



## Isolation and Characterization of Mesenchymal Stem Cells from Human Fetal Liver; Potential Candidates for Replacement Therapy in Liver Disease

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### Abstract

**Background:** Cellular replacement therapy is extensively studied as an alternative treatment for liver disease. Lack of liver donors and problems with *in vitro* expansion and cryopreservation are limiting factors for hepatocyte transplantation. Stem cells emerge as a potential candidate and particularly mesenchymal stem cells (MSCs) gain special interest due to their potential to differentiate along many lineages, their non-immunogenic profile, anti-inflammatory and anti-apoptotic actions in combination with a low oncogenic risk.

**Methods:** Immunochemical staining for CD271 expression in the liver was performed. CD271-labeled magnetic beads were used for isolating CD271<sup>+</sup> cells, and the surface protein expression on the cells was analyzed by flow cytometry. The cells were also tested for their differentiation potential towards osteogenic, adipogenic, and hepatogenic lineages. Immunogenicity and immunomodulatory potential of CD271<sup>+</sup> cells were tested in co-cultures.

**Results:** Fibroblastoid and hepatoblast-like cells in the human fetal and adult liver expressed CD271, with higher expression in the fetal liver. Isolated cells expressed characteristic mesenchymal markers and showed multipotency by being able to differentiate to osteogenic and adipogenic cells *in vitro*, confirming the presence of a MSC population in the human fetal liver (hFL-MSCs). hFL-MSCs expressed the hepatic markers CK18, CK19, AFP, and ALB, but did not differentiate to functional hepatocytes *in vitro*. Upon co-culture, hFL-MSCs did not induce proliferation among allogeneic lymphocytes. hFL-MSCs inhibited lymphocyte allogeneic responses, and when added to co-cultures of lymphocytes and HepG2 cells, they inhibited the directed HepG2 allogeneic response at variable degrees.

**Conclusions:** Human fetal liver contains a population of CD271<sup>+</sup> cells that express mesenchymal and hepatic markers. hFL-MSCs were able to modulate allogeneic responses when co-cultured with allogeneic lymphocytes or with HepG2 cells, which makes them promising co-transplantation candidates at hepatocytes transplantation in the aim to reduce rejection.

**Keywords:** Mesenchymal Stem Cells; Cell therapy; Liver disease; Hepatocytes; Co-transplantation

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**Abbreviations:** LCT: Liver Cell Transplantation; LSPC: Liver Stem/Progenitor Cell; HSCs: Hepatic Stem Cells; MSCs: Mesenchymal Stem Cells; NGFR: Nerve Growth Factor Receptor; hFLCs: Human Fetal Liver Cells; hFL-MSCs: Human Fetal Liver MSCs; CYP: Cytochrome P450; ALB: Albumin; CK: Cytokeratin; AFP: Alpha-fetoprotein; MLCs: Mixed Lymphocyte Cultures; PBLs: Peripheral Blood Lymphocytes.

### Introduction

Liver cell transplantation (LCT) is emerging as an alternative to organ transplantation in the treatment of liver disease since tissue unavailability and immunological incompatibilities are still major obstacles [1,2]. Primary human hepatocytes are the first choice for LCT [3]. However the unavailability of liver donors, the need for high numbers of hepatocytes for transplantation, immunological rejection, and low activity and inability to expand hepatocytes *in vitro* are limiting factors [4]. Therefore stem cells may be an alternative in LCT. Stem cells are defined as undifferentiated cells capable of high proliferation, self-maintenance, production of differentiated functional progeny, and regenerating tissues [5]. This definition has been applied for a possible "liver stem/progenitor cell" (LSPC) population. An LSPC population should similarly have the above-mentioned characteristics and be able to repopulate the liver after injury.

In the fetal liver, cells of the ventral foregut endoderm and the hepatic diverticulum are considered as pluripotent LSPCs. It is suggested that some pluripotent LSPCs exit the proliferative state and remain quiescent in the liver until they are needed [6]. Several LSPC populations have been identified in the human liver that could meet the above-mentioned criteria: hepatic stem cells (HSCs) and hepatoblasts. Both cell populations have their specific size, morphology, and gene expression pattern making them distinct from one another and from mature hepatocytes [7]. HSCs are located in the ductal plates, a rim of cells around the portal triads, and are believed to be progenitors to hepatoblasts. Hepatoblasts are considered as transit-amplifying cells in the liver and are capable of differentiating to hepatocytes or cholangiocytes [8]. HSCs and hepatoblasts are dominant in fetal and neonatal livers while in adult liver mature hepatocytes are the principal cells (>98%) in comparison to HSCs (0.3-0.7%) and hepatoblasts (<0.1 %) [9].

Mesenchymal stem cells (MSCs) have in recent years become a promising source for cell therapy since they have characteristics favourable for transplantation: their potential to differentiate along many lineages, their non-immunogenic profile, having anti-inflammatory and anti-apoptotic actions in combination with a low oncogenic risk [10-14]. This makes MSCs of great clinical interest in the form of allogeneic "off-the-shelf" cells. MSCs have successfully been used to treat graft-versus-host disease in patients [15], and ameliorate healing of injured lung, kidney and central nervous system in experimental animal models [16-18]. MSCs were also able to support hepatocyte survival and function both *in vivo* and *in vitro* [19].

Cells derived from the fetus present characteristics suggestive of

stronger therapeutic potentials than those for cells derived from adult tissues. Fetal MSCs have marked expansion capacity, cycle faster than adult MSCs without expressing a differentiated phenotype; have longer telomeres and express pluripotency markers [20,21]. Fetal MSCs can differentiate into at least three different mesenchymal tissues: bone, cartilage and fat [22] and differentiate outside conventional lineage boundaries into skeletal muscle and oligodendrocytes [23,24].

The characteristics of MSCs are not definite yet. However, it is widely accepted that MSCs should be negative for hematopoietic and endothelial markers while expressing a panel of surface markers [25]. Commonly used methods for MSC isolation are plastic adherence and negative selection using a variety of cell depletion antibody cocktails [26,27]. Nerve growth factor receptor (NGFR) family was first described as a MSC marker by Chesa et al. [28], and cells expressing those markers are exclusively stromal and not hematopoietic or endothelial. CD271 is a member of the NGFR family. Recently, many studies have proved CD271 as a good marker for MSCs and it has been used for immunoselection of MSCs from a variety of tissues like bone marrow and placenta [29,30].

In this study, we show for the first time that cells in the human liver express the MSC marker CD271. Isolated CD271 positive cells showed the characteristic MSC immunophenotype and were able to differentiate to adipocyte- and osteoblast, but not hepatocyte-like cells. However, the cells exhibited immunomodulatory potential, inhibiting immune reactions towards HepG2 cells, making them promising candidates for hepatocyte co-transplantation purposes.

## Material and Methods

### Ethics statement

Fetal MSCs were isolated from liver samples from fetuses that were aborted in the first trimester after modified vacuum curettage where patients had volunteered to donate fetal tissue. Adult MSCs were isolated from bone marrow aspirated from the iliac crest from healthy volunteers. Adult liver sections and primary hepatocytes used in this study were obtained from deceased donors. Peripheral blood was taken from healthy individuals that had volunteered to donate blood. Informed written consent was obtained from all donors participating in this study and it was approved by the Regional Ethical Review Board in Stockholm.

### Analysis of CD271<sup>+</sup> cells within the human fetal and adult liver

To localize CD271<sup>+</sup> cells within the human liver, immunoperoxidase staining of CD271 (Stem Cell Technologies, Vancouver, Canada) in fetal and adult liver sections was performed. Human fetal and adult liver pieces were covered in tissue tek OCT (Sakura Finetek Europe, Zoeterwoude, The Netherlands) and fresh-frozen in liquid nitrogen. Cryosections of 6 µm thickness were prepared using a cryostat (Leica, Köln, Germany). Sections were allowed to air dry for 10 minutes at room temperature before staining. ImmPRESS REAGENT KIT (Vector Laboratories, Burlingame, CA, USA) was used according to the manufacturer's instructions. Briefly, sections were fixed in 30 % acetone in methanol. 0.3% hydrogen peroxide in H<sub>2</sub>O was used to quench any endogenous peroxidase activity. Horse serum from the kit was used for blocking nonspecific binding. DAB substrate kit (Vector Laboratories) was used for color development.

## Preparing single cell suspension of human fetal liver

Fetal liver samples from terminated pregnancies were used to isolate human fetal MSCs. Estimating the gestational age of the fetuses was done according to anatomical markers. Pregnancy terminations with no apparent medical problems were selected, and fetuses with no obvious congenital anomalies were used. Liver samples were dissected and kept in a sterile tube containing RPMI 1640 medium (Invitrogen, Lidingö, Sweden). The liver tissue was perfused using phosphate buffered saline (PBS) to discard circulating cell contaminants. A single cell suspension was prepared by gently pushing the liver tissue through a 70 µm nylon cell strainer (BD Bioscience, Stockholm, Sweden) with continuous flushing using RPMI 1640. The cell suspension, now called human fetal liver cells (hFLCs), was centrifuged at 200 g for 10 minutes to pellet the cells.

## Positive isolation of CD271<sup>+</sup> hFL-MSCs

Three human fetal liver samples (7.5, 9, and 9.5 wk) were used in this study. hFLCs were cultured in T-25 flasks in low glucose Dulbecco's Modified Eagle Medium (DMEM-LG) supplemented with 50 U/ml penicillin, 50 µg/ml streptomycin, and 10% FBS (all from GIBCO), herein named MSC medium. Three days later, the cells were harvested using Trypsin-EDTA 0.05% (GIBCO). EasySep<sup>®</sup> Human CD271 Selection Kit (Stem Cell Technologies) was used to enrich for CD271<sup>+</sup> hFL-MSCs according to the manufacturer's instructions. Briefly, hFLCs were suspended at a concentration of 1×10<sup>8</sup> cells/ml in cold PBS containing 2% FBS and 2 mM EDTA. The cell suspension was passed through a 70 µm nylon cell strainer to ensure a single cell suspension and transferred to 12×75 mm polystyrene tubes in a volume of 1 ml. Human Fc blocker, anti-human CD271 antibody, and magnetic particles were added according to the protocol. The cell suspension was brought up to a volume of 2.5 ml/tube. Three magnetic separations of 5 minutes each were carried out using the EasySep<sup>®</sup> Magnet (Stem Cell Technologies). Isolated hFL-MSCs were plated at a density of 4×10<sup>3</sup> cells/cm<sup>2</sup> in 6-well plates in MSC medium. The medium was changed every 3-4 days. Cells were passaged as described above when reaching 70-80% confluence, which was done 3-5 times before being used for further analysis.

## Characterization of hFL-MSCs

**Flow cytometry:** Flow cytometry characterization of hFL-MSCs was performed as previously described [22]. Cells were stained with fluorescently conjugated antibodies against CD271 (LNGFR) (Miltenyi Biotech, Bergisch, Germany), CD31, CD73 (NT5E), CD90 (THY1), CD80, CD86 (Becton Dickinson, San Jose, CA, USA), CD105 (SH2) (Ansell, Bayport, MN, USA), CD44 (Immunotech, Marseilles, France), CD14, CD34, and CD45 (PTPRC) (Becton Dickinson), HLA-DR, HLA class I, and HLA class II (DakoCytomation, Glostrup, Denmark). Non-specific fluorescence was determined by using equal aliquots of cell preparations incubated with rabbit-anti-mouse monoclonal isotype antibodies (Becton Dickinson). Fluorescence was analyzed in a flow cytometer (FACSsort, Becton Dickinson).

**Immunohistochemical staining:** hFL-MSCs were plated in 8-well chamber culture slides at a density of 4×10<sup>3</sup> cells/cm<sup>2</sup> in MSC medium. Medium was changed every 3-4 days until 90% confluence. Immunoperoxidase staining was carried out using ImmPRESS REAGENT KIT (Vector Laboratories) as mentioned above. hFL-MSCs were stained with non-conjugated monoclonal antibodies against: CD271 (Stem Cell Technologies), CD90 (eBioscience, San

Diego, CA, USA), CD34, CD45, CD73, CD105 and CD166 (all from Becton Dickinson). The AEC substrate kit (Vector Laboratories) was used for color development. Cells were counterstained with haematoxylin and mounted in aqueous mounting medium (Vector Laboratories).

**Differentiating hFL-MSCs to osteogenic, adipogenic and hepatogenic cells *in vitro*:** hFL-MSCs from passages 3-5 were used. The differentiative capacity of hFL-MSCs along adipogenic and osteogenic lineages was assessed as previously described [22,31]. For osteogenic stimulation, hFL-MSCs were plated at a density of  $3.1 \times 10^3$  cells/cm<sup>2</sup> in 48-well plates, let adhere overnight and incubated with DMEM-LG supplemented with 50 U/ml penicillin, 50 µg/ml streptomycin, 10% FCS, 10 nM dexamethasone, 10 mM glycerophosphate, and 0.05 mM ascorbic acid-2-phosphate (all from Sigma Aldrich, Stockholm, Sweden). Control hFL-MSCs were cultured simultaneously in MSC medium. The media was changed every 3-4 days for 3 weeks. Von Kossa and Alizarin red S staining (Sigma Aldrich) was performed to detect for calcium deposition as a sign for bone differentiation. Quantification of Alizarin red S staining was performed by eluting the stain in 10% (w/v) cetylpyridinium chloride (Sigma Aldrich). Absorbance was measured at 562 nm in a spectrophotometer.

For adipogenic stimulation, the cells were seeded at a density of  $2.1 \times 10^4$  cells/cm<sup>2</sup> in 48-well plates, let adhere overnight and cultured alternatively with induction or supportive media every 3-4 days for 3 cycles. Control hFL-MSCs were cultured simultaneously in MSC medium. The induction medium contained: Dulbecco's high glucose DMEM (DMEM-HG) (GIBCO), 50 U/ml penicillin, 50 µg/ml streptomycin, 10% FCS, 0.5 mM 1-methyl-3-isobutylxanthine, 10 mM insulin, 0.2 mM indomethacin and 1 µM dexamethasone (all from Sigma Aldrich). The supportive medium contained: DMEM-HG, 50 U/ml penicillin, 50 µg/ml streptomycin, 10% FCS, and 10 mM insulin. Morphological analysis and staining with Oil red O (Sigma Aldrich) were carried out to detect lipid accumulation as a sign for successful differentiation. Quantification of the staining was performed by eluting the Oil red O using 100% isopropanol. Absorbance was measured at 500 nm in a spectrophotometer.

Hepatogenic differentiation was carried out as previously described [32,33]. hFL-MSCs were plated in 48-well plates at a density of  $1.5 \times 10^4$ /cm<sup>2</sup> in MSC medium. The differentiation started 24 hours later by shifting to an induction medium for 10 days followed by a maturation medium for 21 days with medium change every 3 days. Control hFL-MSCs were cultured simultaneously in MSC medium. The induction medium contained: Iscove's modified Dulbecco's medium (IMDM) (GIBCO) supplemented with 20 ng/ml hepatocyte growth factor (Peprotech, Paris, France), 10 ng/ml basic fibroblast growth factor, 0.61 g/l nicotinamide (Sigma Aldrich), and 1% insulin-transferrin-selenium (GIBCO). The maturation medium contained: IMDM supplemented with 20 ng/ml oncostatin M (Peprotech), 1 µM dexamethasone, and 1 % insulin-transferrin-selenium (Sigma Aldrich).

**Evaluating hFL-MSC differentiation towards hepatocytes:** hFL-MSC's ability to differentiate to hepatocytes was evaluated by immunochemical staining for hepatic markers and by measuring cytochrome P450 (CYP) activity. Undifferentiated hFL-MSCs in addition to the control hFL-MSCs were included for comparison. Immunoperoxidase staining was performed using the

VECTASTAIN Universal Quick kit (Vector Laboratories) according to the manufacturer's instructions. The cells were analyzed for alpha-fetoprotein (AFP) (DakoCytomation) 1:100, albumin (ALB) (Bethyl Laboratories, Montgomery, TX, USA) 1:200, cytokeratin 19 (CK19) 1:100 and cytokeratin 18 (CK18) 1:100 (both from Abcam, Cambridge, MA). Frozen sections of human fetal and adult livers were included for comparison. DAB substrate kit (Vector Laboratories) was used for color development.

The activity of the following CYPs; CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4, and CYP3A7, was assessed using specific P450-Glo substrates and their specific Luciferin Detection Reagents (Promega, Madison, WI, USA) according to the manufacturer's instructions. hFLCs and primary adult hepatocytes were included for comparison. hFLCs were prepared as described above, and cultured in 96-well plates and cultured in MSC medium for 2-3 days before running the functional assay. Primary human adult hepatocytes were isolated using a three-step collagenase perfusion procedure as described elsewhere [34], and plated at a density of  $4 \times 10^4$ /well in collagen-coated 96-well plate and cultures in William's E Medium (Lonza, Denmark) for 2-3 days before running the functional assay. CYP activities were normalized to the amount of double-stranded DNA per well using the Quant-iT PicoGreen dsDNA Reagent and Kit (Molecular Probes, Eugene, USA) according to the manufacturer's instructions. PicoGreen fluorescence was measured using a fluorescence plate reader (TECAN, infinite F500, Grödig, Austria).

**Evaluating hFL-MSCs immunogenicity and immunomodulatory potential:** Mixed lymphocyte cultures (MLCs) were performed as described previously to analyze the immunogenicity and the immunomodulatory potential of hFL-MSCs isolated by selection for CD271 [22]. Adult peripheral blood lymphocytes (PBLs) were collected by centrifugation on a Ficoll gradient (Lymphoprep, Nycomed Pharma, Oslo, Norway). Triplicate samples of  $2 \times 10^5$  PBLs were cultured with  $2 \times 10^5$  irradiated (20 Gy) autologous or allogeneic PBLs pooled from five donors with or without  $2 \times 10^4$  (10 %) irradiated hFL-MSCs. PBLs were also co-cultured with irradiated HepG2 cells with or without  $1 \times 10^4$  irradiated hFL-MSCs. The cells were cultured in a volume of 0.2 ml RPMI 1640 medium supplemented with, 100 IU/ml penicillin, 100 µg/ml streptomycin, 20 mM L-glutamine (Invitrogen), and 10% pooled human AB serum in 96-well plates. The cultures were incubated at 37°C in humidified 5% CO<sub>2</sub> air for 6 days. On day 5, 1 µCi/ml [<sup>3</sup>H]-thymidine (Amersham Pharmacia Biotech, Little Chalfont, UK) was added. After 24 hours, the cells were harvested on a glass-fiber filter (Wallac, Turku, Finland), using a semiautomatic harvesting machine (Harvester 96, Tomtec, Orange, Conn., USA). Radioactivity was determined as counts per minute (cpm) with an Intertechnique beta-counter (Wallac).

### Statistical analysis

Paired Student T test was carried out between the control and samples in MLCs. Results were considered significant when P values were  $\leq 0.05$ .

## Results

### CD271 is expressed in human fetal and adult livers

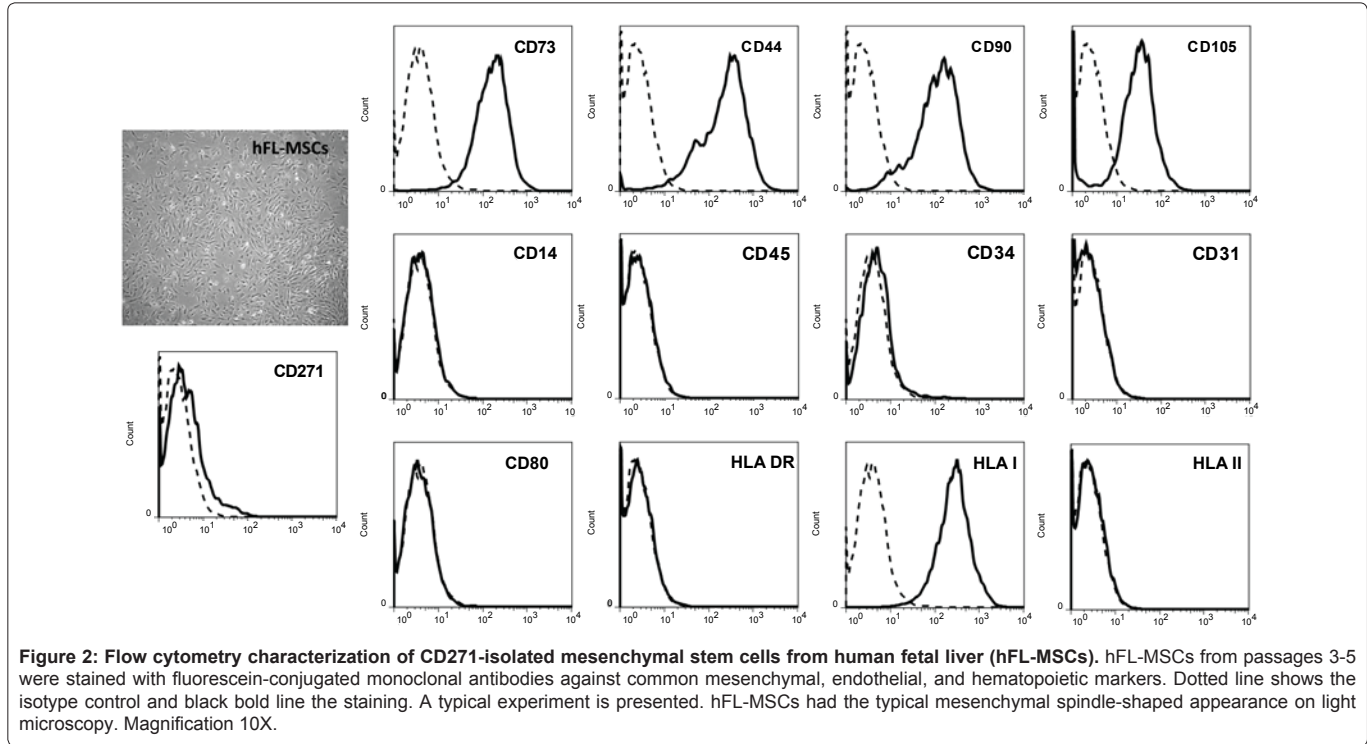
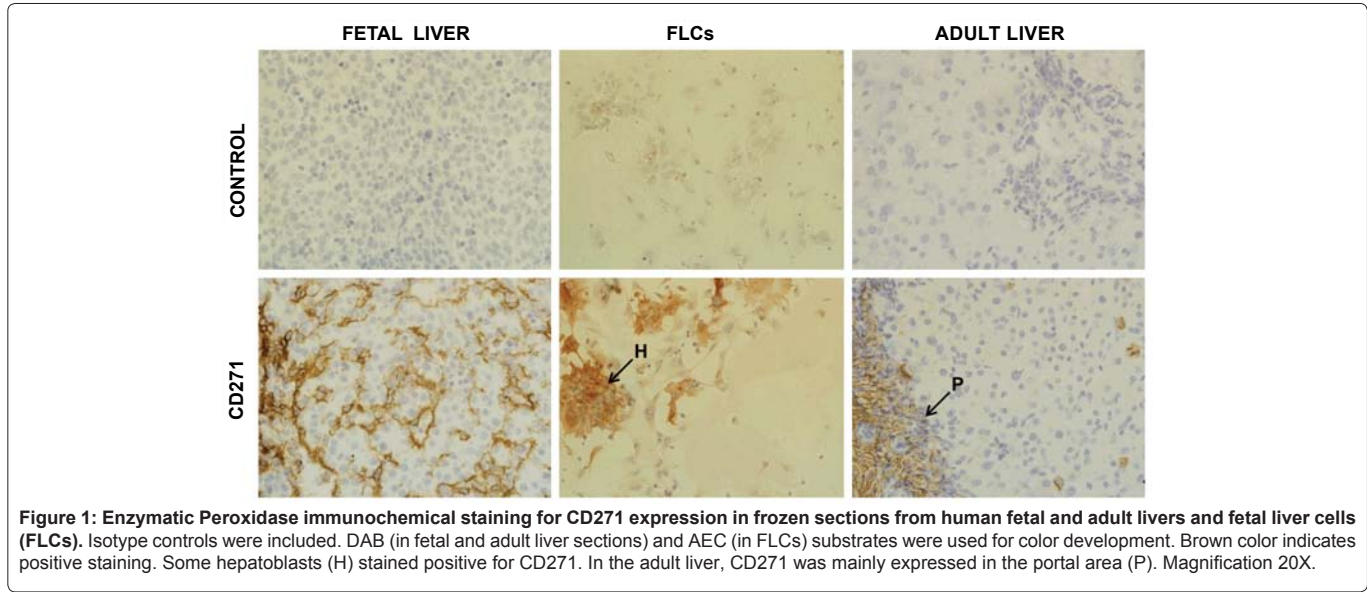
CD271 was tracked by immunochemical staining in sections from fetal and adult livers as well as in FLCs cultured for 3 days (Figure 1). In the fetal liver, expression of CD271 was evenly distributed (70.1% cells in gestation weeks (4+0)-(7+6) were positive for CD271

in the human fetal liver), not limited to mesenchymal-like cells but also expressed in some hepatoblast colonies as seen in FLC staining. CD271 expression was lower in the adult (23.4%) compared to the fetal liver (63.8-70.1%) and was limited to the portal areas except for a few scattered cells within the hepatic lobules.

**hFL-MSCs are positive for typical surface proteins**

hFL-MSCs were successfully isolated with selection for CD271 from 10 fetal liver samples and three were further culture expanded for characterization. Cultured hFL-MSCs were a phenotypically homogenous population having the typical mesenchymal spindle-

shaped appearance (Figure 2). When characterized by flow cytometry at passages 4-6, hFL-MSCs showed the typical immunophenotype common for MSCs [22]. hFL-MSCs were positive (more than 90% positive cells) for: CD105 (SH2), CD44, CD73 (SH4), CD90 (Thy1), and HLA class I, partially positive (17-20%) for CD271, and negative (less than 5% positive cells) for: CD14, CD45, CD31, CD34, CD80, CD86, HLA class II and HLA-DR (Figure 2). The immunochemical characterization confirmed the flow cytometry characterization (data not shown). Data from flow cytometry analysis and immunochemical staining showed that hFL-MSCs lost CD271 expression at passage four to six.



**hFL-MSCs differentiated to osteogenic and adipogenic cells *in vitro***

hFL-MSCs also showed an ability to differentiate towards the osteogenic lineage when induced with osteogenic medium for 3 weeks. Von Kossa and Alizarin red S staining had showed calcium deposition as a sign for successful osteogenic differentiation (Figure 3D and figure 3E) compared to the control (Figure 3A and figure 3B) respectively. The three hFL-MSC samples showed little variation in their osteogenic differentiation potential, which is evident from Alizarin Red S quantification data (Figure 3G).

Oil red O staining of hFL-MSCs induced for three cycles with adipogenic media showed deposition of fat vacuoles as a sign for their adipogenic differentiation (Figure 3F) compared to the control (Figure 3C). The differentiation potential towards adipocytes varied between samples with one sample showing greater adipogenic differentiation ability than the other two. This was clearly seen in Oil red O quantification data (Figure 3H).

**hFL-MSCs did not differentiate to functional hepatogenic cells *in vitro***

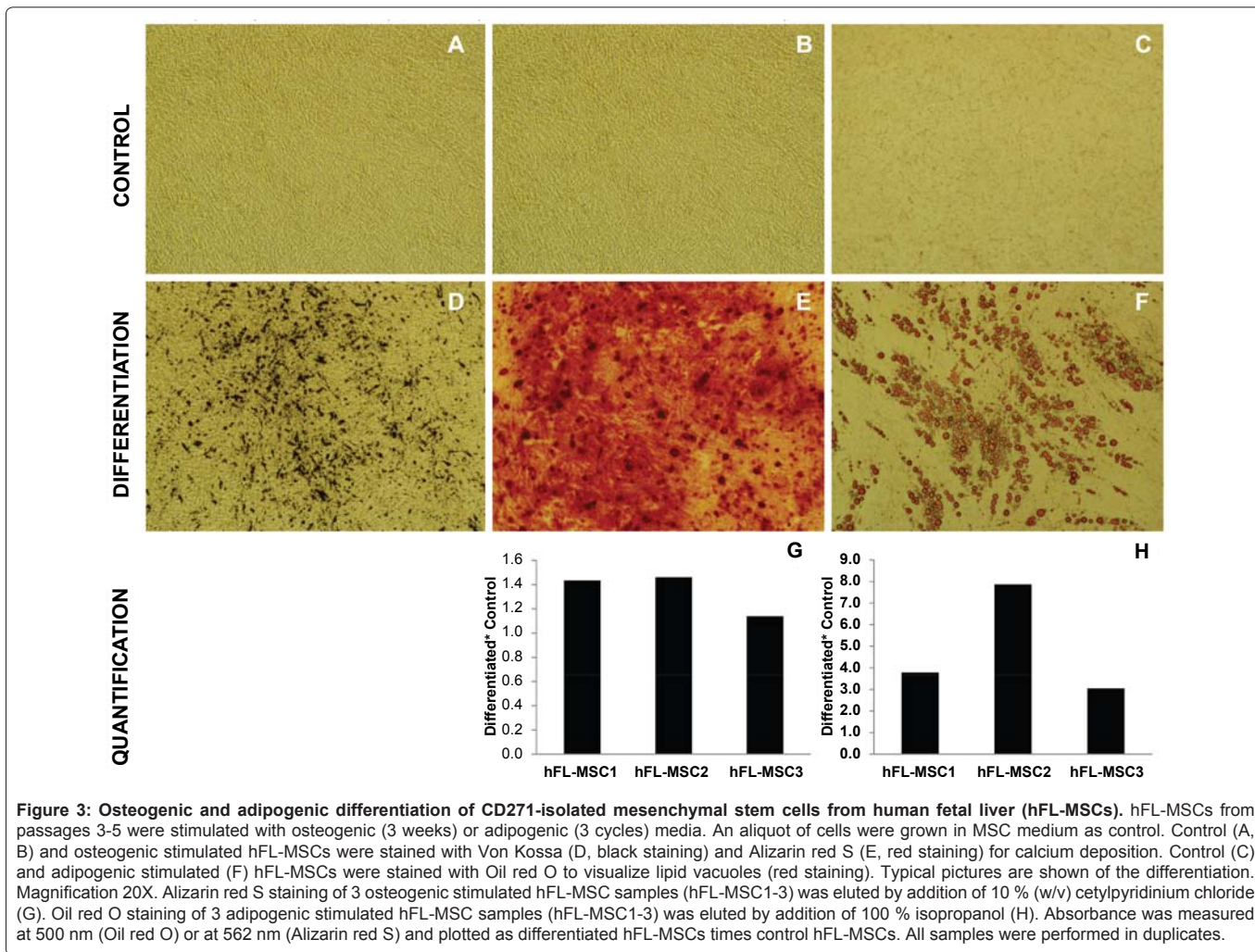
hFL-MSCs cultured in hepatogenic media for 30 days showed morphological changes in the form of expanded cell size with

highly granular cytoplasm and large nuclei (Figure 4). However, the stimulated cells did not show increased expression of the hepatic markers AFP, ALB, CK18, or CK19 compared to the control cells (hFL-MSC cultured in MSC medium for 30 days) upon immunochemical staining. The stimulated cells even showed reduced expression of their hepatic markers, mostly obvious in the case of CK18 and CK19. In contrast, these markers were surprisingly expressed in a higher degree in the undifferentiated hFL-MSCs at the beginning of the experiment (Figure 4).

Furthermore, the functional analysis showed no CYP activity in the hepatogenic stimulated hFL-MSCs compared to FLCs and adult hepatocytes. This included 6 major CYPs tested (CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4, and CYP3A7). FLCs showed activity for CYP1A2, CYP3A7, and CYP3A4 compared to adult hepatocytes in which activity of all the CYPs was clearly detected.

**hFL-MSCs did not elicit an immune response *in vitro***

To determine whether hFL-MSCs isolated by CD271 separation can induce a proliferative response among allogeneic lymphocytes, we performed mixed lymphocyte cultures (MLC). As seen in (Figure 5A), 10 % hFL-MSCs co-cultured with peripheral blood lymphocytes (PBLs) *in vitro* did not elicit an immunological response, although a



**Figure 3: Osteogenic and adipogenic differentiation of CD271-isolated mesenchymal stem cells from human fetal liver (hFL-MSCs).** hFL-MSCs from passages 3-5 were stimulated with osteogenic (3 weeks) or adipogenic (3 cycles) media. An aliquot of cells were grown in MSC medium as control. Control (A, B) and osteogenic stimulated hFL-MSCs were stained with Von Kossa (D, black staining) and Alizarin red S (E, red staining) for calcium deposition. Control (C) and adipogenic stimulated (F) hFL-MSCs were stained with Oil red O to visualize lipid vacuoles (red staining). Typical pictures are shown of the differentiation. Magnification 20X. Alizarin red S staining of 3 osteogenic stimulated hFL-MSC samples (hFL-MSC1-3) was eluted by addition of 10 % (w/v) cetylpyridinium chloride (G). Oil red O staining of 3 adipogenic stimulated hFL-MSC samples (hFL-MSC1-3) was eluted by addition of 100 % isopropanol (H). Absorbance was measured at 500 nm (Oil red O) or at 562 nm (Alizarin red S) and plotted as differentiated hFL-MSCs times control hFL-MSCs. All samples were performed in duplicates.

small but significant increase in proliferation was noted when hFL-MSC were added (n=6).

**hFL-MSCs inhibited proliferation of allogeneic lymphocytes *in vitro***

To determine whether hFL-MSCs affected the proliferation of lymphocytes in response to alloantigens, PBLs were stimulated with PBLs from five donors in MLCs. Addition of 10% hFL-MSCs to the allogeneic response significantly inhibited lymphocyte proliferation in the three tested samples compared to the positive control (MLC

(n=6), (Figure 5B). When 10% hFL-MSCs was added to co-cultures of PBLs and HepG2 cells, hFL-MSCs had variable effects on allogeneic lymphocyte responses towards HepG2 cells: one hFL-MSC sample could significantly inhibit lymphocyte proliferation while the other two seemed to stimulate lymphocyte proliferation compared to MLC with HepG2 (n=6), (Figure 5C).

**Discussion**

The use of stem cells in LCT is attractive to overcome the shortage of liver tissue. For this purpose, many adult and fetal

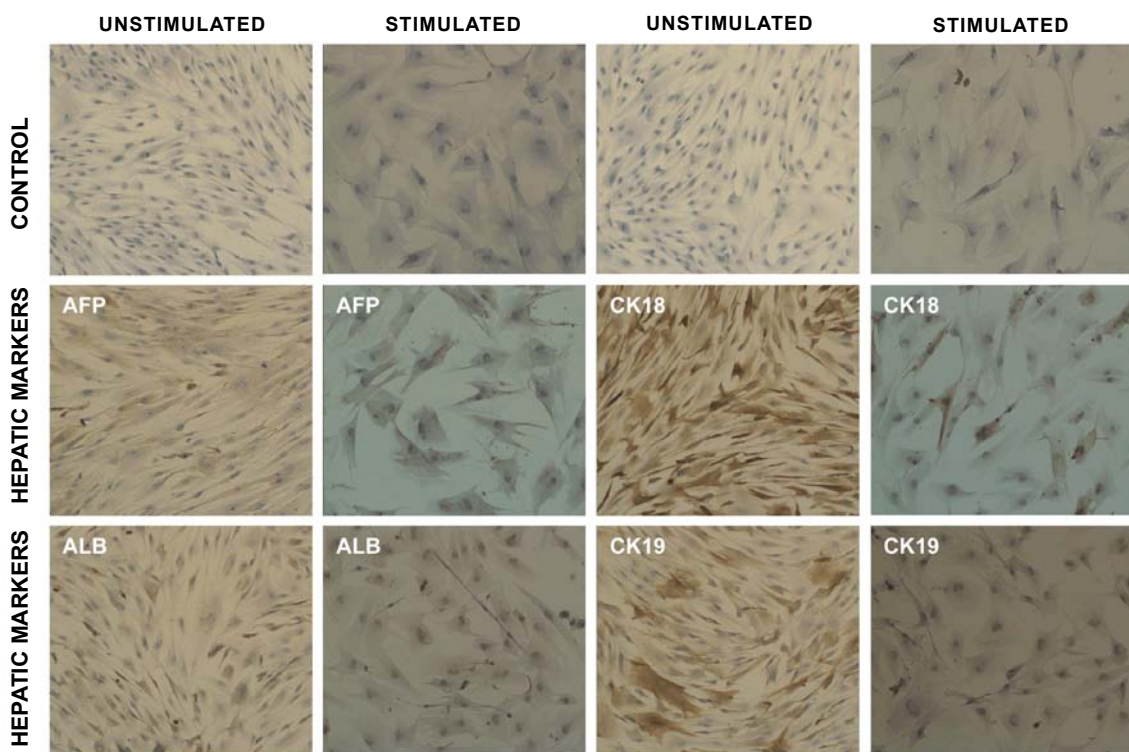


Figure 4: Enzymatic Peroxidase immunochemical staining of hepatogenic stimulated CD271-isolated mesenchymal stem cells from human fetal liver (hFL-MSCs). hFL-MSCs were cultured for 30 days with hepatogenic or MSC medium. hFL-MSCs were thereafter stained for alpha-fetoprotein (AFP), albumin (ALB), and cytokeratin 18 and 19 (CK18 and 19). Isotype controls were included. DAB substrate was used for color development and brown color indicates positive staining. Representative pictures of the stainings are shown. Magnification 20X.

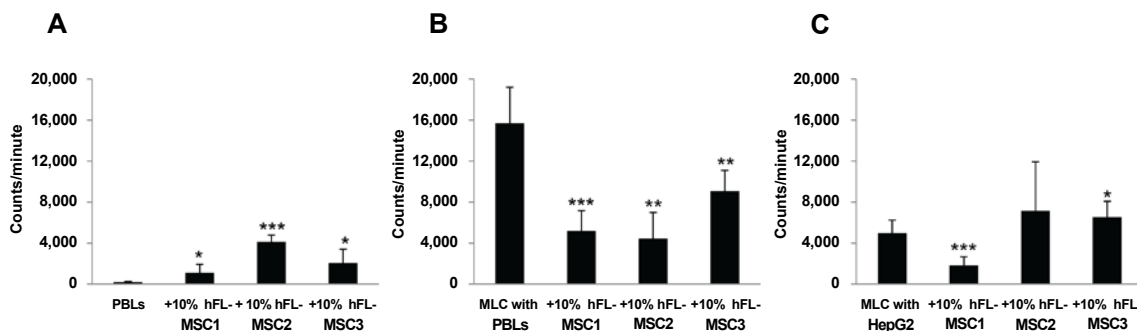


Figure 5: Mixed lymphocyte cultures (MLCs) with mesenchymal stem cells from human fetal liver (hFL-MSCs). 10 % irradiated third party irradiated hFL-MSCs were co-cultured for six days with 200,000 responder and/or stimulator cells. Counts/minute depicts thymidine incorporation in the responder cell DNA. Mean  $\pm$  STD of 6 experiments. (A) Lymphocyte immunological reaction towards hFL-MSCs at 6 days. (B) hFL-MSCs were co-cultured for six days with responder peripheral blood lymphocytes (PBLs) and an irradiated pool of stimulator PBLs, respectively. (C) hFL-MSCs were co-cultured for six days with responder PBLs and irradiated stimulator HepG2 cells, respectively. (\*) =  $P \leq 0.05$ , (\*\*) =  $P \leq 0.005$ , (\*\*\*) =  $P \leq 0.001$ .

stem cell populations are under investigation. In human fetal liver, different stem cells have been identified that have high proliferative and differentiative capacities [22,35]. Human liver progenitor cells are usually transplanted as a mixture of heterogeneous cells [36-38], however, transplantation of a heterogeneous cell population does not state the exact role exhibited by a specific cell type and it may be less efficient [39,40]. This is why we in this study aimed to find a simple, quick and efficient procedure to isolate a homogenous population of MSCs from human fetal liver as a potential off-the-shelf product for LCT. Our choice for fetal MSCs was for their suggested stronger therapeutic potentials being more primitive and having marked proliferative [20,21] and differentiative [23,24] capacities compared to their adult equivalents. We also believe that it is of benefit to use cells that are originally from the liver.

Previously, methods for isolating MSCs have mainly been based on nonspecific cell characteristics like plastic adherence, density gradient centrifugation, or cell depletion antibody cocktails. Therefore, contamination by other cell types cannot be rigorously discounted. Isolation of MSCs through clonal culture has been reported [41], but this method involves tedious laboratory work and cell yield is low. In contrast, the herein described isolation method is simple, fast, and directly enriches for a pure MSC population.

CD271 is a well-established MSC marker and has been successfully used in enrichment of MSCs from different tissues e.g. bone marrow [42], term fetal membranes [43], trabecular bone [44], and adipose tissue [45]. CD271<sup>+</sup> MSCs showed higher colony forming unit-fibroblast activity than MSCs isolated using density gradient centrifugation, RosetteSep technique, or CD133-positive selection [30,46]. In this study, MSCs isolated from human fetal liver by selection for CD271 showed similar characteristics to bone marrow-derived MSCs in terms of fibroblastic morphology, adherence to plastic, immunophenotype, and mesodermal differentiative capacity towards osteogenic and adipogenic cells. hFL-MSCs were easily isolated using CD271 and propagated *in vitro* for at least 10 passages. We did not investigate whether they can continue growing beyond that since at that point one would have enough cells for clinical transplantation. In comparison, MSCs from human fetal membranes and placenta could only proliferate to the 5<sup>th</sup> passage [43,47]. hFL-MSCs lost their CD271 expression after propagation (between passage 4-6). The reason for this is not clear to us, and may limit the value of CD271 to more of an enrichment marker. However, bone marrow MSCs markedly increased expression of CD271 after cryopreservation and culture [48], which implies that a change in CD271 expression upon culturing and cryopreservation could be a possible explanation. hFL-MSCs induced towards the hepatogenic lineage showed similar morphological changes to those described before for hepatocyte-like cells differentiated from different cell types of mesodermal origin using the same protocol [32,33,49,50]. However, in our hands the stimulated cells did not acquire hepatocyte-like features on protein level or achieved any CYP activity. It is possible that hFL-MSCs isolated by CD271 do not have hepatogenic differentiation ability or that the differentiation protocol needs modification for hFL-MSCs. Since the previous publications using the same protocol did not extensively analyze for CYP activity, we can only speculate on this. On the other hand, a summary on the regenerative potential of MSCs concluded that so far there is no convincing evidence for MSC differentiation to hepatocyte-like cells with a broad range of mature

hepatocyte functions [51] debating true MSC trans-differentiation towards hepatic lineages [52].

The early events in human liver development depict a concerted interplay between endoderm and mesoderm. A population of cells expressing both mesenchymal and hepatic markers was recently isolated from the human fetal liver. These cells were suggested to be progenitors to hepatoblasts [53]. Furthermore, a mesodermal origin of the liver parenchyma has been postulated [54]. The marked expression of CD271 in the human fetal liver, its continued but lower expression in the adult liver and its expression by hepatoblast-like cells suggests a potential role for MSCs in liver development and regeneration *in vivo*. Use of MSCs in LCT is under extensive investigation and the potential role of MSCs in supporting hepatocytes both *in vivo* and *in vitro* [19] has been recently reviewed [55]. Allogeneic MSC transplantation has been suggested to support hepatocyte survival, proliferation, and function both *in vivo* and *in vitro* and to decrease the host allogeneic response to transplanted hepatocytes and modulate stellate cell activation in liver cirrhosis causing its regression [19,55]. Promising phase-I/II clinical trials strongly supported MSC use in patients with decompensated liver cirrhosis [56-58] and liver failure [59]. With the present study we expand this proposition to include MSCs from fetal liver since we herein describe that hFL-MSCs isolated by means of CD271 selection are non-immunogenic and immunomodulatory. The immunomodulatory effect of hFL-MSCs is supported by data from fetal MSCs isolated from human fetal liver by plastic adherence [22]. The immunomodulatory characteristics of MSCs may have therapeutic effects for example tissue repair, which could be mediated by the expression of different factors by MSCs that in turns promotes the survival and proliferation of endogenous cells, inducing angiogenesis, inhibiting inflammatory and immune responses and reducing apoptosis [60]. The inhibitory effect of hFL-MSCs on lymphocyte allogeneic responses in co-cultures of lymphocytes and HepG2 cells, suggests their potential immunosuppressive role in hepatocyte co-transplantation. Whether hFL-MSCs present a better alternative over other MSC population in hepatocyte co-transplantation because of their fetal and liver origin needs further evaluation. Furthermore, before MSC routine use in clinical, more information are still needed regarding their fate and long-term survival in host liver, their mode of action, and safety of their use in terms of tumor formation or propagation.

In conclusion, we show for the first time positive isolation of MSCs from 1<sup>st</sup> trimester human fetal liver using CD271. hFL-MSCs showed mesodermal differentiation and expressed both mesenchymal and hepatic markers, although it was not possible to induce them into functional hepatocytes *in vitro* under the herein described conditions. hFL-MSCs did not induce an immune response and exhibited immunomodulatory properties, making them interesting as co-transplants with hepatocytes in LCT, and in research on LSPCs and human liver development.

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