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Chemical Induction of Human Adipose Stromal Cells Into Hepatocyte-Like Cells under Various Differentiation Conditions

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Abstract

Background: A shortage of donor livers for transplant has led to an increased interest in cell therapeutics as an alternative to whole organ transplant to treat end-stage liver disease. Primary human hepatocytes have been used in cell-based therapies. However, hepatocytes do not proliferate *in vitro* so it is challenging to grow enough cells for a successful transplant. Many have suggested using hepatocyte-like Adipose-derived mesenchymal Stromal/stem Cells (ASCs) differentiated into hepatocyte-like cells as a substitute. Here we evaluate how closely these cells resemble primary hepatocyte cell morphology and function.

Methods: Human ASCs were mechanically isolated from lipoaspirates. The stem cell nature of ASCs was characterized using flow cytometry and tri-lineage differentiation into osteocytes, adipocytes, and chondrocytes. ASCs were differentiated into hepatocyte-like cells in culture using various protocols that included combinations of growth factors and small molecules. Primary ASCs quickly attached and proliferated *in vitro,* forming a homogeneous spindle-like cell monolayer. Mesenchymal stem cells showed high expression of the markers CD73, CD90, CD271, CD44, CD166, CD105, and successfully differentiated into osteocytes, chondrocytes, and adipocytes. ASCs were cultured on type I collagen coated plates and differentiated into hepatocyte-like cells using 5 different protocols.

Results: The ASCs differentiated into hepatocyte-like cells, using protocol C (induction with FGF4 and maturation with HGF, ITSPre, Dex, OncM and 2% serum), displayed a cuboidal morphology. Bioactivity assays demonstrated their ability to synthesize urea, uptake LDL, and metabolize glucose; all cardinal characteristics of hepatocytes, not present in undifferentiated ASCs. Gene expression analysis also showed the expression of several genes known to play an important role in liver function including, TDO2, ALB, HNF1B1, HNF6b, HNF4a, and AFP. However, even the best hepatocyte-like induced ASC obtained in this study had much inferior hepatocyte-related gene expression levels compared to primary human hepatocytes.

Conclusion: We successfully differentiated ASCs into hepatocyte-like cells; Protocol C produced the best hepatocyte-like cells based on morphology and function typically seen in primary hepatocytes. Although the results displayed some hepatocyte-related function, comparison of bioactivity and gene expression of hepatocyte-like cells were drastically lower than those of primary human hepatocytes, suggesting that caution should be taken when considering using differentiated hepatocyte-like ASCs to replace hepatocytes. Further studies are needed to better understand the functional capacity of hepatocyte-like ASCs, and which specific metabolic function could potentially offer therapeutic applications.

Keywords: Adipose-derived stem cells; Cell-based therapy; Hepatocyte-like cells; Mesenchymal stromal/stem cells; Adipose tissue

Background

Liver transplantation is a commonly accepted life-saving surgical procedure for a variety of irreversible acute and chronic liver diseases. Advances in the management and surgical techniques have been made since the first human liver transplant [1]. Over the years, this procedure has become routine with an excellent outcome in terms of both quality and extension of life [2,3]. However, the major challenge facing the transplant community, a consequence of their great success, is that more organs for transplantation are now needed. The limited availability of organ donors has led to the search for alternatives to whole-organ transplantation such as cell-based therapies. Hepatocyte transplantations in particular have been successfully performed to treat acute liver failure, end-stage liver disease, and inborn errors of metabolism [4-7]. However, the shortage of organ donors, immune rejection of allogeneic cells, and the fact that primary hepatocytes do not proliferate and lose their function *in vitro* pose great challenges in obtaining enough high quality primary hepatocytes for clinical treatments [8,9]. These difficulties have led to a search for other sources of cells, such as stem cells, that could be substituted for functional hepatocytes [10-13].

Adipose-derived mesenchymal stromal/stem cells have been considered a candidate as a liver progenitor cell source for therapeutic use. Within the mesenchymal stem cell family, ASCs can be isolated from adipose tissue; usually found in excess, thus providing a simple and accessible autologous source of stem cells [14]. In addition, ASCs have

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lower therapeutic risk associated with immune rejection and teratoma formation compared to other types of stem cells like induced Pluripotent Stem Cells [15-20]. In culture, ASCs can be expanded without losing their stemness, compared to the non-proliferative hepatocytes, leading to the advantage of obtaining high cell yields capable of differentiating into hepatocyte-like cells [21]. ASCs from different origins and species can be chemically induced to differentiate into hepatocyte-like cells that demonstrate bioactivity and morphology otherwise exclusive to that of native hepatocytes [22-28]. Despite published results showing hepatocyte-like cells derived from differentiated ASCs, only a limited group of unique molecules/functions are shown to be up-regulated compared to the plethora of molecules/functions expressed in true hepatocytes [13,22,24,25,29-31].

There have been extensive efforts to improve the conditions in which hepatocyte and hepatocyte-like cells can be cultured *in vitro* [32,33]. These efforts include optimizing the culturing conditions, by the addition of cytokines, use of various media, coatings, gels, and sandwich methods, as well as 2-dimension (2-D) or 3-dimension (3-D) culturing conditions [34-40]. More recent approaches in hepatocyte culture conditions have included mimicking the native physiological niche where hepatocytes are found, with the aim to emulate the proteins and signals found *in vivo* [38,39,41]. However, the ideal combination of different proteins as well as different cocktails of cytokines for optimal culture conditions are yet to be achieved [37]. Of the currently available matrices, such as type I collagen, fibronectin, and matrigel, none has demonstrated long-term success [34,36,39,42-46]. Better differentiation of ASCs into hepatocyte-like cells will facilitate long-term functional hepatic integration when applied *in vivo* [27]. Our goal is to optimize the culture conditions for the differentiation of ASCs into functional hepatocyte-like cells *in vitro*.

Materials and Methods

Mechanical isolation of adipose derived mesenchymal stromal/stem cells

Adipose tissues obtained from human liposuction were processed using Lipogems®, which is a class II, single-use medical device for the processing of aspirated adipose tissue. The unit consists of a transparent plastic cylinder with filters and beads for the micro fracturing of adipose tissue. Lipoaspirates from healthy individuals donated by Dr. Lindsey Coombs were received and added into the Lipogems device. Permission for the collection of tissue and subsequent research was unnecessary in Los Angeles because fat was collected under an institutional review board exemption as medical waste. The device further processed the lipoaspirate by washing and mechanically disrupting it into micronized adipose tissue. The resulting micronized adipose tissue was collected and seeded in tissue culture treated plastic-ware at proportions of 2 mL per 25 cm^2 and placed in a 5% CO_2 humidified incubator at 37°C for 3 hours, then 3 mL of Mesempro RS® media was added. Fresh media was added (3 mL) every 3 days without replacing the old media until day 9. On day 9 a total media replacement was performed without disturbing the attached pieces of micro-fat. On day 12, complete media replacement was repeated after removing non-adhered pieces of micro-fat leaving only attached ASCs in the flask. The cells were trypsinized (0.25%Trypsin-EDTA Gibco Cat.#25200056) when they reached confluency, washed, and sub-cultured for further analysis.

Characterization of ASCs by flow cytometry

Collected cells were washed in running buffer (MACS Cat. #130-091-221) and centrifuged at 200 × g, 4°C, for 5 minutes and re-suspended in BlockAid (Thermo Cat. # B10710) × 10 for a final concentration of 15 cells/test/100 µL. Cells were incubated in BlockAid for 15 minutes at 4°C, followed by addition of primary-conjugated antibodies according to manufacturer's instructions, and incubated at 4°C, in the dark, for 1 hour. Stained samples were washed with 1 mL running buffer (200 × g for 5 minutes at 4°C) twice; then reconstituted in 200 µL/sample running buffer before measurements were taken. Flow cytometry analysis was performed with a CytoFLEX 2-L (Beckman-Coulter). Commercially available adipose derived stem cells from lipoaspirates (Thermo Cat. # R7788115) were used as positive controls. Results were quantitated by CytExpert software (Beckman-Coulter). Antibodies used for flow cytometry: Anti-HLA-DR-FITC-Class II (Molecular Probes #MHLDR01), CD44-FITC (Thermo #MEM-85), CD271-VioBright FITC (MACS #130-110-115), CD166-PE (MACS #130-106-618), CD105-PE (StemCell Technologies #60039PE), CD90-PerCP-Cy5.5 (Molecular Probes #A16425), CD19-PE (StemCell Technologies #60005PE), CD11b-PE (StemCell Technologies #60040PE), CD34-PE (StemCell Technologies #60013PE), CD45-PE (StemCell Technologies #60018PE), CD73-PE (StemCell Technologies #60044PE).

Tri-lineage differentiation

Multipotency of isolated mesenchymal stem cells from adipose tissue aspirates was confirmed by tri-lineage differentiation (i.e., adipocyte, osteocytes, and chondrocytes), phenotypic expression, and by flow cytometry. In order to differentiate mesenchymal stem cells into chondrocytes, it was necessary to generate micromass cultures, by seeding 5-µL droplets × 10of cell solution (1.6 7 cells/mL) in a 6 well plate (approximately 10-15 droplets per well) and culturing for 2 hours under standard culture conditions. Prewarmed StemPro® Chondrogenesis Differentiation Kit (Thermo Cat.#A1007101) media was added to the culture vessels and incubated at 37°C, 5% CO_2. Cells for adipose and osteogenic differentiation were seeded in 6-well plates at 10 × 103 cells/cm^2 and incubated at 37°C, 5% CO_2 in MesenPRO RS™ Basal Medium supplemented with MesenPRO RS™ Growth Supplement (Gibco Cat.#12746012), antibiotic-antimycotic (Thermo Cat.#15240062), and Glutamax (Thermo Cat.#35050061), until they reached 80% confluency, then the pertinent differentiation media was added, StemPro® Adipogenesis Differentiation Kit (Thermo Cat.#A1007001) and StemPro® Osteogenesis Differentiation Kit (Thermo Cat.#A1007201), respectively. The differentiation media were replaced every 2 days according to manufacturer's instructions. Alizarin Red S (Sigma Cat.#A5533), Oil Red O (Sigma Cat.#O1391) and Alcian Blue (Sigma Cat.#B8438) staining were used to confirm presence of the adipose-derived osteocytes, adipocytes, and chondrocytes, respectively. Specifically, calcium deposition by adipose-derived osteocytes was visualized by Alizarin Red staining or Van Kossa staining. Cells were fixed with 4% buffered formaldehyde for 30 minutes and washed twice with distilled deionized water. When stained with Alizarin Red S, the cells were treated with a 0.5 M pH 4.2 Alizarin Red solutions for 2-3 min, then washed again with distilled deionized water. Von Kossa stain was performed as follows: 1% silver nitrate solution was added to the cells and the plates were placed on a transluminator to be irradiated with UV light for 1-2 h. Each well was washed with distilled deionized water and then treated with 5% sodium thiosulfate for 1 min, followed by several rinses with distilled deionized water. Proteoglycan production by adipose-derived chondrocytes was visualized by Alcian blue. All cells were fixed with 4% buffered formaldehyde for 30 minutes, washed twice with distilled deionized water, then stained with 1%Alcian Blue in 3% Acetic Acid for 1-2 hrs at room temperature, followed by the addition of 0.1N HCl solution for 30 minutes, then three rinses with distilled deionized water to neutralize the acid. Chondrogenic pellets

were fixed in 4% buffered formaldehyde, dehydrated through graded alcohol washes (70, 95, and 100 %), cleared with xylene (EMD, Millipore, USA), and embedded in paraffin. The sectioned (10 μm thick) chondrogenic pellets were placed on glass slides, then de-waxed to undergo Alcian Blue staining. Lastly, oil production (droplets) in ASC-derived adipocytes was visualized by Oil Red O staining. Briefly, cells were fixed with 4% buffered formaldehyde for 30 minutes, washed twice with distilled deionized water, then stained with Oil red O (0.5% in isopropanol) diluted with water (3:2) and incubated for 15 minutes at room temperature. Cells were then washed with water and the stained fat droplets were visualized by phase-light microscopy.

Differentiation of ASCs into hepatocyte-like cells

ASCs were cultured on type I collagen substrate and differentiated into hypatocyte-like cells using five different chemically induced differentiation protocols. ASCs were seeded in a 6 well plate (5×10^5 cells/well) pre-coated with type I collagen (Rat Collagen I Coated Plates Gibco® Cat.#A1142801) optimized for hepatocyte culture according to manufacturer's instructions. ASCs were cultured at 37°C, 5% CO_2 in MesenPRO RS™ Basal Medium supplemented with MesenPRO RS™ Growth Supplement (Gibco Cat.#12746012), antibiotic-antimycotic (Thermo Cat.#15240062), and Glutamax (Thermo Cat.#35050061), until they reached 80% confluency. The ASC media was then changed to serum-free low glucose DMEM (Gibco Cat.#10567) plus antibiotics for 24 hrs (pre-induction step), followed by their corresponding protocol, see (Table 1). The concentration of each growth factor was: 20 ng/mL human recombinant Epidermal Growth Factor (EGF Gibco Cat.#PHG0311), 10 ng/mL basic Fibroblast Growth Factor (bFGF Gibco Cat.#13256029, 10 ng/mL Fibroblast Growth Factor (FGF4 Gibco Cat.#PHG0154), 20 ng/mL Hepatocyte Growth Factor (HGF Gibco Cat.# PHG0324), 0.61 g/L nicotinamide (Sigma Cat.#N0636), 20 ng/mL OncostatinM (OSM Gibco Cat.#PHC5015), 1 mmol/L Dexamethasone (Dex) (AlfaAesar Cat.#A17590), 50 mg/mL Insulin-Transferrin-Selenium (ITS Gibco Cat.#41400045), 2% Knockout Replacement Serum (Gibco Cat.#10828028), μMPalmitoyl20ng/mLcoenzymeActivin A (Thermo Cat.#PHC9564), 2 lithium saltμM Tr (Sigmacostatin Cat.A#(TSAP9716), Sigma1 Cat.#T8552).

Urea synthesis

Urea secretion was measured by collecting the supernatant from the cultured cells at various time points and using a colorimetric assay kit (QuantiChromTM Urea Assay Kit) according to the manufacturer's instructions. Known concentrations of urea were tested in parallel with the experimental samples and all of the measurements were performed in a Synergy HTX Multi-Mode Reader (Biotek®, VT, USA). Sample values were normalized by cell number, quantified by MTT.

Cytochrome P450 (C3A4) metabolism

Cytochrome P450 (CYP3A4) was measured at the end of the hepatocyte-differentiation protocol using a P450-GloTM CYP3A4 Assay with Luciferin- isopropyl acetal (IPA, Promega). After incubation, an aliquot of 50 μL medium was transferred from each well to a 96-well opaque white luminometer plate at room temperature. Then, luciferin detection reagent (50 μL) was added to each well and the plate was allowed to stand for 20 min in the dark to initiate a luminescent reaction. The resulting luminescence was read using a Synergy HTX Multi-Mode reader (Biotek®, VT, USA). Sample values were normalized by cell number, quantified by MTT.

Periodic Acid-Schiff (PAS)

Glycogen storage of cultured hepatocytes was evaluated by Periodic Acid-Schiff (PAS) staining. Cells in culture were fixed in 4% buffered formaldehyde (Fisher Scientist Cat.#F79-4) for 15 minutes, then treated with 1% periodic acid solution for 5 minutes at room temperature, rinsed three times in distilled deionized water and exposed to Schiff's reagent for 15 minutes for subsequent color development. Finally, wells were washed with distilled deionized water for 5–10 min and observed by bright-field microscopy (EVOS FL, Life Technologies).

Uptake of low-density lipoprotein

The uptake of low-density lipoprotein (LDL) was detected with the Dil-Ac-LDL staining kit (Biomedical Technologies, Stoughton, MA, USA) according to the manufacturer's instructions. ASCs and hepatocyte-like cells were incubated in serum-free DMEM-LG containing 10 μg/mL 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate acetylated-LDL (Dil-Ac-LDL) for 4 h at 37°C. Cells were then washed and visualized under a fluorescence microscope (EVOS FL, Life Technologies).

Viability and proliferation

An MTT (3-(4, 5-dimethylthiozole-2-yl)-2, 5-diphenyltetrazolium bromide, Molecular Probes M6494) assay was used to evaluate cell number/viability. A standard curve with known cell number was used at each time point evaluated 5 MTTmg/mL)solutionwas added (25 to each well and incubated for 5 hrs in a 5% CO_2, at 37°C, humidified incubator. After incubation, the formazan complex was extracted with DMSO and the absorbance was measured at 570 nm.

Characterization and Gene Analysis of Hepatocyte-like Cells by RT-PCR

To confirm gene expression and function of ASCs-derived hepatocyte-like cells, we studied the expression of genes specific to key regulatory functions, metabolic, nuclear transcription, and protein expression of hepatocytes/hepatoblasts. Total ribonucleic acid (RNA), from cells cultured in 6 well plates, was isolated using TRIzol® LS Reagent (Thermo Cat. #10296010) according to manufacturer's instructions for real-time polymerase chain reaction. The cells were rinsed with HBSS and carefully scrapped while adding 2 mL of TRIzol® LS Reagent, followed by incubation on ice for 15 min. To separate the aqueous from the solvent phase 1 mL of chloroform was added to each sample and mixed well followed by centrifugation (13,000 × g). Total RNA was purified using Qiagen's mini spin columns (Qiagen Cat. #27115). The concentration and quality of the purified RNA was determined at 260/280-wavelength optical density ratio using a NanoDrop spectrometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). Complementary deoxyribonucleic acid (cDNA) was synthesized from 150 ng of total RNA using SuperScript III first-strand synthesis supermix with oligo-dT primers (Invitrogen Cat. #11752250). A negative control sample (H_2O) was used to assess random production of cDNA through contaminants. Oligonucleotide primer sequences were designed using Invitrogen's OligoPerfectTM Designer and National Center for Biotechnology Information (NCBI) gene database and manufactured by Thermo- Invitrogen Custom DNA Oligos for the following hepatocyte-specific genes; Alphafetoprotein (AFP), Coagulation Factor IX (F9), G-glutamyltranspeptidase, Hepatocyte Nuclear Factor 4 alpha (HNF4A), Hepatocyte Nuclear Factor 6b (HNF6B), Hepatocyte Nuclear Factor 1 B1 (HNF1B1), phosphoenolpyruvate carboxykinase (PCK), apolipoprotein A-I (APOA1), apolipoprotein C-III (APOC3),

fibrinogen beta chain (FGB), Glucose-6-phosphatase (G6P), tyrosine aminotransferase (TAT), tryptophan-2,3-dioxygenase (TDO), α-antitrypsin (AAT), albumin (ALB), cytochrome P450/3A4 (C3A4), cytokeratin 18 (CK18), cytokeratin 7 (CK7), forkhead box protein A2 (FOXA2), and Transferrin. Master mixes contained 200 nM of forward and reverse primers containing SYBR Green-ER, and qPCR supermix (Invitrogen Cat.# 4309155); synthesized cDNA was added to the appropriate wells. Real-time polymerase chain reaction (RT-PCR) was carried out using a Bio-Rad CFX96 thermal cycler system (Bio-Rad, Hercules, CA, USA). Message RNA expression levels were normalized to glyceraldehye-3-phosphate dehydrogenase (GAPDH). Fold increase in expression levels for each specific gene was normalized to the expression levels of control conditions and reported−Ctmethodbythe.

Statistical Analysis

Numerical data were expressed as the mean SD (standard deviation). Statistical significance was determined using GraphPad Prism 5.00 (GraphPad Software, San Diego California USA). Student's t test was used to determine significant difference between control and test group. Analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison tests was used for analysis of multiple groups, and p values less than 0.05 were considered statistically significant.

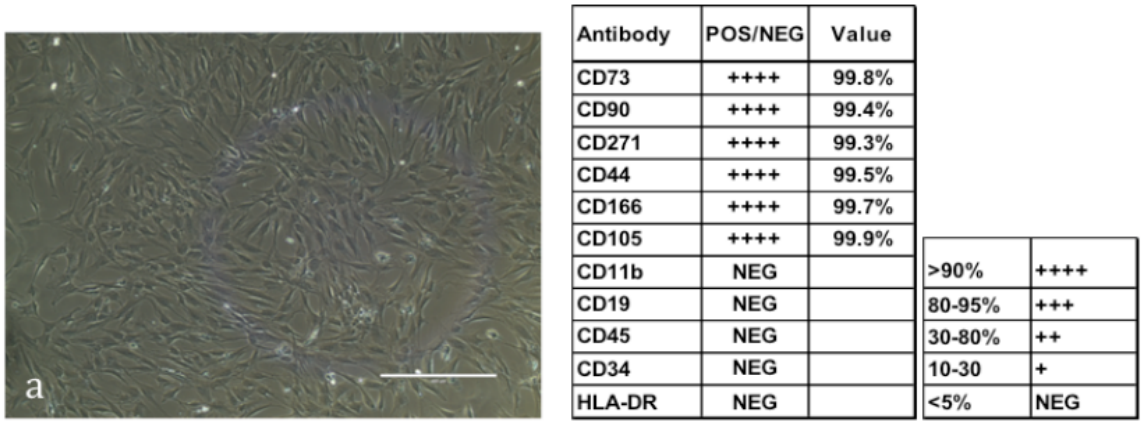
Results

Characterization of ASCs

Freshly isolated ASCs quickly adapted *in vitro* and adhered to the surface of the cell culture plates after the initial seeding. Subsequent days in culture of the ASCs were characterized by rapid cell proliferation forming a homogenous spindle-like cell monolayer (Figure 1a). ASCs showed all required characteristics of multipotent mesenchymal stromal ("stem") cells (MSC) defined by the committee of International Society for Cellular Therapy (ISCT) [47]. First, ASCs adhered to plastic under standard culture conditions. Second, ASCs expressed (>99%) CD105, CD73, CD90, and CD271, CD44, and CD166 and lacked expression (<5%) of the hematopoietic markers; CD45, CD34, CD11b, CD19, and HLA-DR (Figure 1b). Third, ASCs differentiated to osteoblasts, adipocytes and chondrocytes *in vitro*. Specifically, adipogenic differentiation was demonstrated by the accumulation of neutral lipid (triglycerides) vacuoles determined by the positive Oil Red O staining (Figure 1c). Similarly, chondrocyte aggregates showed generation of a proteoglycan-rich extracellular matrix, a hallmark of chondrogenesis (Figure 1e). Histochemical staining of tissue with Alcian blue was a confirmatory adjunct to immune-localization of collagen type II, a chondrocyte-specific molecule within the cartilage matrix (Figure 1e). In contrast to undifferentiated ASCs, differentiated cells using the osteoblast differentiation protocol presented mineralization, accompanied by the development of nodules that stained positive for phosphate, using Von Kossa staining (Figure 1d) and calcium using and Alizarin Red S staining (data not shown).

Urea Synthesis by hepatocyte-like cells

To evaluate the efficiency of hepatocyte differentiation exerted by the various protocols (Table 1), we measured urea synthesis, a key function of hepatocytes. Conditioned media from hepatocyte-like cells cultured under the various differentiation conditions showed high levels of urea production only in the conditioned media of cells cultured using protocols C, D, and E (~2000-5000 ug/Dl/1 × 10^6) compared to undifferentiated ASCs (control) (Figure 2a).



Antibody	POS/NEG	Value
CD73	++++	99.8%
CD90	++++	99.4%
CD271	++++	99.3%
CD44	++++	99.5%
CD166	++++	99.7%
CD105	++++	99.9%
CD11b	NEG	
CD19	NEG	
CD45	NEG	
CD34	NEG	
HLA-DR	NEG	

>90%	++++
80-95%	+++
30-80%	++
10-30	+
<5%	NEG

Figure 1: (a) ASCs in culture (b) Flow cytometry marker (panel) results for adipose derived cells. N ≥ 3. Adipose derived stem cells differentiated into; (c) adipocytes showing Oil Red O staining scale bar=400 μm, (d) osteocytes showing Van Kossa staining scale bar=1mm, and (e) chondrocytes showing Alcian Blue staining. Scale bar=500 μm.

Condition	Pre-Induction (48hrs)	Induction (48hrs)	Differentiation (7days)	Maturation (7days)
DMEM, Low-glucose				
A	DMEM, Low-glucose	EGF+bFGF+Activin A	HGF+bFGF+FGF4+ Nicotinamide+5% Serum	Oncostatin M+ITS+Dexamethasone + 2% Serum
B	DMEM, Low-glucose	EGF+bFGF+FGF4		
Condition	**Pre-Induction (48hrs)**	**Induction (48hrs)**	**Differentiation (7days)**	**Maturation (7days)**
Control	DMEM, Low-glucose , 2%Serum			
C	DMEM, Low-glucose	FGF4	HGF	HGF+ITSPre+Dex+OncM
D	DMEM, Low-glucose	FGF4		HGF+ITSPre+Dex+TSA
E	DMEM, Low-glucose	FGF4		Palmitoyl+ITSPre+Dex+TSA

Table 1: Various methods for the differentiation of ASCs into hepatocyte-like cells

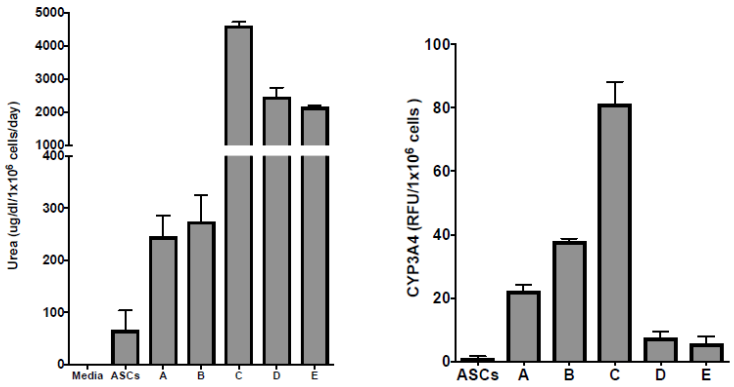


Figure 2: (a) Urea production per unit number cells by ASCs and Hepatocyte-like cells under different differentiation conditions. (b) CYP3A4 activity by ASCs and hepatocyte-like cells under different conditions Data is expressed as the means ± SD of independent measurements.

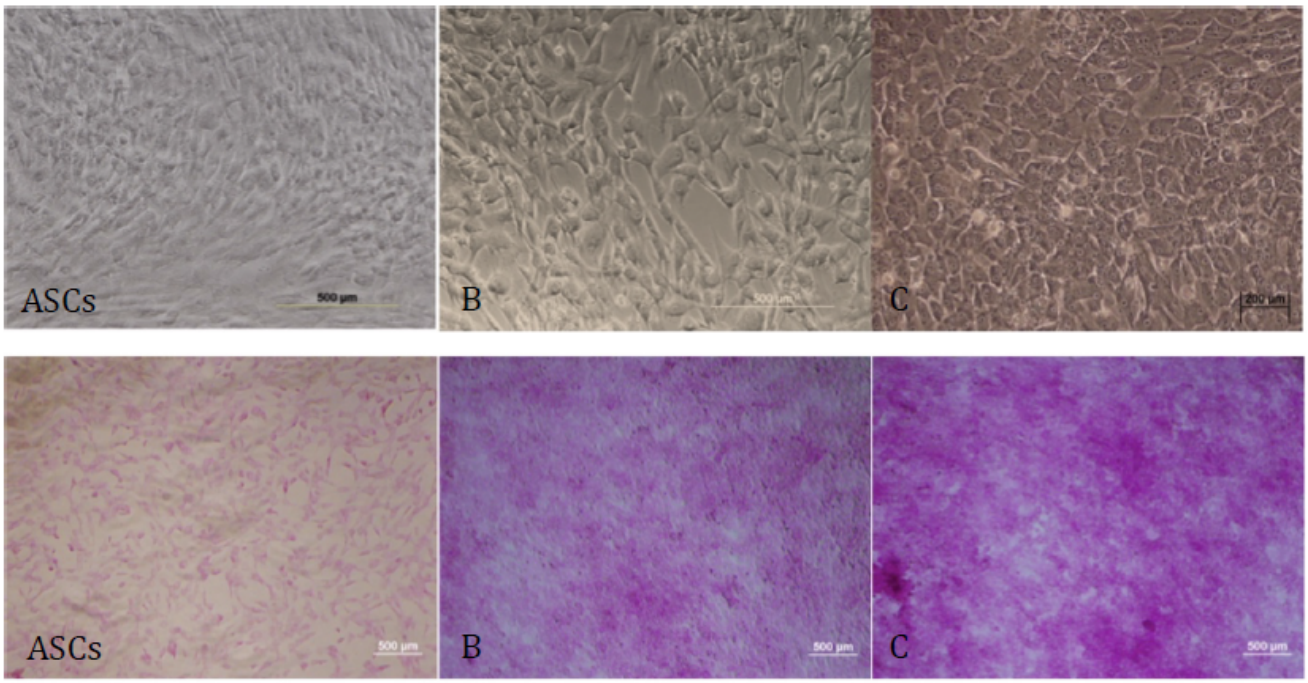


Figure 3: Periodic Acid-Schiff (PAS) staining (bottom images) on adipose derived mesenchymal stem cells differentiated into hepatocyte-like cells using protocol Condition B and Condition C. Top images are bright-field images displaying morphological changes. Bottom images are PAS stained cells. Scale bar =500 µm.

Cytochrome P450 (C3A4) activity in hepatocyte-like cells

Cytochrome P450 activity is a highly hepatocyte-specific function and is one of the main enzymes responsible for drug metabolism. ASCs differentiated under condition C and condition B, displayed the highest values for cytochrome P450 (C3A4) activity (Figure 2b).

Ability of Hepatocyte-like cells to store glycogen and LDL Uptake

PAS staining detected the highest glycogen storage under condition C (Figure 3). In addition, the differentiated ASCs under condition C displayed the greatest degree of morphological change, from spindle

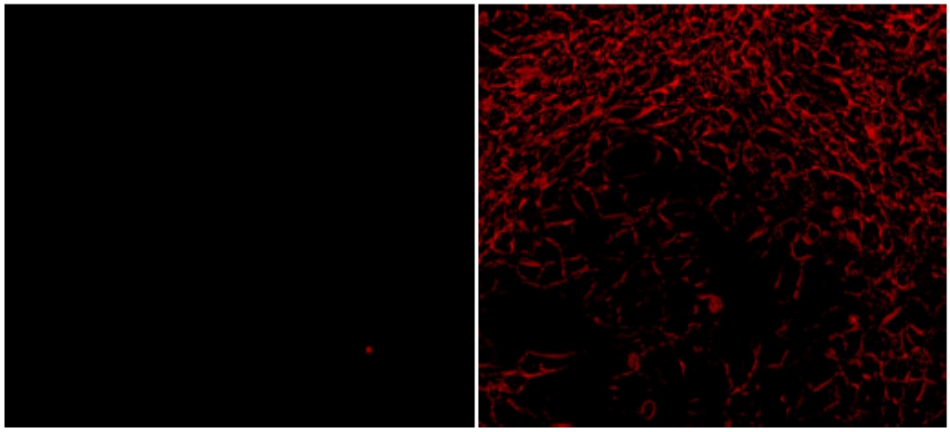


Figure 4: Low-density lipoprotein (LDL) staining on adipose derived mesenchymal stem cells differentiated into hepatocytelike cells using protocol Condition C (Right) and its respective non-differentiated control (Left) 100X.

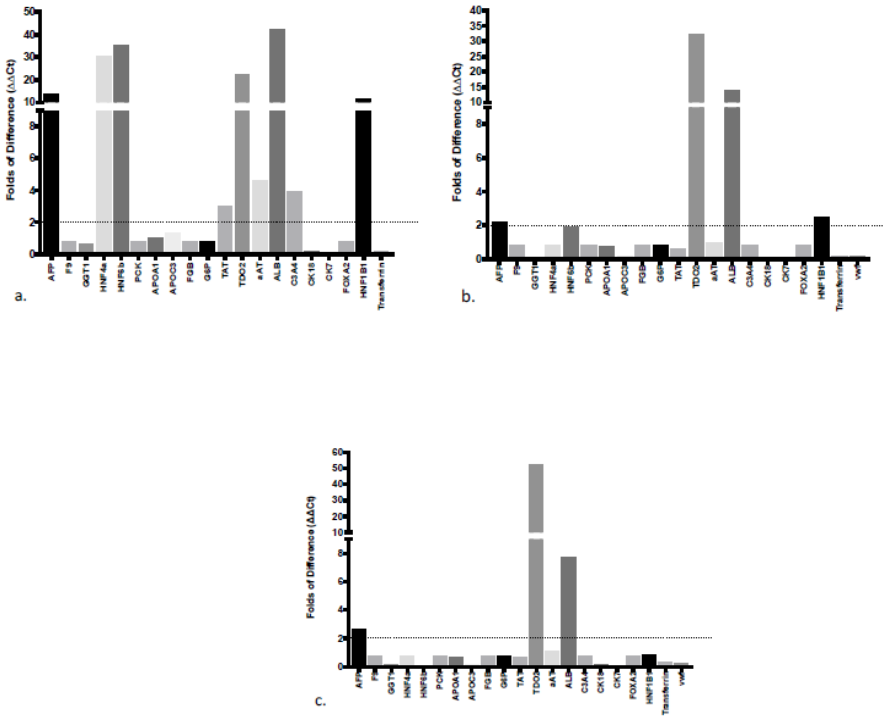
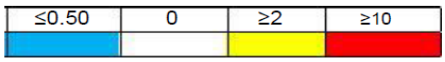


Figure 5: Hepatocyte-related gene expression of adipose derived mesenchymal stem cells after being differentiated into hepatocytelike cells using (a) Condition C (b) Condition A, and (c) Condition B. Fold changes (ΔΔCt) higher than 2 were considered upregulated, indicated by the dotted horizontal line. Abbreviations: AFP=Alphafetoprotein; F9=Coagulation Factor IX; GGT1=Gglutamyltranspeptidase; HNF6b=Hepatocyte Nuclear Factor 6b; PCK=phosphoenolpyruvate carboxykinase; APOA1=apolipoprotein A-I; APOC3=apolipoprotein C-III; FGB=fibrinogen beta chain; TAT=tyrosine aminotransferase; TDO2=tryptophan-2,3-dioxygenase; aAt=α-antitrypsin; ALB, albumin; C3A4=cytochrome P450/3A4; CK18, cytokeratin 18; CK7, cytokeratin 7; FOXA2=forkhead box protein A2; HNF1B1=Hepatocyte Nuclear Factor 1 B1; Transferrin=Transferrin.

cell type to a more "cuboidal" phenotype compared to condition B (Figure 3). Lastly, the ability of hepatocyte-like cells to uptake LDLs, a cardinal characteristic of true hepatocytes to clear LDLs from the blood, was evaluated by fluorescence microscopy. The results displayed that hepatocyte-like cells incorporated significantly more Dil-LDL that ASCs in culture (Figure 4).

Hepatocyte-like gene expression analysis

Comparison of gene expression of the hepatocyte-like cells and non-differentiated ACSs across the three treatments. The 21 genes we examined are drug metabolism related genes (drug transporter, Phase I metabolizing enzymes, Phase II Metabolizing, and Mesoderm) and



Function		Gene	ASCs vs ASCs Hep	ASCs Hep vs Hep
			Ct	
Drug Transporters	Metallothioneins	MT2A	1.15	0.99
		MT3	3.01	0.54
	P-Glycoprotein Family Members	ABCB1	1.51	179.11
		ABCC1	1.06	0.00
		GPI	0.59	0.41
Phase I Metabolizing Enzymes		CYP11B2	3.01	3.23
		CYP17A1	3.01	32.27
		CYP19A1	0.50	0.00
		CYP1A1	0.11	219.45
		CYP2B6	1.51	6039.70
		CYP2C19	1.51	695.46
		CYP2C8	1.56	2557.55
		CYP2C9	3.01	1888.55
		CYP2D6	4.02	402.43
		CYP2E1	11.35	1488.86
		CYP2F1	3.01	1.61
		CYP2J2	0.65	266.24
		CYP3A4	3.01	914.91
		CYP3A5	37.18	1358.65
Other Drug Metabolizing Enzymes		AHR	0.73	0.89
		ARNT	0.31	1.28
		GCKR	31.79	3.39
		SNN	2.88	0.52
Mesoderm		BMP4	0.25	0.07
		CD34	556.06	0.30
		DCN	0.00	22.31
		GATA2	0.54	2.78
		HAND1	1.84	1.48
		IGF2	2.70	1.67
		MIXL1	1.61	1.00
		PDGFRA	0.00	1.51
		RUNX1	0.02	1.36
		T	1.61	0.75
Endoderm		FOXA1	158.13	3.01
		GATA1	1.61	3.01
		GATA6	0.56	1.51
		HNF4A	2.93.37	3.01
		SOX17	1.61	81.39
		SOX7	13.72	1.77
Hepatocyte Progenitor		APOH	4581.47	1.51
		DPP4	0.97	4.01
		MAP3K12	0.01	1.00
Hepatocyte		AFP	1.61	3.01
		ALB	5097.09	3.01
		G6PC	1657.17	3.01
		GGT1	0.53	0.68
		HNF1B	35.50	3.01
		HNF4A	1206.97	3.01
		ITGB4	1.61	0.81
		KRT19	2.09	2.11
		KRT7	0.22	1.30
		SERPINA1	839.27	10.74
		TAT	6330.69	0.75
		TDO2	71.07	0.02

Phase II Metabolizing Enzymes	Carboxylesterases	CES1	556.06	0.30
		CES2	3.72	7.65
		CES3	84.71	0.95
	Decarboxylases	GAD1	1.61	0.58
		GAD2	1.61	1.51
	Dehydrogenases	ADH1B	231.66	0.60
		ADH1C	2.74	3.01
		ADH4	335.63	3.01
		ADH5	3.90	0.67
		ADH6	263.82	1.00
		ALAD	2.90	0.65
		ALDH1A1	767.27	2074.05
		HSD17B1	0.58	3.27
		HSD17B2	1206.97	4.53
		HSD17B3	19.36	3.66
	Glutathione Peroxidases (GPx)	GPX1	0.53	0.68
		GPX2	1957.30	6.03
		GPX3	0.58	2.13
		GPX4	2.16	1.43
		GPX5	1.61	3.01
		GSTA3	1.61	1.51
		GSTA4	0.21	2.06
		GSTM2	0.68	0.47
		GSTM3	0.16	0.17
		GSTM5	0.08	3.01
		GSTP1	0.00	1.65
		GSTZ1	2.05	1.01
		LPO	1.61	1.51
		MPO	1.61	3.01
	Lipoxygenases	ALOX12	0.40	0.10
		ALOX15	1.61	0.11
		ALOX5	1.61	61.97
		APOE	4.63	0.12
	Hydrolases	ASNA1	0.19	0.87
		EPHX1	3.09	0.14
		FAAH	14.70	0.52
		FBP1	1274.74	0.50
	Kinases	HK2	0.01	0.20
		PKLR	287.22	1.21
		PKM	0.01	0.82
	Oxidoreductases	AOC1	3.23	1.51
		BLVRA	0.02	1.21
		BLVRB	10.19	1.18
		CYB5R3	0.44	1.55
		GPX1	0.53	0.68
		GPX2	1957.30	6.03
		GSR	0.83	2.47
		MTHFR	1.26	2.35
		NOS3	1.61	3.01
		NQO1	0.09	0.13
		SRD5A1	5.38	1.21
		SRD5A2	16.14	3.01
	Paraoxonases	PON1	1513.56	39.19
		PON2	5.69	3.32
		PON3	131.19	7.61
	Glutathione S-Transferases	GSTA3	1.61	1.51
		GSTA4	0.21	2.06
		GSTM2	0.68	0.47
		GSTM3	0.16	0.17
		GSTM5	0.08	3.01
		GSTP1	0.00	1.65
		MGST1	2.36	3.17
		MGST2	2.98	0.77
		MGST3	0.30	0.46
	Other Phase II Metabolizing Enzymes	CHST1	0.05	0.11
		COMT	1.77	0.77
		NAT1	0.65	0.91
		NAT2	417.92	15.07

Table 2: Gene expression of hepatocyte -like cells (ASCs Hep) compared to undifferentiated ASCs (first column) and hepatocyte-like cells (ASCs Hep) compared to primary hepatocytes (second column).

developmental genes (endoderm, mesoderm, Hepatocyte progenitor, and hepatocytes). All three treatments resulted in increased expression of Alphafetoprotein (AFP), tryptophan-2,3-dioxygenase (TDO2), and albumin (ALB). However, the highest number of up-regulated genes corresponding to mature hepatocyte functions was 9 (of 21), observed with condition C (Figure 5a). More comprehensive gene analysis comparing our protocol C to differentiate ASCs to hepatocyte-like cells resulted in the upregulation of 50 hepatocyte-related genes compared to non-differentiated ASCs (Table 2- First column ASCs vs. ASCs Hep). With the highest (>10 fold) up-regulation detected in the following genes: CYP2E1, CYP3A5, ALDH1A1, ALOX5, PON1, NAT2, DCN, SOX17, SERPINA1. Followed by genes showing upregulation (>2 fold and <10 fold): MT3, CYP11B2, CYP17A1, CYP2C9, CYP2D6, CYP2F1, CYP3A4, CES2, ADH1C, ADH4, HSD17B1, HSD17B2, HSD17B3, GPX2, GPX3, GPX5, GSTA4, GSTM5, MPO, GSR, MTHFR, NOS3, SRD5A2, PON2, PON3, GSTA4, GSTM5, MGST1, GCKR, GATA2, FOXA1, GATA1, DPP4, AFP, ALB, G6PC, HNF1B, HNF4A, KRT19. Furthermore, when differentiated ASCs into hepatocytes-like cells where compared to primary hepatocytes, primary hepatocytes showed upregulation of 60 hepatocyte-related genes (Table 2- Second column ASCs Hep vs. Hep). With the highest (>10 fold) up-regulation detected in the following genes: ABCB1, CYP17A1, CYP1A1, CYP2B6, CYP2C19, CYP2C8, CYP2C9, CYP2D6, CYP2E1, CYP2J2, CYP3A4, CYP3A5, CES1, CES3, ADH1B, ADH4, ADH6, ALDH1A1, HSD17B2, HSD17B3, GPX2, FAAH, FBP1, PKLR, BLVRB, SRD5A2, PON1, PON3, NAT2, GCKR, CD34, IGF2, FOXA1, HNF4A, SOX7, APOH, ALB, G6PC, HNF1B, SERPINA1, TAT, TDO2. Followed by genes showing upregulation (>2 fold and <10 fold): CYP11B2, CES2, ADH1C, ADH5, ALAD, GPX4, GSTZ1, APOE, EPHX1, AOC1, SRD5A1, PON2, MGST1, MGST2, SNN, KRT19.

Even though hepatocyte-like ASCs expression of hepatocyte-related genes was much inferior than those of primary hepatocyte like cells, some genes in hepatocyte like -ASCs cells were higher (upregulated) when compared to primary hepatocytes, mainly those related to Glutathione S-Transferase functions (Table 2).

Discussion

Human ASCs have been in the spotlight of regenerative medicine for a long time. Primarily, because adipose tissue is readily available, they are acquired with minimally invasive surgery, can be easily isolated, cultured *in vitro*, and have a broad differentiation potential including tri-lineage differentiation into mesodermal lineage cells like: osteocytes, chondrocytes, and adipocytes. More recently, there have been many studies attempting to differentiate ASCs into hepatocyte-like cells, with the intention of replacing the need of primary hepatocytes and use them for clinical applications related to liver diseases. Some of the investigated hepatocyte differentiation methods require long time (months) to induce differentiation and some even included vector-integrated transcription factors to reprogram ASCs into hepatocyte-like cells rendering the later unsafe to recipient [48]. In order to study the differentiation of ASCs into hepatocyte-like cells, we compared different methods of differentiation and evaluated three key hepatocyte-related functions: ureagenesis, P450 metabolism, and glycogen storage. Ureagenesis from ammonia occurs exclusively in the liver and involves both cytosolic and mitochondrial reactions and is a valuable global indicator of hepatic performance [49]. Cytochrome P450 (C3A4) metabolism is a functional process that represents the capability to biotransform xenobiotics, which is one of the most relevant characteristics of mature hepatocytes.

Therefore, there is a great need to ensure the appropriate metabolic performance of cells when cultured *in vitro*. Cytochrome P450 enzymes are essential for the metabolism of many medications. Although this class has more than 50 enzymes, six of them metabolize 90 percent of drugs, with the two most significant enzymes being CYP3A4 and CYP2D6 [50]. Glycogen storage/glucogenesis is a central function performed by mature hepatocytes in the liver and is an essential mechanism in survival because it provides readily available glucose in order to supply the tissues with an oxidizable energy source [51,52].

In addition to glycogen storage, hepatocytes play a protagonist role in cholesterol homeostasis, critical for normal physiological functions. Hepatocytes are responsible for cellular cholesterol metabolism and clearance of plasma cholesterol. Hepatocyte mediated cholesterol metabolism is dependent on hepatocyte LDL receptor mediated uptake, rendering the evaluation of LDL uptake a function of great significance [53]. Although these functions (Urea, P450, glycogen storage, morphology and LDL uptake) and 9 genes do not completely cover all functions of hepatocytes, they are considered reliable functional assays to show hepatocyte specificity [24,27,28]. Moreover, these are rational and practical elements to compare the hepatocyte nature of differentiated cells. It is important to clarify that even the highest values of our hepatocyte-like obtained for urea, glycogen, and P450 metabolism are not necessarily comparable to those values found of mature primary hepatocytes. However, obtaining the highest values possible from all protocols can provide information about the hepatocyte-functional limit conditional to the stem cell nature. The same stands for gene expression analysis of ASC-derived hepatocyte-like cells which demonstrated an average upregulation of 47 hepatocyte related genes after hepatocyte differentiation (Table 2) compared to the original state of the cells. Some generalized trends can be seen by comparing the two groups. First, it is clear that in Phase 1 metabolic enzymes, the expression levels in the primary hepatocytes are much greater than in the differentiated ASCs (see the predominance of red cells in Table 2 on the second column). This trend is reversed in Endoderm, Hepatocytes, and Dehydrogenases comparisons, with more red cells in the first column (ASCs differentiated to hepatocyte-like cells).

The protocols used in this study were carefully chosen from published literature with the intention to consolidate, with minor modifications, the most common strategies utilized to differentiate ASCs into hepatocyte-like cells in order to elucidate which methodology could be more appropriate to generate hepatocyte-like cells [27,28,30].

Although the functional values of hepatocyte-like cells acquired with the different protocols were not as high as those of primary hepatocytes, protocol C (Table 1) produced the highest values. We hypothesize the combination of Oncostatin M (oncM) and HGF in the presence of Dex in protocol C, not included in the other protocols, is responsible for the higher functional values and better hepatocyte-like cell morphology. OncM is a cytokine that is involved in hepatocyte development mediated through the STAT3 pathway during the fetal stages of growth. In contrast, HGF is upregulated after birth and plays an important role in hepatocyte development and maturation through a STAT3-independent pathway, not yet elucidated. It has been shown that the combination of OncM and HGF can induce hepatocyte maturation in the presence of Dex *in vitro* [54].

Though ASCs may not be the best candidates to generate high quality hepatocyte-like cells they are not the only stromal cells available. We are currently in process of studying various stromal cells from different tissue origins in hope to find the best cell type to generate hepatocyte-like cells.

The above results may be discouraging at first, but looking closer, there are some genes that were similar (between -2 and 2 folds) or even higher to those of primary hepatocytes. Although, ASC-derived hepatocyte-like cells were not comparable overall to primary hepatocytes, some specific genes were comparable or even superior than those of hepatocytes, for example those relevant to Phase II metabolism genes family: CYP19A1, glutathione peroxidase gene family (GSTA4, GSTM3, GSTM5, GSTP1, kinases (HK2, PKM), which could represent an opportunity to identify which cases these cells could provide therapeutic benefits for some of the most troublesome liver pathologies.

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