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Comparison of hepatic nuclear factor-4 expression in two- and three-dimensional culture of Wharton's jelly-derived cells exposed to hepatogenic medium

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Abstract

The research on Wharton's jelly-derived mesenchymal stem cells (WJ-MSCs) from the umbilical cord suggests promising therapeutic use for hepatocyte replacement therapy. One of the highly conserved members of the nuclear receptor superfamily in the liver is hepatocyte nuclear factor-4α (HNF4α), involved in hepatocyte differentiation. The objectives of this study were to determine the effects of two- and three-dimensional (2D and 3D) cultures of WJ-MSCs on hepatocyte differentiation. MSCs were isolated from human Wharton's jelly, characterized by flow cytometry, and differentiated toward osteogenic and adipogenic lineage. WJ-MSCs were cultured in 2D collagen films and 3D collagen scaffolds in the presence of hepatogenic media with or without pre-treatment with fibroblast growth factor-4 (FGF4) for 21 days. The expression of HNF4α was evaluated using quantitative real-time polymerase chain reaction (qRT-PCR). According to flow cytometry data, the cells isolated from Wharton's jelly were shown to express MSC markers. HNF4α expression analysis revealed that pre-exposing the cells with FGF4 was more effective in hepatocyte differentiation. 3D cultures also improve the expression of HNF4α compared with 2D culture system. In conclusion, the combination of FGF4 and 3D culture improved hepatocyte differentiation. It seems 3D interaction of the cells improved the hepatogenesis.

Keywords: mesenchymal stem cell, 2D, 3D, collagen, fibroblast growth factor-4, hepatocyte nuclear factor-4α.

Introduction

Chronic liver failure such as cirrhosis is one of the life-threatening liver diseases with severe complications [1]. The most effective therapy for end-stage liver disease is liver transplantation. Due to the limited availability of donors, the complex surgical procedure, immunological rejection, and high costs, new treatment modalities, such as stem cell therapy, were explored. Hepatocyte transplantation is the best therapy for end-stage liver disease [2].

Different sources of stem cells have been used for differentiation of the hepatocyte-like cells. Umbilical cord is an available and non-invasive source of stem cells with no ethical concern. Mesenchymal stem cells (MSCs) derived from the human umbilical cord, Wharton's jelly, can be considered as one of the best cell sources for hepatocyte differentiation and transplantation among various therapeutic strategies and stem cells therapy [3–7]. Wharton's jelly MSCs (WJ-MSCs) have more self-renewal capacity as well as the ability to differentiate into various cell types, including adipocyte, osteocytes, and chondrocytes [8, 9]. These cells have capability to express some early and mid-stage liver specific markers such as albumin, α-fetoprotein, cytokeratins 18 and 19 [10, 11].

One of the highly conserved members of the liver nuclear receptor super family is hepatocyte nuclear factor-4α (HNF4α) [12]. The role of HNF4α in development, organogenesis, and hepatocyte functions has been confirmed by its conserved expression pattern. HNF4α is expressed at the highest level in the liver. It is also expressed in the kidney, intestine, and pancreas of mammals [13, 14]. It is considered as a mediated transcriptional gene that regulates the expression of many hepatocyte genes [15]. It has a significant role in differentiation of the hepatocyte lineage. In the mouse embryo, the first expression of HNF4α is in the liver bud and gut tube at embryonic day 8.5, and then in the developing pancreas, kidney and in extra-embryonic visceral endoderm of the yolk sac [16]. The initial stages of hepatocyte differentiation are started by HNF4α that is accompanied by reduction in chromatin-mediated repression, resulting in a basal expression level of hepatocyte genes [17].

Many protocols are available for induction of the stem cells to differentiate into hepatocytes. It was shown that the combination of hepatocyte growth factor (HGF), insulin-like growth factor-I (IGF-I), and oncostatin M (OSM) induced the bone marrow-derived MSCs toward hepatocyte-like cells [18, 19]. Fibroblast growth factor-4

(FGF4) is one of the important factors in hepatocyte differentiation. FGF4 acts as a mitogenic and angiogenic factor. The proliferation and differentiation of MSCs toward the endoderm and subsequently hepatogenic fate are induced in the presence of FGF4 [20, 21]. Moreover, collagen type I plays an important role in regulation of hepatocyte cell cycle and differentiation [22].

Three dimensional (3D) culture system mimics *in vivo* environmental condition; moreover, it can improve cell–cell contact, cell migration, and gene expression pattern. It was shown that the functional efficiency of the cells can be enhanced by *in vitro* usage of extracellular matrix (ECM) constitutions, such as collagen [8, 23]. Furthermore, 3D culture system has previously been shown to enhance the expression of liver-specific markers [11]; however, we hypothesized that a combination of FGF4 and hepatogenic medium with 3D culture system can improve hepatocyte differentiation. With regard to these considerations, the current study aimed to differentiate the WJ-MSCs toward hepatocyte-like cells on the collagen scaffold in the presence of hepatogenic factors, and also compare the expression level of HNF4α in 2D and 3D culture systems.

Materials and Methods

Isolation of mesenchymal stem cells

The mesenchymal stem cells were isolated from the umbilical cord of the newborn infants born through cesarean section after getting written informed consents from their parents. Thereafter, the umbilical cords were transported to the laboratory in cold phosphate-buffered saline (PBS) containing 100 µg/mL Penicillin and 100 U/mL Streptomycin. The arteries and amnioblast were completely removed and the umbilical vein was incised lengthwise and crushed. For generation of explants with 5 mm dimension, the Wharton's jelly was cut into small pieces. After 15 minutes, the complete α-MEM (Minimum Essential Medium, Gibco), containing 10% Fetal Bovine Serum (FBS), 1% L-Glutamine, 100 µg/mL Penicillin, and 100 U/mL Streptomycin, was also added to the explants. The cells derived from Wharton's jelly reached 70–80% confluence after two weeks.

Surface marker characterization

The WJ-MSCs were characterized by expression of cell surface markers with flow cytometry at the third passage. The cell suspension (1×10^6 cells/mL) was washed in blocking solution, cold PBS containing 10% FBS, for 20 minutes. After that, the cells were labeled with FITC (Fluorescein isothiocyanate)-conjugated anti-CD44, anti-CD90, and anti-CD144, Phycoerythrin-conjugated anti-CD34, anti-CD73, and anti-CD106, and PerCP (Peridinin Chlorophyll Protein complex)-conjugated anti-CD105 antibodies (all from Abcam, UK, Cambridge). They were then washed with cold PBS, resuspended in PBS containing 10% FBS, and analyzed by a flow cytometer (FACS Calibur™, BD Biosciences).

Osteogenic and adipogenic differentiation potential of WJ-MSCs

To assess multipotency capability of the WJ-MSCs, their ability to differentiate into osteoblasts and adipocytes were also evaluated. The osteogenic and adipogenic differentiation of the WJ-MSCs were performed in the presence of osteogenic (MACS, Germany) and adipogenic media (StemCell Technologies, Inc., Canada) for four and three weeks, respectively. The differentiated cells were first fixed with 4% paraformaldehyde and then stained with Alizarin Red S (Sigma, USA) and Oil Red O (Sigma, USA), respectively.

Collagen film preparation

The working solution (1 mg/mL) was prepared from stock solution of collagen type I, rat-tail (3 mg/mL) (code A10483-01, Gibco). Ten times (10×) DMEM (Dulbecco's modified Eagle's medium) were mixed with collagen at 1:8 ratios on ice by slow and gentle pipetting. Then, 125 µL of the working solution was added per well of 4-well plate and polymerized at 37^0C for one hour. Thereafter, 1×10^3 cells were seeded per well.

3D culture

The working solution of collagen was prepared by the method mentioned in the previous section. Wharton's jelly-derived MSCs at a density of 7×10^5 cell/mL were mixed with working solution of collagen. The final concentration of the collagen was 1 mg/mL. The collagen gel was polymerized at 37^0C for one hour. After polymerization, the complete DMEM (Gibco), low glucose, containing 5% FBS + Penicillin/Streptomycin, was added.

Hepatocyte differentiation

First, the cultures in both 2D and 3D were divided into three groups. The control group was incubated at DMEM supplemented with 5% FBS, 100 µg/mL Penicillin, and 100 U/mL Streptomycin. The first experimental group was exposed to DMEM supplemented with 5% FBS, 20 ng/mL HGF, 10ng/mL IGF-I, 100 nM Dexamethasone, 100 µg/mL Penicillin, and 100 U/mL Streptomycin for seven days. Then, 10 ng/mL OSM was added to the hepatocyte differentiation medium and the cells were incubated for an additional two weeks. Finally, the second experimental group was first incubated with DMEM supplemented with 5% FBS and 10 ng/mL FGF4 for two days and thereafter the culture medium was replaced with DMEM supplemented with 5% FBS, 20 ng/mL HGF, 10 ng/mL IGF-I, 100 nM Dexamethasone, 100 µg/mL Penicillin, and 100 U/mL Streptomycin and incubated for an additional five days. The differentiation protocol was continued up to day 21 (Figure 1).

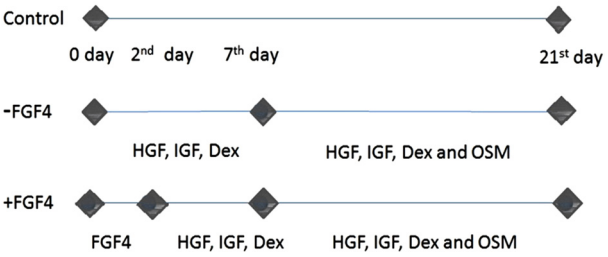

Figure 1 – ***Schematic diagram of the protocol steps for each experimental group.***

During the 14 days of differentiation, 10 ng/mL OSM was added. Fifty percent of the culture medium was changed every three days.

RNA extraction and quantitative real-time PCR analysis

Biazol RNA isolation protocol (Bioflux, Japan) was used for the extraction of RNA from WJ-MSCs. cDNAs were synthesized using 5 µg of total RNA and Applied Biosystems™ (ABI) cDNA Synthesis Kit (USA). The human specific primers were designed in NCBI (National Center for Biotechnology Information) Primer-BLAST (Basic Local Alignment Search Tool) [24] (Table 1). The amplification of cDNA for real-time PCR reactions was performed using the SYBRs Green PCR Master Mix (Applied Biosystems, Rotkreuz, Switzerland) during 50 cycles. First, the melting curve was analyzed to ensure the specificity of the PCR products and the accuracy of the reactions was established. The reference gene, 18S rRNA, was compared to each target gene, for normalizing of qPCR data. Each data point was calculated by ΔΔCt values (ΔCt experiment – ΔCt control).

Table 1 – ***The sequences of primers used for qPCR/real-time PCR***

Gene	Forward primer	Reverse primer	Annealing temperature [^{0}C]
HNF4	5'-AAGAAATGCTTCCGGGCTGG-3'	5'-GACGGGGGAGGTGATCTGTC-3'	57
18S	5'-GTTGATTAAGTCCCTGCCCT-3'	5'-TCCGAGGGCCTCACTAAACC-3'	60

Statistical analysis

All the statistical analyses were performed using GraphPad Prism 5 software (GraphPad, USA) and Mann–Whitney test. *P*-value <0.05 was considered as statistically significant. All the experiments were performed in triplicate.

Results

Cell morphology

The cells cultured on 2D collagen film showed spindle fibroblast-like morphology that is a typical characteristic of MSCs. The morphology of the cells exposed to hepatogenic media modified the cell shape from spindle fibroblast-like into a polygonal- and epithelial-like. The morphology of WJ-MSCs in 3D culture system was spindle or star-shaped; however, the cells treated with hepatogenic media were changed into a round shape. Also, some cells had a tendency to enclose a luminal space in the 3D scaffolds. In both culture conditions, WJ-MSCs proliferated more rapidly in response to the growth factors in the hepatogenic media as indicated by the higher density of the cells compared to the control cells (Figure 2).

Characterization of WJ-MSCs

The results of the flow cytometry analyses showed that the WJ-MSCs were positive for the MSC surface markers, such as CD90 (thy-1) (85.3%), CD73 (ecto-5'-nucleotidase) (99.8%), CD44 (adhesion molecule) (92.4%), CD106 (31.2%), and CD105 (51.5%) (cell adhesion molecules). However, these cells were negative for CD34 (hematopoietic stem cells) (0.93%) and CD144 (endothelial cells) (0.99%) markers (Figure 3).

Osteogenic and adipogenic differentiation potential of WJ-MSCs

The osteogenic and adipogenic differentiation of WJ-MSCs were assessed by Alizarin Red S and Oil Red O staining, respectively (Figure 4).

The cells cultured in osteogenic media could differentiate into osteoblast and restore Ca^{2+}. WJ-MSCs also could store lipid droplet in the presence of adipogenic media. These results confirmed the capability of the cells to differentiate into various lineages that indicated its pluripotency.

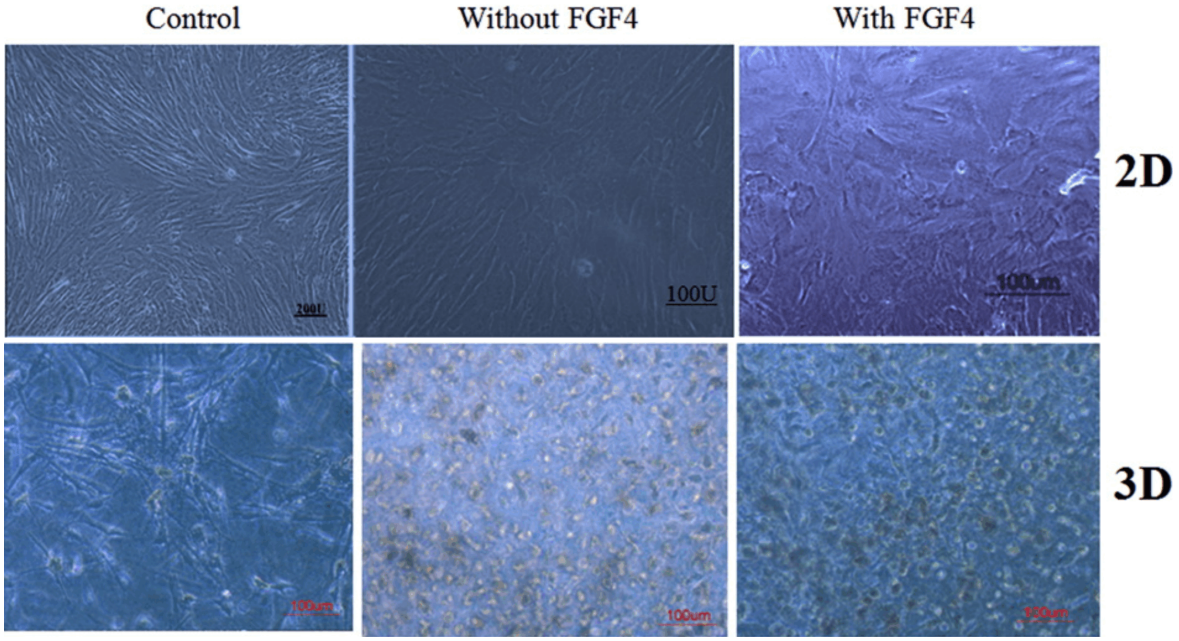

Figure 2 – ***The inverted microscopy of the cells cultured in 2D collagen film and 3D collagen scaffold showed that the cell morphology was changed in the cultures exposed to hepatogenic media with or without FGF4 compared to the control cultures.***

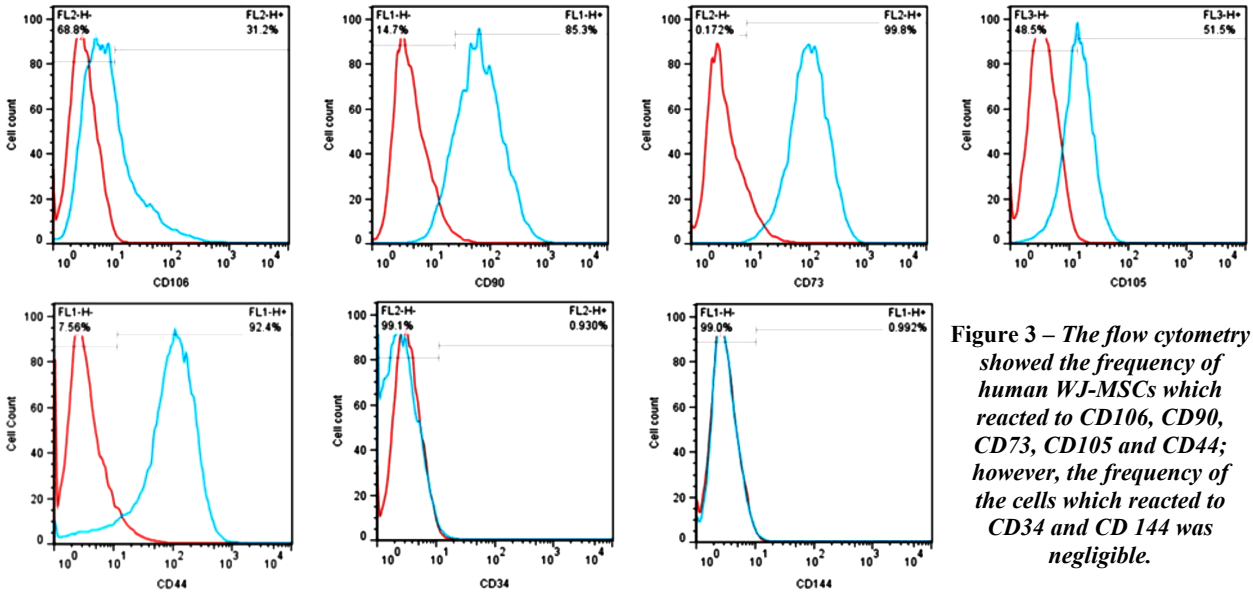

Figure 3 – ***The flow cytometry showed the frequency of human WJ-MSCs which reacted to CD106, CD90, CD73, CD105 and CD44; however, the frequency of the cells which reacted to CD34 and CD 144 was negligible.***

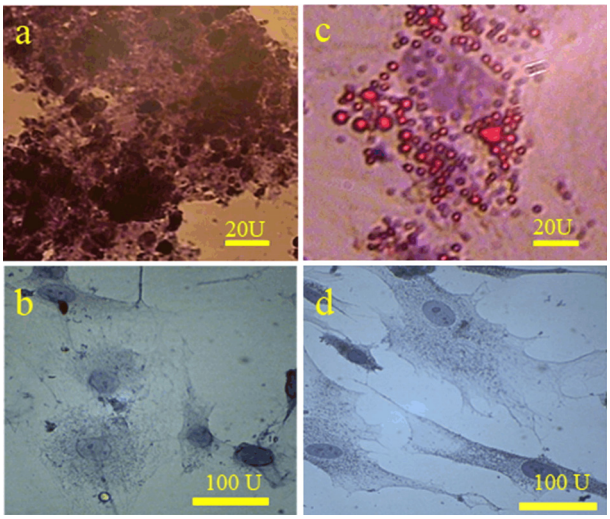

Figure 4 – ***The cells deposited Ca^{2+} in the presence of osteogenic medium stained with Alizarin Red S (a) and control (b); Store lipid droplets in the presence of adipogenic medium stained with Oil Red O (c), and control (d).***

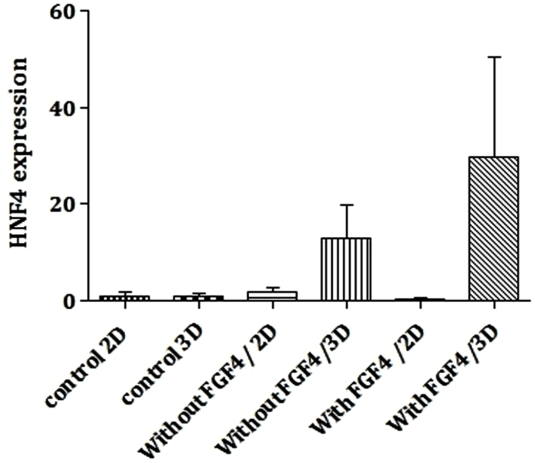

Figure 5 – ***RT-PCR showed the expression of HNF4α in 2D culture films, and 3D collagen scaffolds.***

Quantitative real-time PCR (qRT-PCR)

HNF4α expression was assessed in the cells cultured in all conditions for 21 days. The expression of HNF4α by naïve human WJ-MSCs were negligible both in 3D and 2D culture conditions. In 3D culture conditions, the highest level of the HNF4α mRNA expression was identified in differentiated cells pre-exposed to FGF4 (29-fold) compared with related control cultures, while the cells exposed to hepatogenic media without FGF4 showed 17-fold more HNF4α compared to corresponding control cells. The expression of HNF4α did not change in differentiated cells pre-exposed to hepatogenic media with or without FGF4 compared to control in 2D culture condition (Figure 5).

Discussion

The culture system was designed by growing the FGF4 pre-exposed cells in 3D and 2D environment using collagen in the presence of the growth factors such as HGF, IGF-I, and OSM. HNF4α can be considered as a gene with critical role in hepatocyte differentiation [14]. Despite changes in cell morphology in presence of collagen, our results showed that a negligible amount of HNF4α is expressed by naïve human WJ-MSCs in control cells. Also, some cells had a tendency to enclose a luminar space in the 3D scaffolds. There is a controversy regarding the expression of HNF4α in naïve human WJ-MSCs. The capability of the naïve human WJ-MSCs to express HNF4α was confirmed by some researchers [10, 25] while the other one has shown that these cells could not express HNF4α [26].

Human WJ-MSCs were considered as an appropriate source for liver cell therapy. Various culture systems influence the cell gene expression pattern and especially expression of the liver-specific markers. The previous study showed that culturing of embryonic stem cells within 3D alginate scaffold in the presence of the hepatogenic medium improved the efficiency of the hepatocyte differentiation and function [23]. It was also reported that the cell attachment, hepatocyte phenotype [27], and maturation of the hepatocytes differentiated from human pluripotent stem cells also improved in 3D collagen culture [28]. Our previous work showed that the 3D culture of the WJ-MSCs in collagen scaffold led to an increase in early and mid-stage liver specific markers, such as albumin,

α-fetoprotein, cytokeratins 18 and 19 but not HNF4α compared to the cells cultured on 2D collagen film [11].

The data from the comparison of the 3D and 2D culture system showed the positive influence of 3D culture in the expression of HNF4α. The data also revealed the combination of the FGF4 and 3D culture improved the expression of the liver specific marker, HNF4α. An increase in the expression level of HNF4α, as a key regulator of the liver development [16], was shown in the cells pre-exposed to both FGF4 and cultured in 3D condition compared with corresponding control cultures and the cells exposed to hepatogenic media without FGF4. It was demonstrated that the differentiation of human embryonic stem cells toward the hepatic fate correlates with the nature of the culture conditions. The loss of signals, such as matrix interactions and 3D structure in the culture system, leads to lack of HNF4α expression in the hepatocyte-like cells differentiated from human embryonic stem cell [17]. The beneficial impact of FGF family on the endothelial cells cultured on the collagen/heparin film was shown previously [29, 30]. It indicated the combination of the FGF4 and 3D culture exerted a condition to accelerate the hepatogenic differentiation.

The expression of HNF4α, as the main hepatocyte gene, was increased in differentiated cell into hepatocyte-like cells [13]. Moreover, the other investigation showed that HNF4α was required for expression of several factors that were secreted from the visceral endoderm, and it also generates an environment that could support gastrulation within the embryo [31]. The investigations demonstrated that FGFs could induce the human embryonic stem cells to express HNF4α [13, 31]. Liver-enriched transcription factors, such as HNF4α, induce transcription of genes involved in various liver specific functions including postnatal lipid, cholesterol, and bile acid homeostasis. Moreover, the transfection of the cells with HNF4α caused the cells to express the other liver-specific markers [32]. Therefore, increase in the HNF4α expression may lead the cells to differentiate into more functional hepatocyte-like cells.

Conclusions

The results of this study demonstrated that both FGF4 and 3D collagen scaffold are useful in increasing the expression level of HNF4α, as a pivotal gene in inducing the cells toward hepatocyte-like cell.

Conflict of interests

The authors declared a potential conflict of interests regarding employment contracts.

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