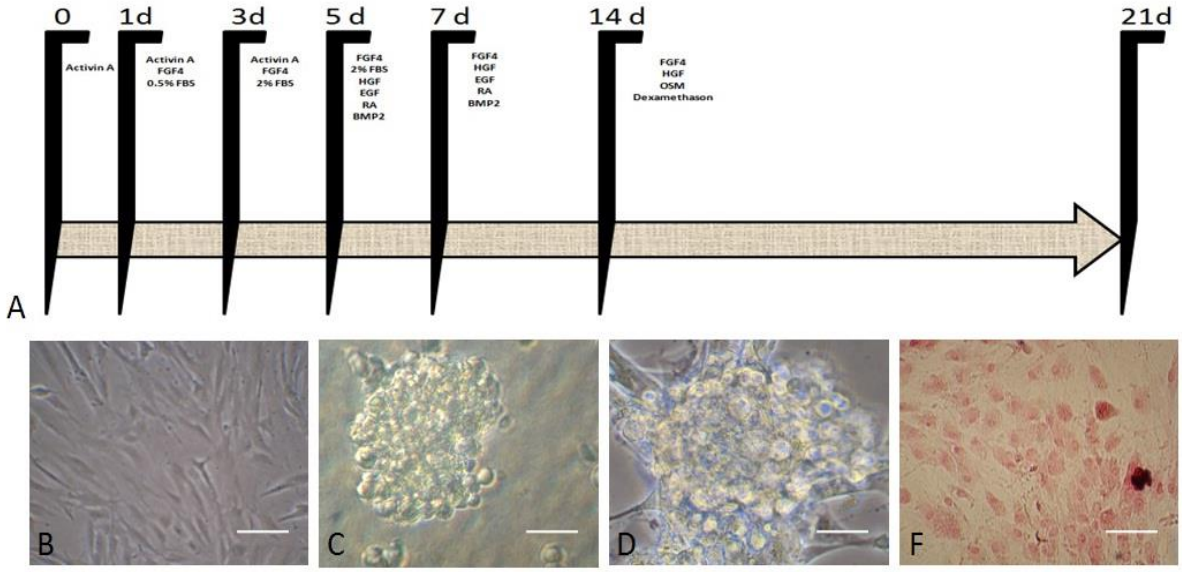

Figure 1. (A) Schematic overview of the (days1, 3, 5, 7, 14, and 21) protocol for hepatic differentiation. (B) Fibroblast like morphology of isolated USSCs and (C) The encapsulated cells on fibrin gel on 7th days and (D) 14th days of continually culture (F) Glycogen storage in differentiated USSCs clusters, as determined by PAS staining. The scale bar is 20 µm.

USSC appeared in the culture as adherent growing colonies at about 1–2 weeks after isolation. All cells were uniform and spindle-shaped (Figure 1b). Surface marker analysis by flow cytometry showed that the cells were positive for CD105 (98%), CD90 (97%), CD166 (86%), and CD44 (99%) but were negative for CD34 (0.004) and CD45 (0.005%) (Figure 2). More extensive characterization of these cells is available in our previous publications (1-5).

Morphology and differentiation of USSCs into cells expressing hepatocyte-like cells markers

After USSCs treating with hepatocyte cell's differentiation media for 48 hours of in the 2D and 3D cultures, cells were induced through hepatocyte-like cells. Their morphology started to change gradually to cuboidal-like cell especially after day seven. (Figure 1C and D). In 3D cultures, cells more clamped than conventional 2D cultures (Figure 1C and D). Immunofluorescence data revealed differentiated cells expressing hepatocyte markers as well as *KRT18, KRT19, albumin, Sox17, FoxA2* and also *Prox 1* as one of selective markers of hepatocyte cell in the both 2D conventional culture and 3D fibrin scaffold (Figure 3A and B). The real-time PCR analysis for neuronal cell markers as well as *KRT18, KRT19, albumin, Sox17, FoxA2* and also *Prox 1* in USSCs derived hepatocyte-like cells revealed from the 7th day after treatment with hepatocyte cell's induction media. The results demonstrating hepatocyte morphology expected to direct differentiation.

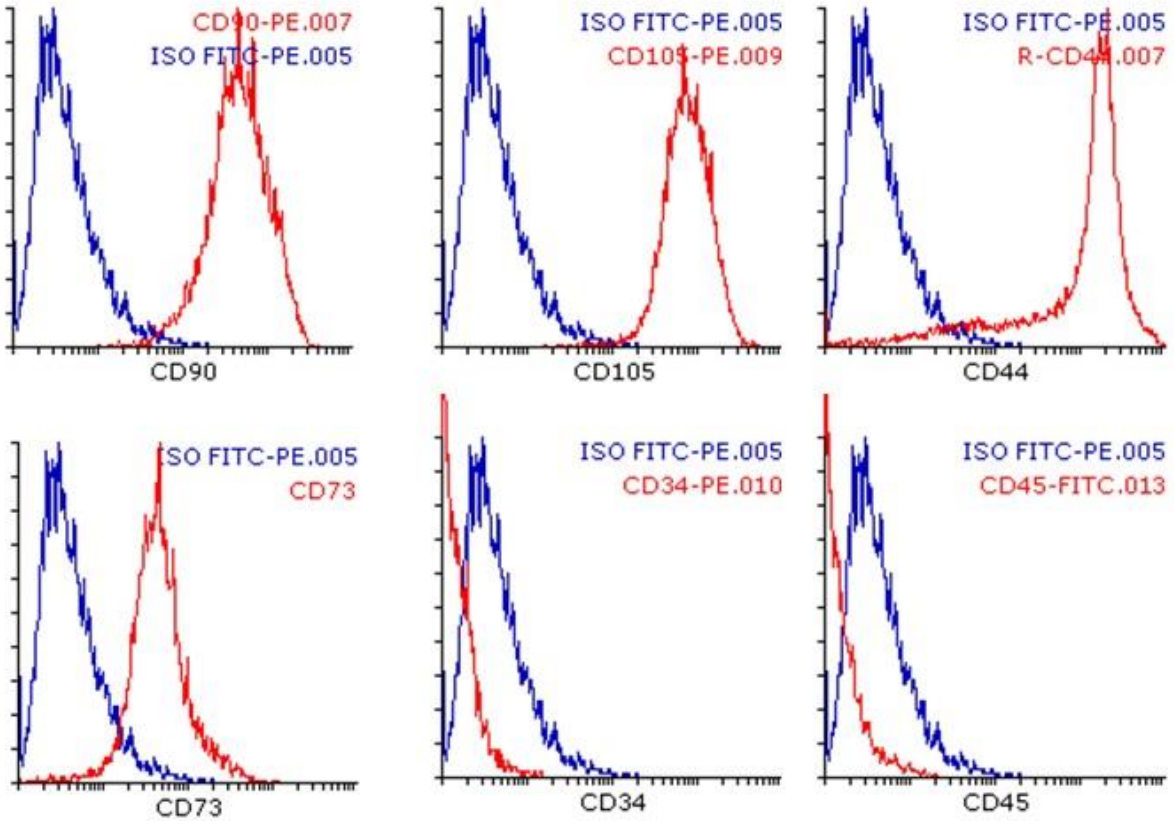

Figure 2. Flowcytometric analysis; CD90, CD105, CD73, CD44 were positive and CD34, CD45 and CD31 were negative. The red line indicates for positives and blue one for negatives.

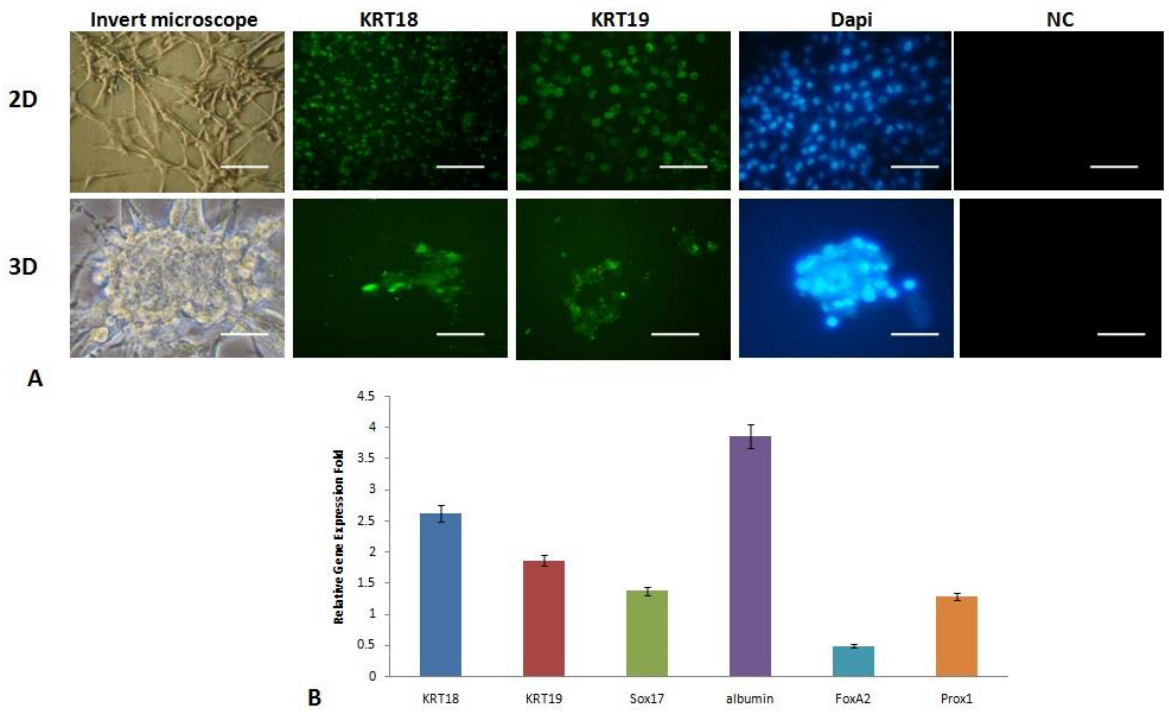

Figure 3. (A) Immunofluorescent staining for expression of Hepatocyte markers in cord blood USSCs after 21 days post-treatment by induction media in 2D and 3D culture. Nuclei are stained with DAPI. (B) Relative gene expression of hepatocyte cell mRNA in 3D environment compared to 2D. The scale bar is 20 µm.

According to Figure 3B mRNA expression level showed that the expression of *KRT18, KRT19, albumin, Sox17, FoxA2* and also *Prox 1* was elevated significantly after differentiation in 3D fibrin culture compared with 2D conventional culture 2.63-fold, 1.87-fold,3.87, 1.38, 0.497 and 1.29-fold respectively.

Discussion

In this research stem cells were isolated from human cord blood by plastic adherence, and only cells at passage 3 were applied in the study. The results showed that in addition to presenting morphological features and phenotypical characteristics of CD44+,CD105+, CD73, CD90+ and CD34-, CD45- (Figure2), the obtained cells were stem cells, and originally non-hematopoietic (9-11).

Here, we have developed a generation of hepatocyte-like cells from USSCs in a 3D network of fibrin scaffold which may eventually have a great alternative potential in liver-associated disease treatment. So this research could help to figure out the effect of fibrin gel providing a 3D network resembling the natural environment of hepatocyte cell layer on hepatocyte-like cell differentiation.

Unrestricted somatic stem cells (USSCs) are derived from human umbilical cord blood, placental blood and/or blood samples from new-borns. Somatic stem cells are distinct but capable to differentiate into mesenchymal cells (bone cells, chondrocytes), endodermal cells, neural cells, but not into endothelial stem or progenitor cells and hematopoietic lineage stem or progenitor cells and adipocytes characterized in that the USSC expresses the DLKl -gene the

expression being preferably detectable and measurable using RT-PCR assay and/or on the protein level and that it is a clonally selected and expanded cell (10, 16, 17). Unrestricted adherent pluripotent isolated cells from umbilical cord blood (USSCs) have the capability to differentiate into osteoblasts, blood cells, chondrocytes, neurons, hepatocytes, and heart tissue under *ex vivo* conditions (10, 17). USSCs express different factors, including growth factors, adhesion molecules and various cytokines such as SCF, TGF-1b, IL-6, G-CSF, VEGF, GM-CSF, M-CSF, LIF, Flt3 ligand, TPO, SDF-1a, IL-15, IL-12, HGF, IL-8, and IL-1b (10). They can also be applied as a reliable feeder layer. Moreover, human umbilical cord blood (CB)-derived USSCs have previously been proved to have a vast differentiation potential and regenerative benevolent effects when applied in animal models of multiple degenerative diseases. It has also been suggested that CB-derived USSCs could significantly contribute in wound healing and be potentially used in cell therapy in some genetic skin diseases including recessive dystrophic epidermolysisbullosa (10, 11).

The human cord blood unrestricted somatic stem cells differentiation capability toward hepatocytes was shown previously (24, 25). The findings of this study along with the others have been revealed that umbilical cord USSCs could express levels of hepatocyte markers (24, 25). It has been documented that other MSCs from cord derived such as Wharton's Jelly-derived mesenchymal stem cells could not express adult hepatic markers; however, the constitutive expression of liver progenitor markers along with transcription factors involved in liver development were reported in this type of cord derived stem cell (26).

Then the appearance of genetic characteristics of hepatic differentiation of USSCs at the mRNA level was confirmed by testing total RNA developed from undifferentiated and differentiated USSCs by Real Time-PCR for mRNAs of KRT18, KRT19, Sox17, FoxA2 and Prox1 (early and middle marker), and albumin (middle and late marker). The results showed the appearance of the hepatic lineage after differentiation.

We therefore deduced that the differentiated cells at that time had both stem cells and hepatic cells characteristics and were at the borderline between immature and mature states. Thirdly, the immunocytochemistry results revealed that albumin, absent in freshly isolated USSCs, appeared after differentiation.

In addition, to examine the function of the differentiated cells, PAS staining performed to test their ability to synthesize and store glycogen, and tested albumin and urea in the supernatants, three characteristics of functional hepatocytes. The results show that most cells stained pink after 3 weeks of differentiation, showing that the cells had finally differentiated into functional hepatocyte-like cells.

Conclusion

This study demonstrates that USSC explain a substantial hepatic differentiation potential and represent an attractive cell type for clinical applications. USSC have several advantages including high telomere length, Pluripotency ability, GMP grade generation, extensive proliferation potential in addition to positive immunomodulatory effects accompanied by low inflammatory signaling compared to MSCs and other adult stem population cells.

Conflict of Interest

Not Declared.

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