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Characterization and hepatic differentiation of skin-derived precursors from adult foreskin by sequential exposure to hepatogenic cytokines and growth factors reflecting liver development

Joery De Kock [a,*], Tamara Vanhaecke [a], Jeffrey Biernaskie [b], Vera Rogiers [a], Sarah Snykers [a]

[a] Department of Toxicology, Vrije Universiteit Brussel (VUB), Laarbeeklaan 103, B-1090 Brussels, Belgium
[b] The Hospital for Sick Children MaRS Centre, Development and Stem Cell Biology, 101 College Street, Toronto, ON, Canada

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ABSTRACT

In the present study, we investigated whether precursor cells isolated from the dermis of infant human foreskin are capable to differentiate into hepatocyte-like cells upon sequential and gradual exposure to hepatogenic factors [fibroblast growth factor (FGF)-4, hepatocyte growth factor (HGF), insulin–transferrin–selenite (ITS), dexamethasone and oncostatin M (OSM)], mimicking the liver embryogenesis *in vivo*.

Undifferentiated human skin-derived precursors (hSKP) are characterized by a fibroblast-like shape. Yet, they already express typical hepatic proteins, including cytokeratin (CK)-18, hepatocyte nuclear factor (HNF)-4 and HNF-1α. Microarray analysis further reveals gene expression of (i) the stemness markers nestin, POU5F1 (OCT-4), telomerase reverse transcriptase (TERT) and thymocyte differentiation antigen (THY)-1, (ii) biliary CK14 and CK19, (iii) biliary/foetal hepatic connexin (Cx)-43, and (iv) adult hepatic CK18, HNF-4 and HNF-1α. Upon differentiation, cells undergo morphological and phenotypic changes. As such, hSKP adopt a more polygonal-to-cuboidal cell shape. At the protein level, Cx43 expression is downregulated whereas typical hepatic markers, including alfa-foetoprotein (AFP), prealbumin (TTR) and albumin (ALB), become expressed in accordance to *in vivo* patterns observed during hepatogenesis. In conclusion, these data show for the first time that hSKP are capable to "trans" differentiate into hepatocyte-like cells upon mimicking the *in vivo* micro-environment of developing hepatocytes *in vitro*.

1. Introduction

The predisposition of a reliable model that can be applied during early preclinical toxicological research to generate accurate data on metabolism, efficacy and safety of candidate drugs in humans is of utmost importance. In our previous research, adult rat and human bone marrow stem cells and neonatal rat biliary progenitor cells were successfully triggered to differentiate into a homogeneous population of functional hepatocyte-like cells by exposing them to hepatogenic factors in a gradual and sequential time-dependent manner mimicking their secretion pattern during *in vivo* hepatogenesis (Snykers et al., 2006, 2007; Patent Submission PCT-EP2004-0012134; Patent Submission PCT/EP2006/005622). The question is now whether adult stem cells, obtained from an easily accessible human source, such as human skin, could also be used to produce an unlimited amount of functional human hepatocytes. A promising type of stem cells are the human skin-derived precursors (hSKP) isolated from the dermis of infant human foreskin (Biernaskie et al., 2006). These potential stem/progenitor cells exhibit a high self-renewal capacity and plasticity (Buranasinsup et al., 2006; Biernaskie et al., 2006; Fernandes et al., 2004). In the present study, the geno/phenotype of these hSKP is analysed and it is investigated whether these cells are capable to differentiate into hepatocyte-like cells when cultured under the same conditions as optimised for postnatal bone marrow and biliary stem/progenitor cells.

2. Materials and methods

2.1. Isolation and subcultivation of undifferentiated hSKP

hSKP were isolated and subcultivated as previously described (Biernaskie et al., 2006). Briefly, the hSKP isolation procedure is as follows. Freshly collected human skin samples are separately

Abbreviations: ALB, albumin; AFP, alpha-foetoprotein; CK, cytokeratin; Cx, connexin; D, differentiation day; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; HNF, hepatocyte nuclear factor; hSKP, human skin-derived precursors; ITS, insulin–transferrin–selenite; LETFs, liver-enriched transcription factors; MRP, multidrug resistance protein; OSM, oncostatin M; TERT, telomerase reverse transcriptase; THY, thymocyte differentiation antigen; TTR, transthyretin.

* Corresponding author. Tel.: +32 2 477 45 17; fax: +32 2 477 45 82.
E-mail address: jdekock@vub.ac.be (J. De Kock).

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incubated with 25 ml of Blendzyme solution (Roche Applied Science, Vilvoorde, Belgium) and gradually cut into small samples of 5–8 cm^2 and 3–5 mm^2. Upon 20 h incubation with Blendzyme solution, the epidermis, blood vessels and remaining adipose tissue (if present) are removed. The samples are then cut into smaller pieces (1–2 mm^2) and incubated at 37 °C for 10–20 min depending on the sample size. During the last minute, 400 μl DNase I (Sigma, Bornem, Belgium) is added to the samples. In order to inactivate all enzymes, samples are next incubated for 5 min at 4 °C with 10% foetal bovine serum (FBS) (Perbio Science, Hyclone, Erembodegem, Belgium). We then grind the samples for ±2 min and shortly spin the cells to pellet down the larger pieces. The supernatant is next brought through a cell strainer and centrifuged for 6 min at 600 g, after which the pellet is resuspended in 1 mL of growth medium. Upon the isolation procedure, viable cells are counted using trypan blue staining. About 5–15 × 10^6 viable cells are obtained per 5–8 cm^2 piece. For cultivation, a cell density of 20,000 cells/cm^2 is applied. Growth media is refreshed every 2–3 days. Spheres are being formed from days 5–7 on. Growth medium for hSKP consists of Dulbecco's Modified Eagle's Medium (DMEM) + Glutamax and F12 Nutrient Mixture (3:1) (all from Invitrogen, Merelbeke, Belgium) supplemented with 50 μg/mL streptomycin sulphate, 733 IU/mL benzyl penicillin, 2.5 μg/mL fungizone, 2% B27 supplement, 40 ng/mL basic FGF-2, 20 ng/mL epidermal growth factor (EGF) (all from Invitrogen).

2.2. Hepatocyte differentiation

hSKP between 1 and 3 passages were cultivated at 90% confluency on 100 μg/ml collagen type I in base medium and sequentially exposed to hepatogenic factors. Base medium consisted of Dulbecco's Modified Eagle Medium + Glutamax/F12 nutrient supplement (3:1) (all from Invitrogen), supplemented with 733 IE/ml penicillin (Continental Pharma, Diegem, Belgium), 50 μg/ml streptomycin, 0.1 mM L-ascorbic acid, 0.03 mM nicotinamide, 0.25 mM sodium pyruvate (all from Sigma) and 2.5 μg/mL fungizone (Invitrogen). The procedure was as follows: day 0: base medium + 10 ng/ml FGF-4 (R&D Systems, Minneapolis, MN); days 1–2: base medium + 10 ng/ml FGF-4 + 20 ng/ml HGF (all from R&D Systems); days 3–5: base medium + 5 ng/ml FGF-4 + 30 ng/ml HGF + 0.5 × ITS (Sigma); days 6–8: base medium + 30 ng/ml HGF + 0.25 × ITS + 20 μg/l dexamethasone (Sigma); days 9–11: base medium + 20 ng/ml HGF + 20 μg/l dexamethasone; and from day 12 onwards: base medium + 20 ng/ml HGF + 20 μg/l dexamethasone + 10 ng/ml OSM (R&D Systems). Unless otherwise defined, media were changed every 3 days.

2.3. Immunocytochemistry

Immunofluorescence was performed as previously described (Snykers et al., 2006). The primary antibodies against cKit, AFP, HNF-3β, HNF-4, TTR, HNF-1α and multidrug resistance protein (MRP)-2 were purchased from Santa Cruz (Heidelberg, Germany). Anti-CK18, anti-CK19, anti-Cx43 and anti-Cx32 antibodies were purchased from Sigma and anti-ALB antibody from Bethyl Laboratories (Montgomery, TX). Respective secondary antibodies were purchased from Jackson Immunoresearch (De Pinte, Belgium).

2.4. RNA isolation and microarray analysis

Undifferentiated hSKP were subjected to microarray analysis. Cell samples were lysed using SuperAmp™ lysis buffer and stored according to the instructions of the SuperAmp preparation kit (Miltenyi Biotec, Germany). SuperAmp RNA amplification was performed according to Miltenyi Biotec's undisclosed procedure. Briefly, mRNA was isolated via magnetic bead technology, and next reverse transcribed to cDNA and amplified. Quantification of the cDNA samples was performed using the ND-1000 Spectrophotometer (NanoDrop Technologies). The integrity of the cDNA was checked via the Agilent 2100 Bioanalyzer platform (Agilent Technologies). Next, 250 ng cDNA was labeled to Cy3 according to Miltenyi Biotec's undisclosed protocol. The labeled cDNAs were then hybridized overnight (17 h, 65 °C) to an Agilent Whole Human Genome Oligo Microarray using Agilent's recommended hybridization chamber and oven. Finally, the microarrays were washed once with 6× SSPE buffer containing 0.005% *N*-lauroylsarcosine for 1 min at room temperature followed by a second wash with preheated 0.06× SSPE buffer (37 °C) containing 0.005% *N*-lauroylsarcosine for 1 min. The last washing step was performed with acetonitrile for 30 s. Fluorescence signals of the hybridized Agilent Microarrays were detected using Agilent's Microarray Scanner System (Agilent Technologies).

2.5. Microarray data analysis

The Agilent Feature Extraction Software (FES) was used to read out and process the microarray image files. For determination of differential gene expression, FES derived output data files were further analysed using the Rosetta Resolver® gene expression data analysis system (Rosetta Biosoftware). Genes with a fold change >2 and p-value <0.01 were selected as putative candidate genes.

2.6. Statistical analysis

Results are expressed as mean ± standard deviation. Statistical analyses were performed using one-way ANOVA and Student's t-test. The significance level was set at 0.05.

3. Results

3.1. Characterization of undifferentiated hSKP

Undifferentiated hSKP are characterized by a typical fibroblast-like shape (Fig. 1). Yet, they already express proteins which are

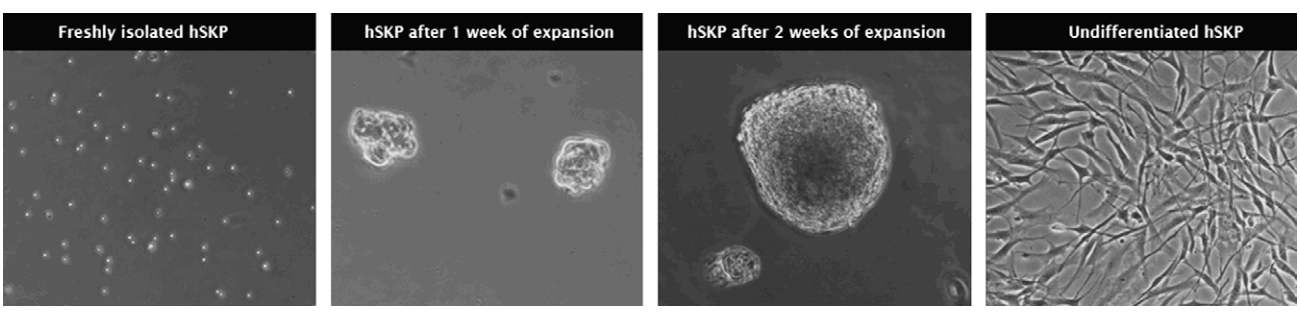

Fig. 1. Light-microscopic analysis of undifferentiated hSKP at different stages upon isolation. Images are representative for 1–3 passages. Final magnification 10 × 10, phase contrast (n > 3).