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REGULAR ARTICLE

Induction of a mature hepatocyte phenotype in adult liver derived progenitor cells by ectopic expression of transcription factors

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Abstract **Background/aims**: By ectopic expression of a distinct combination of transcription factors we aimed to induce a mature hepatocyte phenotype in an adult liver derived progenitor cell population (ALDPC). **Methods**: The open reading frames encoding murine Foxa2, Hnf4α and C/ebpα were cloned into lentivirus vectors and sequentially expressed in target cells. After seven days of culture, cells were analysed for expression of liver specific genes, and functional assays were performed. Fresh primary hepatocytes, twenty four hours in culture, served as positive controls. **Results**: Untransduced ALDPC under established differentiation conditions exhibited moderate signs of maturation, in particular in comparison with fresh hepatocyte controls. In transcription factor transduced cells, fifteen mRNA´s coding for secreted proteins, cytochrome p450 isoenzymes, liver metabolic enzymes were detected by RT-qPCR at levels close to controls. Albumin secretion increased incrementally in single (Foxa2), double (Foxa2, Hnf4α) and triple-transduced cells (Foxa2, Hnf4α, C/ebpα) and reached levels observed in primary

Abbreviations: ALDPC, adult liver-derived progenitor cells; iPS, induced pluripotent stem cells; LETF, liver enriched transcription factors; LD, liver differentiation medium; HBM, hepatocyte basal medium; MM, maintenance medium; ESC, embryonic stem cells; Foxa2, forkhead box protein A2; Hnf4α, hepatocyte nuclear factor 4 alpha; C/ebpα, CCAAT/enhancer binding protein alpha; Alb, Albumin; Aat, alpha-1-antitrypsin; Tat, tyrosine aminotransferase; Tdo2, tryptophan 2,3-dioxygenase; G6p, glucose-6-phosphatase; Cldn1, claudin 1; Cps1, carbamoyl-phosphate synthetase 1; Gys2, glycogen synthase 2; Fah, fumarylacetoacetate hydrolase; Cyp, cytochrome P450; Pxr, pregnane X receptor; Apo, apolipoprotein

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hepatocytes. Glycogen storage as determined by PAS staining was detectable in double and triple transduced cells, comparable to controls. Ureagenesis was also induced in triple transduced cells, but at lower levels compared to primary hepatocytes.
Conclusions: Sequential expression of Foxa2, Hnf4α and C/ebpα induces a mature hepatocyte phenotype in an expandable liver derived progenitor cell line.

Introduction

The isolation of high quality hepatocytes from organs and tissues for toxicological studies and clinical therapies remains a challenge and alternative cellular resources are urgently needed. Various protocols for directed hepatic differentiation of stem cells have been developed as alternatives for large scale hepatocyte production. Embryonic and, more recently, induced pluripotent stem (iPS) cells where shown to substantially expand and differentiate into hepatocyte-like cells in culture [1–3]. Their maintenance, growth and differentiation using cytokine and growth factor supplemented differentiation media, however, are time consuming and expensive. Furthermore, pluripotent stem cells, for now, are not considered for cell therapy applications due to risks of teratoma formation.

Adult extrahepatic tissues and normal postnatal liver provide an alternative potential source for the generation of hepatocytes for in vitro studies and cell therapy. Evidence has been provided that adult stem cells from various extrahepatic sources could occasionally overcome lineage borders and differentiate into hepatic lineage cells, upon coordinated in vitro stimulation [4,5]. The number of stem/progenitor cells, that can be isolated from normal adult liver, is generally low and not sufficient for most of the in vitro and in vivo applications.

Protocols have thus been developed to expand colonies of primitive cells with bipotential progenitor characteristics from cell surface marker enriched populations or directly from primary hepatocyte cultures. Those cells being designated here as adult liver-derived progenitor cells (ALDPC), have first been described as BAML (bipotential adult mouse liver cells) [6,7] or, from humans, as liver epithelial cells (LEC [8]), nonparenchymal epithelial cells (NPE [9]) or human liver progenitor cells [10]. ALDPCs express cytokeratins characteristic both for the hepatic (CK8 and CK18) as well as for the cholangiocytic lineage (CK7 and 19), but lack many hepatocyte specific functions. Most of the conventional differentiation protocols for stem/progenitor cells aim to induce transcriptional changes by external stimuli and signals. Target cell populations are cultured on various extracellular matrix components and in the presence of media supplemented with cytokines and hormones for a prolonged period of time [11]. Despite remarkable progress and refinement of the protocols, adult stem/progenitor cells often fail to achieve complete function sufficient for regenerative therapy and toxicological assays, remaining only "hepatocyte-like".

An alternative experimental approach that "engineers" cell fate and phenotype changes by single or combinatorial ectopic over-expression of key transcription factors in target cell populations, evolved over the past several years. Ber et al. were the first to demonstrate that insulin producing beta cells can be generated from developmentally related hepatocytes by transfer of a single transcription factor PDX-1 [12]. Takahashi and Yamanaka successfully re-programmed mouse embryonic/adult fibroblasts into ES-like stem cells, referred to as iPS cells, by retroviral transfer of the transcription factors Oct4, Sox2, c-Myc and Klf4 [13]. Recently, Vierbuchen et al. were able to convert mouse embryonic and postnatal fibroblasts directly into partially functional neuronal cells by ectopic expression of three transcription factors (Ascl1, Brn2 -also called Pou3f2- and Myt1l) thereby bypassing the state of pluripotency [14]. By a similar approach, Ieda et al. have successfully reprogrammed cardiac or dermal fibroblasts directly into cardiomyocyte-like cells by a combination of three developmental transcription factors [15].

Induction and maintenance of hepatocyte differentiation and control of liver-specific gene expression is attributed, in large part, to the coordinated expression of liver enriched transcription factors (LETFs) [16,17]. This class of proteins includes five families of transcriptional regulators: Hnf1, Foxa (Hnf3), Hnf4, Hnf6 and C/ebp [18,19]. While none of these factors is exclusively expressed in the liver, the combinatorial actions of tissue-specific transcription factors lead to the stringency and dynamic regulation of gene expression required for the proper development and function of the organ [20]. In our present study, we identified three transcription factors, which upon sequential ectopic over-expression confer an advanced mature hepatocyte phenotype in an adult liver derived clonal cell population. The three transcription factors expressed in ALDPCs (Foxa2, Hnf4α and C/ebpα) were selected based on their already known functions in liver development and maturation in combination with the finding of their rapid downregulation during the first days of primary hepatocyte culture - thus provoking experiments to reintroduce them to achieve maturation again.

Results

Functional expression of Foxa2, Hnf4α and C/ebpα

First, a series of transfection experiments in HEK293T cells utilizing a luciferase reporter assay was carried out to validate functional expression of the cloned transcription factors Foxa2, Hnf4α and C/ebpα in their final, lentiviral expression plasmid context (Fig. 1).

Basically, the transcription factor under investigation is expected to have a transcription inhibitory (Foxa2) or promoting (Hnf4α, C/ebpα) activity upon the promoter of the respective luciferase expression plasmid, thus resulting in luciferase signal changes compared to controls. A third cotransfected plasmid, carrying a Renilla-luciferase expression

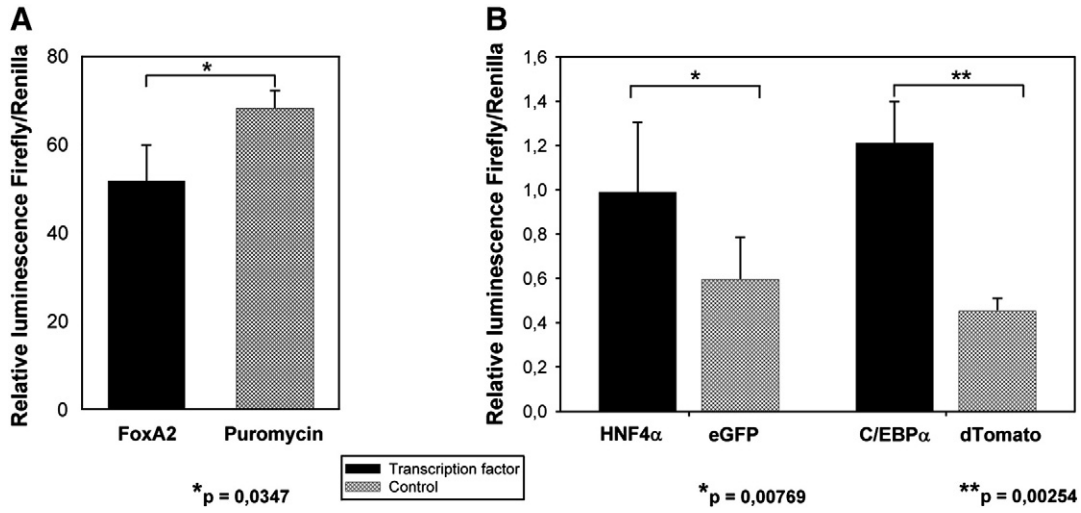

Figure 1 Functional luciferase assay for transcription factors. A) Inhibition of luciferase expression by Foxa2: The lentiviral vector plasmid Foxa2-IRES-puro was cotransfected with the plasmids pTCF-CMVpro-GL4.20. B) Induction of luciferase expression by Hnf4α and C/ebpα: The lentiviral vector plasmids pL-S-Hnf4a-I-egfp and pL-S-Cebpa-I-dTom (Suppl. Fig. 1) were co-transfected with a Ugt1a9-promoter-luciferase construct. In all experiments a cotransfected Renilla-luciferase expression plasmid served to normalize for transfection efficacy. Corresponding lentiviral vector plasmids without transcription factor ORF served as controls (Puromycin, eGFP, dTomato). Data show the mean value (N = 3) and the standard deviation (SD). Statistical analysis: Student's *T* Test.

cassette under control of the TK-promoter, served to normalize for transfection efficiency.

For the Foxa2 plasmid a 25% decrease in relative luminescence ratio was detected from a TCF4-CMVpro promoter reporter plasmid [21] (Fig. 1A) upon transfection. Hnf4α and C/ebpα lentiviral plasmids induced significant luminescence from a Ugt1a9 promoter reporter plasmid [22], resulting in a 66% and 167% increase in Firefly/Renilla luminescence ratio, respectively (Fig. 1B).

Adult liver derived progenitor cells (ALDPC) transduced with each lentiviral vector showed increased mRNA levels of the respective transcription factor (Fig. 2), with expression levels in triple-transduced cells being in the same range as mRNA levels found in freshly isolated hepatocytes (pHc day 0).

Foxa2 transgenic ALDPC were also transduced with C/ebpα only. However, in the absence of elevated levels of (cotransduced) Hnf4α, C/ebpα appeared cytotoxic in our system - dTomato positive cells no longer adhered to the collagen coating and died subsequently in suspension, as indicated by their rapid loss of dTomato-flourescence. Thus the experimental use of ALDPC-F-C for mRNA-expression, protein secretion and metabolic analyses was not feasable in our hands (data not shown).

Characterisation and maturation of untransduced ALDPC

ALDPCs were derived from several primary liver cell cultures in maintenance medium (MM) containing EGF, IGFII and insulin. A tightly packed monolayer of cells was formed after three to six weeks of culture. The ALDPC population we used expressed keratins 7, 8, 18 and 19 mRNAs, detectable, albeit low levels of liver enriched transcription factor mRNA´s including Foxa2, Hnf1α and Hnf4α and no detectable levels of C/ebpα mRNA (Fig. 2, ALDPC-MM and Supplementary Fig. 2). Only trace amounts of albumin and α1-antitrypsin were secreted into the supernatant. Furthermore, no α-fetoprotein (Afp) expression could be detected (data not shown). From the 15 mRNAs chosen to measure differential expression in mouse hepatocytes, none - if at all - was detectable at a range comparable to primary hepatocyte controls (Fig. 3, ALDPC-MM).

Seven days after induction of ALDPC maturation by using collagen I-coating and LD-medium, mRNA levels for transcription factors Foxa2, Hnf4α mRNA were upregulated, also C/ebpα became detectable (Fig. 2, columns ALDPC). Also increased levels of Alb, Aat, Cldn1, Fah, Pxr, Apoa1, Apoa4 and Apoc3 (Fig. 3, columns ALDPC-MM vs. ALDPC) were measured. Furthermore, Tdo2, G6p, Cps1 and Gys mRNAs were consistently - albeit with considerable variation - detected in the ct-range between 35 and 40 under maturation conditions, whereas in MM-conditions mRNAs could not be detected in our PCR-settings (data not shown). With the exception of the cytochrome p450 genes and Tat, all genes tested were upregulated by cultivation of ALDPC under differentiation conditions as compared to standard maintenance conditions - at least 2-fold (Cldn1, Pxr) up to more than 100-fold (Alb, Apoa1) - or became first detectable at all. Apoa1 and Fah could be induced to levels comparable to the primary hepatocyte day 1 controls.

No significant signals for secreted Albumin and Aat were detected after an maturation-induction period of 7 days (Fig. 4). Only marginal ureagenic ability (Fig. 5) and glycogen storage ability (data not shown) could be measured upon induction. The findings indicate limitations of conventional differentiation protocols towards hepatocyte maturity *in vitro*, in particular in comparison with a primary hepatocyte standard.

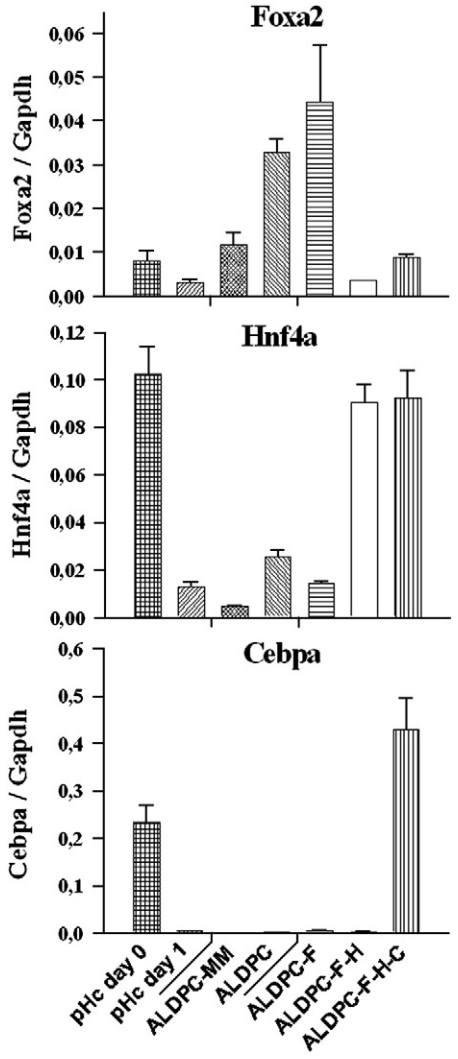

Figure 2 Transcription factor mRNA levels. RT-qPCR analysis at day 7 of the respective treatment. Expression levels of Foxa2 (upper panel), Hnf4α (middle), C/ebpα (lower). Bars are grouped into 1) primary hepatocyte controls: pHc day 0, primary murine hepatocytes freshly isolated without cultivation; pHC day 1, primary hepatocytes in culture 24 hours after isolation; 2) untransduced ALDPC: ALDPC-MM, ALDPC in maintenance medium; ALDPC, ALDPC in liver differentiation medium (LD); 3) transcription factor transduced ALDPC-F, ALDPC-F-H and ALDPC-F-H-C: ALDPC in LD medium, transduced with -F:Foxa2, -H:Hnf4α, -C:C/ebpα. The data show mRNA levels normalized to Gapdh as the internal standard. Data are expressed as mean of three independent experiments ± standard deviation (SD).

Hepatic terminal differentiation of ALDPC by ectopic expression of transcription factors

The concept of maturation induction by sequential expression of defined transcription factors was tested in Foxa2 transgenic ALDPCs by lentiviral transduction of Hnf4α and C/ebpα, using the described vectors and LD medium. At the end of the differentiation protocol (day 7 after transduction), flow cytometry analysis indicated 87% eGFP positive, 60% dTomato positive and 34% double positive cells (mean of a set of 3 independent transduction experiments). The morphology of double and triple transduced cells most closely resembled adult mouse hepatocytes (Fig. 6). ALDPCs in LD medium and Foxa2 over-expressing ALDPCs maintained the proliferative phenotype, consisting of small mononucleated cells. Double and triple transduction induced a major morphological change, which was evident from day three post-transduction. Cells increased significantly in size with an obvious effect on the nucleus-to-cytoplasm ratio. The cytoplasm appeared more complex, some cells became binucleated, resembling primary adult hepatocytes (Fig. 6C and D). Morphological changes were accompanied by a strong proliferation arrest induced by high levels of transduced Hnf4α, as indicated by microscopy and EdU nucleotide incorporation (data not shown) and transduced C/ebpα (albeit difficult to verify because of its above mentioned cytotoxicity in ALDPC-F-C, data not shown). The findings confirm previous reports on proliferation-inhibitory effects of Hnf4α [23–25] and C/ebpα [26,27].

The combinatorial actions of the three transcription factors induced major morphological effects also observed in ALDPC (increase in size, binucleation and growth arrest) in the fibroblast cell line NIH3T3 (Supplementary Fig. 3, A-D).

Liver specific gene expression analysis

All of the 15 genes representing a wide range of hepatocyte functions were significantly induced in at least one of the transcription factor combinations used (Fig. 3). Expression levels similar to hepatocyte controls were obtained for Alb in Foxa2 transduced cells, but lower in double or triple transduced cells. This is consistent with the fact that Foxa2 is a transcriptional activator of Alb and with our observation that Foxa2 levels decreased when cells are cotransduced with Hnf4α (Fig. 2). Aat, Tdo2, Cldn1, Cps1, ApoA4 achieved highest expression levels - comparable to hepatocytes - in the triple transduced group. In the LETF transduction group, expression of G6p, Fah, Pxr, ApoA1 was higher than in primary hepatocytes cultured for 24 hours, approaching levels of freshly isolated hepatocytes (data not shown). Also, Tat, Gys2, Cyp2a5 and Cyp3a11 showed highest expression levels in the triple transduced group - however, reduced compared to the primary hepatocyte day 1 standard. Apart from Apoa1 and Apoa4, expression levels of freshly isolated hepatocytes on day 0 could not be reached by any of the experimental settings.

Transcription factor-transduced NIH-3 T3 cells, exhibiting pronounced changes towards a hepotocyte-like, epithelial morphology and positivity for PAS-staining (Supplementary Fig. 3), upregulated Tdo2 and G6p to levels in the ranges of ALDPC-F-H-C or primary hepatocytes (Supplementary Fig. 4). Alb and Aat, not measurable in untransduced NIH3T3, became detectable.

iPS-derived hepatocyte-like cells, albeit in some cases with considerable variation between the three independent samples, showed Alb-mRNA levels similar to ALDPCs in maturation conditions (Supplementary Fig. 4). Tdo2, G6p, and Cldn1 levels exceeded expression detected in ALDPC-F-H-C or primary hepatocytes.

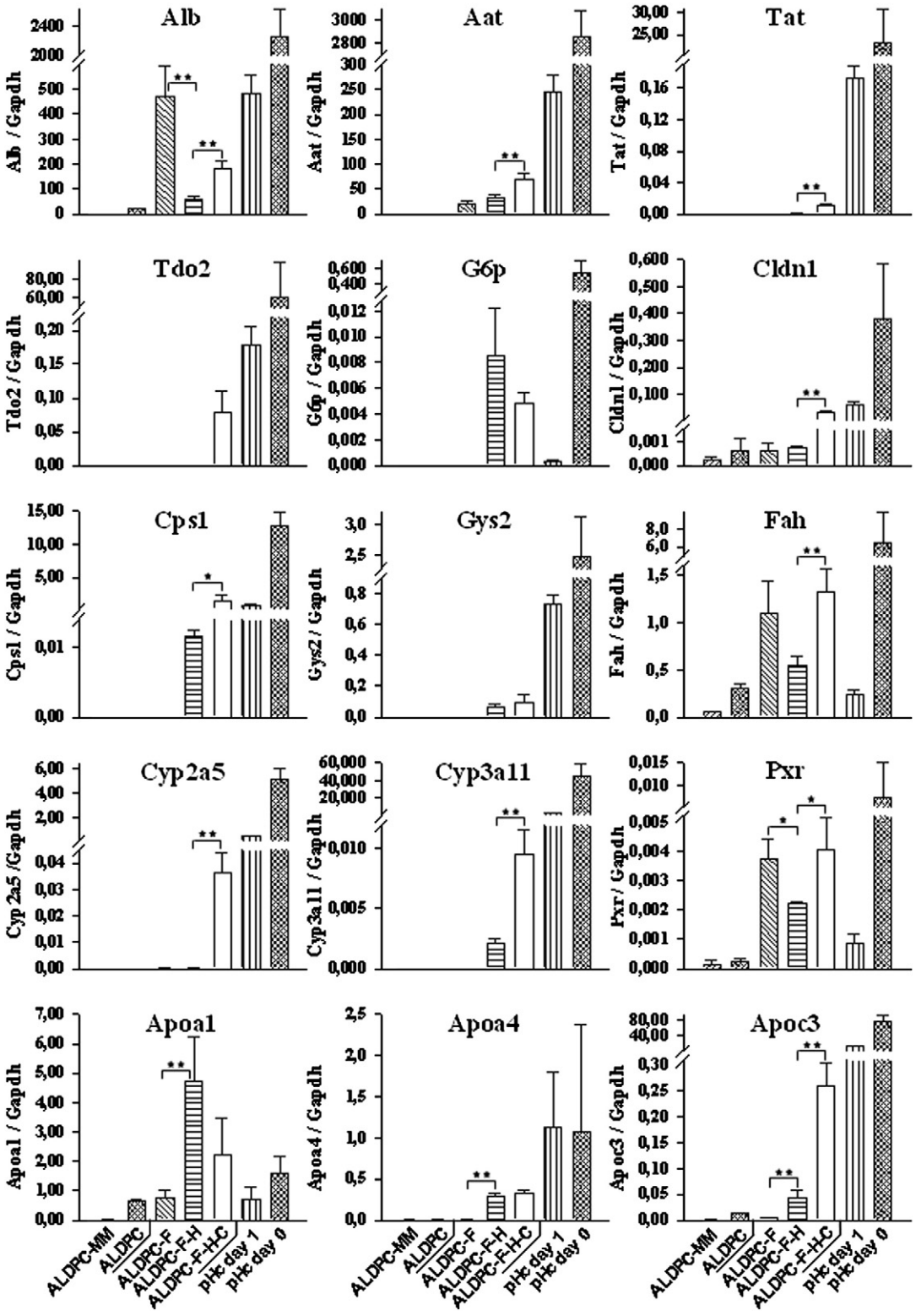

Figure 3 mRNA expression levels of liver genes. RT-qPCR analysis for mRNA levels of the 15 genes indicated at day 7 of the respective treatment. Bars are grouped into 1) untransduced ALDPC: ALDPC-MM, ALDPC in maintenance medium, ALDPC, ALDPC in liver differentiation medium (LD); 2) transcription factor transduced ALDPC-F, ALDPC-F-H and ALDPC-F-H-C: ALDPC in liver differentiation medium (LD), transduced with -F:Foxa2, -H:Hnf4α, -C:C/ebpα; 3) Standard: pHc day 1, primary human hepatocytes in culture (LD medium) 24 hours after isolation and pHc day 0, immediately after isolation. The data show mRNA levels normalized to Gapdh as the internal standard. Data are expressed as mean of three independent experiments ± standard deviation (SD). Statistical analysis: Student Test, *P<0,05, **P<0,01.

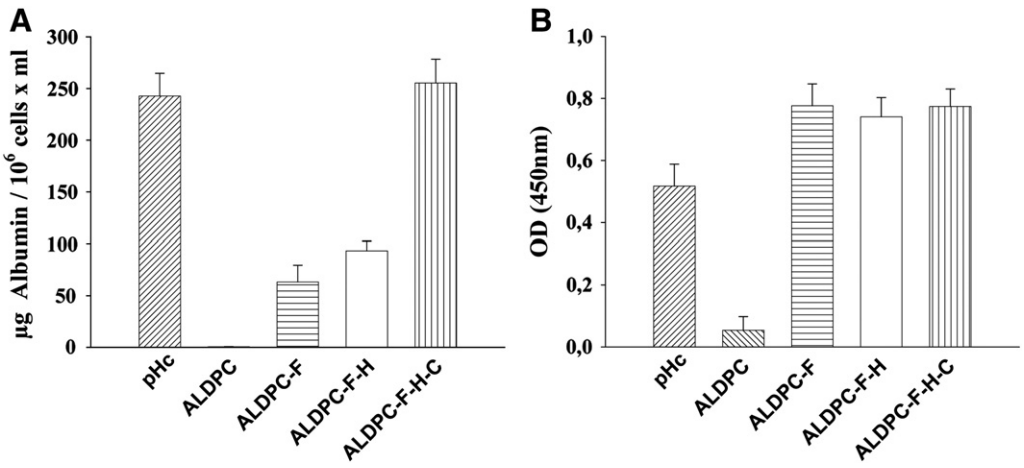

Figure 4 ELISA for protein secretion into the supernatant. A) ELISA for albumin release. B) mouse alpha-1 antritypsin ELISA. The release of $1x10^6$ cells into 1 ml of medium for 24 hours was assayed. The data in A and B show results from three independent experiments (mean value ± SD). pHc: primary hepatocytes in culture 24 hours after isolation; ADLPC, ALDPC-F, ALDPC-F-H and ALDPC-F-H-C: ALDPC in liver differentiation medium (LD), transduced with -F:Foxa2, -H:Hnf4α, -C:C/ebpα.

Protein secretion, ureagenesis and glycogen storage

Compared to baseline, Alb secretion levels increased stepwise in single, double and triple transduced ALDPCs (Fig. 4A). Triple transduced cells secreted albumin at levels comparable to the concentrations measured in supernatants of 24 h cultured primary hepatocytes.

In contrast, Aat protein secretion reached mature hepatocyte levels already following Foxa2 transduction and was not further stimulated in the double or triple transduced cell populations (Fig. 4B).

Ureagenesis was not detectable above background in non-transduced ALDPCs but elevated in transduced cells at day 7 (Fig. 5). Foxa2 transduction of ALDPCs appeared to be sufficient to induce ureagenesis. However, urea production was still considerably lower than in the hepatocyte standard.

Storage of glycogen is a characteristic feature of mature hepatocytes. We thus stained primary mouse hepatocytes and ALDPCs with PAS. Non-transduced or Foxa2 transgenic ALDPCs showed inconsistent PAS staining. Double transduced or triple transduced cells presented a bright PAS positive staining, similar to hepatocyte controls, concordant with findings of Gys2 mRNA upregulation for those settings. Particularly, cells closely resembling adult hepatocytes by morphology also presented the brightest PAS positive staining. (Fig. 7). Also, transcription factor-transduced NIH3T3 fibroblasts stained positive for PAS (Supplementary Fig. 4, F).

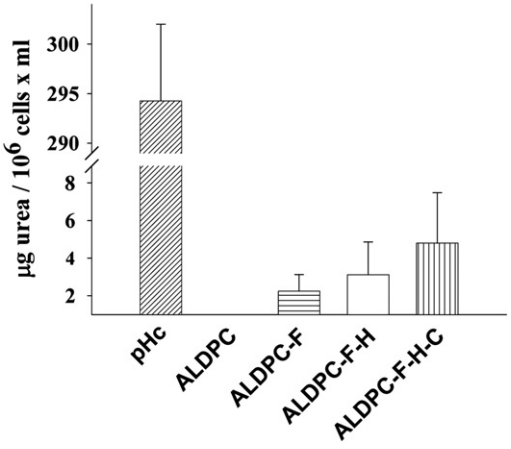

Figure 5 Analysis of urea production. Urea release of $1x10^6$ cells into 1 ml of supernatant over 24 hours. The data show results from three independent experiments (mean value ± SD). pHc: primary hepatocytes in culture 24 hours after isolation; ALDPC, ALDPC-F, ALDPC-F-H and ALDPC-F-H-C: ALDPC in liver differentiation medium (LD), transduced with -F:Foxa2, -H:Hnf4α, -C:C/ebpα.

Discussion

Our data show that terminal hepatic differentiation *in vitro* can be improved by sequential over-expression of the three transcriptional regulators Foxa2, Hnf4α and C/ebpα. Epithelial morphology, hepatic gene expression, secretory function and metabolic activity were reproducibly induced in an expandable adult progenitor cell population after a period of seven days in culture. Protein secretion rates, mRNA expression levels of the 15 representative genes, as well as glycogen production were closing up or even similar to values reached by 24 hours cultured primary mouse hepatocytes. However, RNA expression levels of freshly isolated hepatocytes (day 0) were still superior.

The cells used in our studies appear reliably over time in primary hepatocyte cultures in the presence of growth factor supplemented medium and were first described as BAML cells [6]. Whether they already pre-exist as stem/progenitor cells in the liver or derive from other cell types in the presence of growth factors in culture, is currently not known. Terminal differentiation of those cells under adherent culture conditions *in vitro*, using standard maturation conditions (a variety of conditions from previous reports were tested

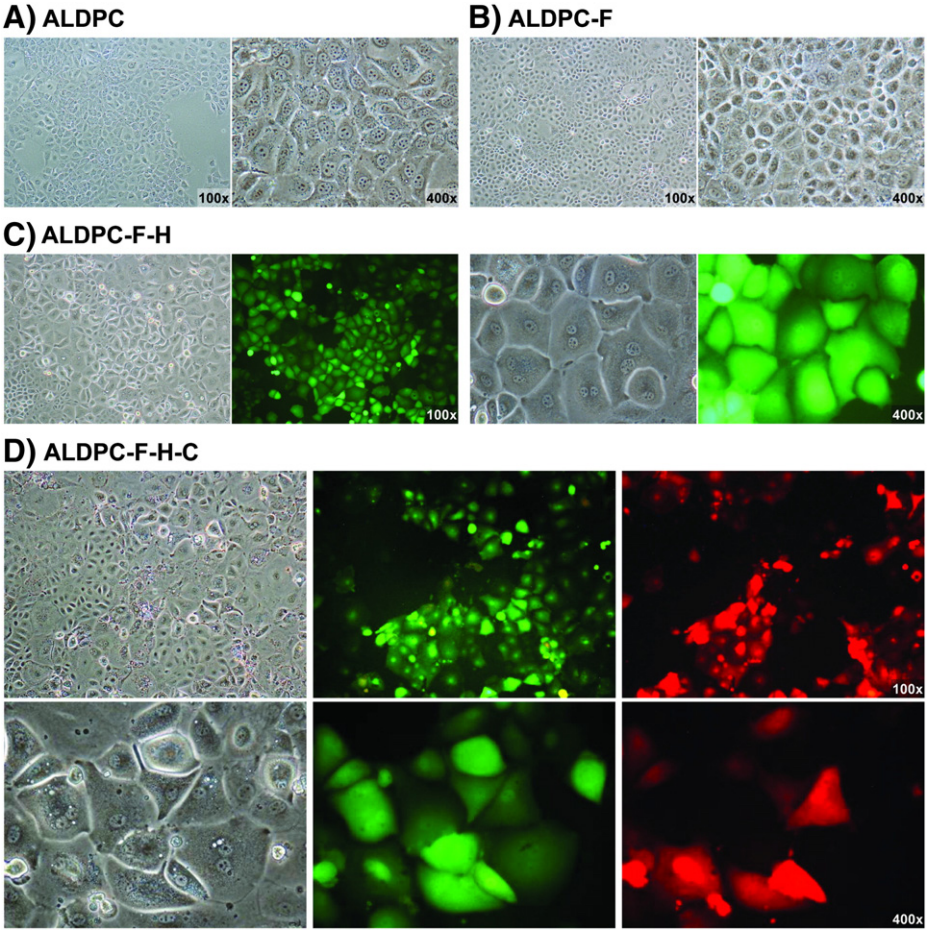

Figure 6 Morphology of untransduced and transduced ALDPC. A) untransduced (MM medium). B) Foxa2 transduced ALDPC (LD medium, day 7). C) Foxa2 transgenic ALDPCs transduced with the lentivirus Hnf4α-IRES-eGFP (LD medium, day 7). D) Foxa2 transgenic ALDPCs co-transduced with the lentiviruses Hnf4α-IRES-eGFP and C/ebpα-IRES-dTomato (LD medium, day 7).

beforehand) was limited, resulting in induction of hepatic gene expression and hepatocyte functions but not reaching primary hepatocyte ranges.

For further improvement of hepatic terminal differentiation, we followed a strategy of ectopic sequential expression of a combination of transcription factors. First, we

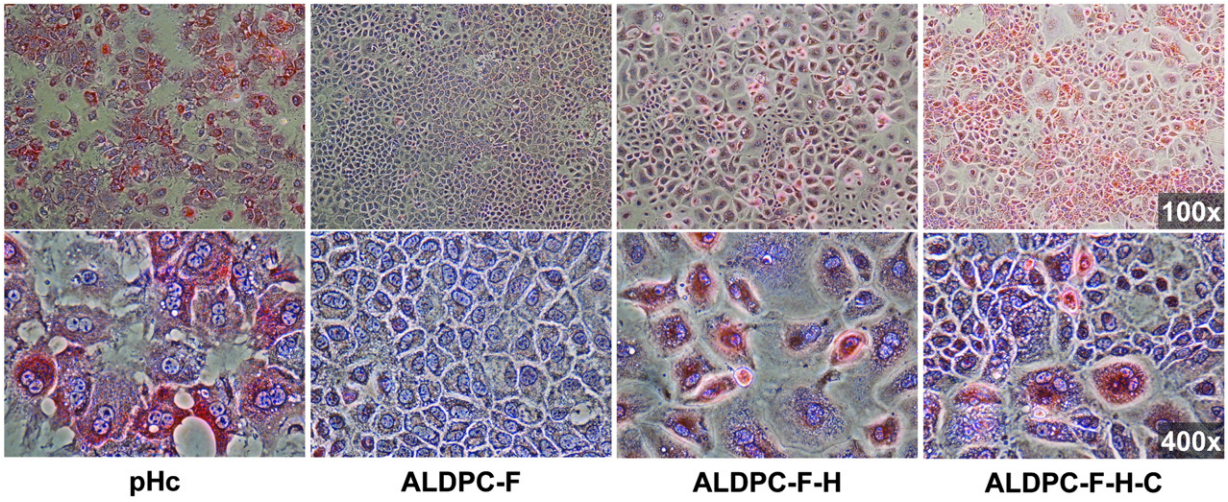

Figure 7 PAS-staining for glycogen storage. pHc: primary hepatocytes in culture 24 hours after isolation; ALDPC, ALDPC-F, ALDPC-F-H and ALDPC-F-H-C: ALDPC in liver differentiation medium (LD), transduced with -F:Foxa2, -H:Hnf4α, -C:C/ebpα.

generated an ALDP cell line, which uniformly expressed Foxa2. Foxa transcription factors are required for normal development of endoderm derived organs such as liver and pancreas [28]. Recent studies identified that Foxa proteins act as "competence factors" and facilitate binding and transcriptional activity of other transcription factors in endoderm cells and tissues [28]. Moreover, it has been shown that Foxa2 is required for normal liver homeostasis also in the adult liver, as >43% of genes expressed in the liver were associated with Foxa2 binding [29]. Standard liver differentiation conditions were sufficient to maximally induce secretion of Aat in Foxa2 transgenic cells. In contrast, we have observed an incremental increase in albumin secretion in single, double and triple transduced cells, whereas albumin-mRNA levels already peaked in the Foxa2-only setting, indicating that maturation of specific secretory functions requires elevated levels of Hnf4α and C/ebpα, too.

Ishii et al. recently demonstrated enhanced expression of Albumin, Afp, Tat and epithelial cell adhesion molecule (Epcam) genes in bone marrow derived mesenchymal stromal cells over-expressing only Foxa2. The differentiated cells also showed hepatocyte-specific functions including glycogen production and urea secretion, compared to HUH-7 or HepG2 controls, but no comparison was made with adult primary human hepatocytes [21].

In a second step, Foxa2 transgenic ALDPC were co-transduced with lentiviral vectors expressing either Hnf4α or C/ebpα. The hepatocyte nuclear factor 4α is known as a key regulator of both hepatocyte differentiation during embryonic development and maintenance of a differentiated phenotype in the adult liver [30,31]. Recent studies using hepatocyte-specific Hnf4α-knockout mice have shown that Hnf4α is essential for the generation of hepatic epithelium [31]. The CCAAT/enhancer-binding protein alpha (C/ebpα) maintains the differentiated state of hepatocytes and directs transcription of many genes expressed in the liver such as albumin or ornithine cycle enzymes involved in urea production. Conditional knockdown of C/ebpα revealed an important role in hepatic glucose, nitrogen, bile acid and iron metabolism - all of which represent highly differentiated hepatocyte functions [28]. Cultures of ALDPC transduced with Foxa2, Hnf4α and C/ebpα adopted an epithelial morphology and contained more cells with two or more nuclei, which is a hallmark of adult hepatocytes (Figs. 6 and 7) and indicative of inhibition of full cell division.

The expression of liver specific genes in triple transduced ALDPCs approached in many cases levels in the range of the primary hepatocyte day 1 standard (Fig. 3). However, some genes like CYP450 enzymes and Apoc3, although maximally induced in triple transduced cells, have proven to be difficult to up-regulate to levels found in primary hepatocytes. Expression of additional transcription factors, the application of histone deacetylase inhibitors, which have been shown to improve hepatic differentiation of ESCs, and in particular three dimensional culture systems allowing the formation of organ-like epithelial structures, might be required in order to maximally induce the expression of those genes.

Although the triple transduced cells exhibit gene expression and protein secretion levels comparable with a mature hepatic phenotype, more complex metabolic functions of hepatocytes such as ureagenesis were still sub-optimal. Expression of carbamoylphosphate synthase-1 (Cps1), a rate-limiting enzyme in urea synthesis, was reported to be strongly decreased in the absence of C/ebpα [32,33]. In triple transduced ALDPCs Cps1 was highly induced, but over-expression of C/ebpα appeared not sufficient to upregulate ureagenesis in the range of primary hepatocytes (Fig. 5).

With our triple transduction protocol, partial induction of a liver phenotype across lineage borders could be observed in NIH3T3 cells, as seen by morphological changes towards an epithelial phenotype (Supplementary Fig. 3) and partial induction of mRNA expression for liver specific genes (Supplementary Fig. 4). The finding, that the three transcription factors led to some changes towards a hepatocyte-like phenotype even in NIH3T3, also indicates their importance for hepatocyte development and terminal differentiation.

iPS-derived hepatocyte-like cells reached either intermediate gene expression levels or in the range of (or even exceeding) primary hepatocytes (Supplementary Fig. 4). It remains to be seen whether transcription factor transduction will improve terminal maturation of iPS-derived hepatocyte-like cells.

The current protocol utilizing constitutive expression of the transcription factors is not compatible with the application of differentiated ALDPCs for cell therapy. Both Hnf4α and C/ebpα inhibit proliferation [23–27] and thus would interfere with organ repopulation *in vivo*. Indeed, a major phenotypic change from a proliferative phenotype to a differentiated hepatocyte-like phenotype was only noted when the transgenic Foxa2 ALDPCs have been further co-transduced with growth inhibitory Hnf4α and C/ebpα. In future studies, using human adult liver progenitor cells and human transcription factors - building upon the studies presented here, vectors with inducible transcription factor expression will be tested. Proliferative Foxa2-transgenic human liver progenitors, already carrying inducible Hnf4α and C/ebpα transgenes might be useful for *in vitro* drug- and drug-toxicity testing as well as for the colonization and subsequent inducible operation of bioartificial liver support devices.

In conclusion, we have shown that sequential ectopic expression of Foxa2, Hnf4α and C/ebpα induces a hepatocyte phenotype of significantly advanced maturity in an expandable liver derived progenitor cell line. Our rapid differentiation protocol might provide the means to generate reliable cell sources for *in vitro* basic cell research, drug testing studies and bioartificial liver support devices in the future.

Materials and Methods

Study design

The murine liver enriched transcription factors Foxa2, Hnf4α and C/ebpα were cloned into lentiviral vectors and expressed in liver derived progenitor cells using a two step protocol. First, a Foxa2 transgenic ALDPC cell line was generated under puromycine selection. Terminal hepatic differentiation was induced in a 7 day protocol by co-transducing the Foxa2 transgenic ALDPCs with Hnf4α and C/ebpα. Hepatic differentiation of transduced and non-

transduced cells was assesed by mRNA expression quantification and standard functional assays and compared to primary hepatocytes 24 hours in culture. In addition, maturation induction in triple transduced ALDPCs was also compared to iPS-derived hepatocyte-like cells and triple transduced NIH-3 T3 cells.

Lentivirus vectors

The sequences of the murine transcription factors were amplified from a commercial full ORF (Open Reading Frame) Shuttle Clone (Foxa2, C/ebpα from ImaGenes, Berlin, Germany) and an adult mouse liver cDNA sample (Hnf4α), and inserted into the BamHI cloning site of a 3 rd generation lentiviral expression plasmid containing a SFFV promoter driven IRES-Puro (Foxa2, vector plasmid pL-S-Foxa2-I-puro), IRES-dTomato (C/ebpα, pL-S-Cebpa-I-dTom) or IRES-eGFP (Hnf4α, pL-S-Hnf4α-I-egfp) expression cassette, respectively (Supplementary Fig. 1). The correctness of inserts and cloning procedures was verified by restriction enzyme digestion and sequencing (SeqLab, Göttingen, Germany) before virus production. Lentiviruses carrying the transcription factor cDNA´s and the respective "empty" controls - encoding solely for the marker genes puro, eGFP, dTomato - were produced by transient four plasmid transfection of HEK293T cells and enriched as described earlier [34]. Virus titers were calculated from transduction of NIH-3 T3 cells (DSMZ, Braunschweig, Germany) using increments of the enriched culture supernatants.

Transcription factor reporter assays

In order to verify functionality of the ectopically expressed transcription factors, transfection assays were performed using the Dual Luciferase reporter assay system according to the manufacturers protocol (Promega). For evaluation of Foxa2 functional activity, the plasmids pTCF4-CMVpro-GL4.20 (luciferase expression under control of a promoter with a negative Foxa2 element) and pL-S-Foxa2-I-puro were used based on a previous report [21]. Using this system it has been shown that overexpression of Foxa2 downregulates Wnt/β-catenin signalling, resulting in a decreased Firefly/Renilla luminescence ratio [21]. For estimation of the Hnf4α and C/ebpα transcriptional activities, a luciferase reporter plasmid (pGL3-basic) containing a 530 bp sequence from the Ugt1a9 promoter [22] was used in combination with the plasmids pL-S-Hnf4a-I-egfp or pL-S-C/ebpa-I-dTom. It has been shown that Hnf4α directly activates the promoter of Ugt1a9 [35]) and that C/ebpα is an essential transcriptional upregulator of UDP glucuronosyltransferase expression in the liver [36].

For the dual luciferase reporter assay, $5x10^4$ HEK293T cells were transfected at 70% confluency in 12-well plates with 1,5 μg lipofectamine (Invitrogen) complexed DNA/well (1:1 ratio of the lentiviral and the respective luciferase reporter plasmid). The plasmids pGL4.74[hRLuc/TK]ΔTCF [21] or pRL-TK (Promega) expressing Renilla luciferase (19,8 ng/well) were co-transfected as internal transfection controls. Control experiments have demonstrated before, that none of the three transcription factors had influence on TK-promoter activity (data not shown). The plasmids pL-S-I-puro, pL-S-I-egfp and pL-S-I-dTom plasmids were used as transcription factor negative controls (Supplementary Fig. 1). All experiments were performed in triplicates. Firefly/Renilla luminescence ratio was analyzed 36 hours after transfection.

Primary hepatocytes and adult liver derived progenitor cells

Adult mouse hepatocytes were isolated by a modified 2-step collagenase perfusion from 2–6 month-old C57BL/6 mice as previously described by Seglen et al. with minor modifications [37]. Fresh hepatocyte RNA samples were taken either immediately for day 0 measurements or after cultivation for 24 hours on collagen I-coated plates in LD medium (described below) for day 1-RT-qPCR.

Adult liver derived progenitor cells (ALDPC) were obtained from cultured adult mouse liver cell suspensions by the "plate and wait" technique, according to published protocols [7]. After separation of distinct colonies by trypsination from other colonies, (see Supplementary Fig. 2A, 30 days) homogeneous cell cultures of ALDPC´s were generated and substantially expanded using Williams' E medium supplemented with 10% FCS (PAA), 50 ng/ml epidermal growth factor (EGF, PeproTech), 30 ng/ml insulin-like growth factor II (IGFII, PeproTech) and 10 ng/ml insulin (Sigma-Aldrich), designated as maintenance medium (MM). For our hepatic differentiation experiments, the ALDPC clone E was used at passage 40. At this passage the cells were actively growing, requiring 1:5 splitting every 3 days. No morphological and changes of growth and gene expression properties could be observed up to at least passage 60.

Hepatic maturation was induced in adherent clone E cells over a period of 7 days. Cells were seeded at a density of 1.5×10^5 cells/well in rat tail collagen I-coated 6 well plates (Roche) using liver differentiation medium (LD), consisting of HBM (Lonza) supplemented with the "standard single quots" of insulin, hydrocortisone, transferrin, ascorbic acid, antibiotics according to the manufacturers instructions (Lonza, HCM single quots) and mouse hepatocyte growth factor (HGF, 20 ng/ml, R&D Systems).

Transcription factor transduction

In a first transduction step, Foxa2 transgenic ALDPC cells were generated by transducing passage no. 40 expanded ALDPCs with the Foxa2 lentivirus at an MOI (multiplicity of infection) of approximately 0.3. Transduction was followed by puromycin selection (2 μg/ml). After selection, cells were kept in MM containing 1 μg/ml puromycin (see 4.4). For experimental use, Foxa2-transduced ALDPC were treated the same as ALDPC for maturation induction, described in 4.4 without further puromycine supplementation.

In a second transduction step, 1.5×10^5 Foxa2 transgenic ALDPC's were seeded on rat tail collagen I-coated (Roche) 6 well plates, cultured in LD medium and co-transduced with either L-S-Hnf4a-I-egfp and L-S-Cebpa-I-dTom or with L-S-I-egfp and L-S-I-dTom control lentiviruses. An MOI of 1,5 was used for transduction. Single transductions with L-S-Hnf4a-I-egfp or L-S-Cebpa-I-dTom were also included. Subsequent

analyses were consistently performed at day 7 post maturation induction and lentiviral transduction.

RNA extraction and quantitative real time PCR

Total RNA from freshly isolated mouse hepatocytes, 24 hours cultured mouse hepatocytes, ALDPCs, was extracted using the RNeasy Mini kit (Qiagen) and subjected to reverse transcription using iScript cDNA synthesis kit (Biorad). The messenger RNA (mRNA) expression levels were determined by quantitative RT-PCR using a LightCycler 480 System (Roche) and gene specific primers (Supplementary Table 1). Absolute quantification analyses were performed using the Second Derivative Maximum Method. PCR reactions were run for 45 cyles, however, ct-values above 35 were regarded as unsafe and not used for graphical depiction, according to the MIQE guidelines [38]. For standardization of the RT-qPCR analysis, the PCR fragment of each gene was cloned into the pCR4-Topo vector (Invitrogen) and sequenced. Standard curves for all template-primer pair combinations were generated by diluting equal starting concentrations of the plasmids in a pooled sample. The mRNA-values were calculated from the standard curves and normalized to Gapdh mRNA expression.

Enzyme linked immunosorbent assay

The amount of albumin secretion was quantified by sandwich enzyme linked immunosorbent assay using a mouse albumin ELISA quantitation kit according to the manufacturers protocol (Bethyl Laboratories, TX, USA).

The mouse alpha-1 antitrypsin (mAat) ELISA was performed using cell culture supernatants in 96 well plates (EIA/RIA 96well, Costar) using a monoclonal antibody against murine Aat (Abcam). After incubation with a secondary HRP labelled antibody (Abcam) the substrate TMB/H_2O_2 was added. The colour development was stopped with 1 N sulfuric acid after 30 min. followed by photometric analysis at 450 nm. Protein secretion studies and mRNA expression studies were performed simultaneously using the same cell samples.

Analysis of urea production

Ureagenic ability of differentiated cells was assessed using the QuantiChromTM Urea Assay kit (BioAssay Systems, CA, USA). ALDPC or hepatocyte cultures were incubated for 24 hours with 5 mM ammonium chloride and the amount of urea secreted into the culture medium was measured according to the manufacturer's protocol.

PAS staining

The glycogen storage capacity of the differentiated ALDPCs and control hepatocytes was assessed by PAS staining. Six-well adherent cell cultures were fixed with 95% ethanol for 10 minutes, treated with periodic acid, stained with Schiff's reagent and finally counterstained with Meyer's Hemalaun solution.

Statistical analysis

All values given represent the mean values of at least three independent experiments and the corresponding standard deviation (SD). Data between study groups were compared using Student's *T*-Test. A p-value$<$0.05 was considered statistically significant.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.scr.2011.02.002.

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