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# Differentiation of human hepatoma cells during confluence as revealed by gene expression profiling

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**Abstract**

Certain human hepatocarcinoma cells undergo differentiation when grown at confluence. In order to understand the basis for this differentiation, we investigated the phenotypic changes occurring during confluent growth of the human hepatoma B16A2 cell line. The global gene expression profile of B16A2 cells grown during confluence for 5 weeks was investigated using microarrays containing complementary sequences corresponding to approximately 10,000 genes, and compared with profiles of adult human liver and HepG2 cells. The major part of gene products detected were shared by all three systems and the hepatoma cell lines expressed surprisingly high levels of liver-enriched transcription factors. During confluence of B16A2 cells, the majority of transcriptional changes monitored were directed towards the phenotype of adult human liver *in vivo*, although the changes accounted for less than 10% of those necessary to acquire a native hepatic phenotype. Several markers of liver differentiation and regeneration were changed in similar manner as observed in developing liver and during liver regeneration. In conclusion, the data indicate that differentiation *in vitro* of the B16A2 cell line during confluence partially resembles that of hepatic differentiation and regeneration *in vivo*, implying a partial normalization of a low differentiated phenotype.


*Keywords:* Microarray; Liver-enriched transcription factor; Cytochrome P450; Wnt signaling; PCA analysis; Cluster analysis

## 1. Introduction

The liver is unique in its ability to regenerate after injury. It has been postulated that the process of regeneration is similar to that of the differentiation of the hepatic lineage from the ventral mesoderm during embryogenesis [1]. Cell lines derived from either embryonic or malignant liver generally express few hepatic functions [2]. However, the process of hepatic differentiation has been difficult to mimic in any *in vitro* system and primary hepatocytes in culture rapidly loose their phenotype. Knowledge about the processes involved in differentiation of hepatocytes is thus central for being able to develop hepatocyte derived *in vitro* systems containing highly differentiated cells, originating from, e.g. stem cells or transformed hepatoma cells, for use in studies of hepatic functions.

The hepatocytes contain the majority of phase I and phase II enzymes in the body responsible for detoxification of xenobiotics [3]. Drug-induced hepatotoxicity, often caused secondary to the metabolic activation of the parent compound, is common and can cause fulminate hepatitis, hepatic failure and death. It limits clinical use of several pharmacologically active potential drugs and causes withdrawal of drugs from the market [4,5]. *In vitro* systems that could predict the potential hepatotoxic effects, and unsuitable pharmacokinetic properties of drug candidates with improved accuracy would therefore facilitate drug development and make efficient tools in rational drug design [6,7]. Primary human hepatocytes constitute a common model for *in vitro* toxicological testing. However, the decline in activity over time of drug metabolizing enzymes after plating of primary hepatocytes is rapid and substantial

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*Abbreviations:* CYP, cytochrome P450; HL, human liver; PC, principal component; PCA, principal component analysis; LETF, liver-enriched transcription factor.

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[8–10] making them unsuitable for such purposes. Due to the lack of human liver (HL) material, human hepatoma cell lines are often used for *in vitro* studies. One of the commonly used, HepG2, does not express important levels of xenobiotic metabolizing enzymes. However, during recent years, the human hepatoma cell line B16A2 has been established, which in contrast to HepG2, expresses more drug metabolizing enzymes like CYP3A and CYP2E1. B16A2 also expresses liver-enriched transcription factors (LETFs), such as HNF-1, HNF-3 and HNF-4 like DNA binding activity [11–13].

As has been previously registered by mainly monitoring drug-metabolizing enzymes, the B16A2 cell line differentiates during culture under confluent conditions, thereby acquiring a more suitable phenotype for studies of drug metabolism [11,13]. Hence, we considered it important to monitor the fundamental transformations in phenotype occurring during confluent growth of B16A2 cells, and in addition, to compare the B16A2 phenotype to that of adult HL and HepG2 cells. This was achieved using microarray techniques, bioinformatics and statistical analyses with complementary analyses carried out by real time PCR for important LETFs. Such a knowledge can be of value for developing other cells lines, possibly based on stem cell research, that would allow better *in vitro* models.

The results interestingly indicate that the changes in gene expression occurring during confluence of the B16A2 cell line, both with respect to genes undergoing increased or decreased expression, to the major part force them phenotypically towards that of HL. The phenotypic changes seen represent the influence of cell–cell interaction on differentiation *in vitro* of a hepatoma cell line, which partially resembles that of hepatic differentiation and regeneration *in vivo*.

## 2. Materials and methods

### 2.1. Chemicals

Phenobarbital was purchased from Apoteksbolaget AB. Rifampicin was purchased from Sigma Aldrich.

### 2.2. Cell culture

Human hepatoma HepG2 cells, purchased from ATCC (American Type Culture Collection) were routinely cultured in MEM supplemented with 10% (v/v) fetal bovine serum, non-essential amino acids, sodium pyruvate (1 mM), penicillin (100 unit/mL), and streptomycin (100 μg/mL). HepG2 cells were harvested when reaching confluence (after 1 week of culture following being split 1/6). All tissue culture reagents were from Invitrogen, Stockholm. B16A2 cells were kindly provided by Drs. Laurent Corcos and Andre Gouillozo (University Rennes). The cell line has previously been characterized and culture conditions established by Le Jossic *et al*. [11]. B16A2 cells were grown to confluence (after 1 week of culture following being split 1/6) and harvested at time points 0, 2 and 5 weeks after reaching confluence. After 5 weeks of confluence B16A2 cells were treated with phenobarbital (1 mM for 54 hr) and rifampicin (50 μM, 54 hr). Cell medium was always changed 7 hr before harvesting for both cell types. Cell experiments were performed on three separate occasions but cells for replicate experiments originated from the same batch.

### 2.3. Liver samples

Human adult liver samples were obtained from the liver banks of Sahlgrenska University Hospital and Huddinge University Hospital. The use of human material was approved by the Stockholm Ethical Committee. The characteristics of these samples have previously been described [14]. Three pools of total RNA (equivalent amount of total RNA from each liver) were made consisting of (A) HL 43, HL 49 and HL 51, (B) HL 124, HL 126 and HL 129, and (C) HL 46, HL 48 and HL 120. These were used for the microarray analysis. For RT–PCR analysis liver RNA samples were used individually.

### 2.4. Isolation of total RNA

Total RNA was isolated from cells using RNeasy Midi Kit (Qiagen) according to the manufacturer's protocol, and from tissue samples by using a CSTFA gradient [15] (CSTFA from Amersham Biosciences).

The integrity of the RNA was confirmed by denaturing gel electrophoresis. RNA samples were quantified using the RiboGreen® quantitation assay, after DNase digestion, according to procedures developed by the manufacturer (Molecular Probes).

### 2.5. Microarray analysis

cDNA was prepared using 5–40 μg of total RNA in a 20 μL reaction using SuperScript™ II RT system (Invitrogen) according to the manufacturers instructions. Synthesis of cDNA and cRNA was performed as described by Waring *et al*. [6]. Labeled cRNA was hybridized to the Affymetrix GeneChip Test 2 Array to verify quality of cRNA. Following this, cRNA was hybridized to the Human Genome U95A v.1 and v.2 Microarrays (Affymetrix) at 45° over night, followed by washing and scanning according to procedures developed by the manufacturer. In total, 22 hybridizations were performed. The arrays were scanned using Hewlett-Packard confocal laser scanner and visualized using Affymetrix® Microarray Suite 5.0 software (Affymetrix). Affymetrix oligonucleotide microarrays are composed of multiple perfect-match and mismatch oligonucleotides. The GeneChip® software (Affymetrix) calculates a measure of the abundance of each transcript based on the measured fluorescence intensity of the oligonucleotide probes,

referred to as Signal in Affymetrix terminology [16]. All probe sets are classified as Present, Absent, or Marginal by the GeneChip® software [17]. All array experiments were scaled to an average signal intensity of 100. Twenty-six probe pairs were removed from the version 1 HG_U95 chip by the manufacturer, and replaced with 25 probe pairs for version 2. All other probe pairs on the HGU_95 chip are identical. The probe sets that were replaced between version 1 and version 2 of the chip were removed from further analysis.

### 2.6. Quality of data

Internal controls present on the Affymetrix array were monitored to ensure proper hybridization of the chips, as well as cRNA integrity and cRNA labeling. Two chips out of 22 were removed from further analysis.

Hybridization experiments were run at three different occasions. Reproducibility of the experiments was evaluated by visual inspection of scatter-plots and by calculating correlation coefficients for the same cell type or treatment at different experimental dates. The average for correlation coefficients was 0.97 with a standard deviation of 0.01.

### 2.7. Data analysis

Data was divided into two subsets. The first dataset included B16A2 samples only, to study effect of confluence time and treatment effects on the cell line. The other data set consisted of B16A2 untreated 5-week samples, HepG2 cells and HL samples to enable comparisons between the different cell types.

Principal component analysis (PCA) was performed on signal intensity values and used as a tool to study sources of variation in the data, as well as to study similarities between samples [18,19]. In a PCA of all samples, the third largest component of variation was between different experimental dates, an effect taken into account in the subsequent ANOVA.

Standard ANOVAs were performed at the level of individual transcripts, to enable selection of genes with an altered level of expression between conditions. Two separate ANOVA models were fitted to the two datasets, both models adjusting for variation between experimental occasions.

Genes were clustered to organize data thus making patterns of expression more easily distinguishable, using two-dimensional hierarchical agglomerative clustering, with Pearson correlation as a similarity measure [20,21], and average (unweighted pair group method with arithmetic mean) as linkage measure. Choosing correlation as a measure of similarity, aids in clustering genes with similar patterns of expression (e.g. increase or decrease over time), while being insensitive to the absolute magnitude of expression of different genes [22]. For all cluster analyses, genes classified as Absent in all experiments were removed.

### 2.8. Reverse transcription and PCR

Reverse transcription reaction was performed with 0.5 μg of total RNA in a final volume of 10 μL, using M-MLV Reverse Transcriptase (Invitrogen) essentially according to procedures developed by the manufacturer. Each PCR reaction contained 2–10 μL of cDNA template, 1× Buffer, 1.25–1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.25 μM of each primer, and 0.625 unit Taq DNA Polymerase (ABGene). For primer sequences, see Table 1. Each PCR consisted of 95° for 1 min followed by 25–40 cycles of 95° for 15 s, 52 or 60° for 20 s, and 72° for 1 min. PCR products were separated on EtBr containing agarose gels. Identity of PCR products was verified by DNA sequencing, using BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) according to the manufacturers instructions.

Table 1
Primer sequences for PCR and quantitative PCR

| Factor | Primers | Designed from | Size (bp) |
|---|---|---|---|
| PXR | Sense 5′-CAA GCG GAA GAA AAG TGA ACG-3′<br>Antisense 5′-CTG GTC CTC GAT GGG CAA GTC-3′ | Dotzlaw *et al.* [43] | 442 |
| CAR | Sense 5′-ATA CTG TCG GCA GAA GCC-3′<br>Antisense 5′-CTA CCT GGA ACC CCA C-3′ | NM_005122.1 | 469 |
| HNF-1α | Sense 5′-CTC TAC AGC CAC AAG CCC GAG GTG-3′<br>Antisense 5′-TCT GAG CTC TGG TAC AGC ACC A-3′ | NM_000545 | 302 |
| HNF-4α | Sense 5′-GCC TAC CTC AAA GCC ATC AT-3′<br>Antisense 5′-GAC CCT CCC AGC AGC ATC TC-3′ | Jover *et al.* [35] | 275 |
| HNF-6 | Sense 5′-CCA TCA ACG GCC TTC CTC-3′<br>Antisense 5′-GAG TTC GAC GCT GGA CAT CT-3′ | U96173 | 471 |
| Oct-1 | Sense 5′-TTG GAG GAG CCC AGT GAC CTT-3′<br>Antisense 5′-CAT AGT GAT CTC TTC CGA GGT-3′ | NM_002697 | 399 |
| DBP | Sense 5′-CTG GCC ACG AGA CCT TTG-3′<br>Antisense 5′-AAC AGG GCG TAA GTC TCA GCA-3′ | NM_001352 | 387 |
| GAPDH | Sense 5′-GCA GGG GGG AGC CAA AAC GG-3′<br>Antisense 5′-TGC CAG CCC CAG CGT CAA AG-3′ | Jung *et al.* [44] | 315 |

"Designed from" refers to either the reference from where primer sequences were obtained, or to the Entrez Nucleotides Database sequence number of the sequence used for designing primers.

### 2.9. SybrGreen real time PCR and primers

PCR was performed using 5 μL of diluted cDNA template in a 25 μL reaction containing 0.25 μM of each primer and 12.5 μL SybrGreen real time PCR MasterMix (Applied Biosystems). Each PCR consisted of 50° for 2 min, 95° for 10 min followed by 40 cycles of 95° for 15 s, 52 or 60° for 20 s and 72° for 60 s. Each PCR was followed by a melt curve analysis, and separation on EtBr containing agarose gels. PCR was performed using the SmartCycler System from Cepheid. For primer sequences see Table 1. All reactions were performed on duplicate samples from two separate cell experiments and from two HLs.

## 3. Results

Transcriptional profiles for approximately 10,000 genes were generated using Affymetrix Human Genome microarrays on mRNA preparations isolated from HL, HepG2 cells and B16A2 hepatoma cells during different times of confluence. Lists of all results mentioned in this publication, with corresponding *P* values, can be found at http://www.imm.ki.se/butura.htm.

### 3.1. Alteration in gene expression in B16A2 cells during culture

We examined differences in gene expression of B16A2 cells cultured for 0, 2 or 5 weeks of confluence in comparison to that of HL by means of ANOVA. When choosing a high degree of significance ($P = 0.001$), analyses revealed 186 probe sets that changed in expression during the time period. These genes were organized using hierarchical clustering as shown in Fig. 1A, where results using mRNA from pools of HL are shown as comparison. The diagram shows B16A2 and HL clustering as separate groups. Different colors illustrate the relative extent of gene expression.

When conducting a profile search for phenotypic changes over time, three major clusters of genes can be distinguished (see Fig. 1B): cluster I contains 47 genes with lower expression in B16A2 than in HL, increasing in expression during confluence, cluster II, where the genes (N = 33) increase in expression over the time of confluence, but where expression in HL is lower than in B16A2 cells at 2 and 5 weeks and cluster III where highly expressed genes (N = 72) decrease their expression to levels more closely matching the HL. As shown by the dendrogram at the top of the image, the major changes in gene expression take place between 2 and 5 weeks of confluence.

Among the genes with increased expression during culture (cluster I), approaching the levels in HL, are those encoding integral membrane proteins (e.g. ERBB-3 receptor protein-tyrosine kinase, heme oxygenase 1), serine proteases (e.g. serine protease HTRA1, complement factor I precursor) proteins involved in lipid and protein metabolism (e.g. sphingomyelin phosphodiesterase, hyaluronan binding protein 2) and inflammatory response (e.g. complement C5 precursor, complement component C6 precursor). Examples of genes with dramatic increase in

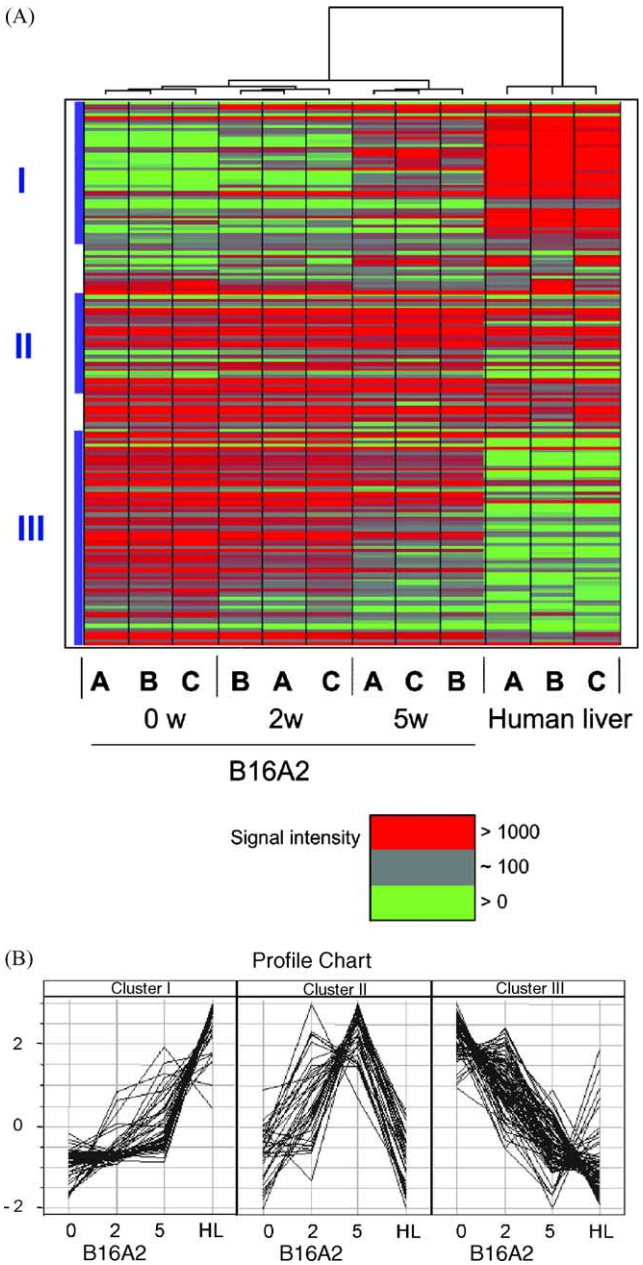


Fig. 1. Changes in gene expression in B16A2 cells over confluence time. (A) Hierarchical clustering of B16A2 cells at 5 weeks of confluence, and HL. Clustering was performed on 186 probe sets which had either a *P* value of ≤0.001 in an ANOVA comparing the B16A2 at 5 weeks of confluence with B16A2 at 0 weeks, or a *P* value of ≤0.001 comparing the B16A2 at 5 weeks with the treated cells. Clustering was performed using UPGMA, with Pearson's correlation as similarity measure and colored after amount of expression. (B) Profiles of individual transcripts from clusters I (47 transcripts), II (33 transcripts) and III (72 transcripts) in the B16A2 time cluster diagram, A. *Y*-axis values represent fold change of transcripts when all averages have been normalized to 0. All cells and tissue are represented by the average of triplicate values. A, B, and C for cells represent three separate biological experiments, and for HL three different pools of three livers each.

expression are presented here. Serum albumin mRNA increases approximately 33-fold during long-term growth of B16A2 cells, and reaches levels of 4.6% of HL. The expression of alcohol dehydrogenase mRNAs (alpha, beta and gamma isoforms) increased up to ~30, respectively ~20-fold during confluence, but the induced expression still only constitutes ~5–7% of the expression in HL. Furthermore, CYP2E1 mRNA increased 7-fold during 5 weeks of confluence, reaching 2.9% of the content in HL. Similarly, CYP3A4 mRNA increased 8-fold, which accounted for 5.8% of the amount in liver. By contrast, the increase in the hepatic form of flavin containing monooxygenase 3 (FMO-3) mRNA expression indeed resulted in levels similar to HL.

Genes down regulated over the time period, towards the low levels seen in HL (cluster III), are encoding membrane transport proteins (e.g. L-type amino acid transporter 1, monocarboxylate transporter-5), cytoskeletal proteins (e.g. tropomyosin-1 fibroblast isoform TM3, actin-binding LIM protein 1), annexins, proteins involved in secretion/trafficking, and signal transduction, (e.g. regulator of G-protein signaling 20). In addition, also genes encoding cancer antigens, and genes involved in sugar–alcohol metabolism (aldose reductase, transaldolase) diminished their expression. Several proteins involved in developmental processes are also represented in this group, such as Frizzled-2, Notch homologue 2, and Disabled homologue 2.

Examples of genes that were up regulated but are expressed at low levels in HL (cluster II), are the ribosomal proteins 40S and 60S, multidrug resistance protein 1, and proteases, such as metalloproteases and aspartic proteases.

Several metabolic pathways (as defined by KEGG, http://www.genome.ad.jp/kegg/) increase significantly during confluent growth of B16A2 cells ($P$ value of <0.01 and fold change >2). The most affected pathways are fatty acid metabolism (e.g. alcohol dehydrogenase 1A class I, CYP2C18), bile acid biosynthesis (e.g. bile acid coenzyme A amino acid $N$-acyltransferase, alcohol dehydrogenase IB class I) and tyrosine metabolism (e.g. monoamine oxidase B, alcohol dehydrogenase 1C class I). These three pathways all increased to levels more closely matching the expression in HL, and all represent important liver functions.

Furthermore, several pathways decreased their expression, such as sterol biosynthesis (e.g. geranyltranstransferase, lanosterol synthase), the Wnt-signaling pathway (e.g. Frizzled-2, and an increase in the inhibitor Dickkopf protein-1), and integrin-mediated cell adhesion as shown by calpain 2 and p21 activated kinase. The phosphatidylinositol signaling system is a part of integrin-mediated cell adhesion, and a decrease was seen in phosphatidylinositol signaling (e.g. dual specificity phosphatase-1) and inositol phosphate metabolism (e.g. serum-inducible kinase, serine/threonine kinase 17a).

Analyses were also carried out on phenobarbital and rifampicin treated B16A2 cells. However, no significant induction of genes previously known to be highly induced by phenobarbital or rifampicin was obtained by either compound.

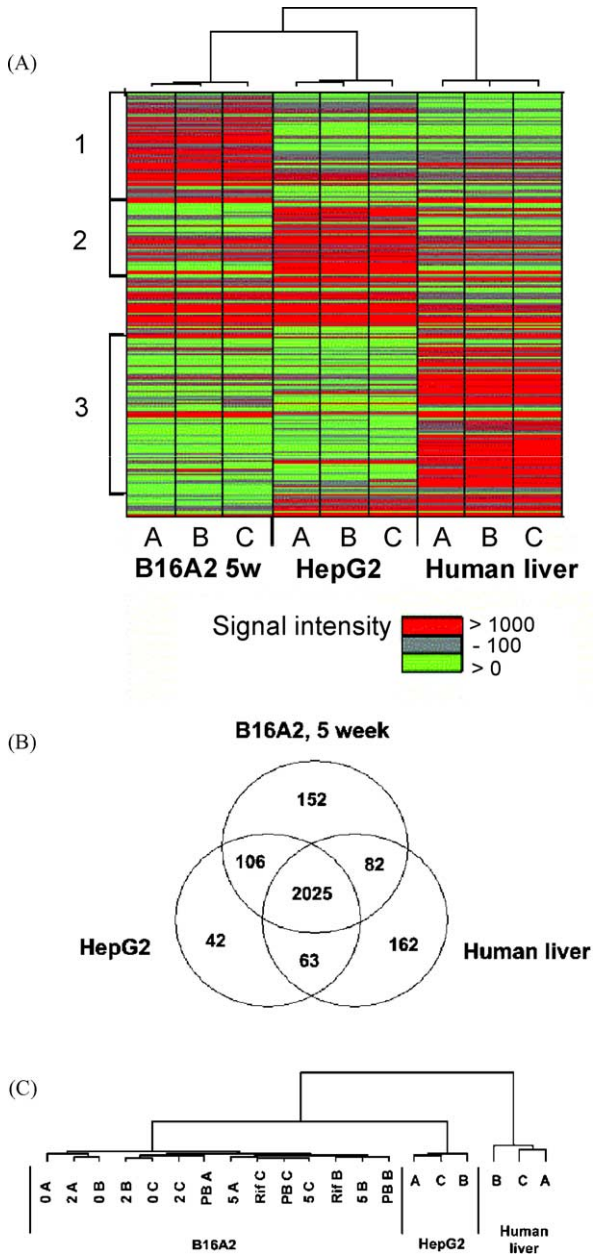


Fig. 2. Comparison of gene expression between B16A2, HepG2 and HL. (A) Hierarchical clustering of B16A2 cells at 5 weeks of confluence, HepG2 cells at confluence, and HL pools. Clustering was performed on 530 probe sets with a $P$ value of $\leq$0.001 in an ANOVA comparing the two different cell lines and liver tissue. Clustering was performed using UPGMA, with Pearson's correlation as similarity measure and colored after amount of expression. (B) Genes classified as Present (P) in all three out of three repeated experiments, using the Affymetrix Detection Call (Affymetrix MAS 5.0). The 2025 probes in the center of this diagram correspond to 1910 genes. For a description of Present (P) classification, refer to Section 2. (C) Hierarchical clustering of all experiments performed, on all probe sets on the Affymetrix Human Genome 95 Chip. Clustering was performed using UPGMA, with Pearson's correlation as similarity measure and colored after amount of expression. PB and Rif indicate B16A2 cells treated with phenobarbital and rifampicin, respectively.

### 3.2. B16A2 cells in comparison to HepG2 and human liver

Genes specifically expressed in each cell type were identified by comparing B16A2 cells (5 weeks of confluence), HepG2 cells and HL using an ANOVA model. Differentially expressed genes ($P$ value of $\leq 0.001$) were selected and clustered hierarchically into those preferentially expressed in B16A2 cells (group 1), HepG2 cells (group 2) and HL (group 3) (Fig. 2A). Some examples of transcripts from Fig. 2A, highly expressed in HepG2 cells, are nuclear proteins and proteins involved in transcriptional regulation. Transcripts enriched in B16A2 cells encode oxidoreductases, receptors (mostly integral membrane proteins) and calcium binding proteins. Generally, the two hepatoma cell lines contain more genes lacking functional annotation as compared to liver. Similarities were found between HL and B16A2 but not HepG2, for genes involved in adhesion and molecular recognition, and genes involved in membrane transport.

To compare the two cell types and HL, we made use of the Absent or Present classification of the Affymetrix MAS 5.0 software and hierarchical clustering. Figure 2B shows gene probe sets detected as present in B16A2 5w, HepG2 and HL. The majority of transcripts (1910, corresponding to 2025 probe sets) are expressed in all three systems. Some more transcripts were common between HL and B16A2 cells as compared to HL and HepG2 cells. (Note that the Absent/Present classification relates to if genes are above a certain threshold but not directly to expression levels.) The clustering of all transcripts, which puts more weight on expression levels than the Absent/Present classification, is illustrated in Fig 2C. In general, this illustrates a rather large gap between the current *in vitro* models, and the *in vivo* situation they are to portray.

Differences in gene expression between the cell lines and HL were visualized using PCA analysis on signal intensity values (Fig. 3). The analysis showed an assemblage of the triplicate experiments for each cell type into readily distinguishable groups. The two major sources of variation were found between different types of cells, while the third largest component of variation was between different experiments performed. Note that none of the groups overlap, nor is one of the two cell lines clearly closer in 3D space to the HL samples.

Focusing on genes of interest for drug metabolism and toxicology, we investigated genes encoding phase I and phase II enzymes. Clustering of P450 genes

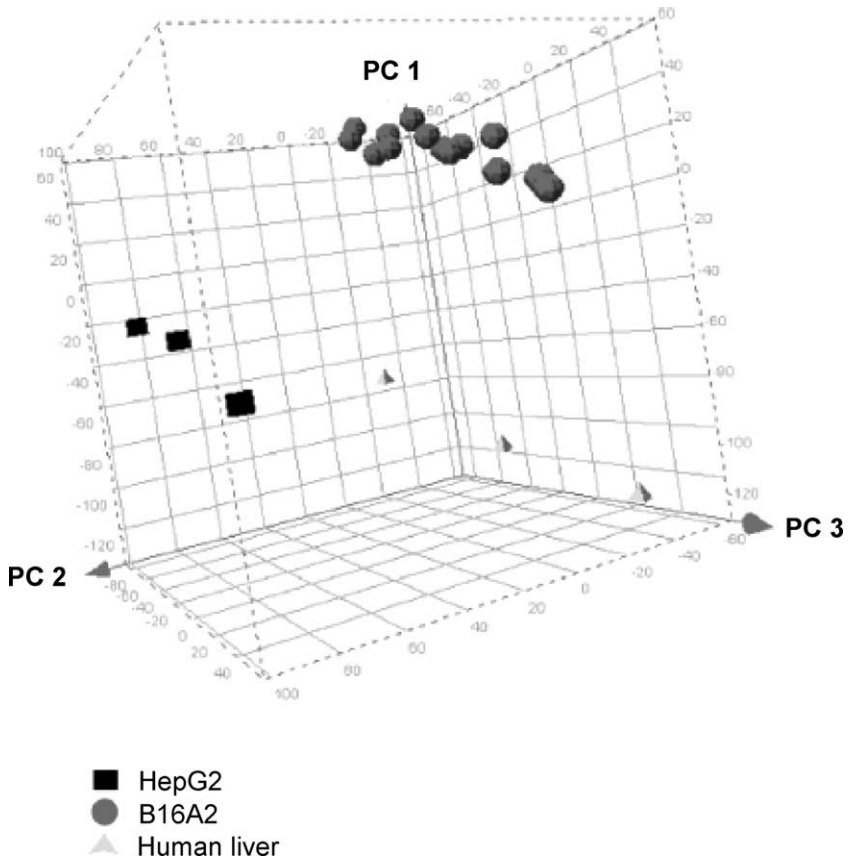


Fig. 3. Principal component analysis. PCA visualization showing the three principal components of the dataset. Triplicates of chips from all cell types were included in this analysis to help pinpoint general trends in the data. Percent variance for the components are (counter clockwise) PC1: 23.1%, PC 2: 14.2% and PC 3: 9.8%, i.e. PC 1 explains 23.1% of the total variation. The B16A2 cells have more samples in the cluster because the whole B16A2 time series, and also treated samples are included.

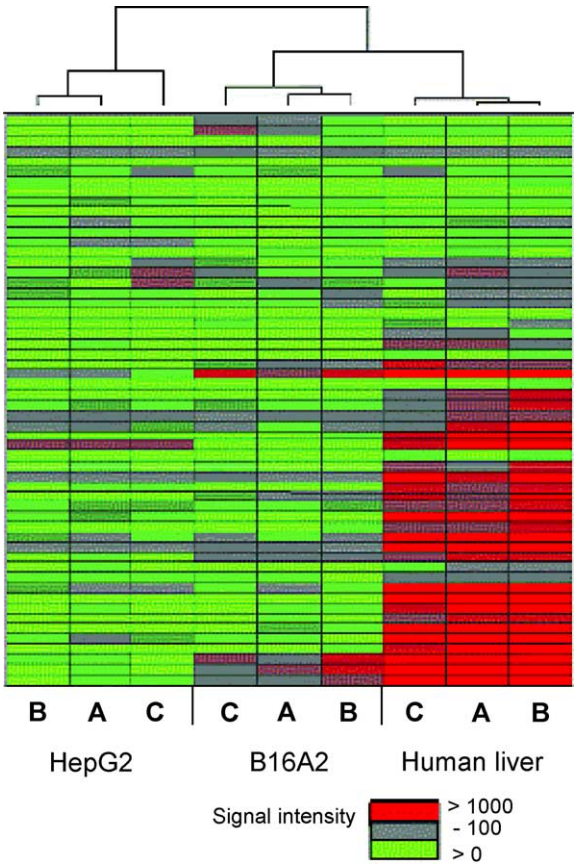


Fig. 4. Comparison of CYP mRNA expression in HepG2 cells, B16A2 cells and HL. Hierarchical clustering of B16A2 cells at 5 weeks of confluence, HepG2 cells, and HL pools. Clustering was performed on 56 probe sets classified as belonging to the CYP family of enzymes. Clustering was performed using UPGMA, with Pearson's correlation as similarity measure and colored after amount of expression.

(Fig. 4) shows that B16A2 cells as compared to HepG2 cells had a more pronounced expression and were in the same branch of the dendrogram as HL. Clustering of transcripts classified to encode products involved in phase II metabolism, also including gene products involved in xenobiotic uptake and distribution, revealed two out of three HL pools and three HepG2 samples in the same branch of the dendrogram (see http://www.imm.ki.se/butura.htm).

### 3.3. Expression of liver-enriched transcription factors

To analyze genes highly relevant for hepatic function, but expressed at very low levels, quantitative SybrGreen real time PCR was performed on representatives of several families of LETFs. Figure 5 summarizes the findings concerning HNF-1α, HNF-4α, HNF-6, Oct-1 and DBP. Unexpectedly, the levels of these LETFs were not much different between the hepatoma cells and HL, except for HNF-4α, which was highly expressed in the HepG2 cells, as compared to HL or B16A2. Additionally, B16A2 cells did not express PXR, and only trace amounts of CAR, whereas HepG2 expressed both these factors to some

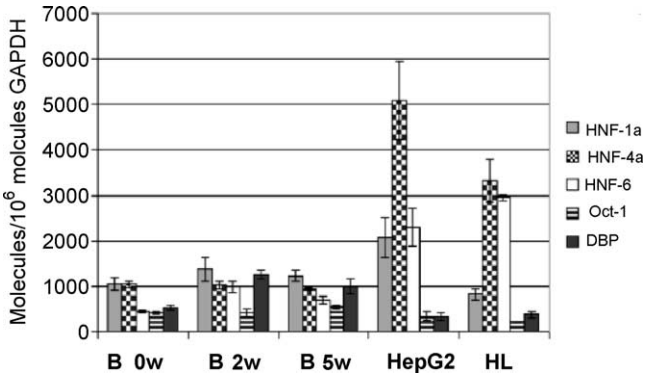


Fig. 5. Expression of LETFs in HepG2 cells, B16A2 cells and HL. SybrGreen real time PCR of LETFs was carried out using cDNA from B16A2 (B 0w to B 5w) and HepG2 cell lines and HL. Expression of PXR was not detected in B15A2 cells at any time point. Expression in HepG2 was 3811 mol./$10^6$ mol. GAPDH. Expression of PXR in HL was 12,578 mol./$10^6$ mol. GAPDH. Detection limit for PXR is 1 mol./$10^6$ mol. GAPDH which is ~100 molecules/reaction. Expression of CAR was 26 mol./$10^6$ mol. GAPDH in B16A2 at 5 weeks, 97 mol./$10^6$ mol. GAPDH in HepG2 and 26,300 in HL mol./$10^6$ mol. GAPDH. Detection limit for CAR was ~5 mol./$10^6$ mol. GAPDH, which is ~100 molecules/reaction. Mol.; molecule.

extent. The real time PCR results were normalized to GAPDH, which was shown to have a stable expression in relation to total RNA between the different cell types.

## 4. Discussion

Our results show that the majority of transcriptional changes monitored in the B16A2 cell system during confluence are directed towards the phenotype of adult HL *in vivo*. The change of phenotype during confluence occurred without addition of DMSO, previously used to induce differentiation [23,24], and is hence possibly linked to cell–cell contact. As cell–cell contact has been shown to be required for induction of adult liver function in fetal hepatocytes [25], increased cell–cell interaction could putatively result in a more differentiated pattern of gene expression in a cell system. Consequently, our study captures the sole influence of cell–cell contact, and its impact on a differentiation processes. However, after 5 weeks of confluence, gene expression in the B16A2 cells reached less than 10% of the expression levels that would result in a phenotype similar to HL.

Genes affected by confluence in B16A2, such as those encoding products involved in lipid metabolism (increasing in expression), and cytoskeletal proteins and enzymes controlling protein turnover (decreasing in expression), are to great extent similarly affected during rat hepatic stem cell differentiation, as showed by Plescia *et al.* [24]. Examples of genes displaying the same changes in both experiments are alcohol dehydrogenase and asialoglycoprotein receptor that increase in expression, and c-*myc*, asparagines synthetase, and several forms of integrin that decrease in expression. Gomez-Lechon *et al.* [13] have

hypothesized that the capability of growth re-initiation after long-term confluence, a feature shared by the BC2 and B16A2 cell line, to some extent reflects the liver-regeneration process *in vivo*. Here we show that a hepatoma cell line, B16A2, does in fact undergo a process corresponding to that of liver differentiation/regeneration, as several key markers for differentiation from stem cell lineage, and from regenerating liver are affected during long-term confluence of the B16A2 cell line.

The CYP3A4 and 2E1mRNA levels in the B16A2 cell line increased ~5–10-fold over long-time confluence but reached only low levels as compared to HL. This has been registered on the level of enzymatic activity in the partially characterized BC2 cell line [13], derived from the same human hepatocarcinoma as the B16A2 line. Phenotypically several CYP:s, among others CYP2E1, are expressed at much lower levels in hepatocellular carcinoma as compared to normal adult liver [26]. In this study, we registered a major gap in the expression of most hepatic P450:s between HL, and the HepG2 and B16A2 cell lines (Fig. 4). However, the study clearly shows that the B16A2 cell line over culture time expresses more metabolic functions important for the normal liver, as seen by the increased expression of genes involved in fatty acid metabolism, bile acid biosynthesis and tyrosine metabolism.

Genes encoding albumin, fibrinogens and proteins involved in lipid metabolism show lower rates of transcription in carcinoma [26]. Interestingly, nearly all transcripts having a lower expression in hepatocellular carcinoma as listed by Xu *et al*. [26] increased in the B16A2 cell line during confluence, such as for example serum albumin, CYP2E1 and adenosine deaminase. Baker *et al.* [8] investigated the de-differentiation of rat primary hepatocytes in culture, and showed decreased expression of CYP2E1, CYP3A isoforms, and methyltransferases. Similarly, the expression of these genes is reversed in the B16A2 cell line over culture time. α-Fetoprotein, a marker of fetal liver, is not expressed in neither B16A2 cells nor HL, but very highly expressed in the HepG2 cell line. Together these results thus indicate a partial reversion to the adult hepatic phenotype of the B16A2 cells during confluence.

Decrease of expression of the Wnt signaling pathway is important for differentiation of mouse hepatoma stem cells *in vitro* [24]. The Wnt signaling pathway, known to be over expressed in several types of tumors, decreased over time in the B16A2 cells, as seen from decreased Frizzled-2 expression. Involvement of the Wnt pathway is important for differentiation in such a diversity of tissues as adipocytes [27] and cardiac myocytes [28], implying that the decreased expression of Frizzled-2 and the phenotypic changes seen in B16A2 over time are part of a general process of differentiation. Further evidence of down regulation of the Wnt-signaling pathway is observed by the increased expression of Dickkopf-1 (Dkk-1), an indirect negative regulator of Wnt signaling [29], over confluence time in B16A2, peaking at 2w of confluence. Furthermore, Ezrin, previously known to be involved in liver regeneration [30], but interestingly a common component of both regeneration and differentiation of hepatocytes [1], was expressed at high levels in B16A2. Two other transcripts decreasing their expression over time, thioredoxin reductase, and four and a half LIM domain protein, have been shown to be enriched in stem cells of several origins [31].

Furthermore, several members of the Notch pathway decreased in expression over confluence time in B16A2 cells, i.e. Notch-2 and disabled homologue 2. The Notch signaling pathway is known to be highly influential on cell fate and determination for a number of cell types, but its role in liver development or liver transformation remains unclear.

Integrin-mediated cell adhesion to substrate is an important step in the development of cancer metastases [32]. In addition, integrin-mediated cell–substrate adhesion inhibits cadherin-mediated cell–cell adhesion and thereby weakens cell–cell interaction [33]. As integrin-mediated cell adhesion shows decreased expression, this would indirectly favor increased cell–cell interaction in the B16A2 system.

Liver-enriched transcription factors have a role in a regulatory network for determination and maintenance of the hepatic phenotype. There are today six known families of LETF:s, namely HNF-1, HNF-3, HNF-4, HNF-6, C/EBP, and DBP [34], of which HNF-1α, HNF-4α, HNF-6 and DBP have been studied here. Many of those are known to control expression of hepatic cytochromes P450 (CYPs) [35]. It has been postulated that these factors in co-operation with ubiquitous transactivating factors such as Oct-1, are necessary and perhaps sufficient to maintain liver-specific transcription [36]. All LETF:s examined were determined to be present at the mRNA level in the cell lines studied, thus providing the prerequisite for hepatic specific gene expression. Our finding of HNF-1α and HNF-4α expression in both HepG2 and B16A2 cells is in line with results by Le Jossic *et al.* [11]. Expression of both factors is expected since HNF-4α is an essential regulator of the HNF-1α gene [37]. However, the ubiquitous expression of all LETF:s analyzed in HepG2 cells was somewhat surprising. PXR, known to be crucial for induction of CYP3A4 by rifampicin and phenobarbital [38] was lacking in the B16A2 cell line. We believe that this could explain the lack of induction in the cell line.

To conclude, this study portrays the effect of cell–cell interaction on a differentiation process of a hepatoma cell line, and shows that although this leads to a partial reversion of the malignant phenotype, changes in gene expression are less than 10% of the expression levels that would result in a phenotype similar to HL. Strategies to enhance liver specific gene expression include for instance transfection of specific genes, co-culturing with epithelial cells [39,40], with transformed stellate cells and Kupffer cells,

or growth of cells on different types of matrices [41,42]. Stem-cell research could also shed light on the possibility of obtaining hepatocyte-like cultures *in vitro* [25]. Thus, further innovative strategies are required to determine what crucial components are lacking in hepatic *in vitro* models in order to obtain stable terminally differentiated *in vitro* systems.

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