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Expression of drug metabolizing enzymes in hepatocyte-like cells derived from human embryonic stem cells☆

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ABSTRACT

Human embryonic stem cells (hESC) offer a potential unlimited source for functional human hepatocytes, since they can differentiate into hepatocyte-like cells displaying a characteristic hepatic morphology and expressing several hepatic markers. Such cells could be used for, e.g. studies of drug metabolism and hepatotoxicity, which however would require a significant expression of drug metabolising enzymes. Thus, we have investigated the expression of cytochrome P450s (CYPs), UDP-glucuronosyltransferases (UGTs), drug transporters, transcription factors and other liver specific genes in hepatocyte-like cells derived from hESC using a simple direct differentiation protocol. The mRNA and protein expression of several important CYPs were determined using low density arrays, real time PCR and Western blotting. Significant CYP expression on the mRNA level was detected in hepatocyte-like cells derived from one out of two different hESC lines tested, which was much higher than in undifferentiated hESC and generally higher than in HepG2 cells. CYP1A2, CYP3A4/7 and low levels of CYP1A1 and CYP2C8/9/19 protein were detected in both lines. The mRNAs for a variety of CYPs and liver specific factors were shown to be inducible in both cell lines, and this was reflected in induced levels of CYP1A2 and CYP3A4/7 protein. This first report on expression of all major CYPs in hepatocyte-like cells derived from hESC represents an important step towards functional hepatocytes, but efforts to further differentiate the cells using optimized protocols are needed before they exhibit similar levels of drug metabolizing enzymes as primary human hepatocytes and liver.

☆ For information on hESC please visit http://www.cellartis.com/.

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1. Introduction

Human embryonic stem cells (hESC) are pluripotent and can give rise to cells of all three embryonic germ layers: endoderm, ectoderm, and mesoderm, and further on, to all somatic and germ cells [1,2]. Thus, in the future, differentiated cells derived from hESC can potentially be used for regenerative therapies that may cure various diseases. HESC derived cells with functional characteristics of hepatic cells do not only have the potential of being used in bioreactors for extra corporal liver support in patients with liver failure [3], but also as a test system for studying hepatic metabolism of xenobiotics and hepatotoxicity. Since hESC have the ability to self-renew, the use of hESC derived hepatocytes can potentially provide an unlimited source of functional human hepatocytes, from the same genetic donor if desired, and thereby improve the predictability of toxicity tests and reduce the need for animal experimentation. However, the toxicity of xenobiotics is often dependent on their biotransformation into toxic and reactive metabolites and, therefore, the presence and distribution of biotransforming systems is required. At present, primary human hepatocytes constitute a common model for *in vitro* drug metabolism and toxicity testing. Nevertheless, the activity of drug metabolizing enzymes and many transporter functions are rapidly lost and/or changed when primary hepatocytes are cultured [4–6]. Moreover, many of the hepatoma cell lines, e.g. HepG2, which are used for *in vitro* studies, lack expression of many important drug metabolizing enzymes [7,8].

Cytochrome P450s (CYPs) are mixed function monooxygenases and the major enzymes in phase I metabolism of xenobiotics. This oxidative metabolism results in, depending on the nature of the xenobiotic, inactivation and facilitated elimination, activation of pro-drugs or metabolic activation. The major site of CYP expression is the liver and CYP3A4 is the most abundant CYP isozyme in human adult liver. The enzymes of greatest importance for drug metabolism belong to the families 1–3, responsible for 70–80% of all phase I dependent metabolism of clinically used drugs [9,10]. CYP expression and activity present large interindividual variations due to polymorphisms. Moreover, CYPs can be induced several fold or inhibited by specific drugs, resulting in additional, although transient, variability of metabolic activity [11].

Many reports on the differentiation of hESC have been published, mainly considering derivation of ectodermal or mesodermal lineages. Directed differentiation into endoderm has been more difficult to achieve, most likely due to a lack of early lineage-specific markers, and there are only few reports on hepatocyte-like cells derived from hESC [12–16]. In these reports, only limited studies of CYP expression and induction in hESC derived hepatocyte-like cells have been included. Schwartz et al. detected phenobarbital-inducible CYP expression as measured by quantitative RT-PCR and pentoxyresorufin-O-deethylase (PROD) activity in hepatocyte-like cells [12], whereas Rambhatla et al. reported inducible CYP1A2 activity as detected by ethoxyresorufin-O-deethylase (EROD) activity in hESC derived cells with hepatocyte-like characteristics [15].

We have previously shown that hepatocyte-like cells derived from hESC analyzed in the present study, display characteristic hepatic morphology and express liver markers such as HNF3β (Foxa2), liver fatty acid binding protein (LFABP), α-1-antitrypsin (α-1-AT), albumin, and cytokeratin 18. Furthermore, the cells accumulate glycogen, a feature typical for hepatocytes, and exhibit glutathione transferase protein expression and activity that closely resembles that of human hepatocytes [17]. In this study, we have evaluated the CYP mRNA and protein expression in hepatocyte-like cells derived from two different hESC lines using real time PCR based methods and Western blotting. We could detect mRNA from most of the CYP enzymes tested in one of the hepatocyte-like cells, as well as CYP1A2 and CYP3A4/7 protein. Inducibility of CYP1A2 and 3A4/7 was also demonstrated. These results are discussed in terms of the maturity of the hepatocytes-like cells and the need for further differentiation towards a suitable phenotype.

2. Materials and methods

2.1. Cell and tissue material

In this study, we have used hepatocyte-like cells derived from hESC lines SA002, SA002.5 and SA167. These cell lines were established, characterized and cultured as previously described [1,18] at Cellartis AB, after approval from the local ethics committee at Gothenburg University. Hepatocyte-like cells were derived using a direct 2D-differentiation protocol as previously described [17]. Briefly, hESC were allowed to differentiate for 18–30 days in VitroHES™ supplemented with 4 ng/ml human recombinant basic fibroblast growth factor (bFGF) in IVF-(*in vitro* fertilization) culture dishes (Becton Dickinson). Medium was changed every 7–10 days, a crucial parameter for differentiation of hepatocyte-like cells. Differentiation of hESC results in a mixed population of different cell types, where the hepatocyte-like cells are located in the periphery, surrounding the core of the colony. In order to perform gene expression profiling and protein analysis, mRNA and protein, respectively, were purified from enriched populations of hepatocyte-like cells. Enrichment of such cells, with hepatic morphology, i.e. large, polygonal and often bi-nucleated, was performed by manual micro dissection. Isolated hepatocyte-like regions from 20 to 25 dishes were collected and pooled for each cell line. Since hepatocyte-like cells are grown on a layer of mouse embryonic fibroblast (MEFs), the isolated cell samples contained a small amount (about 12%) of MEFs [17]. Therefore, MEFs as well as primary human hepatocytes, HepG2 cells and human liver samples were used as controls in the experiments. MEFs were derived and cultured as previously described [19]. Primary human hepatocytes were from In Vitro Technologies (Lot GIU, Leipzig, Germany). HepG2 cells (HB-8065, American Type Culture Collection) were cultured as previously described [8]. Human liver samples were obtained from Sahlgrenska Hospital (Gothenburg, Sweden) and originated from patients undergoing liver resection. All tissues were obtained through qualified medical staff, with donor consent and with the approval of the local ethics committee at Sahlgrenska Hospital.

2.2. Isolation of RNA and cDNA synthesis

Total RNA from the different cells was isolated using Trizol reagent (Invitrogen, Stockholm, Sweden) according to the manufacturer's instructions for isolation of small amounts of RNA, by using glycogen as a carrier for RNA. From human liver tissue total RNA was isolated using a CsTFA gradient (Amersham Biosciences, Piscataway, NJ) [20].

RNA concentrations were determined by UV-spectroscopy and the RNA samples were stored at −80 °C.

Synthesis of cDNA was conducted with 0.5–0.6 μg of total RNA in a final volume of 10 μl, using M-MLV Reverse Transcriptase (Invitrogen) with 5 μM oligo $(dT)_{18}$ and following the manufacturer's protocol. Control reactions for genomic DNA contaminations were made for every sample by excluding the M-MLV Reverse Transcriptase.

2.3. TaqMan low density arrays

The gene expression levels of 45 genes were examined in each sample by using TaqMan low density array (LDA) cards (Applied Biosystems, Foster City, CA). Each sample was repeatedly run on 3–4 LDA cards. The LDA cards were custom made by choosing the primers and probes online from Applied Biosystems Assays-on-Demand Gene Expression Products. The LDA cards were configured into eight identical sets of 48 genes. Each set also contained housekeeping genes, of which glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and hypoxanthine phosphoribosyltransferase 1 (HPRT1) were used for normalization.

To 52 μl of 2× TaqMan Universal PCR Master Mix containing AmpErase uracil-*N*-glycosylase (Applied Biosystems) 52 μl of 2–5-fold diluted cDNA was added, and thereafter gently mixed. After brief centrifugation, 100 μl of the mixture (150–170 ng cDNA) was transferred into one of the eight loading ports on a LDA card. The cards were centrifuged for 2 × 1 min at 1200 rpm, and then sealed according to the manufacturer's instructions. PCR amplification and fluorescence data collection were performed with the ABI Prism 7900HT Sequence Detection System (Applied Biosystems).

The comparative C_t method for relative quantification ($\Delta\Delta C_t$ method), which describes the change in expression of the target gene in a test sample relative to a calibrator sample, was used to analyse the data [21]. Human liver was used as the calibrator sample and all gene expression values in human liver were assigned a relative value of 1.00. Data were analyzed with the 7900HT Sequence Detector System (SDS) software version 2.2.1 (Applied Biosystems).

2.4. Real time PCR

For detection of mRNA expression levels below the detection level of the LDA cards, selected genes were also analyzed by either SybrGreen- (CYP1A1, 1A2 and 2A6) or TaqMan- (CYP3A4 and 3A7) based real time PCR. All PCRs were performed using the ABI 7500 Fast Real-Time PCR System and data was analyzed with 7500 Fast System SDS software version 1.3.1 (Applied Biosystems).

SybrGreen real time PCR was perfomed using 5 μl of 2–3-fold diluted cDNA template in a 25 μl reaction mixture containing 0.30 μM of each primer and 12.5 μl SybrGreen real time PCR Master Mix (Applied Biosystems). The PCR reaction was run as follows; 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 60 °C for 20 s and 72 °C for 60 s. Each PCR was followed by a melting curve analysis. The primer sequences used were as follow: CYP1A1; the forward primer, 5′-CCA CCA AGA ACT GCT TAG CC-3′ and the reverse primer, 5′-CAG CTC CAA AGA GGT CCA AG-3′, designed from Entrez Nucleotides Database sequence number NM_000499, CYP1A2; the forward primer, 5′-AAC AAG GGA CAC AAC GCT GAA T-3′ and the reverse primer, 5′-GGA AGA GAA ACA AGG GCT GAG T-3′ [22], CYP2A6; the forward primer, 5′-CCC TCA TGA AGA TCA GTG AGC-3′ and the reverse primer, 5′-GCG CTC CCC GTT GCT GAA TA-3′ [23], GAPDH; the forward primer, 5′-GAT CCC TCC AAA ATC AAG TG-3′ and the reverse primer, 5′-TGA TGT TCT GGA GAG CCC-3′, designed from Entrez Nucleotides Database sequence number NM_002046.

Taqman real time PCR was performed using ready-to-use TaqMan Gene Expression Assays (Applied Biosystems) for CYP3A4, CYP3A7, and with human TBP (TATA-box binding protein) as an endogenous control, according to the manufacturer's instructions.

2.5. Western blot

Cytochrome P450 protein expression was studied by Western blotting as described elsewhere [17]. Briefly, proteins were extracted from cells using the M-PER protein extraction reagent (Pierce, Rockford, IL) supplemented with 1:100 protease inhibitor cocktail (Sigma–Aldrich) and separated on a 12% SDS polyacrylamide gel by electrophoresis. Antibodies against human CYP1A2 (Biomol, Plymouth Meeting, PA; Chemicon, Temecula, CA), 1A1, 2A6, 2B6, 2C8/9/19, 2D6 (Biomol), 2E1 (Chemicon; Biomol) and 3A4/7 (produced as previously described [24]; Biomol) were used. For Western blot analysis MEFs, undifferentiated hESC and HepG2 cell lysates were used as controls. Cell lysates of primary human hepatocytes were used as positive control in all cases except for the CYP1A1 incubations, where recombinant CYP1A1 enzyme (a kind gift of Prof. Rannug at IMM, Karolinska Institutet) was used.

2.6. Induction

The inducibility of CYP enzymes in hepatocyte-like cells was evaluated by exposing 18–25 days cultures to a cocktail of 25 μM rifampin, 20 μM primidone, 100 μM dexamethasone, 88 mM EtOH, 25 μM omeprazole and 100 μM isoniazid in VitroHES™ for 96 h prior to isolation of hepatocyte-like cells. The cells showed no signs of toxicity following the treatment, as determined by morphological assessment (data not shown). The expression of mRNA and protein in treated and untreated hepatocyte-like cells was evaluated using LDA arrays, real time PCR and Western blotting.

2.7. Cytochrome P450 activity assays

Hepatocyte-like cells were washed twice in PBS and resuspended in cold 50 mM potassium phosphate buffer, pH 7.4. Cells were sonicated and centrifuged at 800 × *g* and 4 °C for 10 min. The supernatant was centrifuged again at 6500 × *g* and

4 °C for 10 min and used to measure metabolic activity. Protein concentrations were measured, using bovine serum albumin (BSA) as standard, either according to the method of Bradford [25] or by using Micro BCA protein determination kit (Pierce, Rockford, IL).

To evaluate CYP1A activity, ethoxyresorufin-O-deethylation (EROD) measurements based on the method described by Lubet et al. [26], were performed. Briefly, the increase in fluorescence due to resorufin formation was measured at excitation λ = 530 nm and emission λ = 586 nm. The reaction mixture contained 50 mM Tris–HCl buffer, pH 7.5 with 25 mM $MgCl_2$, 5 μM ethoxyresorufin (Sigma–Aldrich), 24 pmol NADPH-cytochrome P450 reductase (BD Gentest, Bedford, MA) and 50 μl sample in black 96-well plates (total volume of 250 μl). The samples contained between 50 and 200 μg protein. NADPH (100 μM; Sigma–Aldrich) was used to start the reaction, and the fluorescence was measured every minute for 60 min using a Tecan GENios Pro fluorospectrophotometer and Magellan V6.2 software. A resorufin standard curve was used for quantification.

The CYP3A4 activity was analyzed using the fluorescent substrate dibenzylfluorescein (DBF; BD Gentest), which is converted into fluorescein primarily by CYP3A4 and CYP1A1 [27]. The enzyme activity was assayed by incubating 50 μg protein in a final volume of 100 μl containing 100 mM potassium phosphate buffer, pH 7.4, 48 pmol NADPH-cytochrome P450 reductase (BD Gentest), 48 pmol cytochrome b5 (Invitrogen) and 10 μM of DBF, in black 96-well plates. The reactions were started by addding NADPH (Sigma–Aldrich) at a final concentration of 600 μM. The reaction mixtures were incubated at 37 °C and the fluorescence was measured at the excitation λ = 485 nm and emission λ = 538 nm in a Spectra Max Gemini fluorospectrophotometer (Molecular Devices, Sunnyvale, CA) every 15 min between 0 and 120 min. After the last measurement a known amount of fluorescein standard (Sigma–Aldrich) was added to the reaction mixtures and the fluorescence for the standard was measured.

3. Results

3.1. *Gene expression analysis using low density array*

The mRNA expression of liver specific genes was analyzed in hepatocyte-like cells derived from hESC lines SA002 and SA167, undifferentiated hESC, MEFs, HepG2 cells, primary human hepatocytes, and human liver samples using TaqMan low density arrays (LDAs). The genes selected were in the categories cytochrome P450s (CYPs), UDP-glucuronosyltransferases (UGTs), transporters, transcription factors and other liver specific genes. Two genes, D-site albumin promoter binding protein (DBP) and alpha-1-antitrypsin, were excluded from further analysis because of high background levels in the MEF sample and because the primer set did not work. No gene expressions were detected in the MEF sample except for the DBP gene (data not shown). The relative gene expression levels

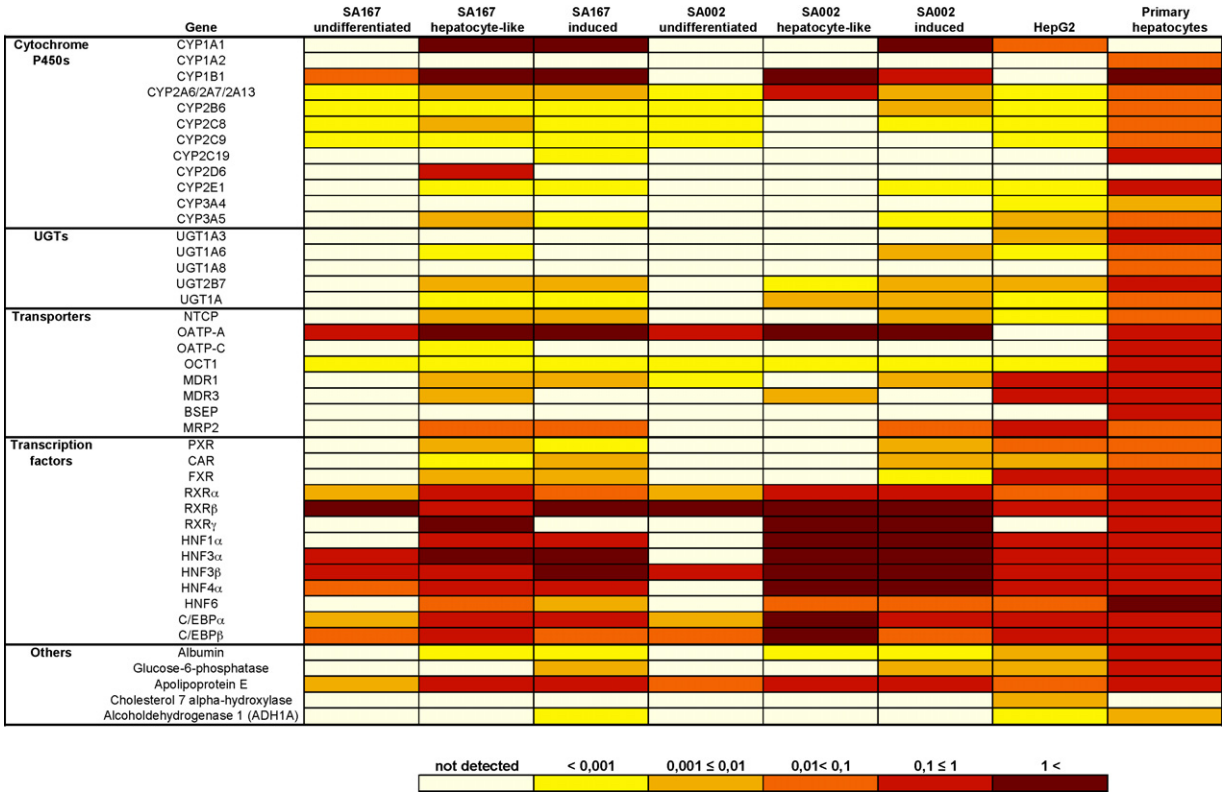

Fig. 1 – Relative gene expression for liver related genes as measured by LDA assay. All samples were run on LDA cards containing different genes associated with drug metabolism in the liver. The expression for all genes is normalized against the expression of GAPDH in each sample. The expression levels in each sample are compared to the expression levels in human liver samples, which are set to 1.0 for all genes. UGTs: UDP-glucuronosyltransferases.

in the different samples in comparison with human liver samples (=1.0) are summarized in Fig. 1.

The LDA results show that gene expression in hepatocyte-like cells from the two hESC lines differ from each other. CYP1A1, 1B1, 2A6/2A7/2A13, 2B6, 2C8, 2C9, 2D6, 2E1 and 3A5 mRNA expression was detected in hepatocyte-like cells from line SA167, whereas only mRNA for CYP1B1 and 2A6/2A7/2A13 was detected in cells from SA002. In general, the relative gene expression levels for the genes investigated were much lower in hepatocyte-like cells from both lines, than in human liver and primary human hepatocytes. However, the relative gene expression levels for CYP1A1, 1B1 and the transporter OATP-A were higher in the hepatocyte-like cells than in human liver samples. Moreover, the relative expression levels for most transcription factors, including HNF1α, HNF3α and β, HNF4α, RXRβ and γ, C/EBP α and β, were also higher in the hepatocyte-like cells, especially from line SA002, than in human liver. Importantly, for several of the investigated genes, including CYP1B1, 2A6/2A7/2A13, 2C8 and 2D6, higher mRNA levels were detected in hepatocyte-like cells from line SA167 than in HepG2 cells.

In the undifferentiated hESC low levels of some CYPs, namely CYP2A6/2A7/2A13, 2B6, 2C8 and 2C9, together with higher levels of RXRα and β, HNF3α and β, HNF4α, C/EBP α and β, and apolipoprotein E were detected. However, in general the relative gene expression levels in the undifferentiated hESC were much lower than in their respective hepatocyte-like cell.

Treatment of hepatocyte-like cells, especially from line SA002, with known cytochrome P450 inducers resulted in increased mRNA levels of CYP1A1, 2B6, 2E1, 3A5, UGT1A6, UGT2B7, NTCP, MDR1, MRP2, PXR, CAR and FXR. However, no induction of the CYPs studied was detected in hepatocyte-like cells from line SA167.

3.2. *Analysis of selected gene expressions using real time PCR*

Since mRNA expression was not detected with the LDA cards for some of the most abundant hepatic CYP genes, CYP3A4 and CYP1A2, we further analyzed expression for these genes, as well as CYP1A1 and CYP3A7, using a more sensitive real time PCR method. CYP2A6 was also analyzed with real time PCR, since no discrimination between expression of CYP2A6, 2A7 and 2A13 could be made with the LDA assay. No mRNA for these CYPs was detected in the MEF sample. Both CYP3A4 and CYP3A7 mRNA was detected in the hepatocyte-like cells, HepG2 cells, primary human hepatocytes and human liver samples using the Taqman real time PCR method (Fig. 2A). The expression level of CYP3A4 was very low in the hepatocyte-like cells from both hESC lines compared to the levels in human liver and primary human hepatocytes, but similar to the level in HepG2 cells. The relative expression level of CYP3A7 in the hepatocyte-like cells was also low in comparison to human liver, but higher than the relative levels for CYP3A4 (Fig. 2A). In addition, mRNA for CYP3A7 was also detected in the undifferentiated hESC line SA167 (Fig. 2A).

CYP1A1 mRNA was detected in all samples (Fig. 2B). The amount of CYP1A1 mRNA was higher in hepatocyte-like cells from line SA167 and similar in cells from SA002, in comparison to the level detected in human liver samples. CYP1A1 mRNA

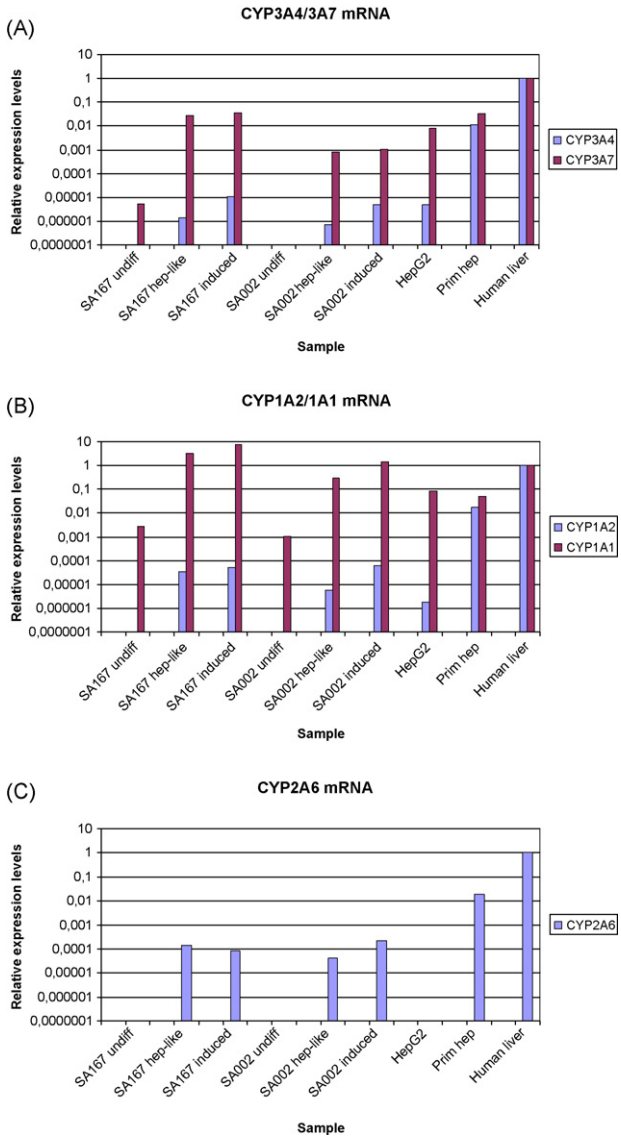

Fig. 2 – Relative gene expression for selected CYPs as measured by real time PCR. The gene expression levels in each sample are compared to the expression levels in human liver samples, which are set to 1.0 for all genes. The expression for all genes is normalized against either the GAPDH (CYP1A1/1A2, CYP2A6) or TBP (CYP3A4/3A7) expression in each sample. Expression of mRNA for CYP3A4/3A7 analyzed by TaqMan real time PCR (A), and for CYP1A1/1A2 (B) and CYP2A6 (C) analyzed by SybrGreen based real time PCR is shown.

levels were also higher than seen in HepG2 cells and primary human hepatocytes. Moreover, CYP1A2 mRNA was detected in all samples except in the undifferentiated hESC. The expression level for CYP1A2 mRNA in hepatocyte-like cells from both lines was much lower than in human liver samples and primary human hepatocytes, but higher than in HepG2 cells (Fig. 2B). CYP2A6 mRNA expression was detected in all samples except for undifferentiated hESC and HepG2 cells (Fig. 2C).

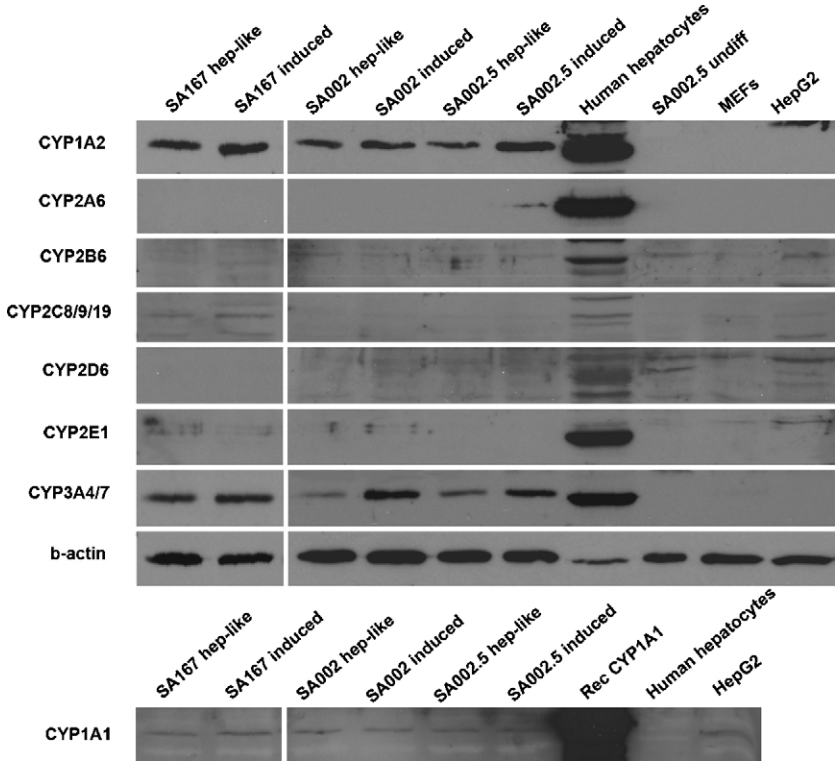

Fig. 3 – Western Blot analysis of CYP1A1, 1A2, 2A6, 2B6, 2C8/9/19, 2D6, 2E1 and 3A4/7 protein expression in untreated and induced hepatocyte-like cells derived from hESC lines SA167, SA002 and SA002.5, primary human hepatocytes Lot GIU (for CYP1A1 Lot MYO), undifferentiated hESC from line SA002.5, MEFs and HepG2 cells. Positive control for CYP1A1 analysis was recombinant CYP1A1.

3.3. *Analysis of cytochrome P450 protein expression*

The CYP protein expression in hepatocyte-like cells from lines SA002, SA002.5 and SA167 was evaluated by Western blotting. The analyses showed presence of CYP1A2 and CYP3A4/7 protein in hepatocyte-like cells, at slightly different levels in cells from the respective lines, as revealed from blots incubated with antibodies from several different sources. The highest CYP protein content was observed in hepatocyte-like cells from SA167, but this was at a much lower level compared to primary human hepatocytes (Fig. 3). The expression of CYP1A2 and 3A4/7 protein was increased in cells exposed to the inducer cocktail (cf. Methods). The CYP1A1 and CYP2C8/9/19 proteins were only very weakly detected in hepatocyte-like cells, whereas no CYP2A6, 2B6, 2D6 and 2E1 protein expression was revealed by Western blotting (Fig. 3). Apart from weak detection of CYP1A1 in HepG2 cells, none of the CYP proteins studied could be detected in MEFs, undifferentiated hESC line SA002.5 or HepG2 cells.

3.4. *Cytochrome P450 dependent enzyme activities*

Examination of EROD activity for detection of CYP1A and DBF substrate fluorescence for detection of CYP3A4 and CYP1A1 activity was performed. No activity could be detected in hepatocyte-like cells, whereas activity in primary human hepatocytes was detected at levels comparable to those reported in the literature. The CYP1A activity in primary hepatocytes as measured by the EROD assay was 0.43 pmol/min/mg protein [28–30] and the CYP3A4/CYP1A1 activity as measured using DBF was 0.73 pmol/min/mg protein. The detection limit for the EROD assay was estimated to 0.03 pmol/min and for the 3A4/1A1 activity assay to 0.02 pmol/min, i.e. about 6% and 3%, respectively, of the activity registered in the primary human hepatocytes.

4. Discussion

Here, we show that hepatocyte-like cells derived from hESC express mRNA and protein for several hepatic CYPs. We also show that there is a clear difference in expression patterns between hepatocyte-like cells from the two hESC lines investigated. Thus, many of the liver specific genes, including most CYPs, were only detected in hepatocyte-like cells from line SA167, and not in cells from SA002, which indicates a difference in the degree of differentiation. It could be speculated whether the fact that SA002 has one extra chromosome 13 [1] could affect the expression of the genes studied in this paper, but so far no such indication has been observed when comparing this hESC line with chromosomally normal lines such as SA167. In general, the relative expression levels for most of the CYPs were much lower as compared to the levels in human liver and primary human hepatocytes. However, in hepatocyte-like cells from line SA167, the mRNA

levels for several of the CYPs were higher than in the commonly used hepatoma cell line HepG2.

Cytochrome P450 gene expression in the hepatocyte-like cells was confirmed by Western blot analysis showing CYP1A2 and CYP3A4/3A7 protein in cells from both line SA002 and SA167. Possible cross-reactivity between CYP1A1 and CYP1A2 protein in the samples was excluded since none of the CYP1A2 antibodies used here detected recombinant CYP1A1 protein (data not shown). In addition, Western blot analysis using a CYP1A1 antibody showed that only very low levels of CYP1A1 can be detected in the hepatocyte-like cells. However, no discrimination between CYP3A4 and 3A7 could be made since the antibodies used detect both proteins.

Although very low levels of CYP1A2 and CYP3A4 mRNA are found in hepatocyte-like cells, Western blot analysis clearly showed corresponding protein expression. This might be due to further posttranscriptional regulation influencing, e.g. RNA polyadenylation and capping, RNA trafficking, RNA stability, translation, protein processing and protein stability.

Inducibility is an important feature of CYP enzymes [11] and here we were able to detect mRNA induction of several CYP genes as well as of CYP1A2 and CYP3A4/7 protein. Although both CYP1A2 and CYP3A4/7 apoproteins were identified by Western blot analysis, no catalytically active enzyme was detected in the hepatocyte-like cells used in this study. It is likely that the enzymes are active, although the activity may be below the lower limit of detection of the assays used here, which was as high as 3–6% of the level seen in primary human hepatocytes. However, it is possible that the CYP enzymes present in the hepatocyte-like cells are not functional due to, e.g. incorrect folding of the protein or lack of incorporation of the heme moiety. Only a few studies have previously shown constitutive and inducible activity of selected CYPs in hESC derived hepatocyte-like cells [12,15]. However, the evaluation of gene and protein expression in these studies is limited or missing and, to our knowledge, the present study is the first extensive investigation of expression of the most important CYP enzymes in such cells.

Although the hepatocyte-like cells seem to express very low amounts of cytochrome P450s or other liver specific genes, such as albumin, glucose-6-phosphatase and cholesterol 7 α-hydroxylase (CYP7A), the mRNA levels for most of the transcription factors investigated here are higher than in the human liver sample. This indicates that these cells have the transcriptional machinery for producing many of the drug metabolizing proteins present in the liver. High levels of some of these transcription factors, especially HNF4α, C/EBPα and C/EBPβ, may suggest an immature phenotype of the hESC-derived hepatocyte-like cells, as these factors have been shown to be involved in liver development and hepatocyte differentiation [31,32].

In the present study, a simple direct differentiation protocol, in which hESC are differentiated on MEFs and in VitroHES medium supplemented with bFGF, was used to derive hepatocyte-like cells from hESC *in vitro* [17]. Despite the simple derivation method, hepatocyte-like cells are repeatedly appearing in the periphery of the colonies and account for 10–50% of the cells in the colony. Although morphological characteristics and liver typical protein expression of the hepatocyte-like cells are similar to human hepatocytes [17], the LDA analysis in the present study is indicating an immature and fetal-like phenotype rather than a fully mature and functional hepatocyte. However, Western blot data clearly demonstrates presence of CYP1A2 and 3A/7, which are abundant CYP enzymes in the human liver and important for drug metabolism, and this suggests a great potential of the present cell population to become a fully functional hepatocyte-like cell type. The differentiation of these cells towards the hepatic lineage will in the future be further improved by finding optimal conditions for cell differentiation and growth by means of, e.g. addition of different cytokines and growth factors.

Cultured primary hepatocytes as well as hepatoma cell lines, such as HepG2 cells, are commonly used today for *in vitro* studies of liver specific drug metabolism and toxicity. However, these cell types entirely lack or have very low levels of many of the drug metabolizing enzymes and transporters found in hepatocytes *in vivo* [5–8]. The mRNA and protein expression data that we present in this study indicate that the hepatocyte-like cells in general express lower amounts of drug metabolizing enzymes than human liver and primary human hepatocytes but higher expression of many liver specific genes, including CYPs, as compared to HepG2 cells.

In conclusion, the results presented here demonstrate that hepatocyte-like cells derived from hESC show mRNA expression of several important CYPs and clear expression of CYP1A2 and CYP3A/7 protein, in contrast to the stem cells from which they are derived that showed no or very low expression. This indicates a great potential of these cells to be further developed by suitable differentiation protocols into hepatocyte-like cells with mature enough phenotype for future use in studies of drug metabolism and drug-induced hepatotoxicity.

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REFERENCES

[1] Heins N, Englund MC, Sjoblom C, Dahl U, Tonning A, Bergh C, et al. Derivation, characterization, and differentiation of human embryonic stem cells. Stem Cells 2004;22(3):367–76.

[2] Geijsen N, Horoschak M, Kim K, Gribnau J, Eggan K, Daley GQ. Derivation of embryonic germ cells and male gametes from embryonic stem cells. Nature 2004;427(6970):148–54.

[3] Gerlach JC, Mutig K, Sauer IM, Schrade P, Efimova E, Mieder T, et al. Use of primary human liver cells originating from discarded grafts in a bioreactor for liver support therapy and the prospects of culturing adult liver stem cells in

bioreactors: a morphologic study. Transplantation 2003;76(5):781–6.
[4] Baker TK, Carfagna MA, Gao H, Dow ER, Li Q, Searfoss GH, et al. Temporal gene expression analysis of monolayer cultured rat hepatocytes. Chem Res Toxicol 2001;14(9):1218–31.
[5] Rodriguez-Antona C, Donato MT, Boobis A, Edwards RJ, Watts PS, Castell JV, et al. Cytochrome P450 expression in human hepatocytes and hepatoma cell lines: molecular mechanisms that determine lower expression in cultured cells. Xenobiotica 2002;32(6):505–20.
[6] Wu DF, Clejan L, Potter B, Cederbaum AI. Rapid decrease of cytochrome P-450IIE1 in primary hepatocyte culture and its maintenance by added 4-methylpyrazole. Hepatology 1990;12(6):1379–89.
[7] Wilkening S, Stahl F, Bader A. Comparison of primary human hepatocytes and hepatoma cell line Hepg2 with regard to their biotransformation properties. Drug Metab Dispos 2003;31(8):1035–42.
[8] Butura A, Johansson I, Nilsson K, Warngard L, Ingelman-Sundberg M, Schuppe-Koistinen I. Differentiation of human hepatoma cells during confluence as revealed by gene expression profiling. Biochem Pharmacol 2004;67(7): 1249–58.
[9] Bertz RJ, Granneman GR. Use of *in vitro* and *in vivo* data to estimate the likelihood of metabolic pharmacokinetic interactions. Clin Pharmacokinet 1997;32(3):210–58.
[10] Ingelman-Sundberg M. Human drug metabolising cytochrome P450 enzymes: properties and polymorphisms. Naunyn Schmiedebergs Arch Pharmacol 2004;369(1):89–104.
[11] Pelkonen O, Maenpaa J, Taavitsainen P, Rautio A, Raunio H. Inhibition and induction of human cytochrome P450 (CYP) enzymes. Xenobiotica 1998;28(12):1203–53.
[12] Schwartz RE, Linehan JL, Painschab MS, Hu WS, Verfaillie CM, Kaufman DS. Defined conditions for development of functional hepatic cells from human embryonic stem cells. Stem Cells Dev 2005;14(6):643–55.
[13] Lavon N, Yanuka O, Benvenisty N. Differentiation and isolation of hepatic-like cells from human embryonic stem cells. Differentiation 2004;72(5):230–8.
[14] Baharvand H, Hashemi SM, Kazemi Ashtiani S, Farrokhi A. Differentiation of human embryonic stem cells into hepatocytes in 2D and 3D culture systems *in vitro*. Int J Dev Biol 2006;50(7):645–52.
[15] Rambhatla L, Chiu CP, Kundu P, Peng Y, Carpenter MK. Generation of hepatocyte-like cells from human embryonic stem cells. Cell Transplant 2003;12(1):1–11.
[16] Soto-Gutierrez A, Navarro-Alvarez N, Rivas-Carrillo JD, Chen Y, Yamatsuji T, Tanaka N, et al. Differentiation of human embryonic stem cells to hepatocytes using deleted variant of HGF and poly-amino-urethane-coated nonwoven polytetrafluoroethylene fabric. Cell Transplant 2006;15(4):335–41.
[17] Söderdahl T, Küppers-Munther B, Heins N, Edsbagge J, Björquist P, Cotgreave I, et al. Glutathione transferases in hepatocyte-like cells derived from human embryonic stem cells. Toxicol In Vitro 2007;21(5):929–37.
[18] Heins N, Lindahl A, Karlsson U, Rehnstrom M, Caisander G, Emanuelsson K, et al. Clonal derivation and characterization of human embryonic stem cell lines. J Biotechnol 2006;122(4):511–20.
[19] Hogan B, Beddington R, Costantini F, Lacy E. Manipulating the mouse embryo: a laboratory manual. Plainview, NY: Cold Spring Harbor Laboratory Press; 1994. p. 260–261.
[20] Okoyama H. Recombinant DNA, part E. In: Wu R, editor. Methods in enzymology. Academic Press; 1987. p. 3–28.
[21] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25(4):402–8.
[22] Perez G, Tabares B, Jover R, Gomez-Lechon MJ, Castell JV. Semi-automatic quantitative RT-PCR to measure CYP induction by drugs in human hepatocytes. Toxicol In Vitro 2003;17(5–6):643–9.
[23] Kiyotani K, Yamazaki H, Fujieda M, Iwano S, Matsumura K, Satarug S, et al. Decreased coumarin 7-hydroxylase activities and CYP2A6 expression levels in humans caused by genetic polymorphism in CYP2A6 promoter region (CYP2A6*9). Pharmacogenetics 2003;13(11):689–95.
[24] Eliasson E, Mkrtchian S, Halpert JR, Ingelman-Sundberg M. Substrate-regulated, cAMP-dependent phosphorylation, denaturation, and degradation of glucocorticoid-inducible rat liver cytochrome P450 3A1. J Biol Chem 1994;269(28):18378–83.
[25] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
[26] Lubet RA, Mayer RT, Cameron JW, Nims RW, Burke MD, Wolff T, et al. Dealkylation of pentoxyresorufin: a rapid and sensitive assay for measuring induction of cytochrome(s) P-450 by phenobarbital and other xenobiotics in the rat. Arch Biochem Biophys 1985;238(1):43–8.
[27] Stresser DM, Turner SD, Blanchard AP, Miller VP, Crespi CL. Cytochrome P450 fluorometric substrates: identification of isoform-selective probes for rat CYP2D2 and human CYP3A4. Drug Metab Dispos 2002;30(7):845–52.
[28] Hengstler JG, Utesch D, Steinberg P, Platt KL, Diener B, Ringel M, et al. Cryopreserved primary hepatocytes as a constantly available *in vitro* model for the evaluation of human and animal drug metabolism and enzyme induction. Drug Metab Rev 2000;32(1):81–118.
[29] Alexandre E, Viollon-Abadie C, David P, Gandillet A, Coassolo P, Heyd B, et al. Cryopreservation of adult human hepatocytes obtained from resected liver biopsies. Cryobiology 2002;44(2):103–13.
[30] Li AP, Lu C, Brent JA, Pham C, Fackett A, Ruegg CE, et al. Cryopreserved human hepatocytes: characterization of drug-metabolizing enzyme activities and applications in higher throughput screening assays for hepatotoxicity, metabolic stability, and drug–drug interaction potential. Chem Biol Interact 1999;121(1):17–35.
[31] Li J, Ning G, Duncan SA. Mammalian hepatocyte differentiation requires the transcription factor HNF-4alpha. Genes Dev 2000;14(4):464–74.
[32] Westmacott A, Burke ZD, Oliver G, Slack JM, Tosh D. C/EBPalpha and C/EBPbeta are markers of early liver development. Int J Dev Biol 2006;50(7):653–7.