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Characterization of hepatic markers in human Wharton's Jelly-derived mesenchymal stem cells

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ABSTRACT

Stem cell technology could offer a unique tool to develop human-based *in vitro* liver models that are applicable for testing of potential liver toxicity early during drug development. In this context, recent research has indicated that human Wharton's Jelly-derived mesenchymal stem cells (hWJs) represent an interesting stem cell population to develop human hepatocyte-like cells. Here, an in-depth analysis of the expression of liver-specific transcription factors and other key hepatic markers in hWJs is evaluated at both the mRNA and protein level. Our results reveal that transcription factors that are mandatory to acquire and maintain an adult hepatic phenotype (HNF4A and HNF1A), as well as adult hepatic markers (ALB, CX32, CYP1A1, CYP1A2, CYP2B6 and CYP3A4) are not expressed in hWJs with the exception of K18. On the contrary, transcription factors involved in liver development (GATA4, GATA6, SOX9 and SOX17) and liver progenitor markers (DKK1, DPP4, DSG2, CX43 and K19) were found to be highly expressed in hWJs. These findings provide additional indication that hWJs could be a promising stem cell source to generate hepatocyte-like cells necessary for the development of a functional human-based *in vitro* liver model.

1. Introduction

Drug-induced liver toxicity, so-called DILI is one of the major causes of post-marketing drug withdrawal (Meng, 2010). Therefore it is of utmost importance to identify the toxicological profile of drug candidates as early as possible during their development (Mandenius et al., 2011). Because of economical, but also ethical and scientific considerations, there is a growing interest from pharmaceutical companies towards the application of *in vitro* methods and in particular human-based *in vitro* models in the preclinical phase of drug development (Baranczewski et al., 2006). In this context, stem cell technology is an emerging science that potentially can deliver functional human hepatocyte-like cells. Human adult stem cells (hASCs) are particularly an interesting source of stem cells since they overcome the ethical debate regarding the use of human embryos for the generation of human stem cells (Leeb et al., 2011). For years, hASCs were considered to be lineage-restricted, but over the last decade several publications showed that postnatal stem cell populations in bone marrow, skin, umbilical cord and adipose tissue can give rise to cell types different from the tissue of origin (Aurich et al., 2009; Campard et al., 2008; Snykers et al., 2007; Toma et al., 2001). This multi-lineage differentiation capacity makes hASCs indeed an attractive source for the development of human-based alternative *in vitro* models that ultimately could replace experimental animal use. However, for hASCs we have some indication that the age of the donor is one of the parameters affecting the quality of the cells in terms of proper pluri-/multipotency (De Kock et al., 2009; Gago et al., 2009). Therefore it seems essential to make use of the youngest adult postnatal non-

Abbreviations: ACTB, beta-actin; AFP, α-foetoprotein; ALB, albumin; B2M, beta-2-microglobulin; CX, connexin; CYP, cytochrome P450; DAPI, 4′,6-diamidino-2-phenylindole; DKK, dickkopf; DMEM-LG, Dulbecco's modified eagle medium-low glucose; DPP, dipeptidyl-peptidase; DSG, desmoglein; EGF, epidermal growth factor; FBS, fetal bovine serum; FGF, fibroblast growth factor; FOX, forkhead box; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GJ, gap junction; hADSCs, human adipose tissue-derived stromal cells; hASCs, human adult stem cells; hBMSCs, human bone marrow-derived stromal cells; HMBS, hydroxy-methylbilane synthase; HNF, hepatocyte nuclear factor; HEPs, human hepatocytes; hSKPs, human skin-derived precursor cells; hWJs, human Wharton's Jelly-derived mesenchymal stem cells; KRT, keratin; K, keratin; MSCs, mesenchymal stem cells; ND, not significantly detected; ONECUT, onecut homeobox; PBS, phosphate buffered saline; PFA, paraformaldehyde; qPCR, quantitative real-time reverse transcriptase polymerase chain reaction; SOX, sex determining region Y-box; UBC, ubiquitin C.

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hematopoietic stem cell source available, being the umbilical cord and more specifically the Wharton's Jelly. Previous research results of our group suggest that human Wharton's Jelly-derived mesenchymal stem cells (hWJs) are among other postnatal stem cell sources the most promising one to generate functional human hepatocyte-like cells (De Kock et al., 2012). More specifically, comparative analysis of the global gene expression profile of hWJs *versus* three different mesoderm-derived stem cell populations, including bone marrow-derived stromal cells (hBMSCs), adipose tissue-derived stromal cells (hADSCs) and skin-derived precursor cells (hSKPs) from the ventral trunk skin, revealed that hWJs showed a significantly higher expression of genes and a significant enrichment of functional gene classes associated with liver development (De Kock et al., 2012). Therefore, in the present study, a more in-depth analysis of the expression of liver-specific transcription factors and other key hepatic markers was performed in hWJs at both the mRNA and protein level. Furthermore, the data was compared to primary human hepatocytes, that represent the "gold standard", and hSKPs that, in contrast to hWJs, do not exhibit an enrichment of functional gene classes in liver development (De Kock et al., 2012).

2. Materials and methods

2.1. Isolation and cultivation of hWJs

After receiving informed consent from the mothers of the neonates involved, umbilical cords ($n = 3$) were collected and processed as previously described (De Bruyn et al., 2011). Only full-term deliveries were included in this study. The mesenchymal stem cells (MSCs) were isolated from the Wharton's Jelly based on the migratory and plastic adhesive properties of MSCs, thus omitting enzymatic digestion or dissection. Briefly, umbilical cord segments of 5–10 cm were cut longitudinally and plated for 5 days in Dulbecco's modified eagle medium-low glucose (DMEM-LG) (Lonza, Braine-l'Alleud, Belgium) supplemented with 15% (v/v) heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine and 0.5% (v/v) antibiotic/antimycotic solution containing penicillin, streptomycin and fungizone (all from Life Technologies, Merelbeke, Belgium). After removing the cord segments, culture was pursued until subconfluence at 37 °C in a 5% (v/v) CO_2 humidified atmosphere. After 48 h, non-adherent cells were removed by washing with phosphate buffered saline (PBS), and the medium was changed twice a week. When subconfluency (80–90%) was achieved, adherent cells were harvested after detachment by 10 min incubation with TrypLE® solution (Life Technologies) and expanded for all subsequent passages by replating at a lower density (1×10^3 cells/cm^2) for 2 weeks. hWJs between passages 1 and 2 were used for further analysis.

2.2. Isolation and cultivation of hSKPs

hSKPs were isolated and subcultivated as previously described (De Kock et al., 2012). Informed consent was obtained from both parents. The median age of the donors was 3 years ($n = 3$; range 2–5). Briefly, freshly collected human male foreskin samples were incubated with 25 mL of 0.2 mg/mL Liberase DH solution (Roche Applied Science, Vilvoorde, Belgium) and incubated for 20 h at 4 °C. Next, the epidermis was removed and the tissue was incubated at 37 °C for another 10–20 min depending on the sample size. After processing the samples, typically 5–15 $\times 10^6$ viable cells were obtained per 5–8 cm^2 foreskin. For cultivation, a cell density of 2×10^4 cells/cm^2 was applied. Growth medium for hSKP consists of DMEM + GLUTAMAX/F12 Nutrient Mixture (3:1) (Life Technologies) supplemented with 7.33 IU/mL benzyl penicillin (Continental Pharma, Puurs, Belgium), 50 μg/mL streptomycin sulphate (Sigma-Aldrich, Diegem, Belgium), 2.5 μg/mL fungizone, 2% (v/v) B27 Supplement (Life Technologies), 40 ng/mL basic fibroblast growth factor (FGF)-2 and 20 ng/mL epidermal growth factor (EGF) (both from Promega, Leiden, The Netherlands). Cell cultures were incubated at 37 °C in a 5% (v/v) CO_2 humidified atmosphere for 2 weeks. Growth media was replenished every 2–3 days. hSKP spheres were passaged every 2 weeks using 0.2 mg/mL Liberase DH solution (Roche Applied Science). hSKP between passages 1 and 4 were used for further analysis.

2.3. Human hepatocytes

Human hepatocytes were obtained from Life Technologies.

2.4. Morphology

The morphology of hWJs was examined by phase-contrast light microscopy (Nikon).

2.5. Isolation of RNA and reverse transcriptase–polymerase chain reaction (PCR)

Total RNA was extracted from all samples using the GenElute Mammalian Total RNA Purification Miniprep Kit (Sigma–Aldrich, Bornem, Belgium) according to the manufacturer's instructions. The isolated RNA was quantified at 260 nm using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, USA). Total RNA was reverse transcribed into cDNA using iScript™ cDNA Synthesis Kit (BioRad, Nazareth, Belgium) followed by cDNA purification with the Genelute PCR clean up kit (Sigma–Aldrich).

2.6. Quantitative real-time PCR (qPCR)

cDNA products were used for quantitative amplification of the target genes. The gene expression assays used in this study are listed in Supplementary Table S1. All samples were done in duplicate and each run included two no template controls and a serial dilution of a pooled cDNA mix from all samples to estimate the qPCR efficiency. The qPCR reaction mix consisted of 10 μL TaqMan Fast Advanced Master Mix (Life Technologies), 1 μl 20× Assay-on-Demand Mix (Life Technologies) and 2 μl of cDNA in a 20 μL volume adjusted with DNase/RNase-free water. qPCR conditions, using the StepOne Plus system (Life Technologies) were as follows: incubation for 20 s at 95 °C, followed by 40 cycles of 1 s denaturation at 95 °C, annealing for 20 s at 60 °C (Life Technologies).

2.7. qPCR data analysis

qPCR efficiency was estimated by the StepOne Plus System's Software and the data were only used when the calculated PCR efficiency ranged from 0.85 to 1.15. Moreover, for selecting reliable reference genes to normalize the qPCR data, we first evaluated the expression stability of five candidate reference genes: glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), beta-2-microglobulin (*B2M*), hydroxy-methylbilane synthase (*HMBS*), beta-actin (*ACTB*) and ubiquitin C (*UBC*). According to geNorm® the most stable reference target was *HMBS* (Supplementary Fig. S1). Due to the very high stability of all reference targets (Supplementary Fig. S1), the geometric mean of *ACTB*, *B2M*, *GAPDH*, *HMBS* and *UBC* was used to normalize the expression data (geNorm®, Biogazelle, Gent, Belgium). Thereafter, to evaluate the relative mRNA expression levels of the target genes, results were expressed *versus* primary human hepatocytes using qbasePLUS® software (Biogazelle). Statistical analyses were performed using a One-way ANOVA. Gene expressions with a fold change of at least 2-fold and a p-value lower or