THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

Polymer Supported Directed Differentiation Reveals a Unique Gene Signature Which Predicts Stable Hepatocyte Performance

OPEN ACCESS

www.MaterialsViews.com

ADVANCED
HEALTHCARE
MATERIALS
www.advhealthmat.de

COMMUNICATION

Polymer Supported Directed Differentiation Reveals a Unique Gene Signature Predicting Stable Hepatocyte Performance

Baltasar Lucendo Villarin, Kate Cameron, Dagmara Szkolnicka, Hassan Rashidi, Nicola Bates, Susan J. Kimber, Oliver Flint, Stuart J. Forbes, John P. Iredale, Mark Bradley, and David C. Hay*

The ability to generate renewable sources of human soma, from defined genetic backgrounds, has enormous potential for modern medicine, with immediate impacts being the delivery of tailor-made models of human tissue "in a dish," and the delivery of cell-based therapies likely to follow. Theoretically, pluripotent stem cells (PSCs) can give rise to all somatic cell types found in the human body,[1] with their self-renewal and differentiation properties offering the prospect of generating unlimited quantities of human cells for biomedical application. Our particular interest lies in the delivery of human hepatocyte like cells (HLCs) derived from PSCs. Numerous hepatocyte differentiation protocols have been established using 2D and 3D cell culture,[2] and encouragingly, the stem cell–derived HLCs produced exhibit many typical hepatocyte characteristics.[3–15] While these attributes are promising, the HLCs produced are immature in status, reminiscent of fetal or neo-natal hepatocytes.[16] One major obstacle to stem cell–derived HLC maturation has been cellular instability in culture. Notably, the use of undefined or xenobiotic culture components has contributed to variable cell performance, making it difficult to unravel the complexity behind hepatocyte differentiation and dedifferentiation.

Therefore, the development of highly defined cell based systems are required if the true potential of stem cell–derived HLCs is to be realized. Such systems should be simple to use, inexpensive, scalable, defined, and capable of delivering a product with predictable performance and long "shelf life." There have been a number of approaches developed, including the use of differential cytokine or chemical combinations, 3D cell aggregation, and perfused devices, to mimic the liver niche and architecture.[17–20] While these approaches marked significant progress, their complexity and/or undefined nature has limited large-scale deployment of the technology. In order to tackle this issue, we hereby report on the use of a defined biopolymer substrate in conjunction with a serum-free differentiation procedure with both research and good manufacturing practice (GMP) grade PSCs. Importantly, those stem-derived populations displayed robust and predictable performance in cell culture which was hallmarked by gene expression of matrix metalloproteinase 13 (MMP13), delta catenin (CTNND2), and thrombospondin 2 (THBS2).

H9 human embryonic stem cells (hESCs), displaying an appropriate morphology and expressing stem cell markers (Oct-4 and Nanog, SSEA-4, TRA-1–60, and TRA-1–81) were expanded and used for the differentiation experiments. Importantly, SSEA-1 expression was minimal in these populations (Figure S1A–D, Supporting Information). Additionally, hESCs were able to spontaneously differentiate to all the three germ layers and directly differentiate to HLCs (Figure S1E, Supporting Information and **Figure 1**). Following validation, hESC populations were directly differentiated to hepatoblasts using a defined procedure.[20] Stem cell–derived hepatoblasts were removed from their substrate and replated, under serum-free conditions, onto a chemically defined polyurethane surface (PU). At 10, 15, and 20 d postreplating, hepatocyte differentiation status was studied by immunostaining for a marker of hepatocyte differentiation, HNF4α, and dedifferentiation, vimentin. Stem cell–derived hepatocytes expressed stable levels of HNF4α on the PU for up to 20 d in culture (Figure 1A). The converse was true for Matrigel (MG) cultures which began to deteriorate from day 15 onward, and by day 20 approximately fourfold fewer cells expressed HNF4α (22% ± 1.8) (Figure 1B). The loss of the hepatocyte phenotype was also corroborated by staining for a marker of hepatocellular dedifferentiation. Stem cell–derived HLCs replated on PU expressed low levels of vimentin, ranging from 23% to 35% (Figure 1A). In contrast, stem cell–derived hepatocytes on MG demonstrated greater dedifferentiation, with staining ranging from 54% to 78% (Figure 1B). These results were further supported by immunostaining studies

B. L. Villarin, Dr. K. Cameron, D. Szkolnicka,
Dr. H. Rashidi, Dr. O. Flint,
Prof. S. J. Forbes, Dr. D. C. Hay
MRC Centre for Regenerative Medicine
University of Edinburgh
5 Little France Drive, Edinburgh EH16 4UU, UK
E-mail: davehay@talktalk.net
N. Bates, Prof. S. J. Kimber
Faculty of Life Sciences
Michael Smith Building
Oxford Road, Manchester M13 9PT, UK
Prof. J. P. Iredale
MRC Centre for Inflammation Research
University of Edinburgh
47 Little France Crescent, Edinburgh EH16 4TJ, UK
Prof. M. Bradley
School of Chemistry
University of Edinburgh
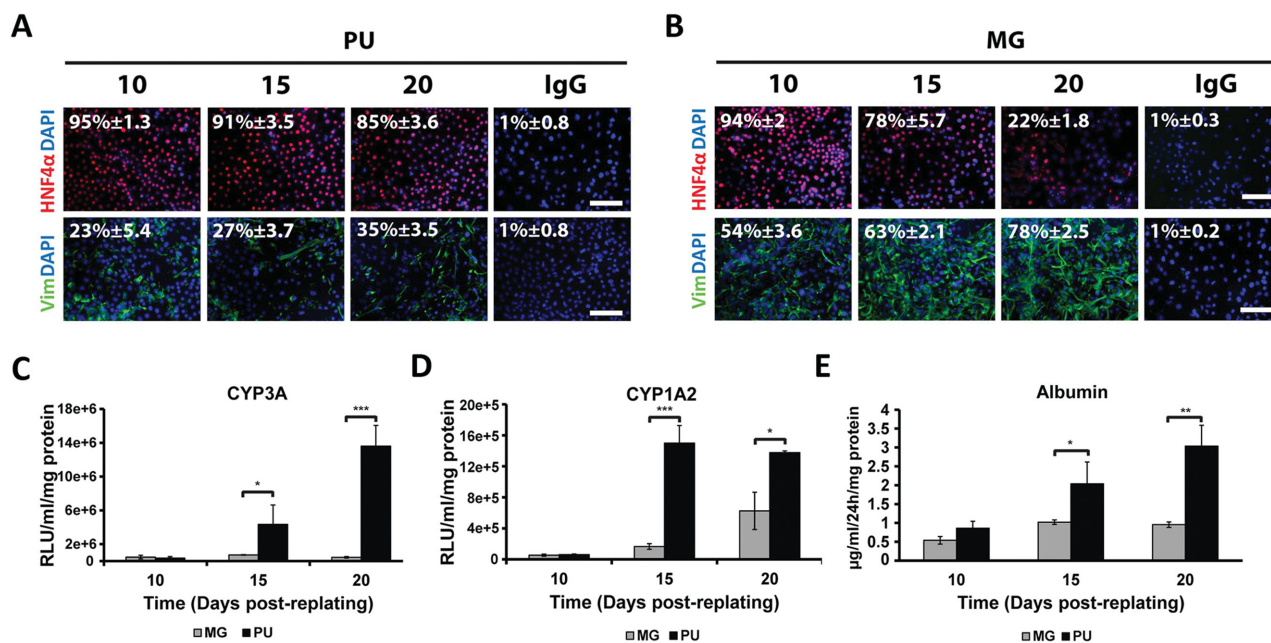West Mains Road, Edinburgh EH9 3FJ, UK

COMMUNICATION

Figure 1. Serum-free hepatocyte differentiation of hESCs on a defined polyurethane surface (PU), and a biological matrix Matrigel (MG). A,B) Expression of hepatocyte nuclear factor 4α, (HNF4α - red), and the dedifferentiation marker vimentin (green) in hESC-derived hepatocytes maintained on PU or MG at different time points during the differentiation procedure. Cell nuclei were counterstained with DAPI (blue). IgG controls demonstrate the specificity of immunostaining. The yield percentage of positive cells was calculated from five random fields of view containing at least 400 cells each. Data are expressed as mean ± SD. Images are taken at 20× magnification. Scale bar represents 100 μm. C,D) HLC cytochrome P450 3A and 1A2 (CYP3A and CYP1A2) activity maintained on PU or MG during differentiation. Units of activity are expressed as relative light units (RLU) mL^{-1} mg^{-1} of protein. E) Albumin production was detected by ELISA and expressed as micrograms of albumin mL^{-1} 24 h^{-1} mg^{-1}. Data represent mean ± SD ($n = 3$).*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Images were taken at 20× and the scale bar represents 100 μm.

examining human albumin and cytochrome P450 3A (CYP3A) expression (Figure S2, Supporting Information). hESC-derived HLCs differentiated on PU generated cell populations that were 86% (±4%) and 88% (±1.7%) positive for albumin and CYP3A, respectively, 20 d postreplating. This was an approximate twofold improvement compared with MG cultures, with 43% (±9.5%) and 44% (±8.8%) cells positive for albumin and CYP3A, respectively. The immunostaining studies were further supported by functional analysis. Analysis of cytochrome P450 activity revealed a 30-fold increase in CYP3A activity and a ninefold increase in the CYP1A2 activity in cells cultured on PU (Figure 1C,D). The ability to secrete albumin was also improved threefold in HLCs differentiated on PU (Figure 1E). The improved differentiation status of the stem cell–derived HLCs generated under defined conditions was further supported by their response to compounds which measure hepatocyte metabolic capacity (Figure S3, Supporting Information). Stem cell–derived HLCs differentiated on PU displayed consistent performance in response to two compounds, BMS-827278-01 and BMS-835981-01, which require metabolic activation, via CYP2D6, to toxic endpoints. This was in contrast to HLCs differentiated using MG surfaces which exhibited less consistent performance, presumably due to deficiencies in the CYP2D6 metabolic pathway (Figure S3, Supporting Information).

Given the superior performance of HLCs under defined conditions, we were keen to identify genes important in this process. We therefore employed PCR array technology to screen for candidate genes. The RT^2 Profiler PCR array

profiled the expression of 84 genes important for cell–cell and cell–matrix interactions. Stem cell–derived HLCs differentiated on MG and PU (20 d postreplating) were compared and from these studies, we identified increased expression of six genes: ADAMTS13, NCAM1, CTNND2, THBS2, MMP10, and MMP13, on PU (**Figure 2**A,B and Table S4, Supporting Information). The output of the array was validated by real time quantitative PCR (qPCR). One of the candidate genes, ADAMTS13, was not amplified to the same extent and was therefore was excluded from further studies (Figure 2C). The remaining genes were found to be enriched three to sixfold in HLCs differentiated on PU. In order to understand the role each gene played in hepatocyte function, we employed small interfering RNA (siRNA) technology to knockdown the target gene products. HLCs were transfected twice over a 48 h period during differentiation (days 13–15). Posttransfection, gene knockdown was assessed by qPCR. Gene knockdown was dependent on the siRNA used. NCAM1 and CTNND2 levels decreased by ≈25%, MMP10 levels fell by ≈40%, MMP13 decreased by ≈60%, while THBS2 levels fell by ≈75% (**Figure 3**A) after transfection. In response to gene knockdown, albumin secretion fell by ≈30% and ≈50% in cells transfected with siRNAs to CTNND2 and MMP13, respectively (Figure 3B). Whereas, CYP3A function was reduced by ≈25% and ≈30% in response to the knockdown of THBS2 and MMP13, respectively (Figure 3C). These studies demonstrated that THBS2, MMP13, and CTNND2 are important in stabilizing HLC phenotype under defined conditions.
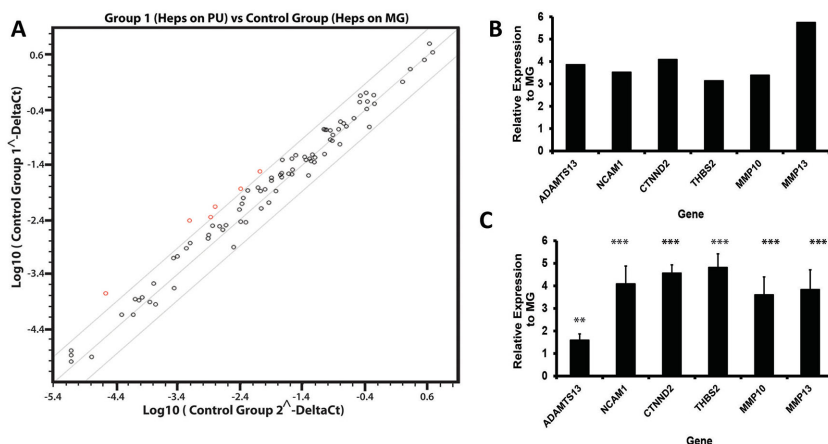
Figure 2. Differential gene expression of HLCs maintained on PU or MG. A) The scatter plot represents expression of the major ECM components and matrix associated receptors expressed in human cells. The scatter plot represents threefold change in gene expression. Symbols outside the boundary areas indicate fold-differences greater or lesser than threefold. B) Graph representing the induced genes in hESC-derived HLCs maintained on PU. C) qPCR was employed to validateon the upregulated genes identified on the array. Data represent mean ± SD ($n = 3$). **$P < 0.01$, ***$P < 0.001$ versus Matrigel.

The manufacture of stem cell–derived somatic cells is likely to play an important part in developing cell based therapies of the future. To be able to deliver clinical grade materials, one must use clinical grade components. In this vein, we employed two hESC lines which were derived under GMP conditions, Man 11 and Man 12. Both Man lines were characterized in detail, displaying typical hESC morphology and expressing Oct-4 and Nanog. hESC surface maker expression was also detected by flow cytometry. Man 11 and Man 12 hESCs expressed SSEA-4, TRA-1–60, and TRA-1–81, but not SSEA-1 (Figure S4A–C, Supporting Information). Additionally, both hESC lines were able to spontaneously form three germ layers and directly differentiate to HLCs (Figures S4D and S5, Supporting Information). Encouragingly, Man 11 and Man 12 differentiation was highly efficient, with 87% and 95% of the Man 11 derived HLCs expressing HNF4α and albumin, respectively. The pattern of expression was similar for Man 12 derived HLCs, with 84% and 99% of derived hepatocytes expressing HNF4α and albumin, respectively (Figure S5, Supporting Information). Following basic characterization, hESCs were differentiated toward the hepatocyte lineage. Cytochrome P450 activity, albumin and alpha-fetoprotein (AFP) production was measurable in HLCs maintained on PU for at least 15 d postreplating (Figure 3D,F,G and Figure S6A, Supporting Information). At this point stem cell–derived HLCs expressed greater levels of MMP13, CTNND2, and THBS2 than their MG counterparts (Figure 3E and Figure S6B, Supporting Information). There were differences recorded in the gene expression between Man 11 and Man 12 HLCs. CTNND2 expression was marginally increased and MMP 13 expression was reduced in Man 11 HLCs in comparison to Man 12 HLCs (Figure 3E and Figure S6B, Supporting Information). THBS2 was expressed at similar levels in both Man 11 and Man 12 HLCs. In addition to changes in gene expression, we also observed differences in the stability of Man 11 and Man 12 HLCs. Man 12 HLCs, like their H9 counterparts, were stable for up to 20 d on the PU surface.

At this point albumin production was stable, while the production of AFP decreased approximately fivefold over the same 5 d time frame. In these experiments, Man 11 derived HLCs were only stable up to 15 d in culture, and therefore could not be characterized past this point (Figure S6A, Supporting Information).

The ability to differentiate and maintain human somatic cells in culture has revolutionary potential. However, the delivery of reliable and stable human models is a major barrier limiting current endeavors. The development of defined differentiation systems, free of xenobiotic materials or undefined additives, is required to achieve this objective. We developed an interdisciplinary approach, blending polymer chemistry, and defined biological differentiation systems. The focus of our studies was to identify novel genetic signatures associated with stable hepatocyte differentiation from both research and GMP grade hESC lines (Figure 1 and Figure S3, Supporting Information). In a focused survey of human cell membrane components and receptors, using a PCR array, we identified five genes which were enhanced in cells differentiated in a defined culture environment. Those genes, which appear to be new markers of hepatocyte differentiation, could be broadly divided in two categories; extracellular matrix (ECM) remodelers (MMP10 and MMP13) or cell–cell and cell–matrix interactors (THBS2, CTNND2, and NCAM1) (Figure 2). The MMPs are a family of proteins known to display substrate specificity for particular ECMs. Their activity is regulated by post-translational modifications and by the tissue inhibitor of matrix metalloproteinases (TIMPs).[21] MMPs are essential for normal remodeling of the extracellular matrix, tissue morphogenesis, and wound healing.[21] Our analysis indicated that both MMP13 and 10 gene expression was increased over cultures differentiated in an undefined manner. It is possible that differences in gene expression may reflect the capacity of the cell to remodel the ECM which is known to be important in the maintenance of hepatocyte phenotype.[22–24] In addition to its role in collagen remodeling, we identified MMP13 as a potential regulator of cytochrome P450 metabolism and human albumin secretion (Figure 3). Our analyses also demonstrated increased expression of genes which code for proteins involved in cell–cell and cell–matrix interactions. THBS2, CTNND2, and NCAM1 were expressed at greater levels in cell populations differentiated under defined conditions. THBS2 and CTNND2 are known to regulate cell adhesion, organization, and migration, in response to growth factor stimulation.[25–27] Along similar lines, NCAM1 has been identified as important in cell motility, liver development and liver regeneration.[28] In addition to their proposed roles in cell attachment, organization, and migration, we report our novel findings: a reduction in THBS2 expression led to reduced cytochrome P450 function, while reduced CTNND2 led to reduced albumin secretion (Figure 3B,C). Our further experiments focused on the functional gene signature MMP13, THBS2, and CTNND2. In these studies, we employed
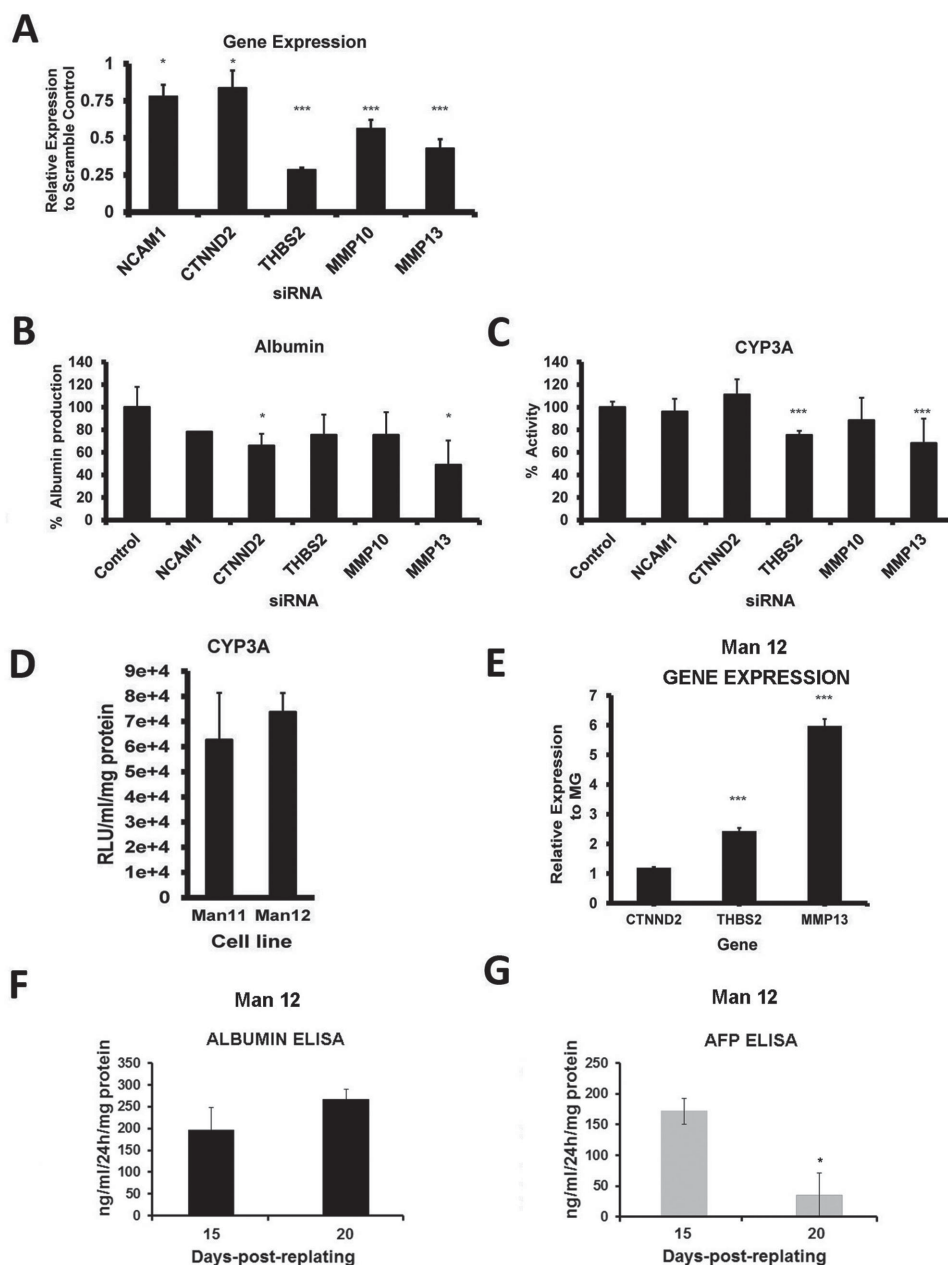
Figure 3. Role of the candidate genes in HLCs function. A) Graph representing the decrease of candidate genes in HLCs post-siRNA transfection. B) HLC albumin production, post-siRNA transfection, was detected by ELISA. Data represent the mean ± SD ($n = 3$). *$P < 0.05$ versus scrambled control. C) HLC CYP3A activity, post-siRNA transfection, was determined using the pGlo system. Units of activity are expressed as relative light units (RLU) mL^{-1} mg^{-1} of protein. Data represent the mean ± SD ($n = 3$). ***$P < 0.001$ versus scrambled control. D) Man 11 and Man 12 derived HLCs maintained on PU display CYP3A activity at day 15 postreplating. Units of activity are expressed as RLU mL^{-1} mg^{-1} of protein. Data represent the mean ± SD ($n = 3$). E) Candidate genes upregulated in Man 12 HLCs on PU at day 15 postreplating. Data represent the mean ± SD ($n = 3$). ***$P \leq 0.001$ versus Matrigel. F,G) Man 12 derived HLCs maintained on PU secrete albumin and AFP at days 15 and 20 postreplating. Units of activity are expressed as nanograms of albumin or AFP mL^{-1} 24 h mg^{-1}. Data represent mean ± SD ($n = 3$). *$P < 0.05$ versus day 15 HLCs.

two hESC lines derived to GMP and subsequently maintained serum free. Notably, defined and directed differentiation of both GMP lines yielded cell populations which expressed increased levels of MMP13, THBS2, and CTNND2 and thereby supporting data derived from the research grade hESC line. Of note the Man 12 derived HLCs performed best in culture, demonstrating stable albumin production and a fivefold reduction

in AFP secretion over the same 5 d period. These results imply that Man 12 HLCs were maturing in culture as HLC phenotype stabilized.

In conclusion, dependable differentiation from pluripotent stem cells requires defined culture parameters. Such systems will permit informative and mechanistic analysis of human biology. The novel gene signature identified in these studies is

one example, which may also serve as important gold standard parameters for quality control and manufacture of GMP grade products at scale.

Experimental Section

Cell Culture and Differentiation: hESCs H9 (WARF), Man 11 and Man 12 (University of Manchester) were cultured and differentiated to hepatoblasts as reported previously.[20] At day 8 in the differentiation process the cells were cultured in HepatoZYME-SFM media (Life Technologies) supplemented with 10×10^{-6} M hydrocortisone 21-hemisuccinate (Sigma) and 2×10^{-3} M GlutaMAX (Gibco) containing 10 ng mL^{-1} hepatocyte growth factor and 20 ng mL^{-1} oncostatin M (Peprotech) for 24 h cells. At day 9 stem cell–derived hepatoblasts were removed from their substrate using 5 min at 37 °C incubation with TrypLE (Life Technologies). Following this, HLCs were plated onto an optimized PU polymer surface[29] or Matrigel (BD Biosciences, San Jose) coated surfaces in HepatoZYME-SFM supplemented with 5% KnockOut Serum Replacement (Life Technologies), containing 20 ng mL^{-1} hepatocyte growth factor, 40 ng mL^{-1} oncostatin M, and 10 ng mL^{-1} EGF (Peprotech). 48 h postreplating the HepatoZYME-SFM was supplemented with 10 ng mL^{-1} hepatocyte growth factor and 20 ng mL^{-1} oncostatin M. The media was changed every 48 h until the experimental endpoint.

RNA Isolation and qPCR: Total RNA was prepared using the RNAeasy Mini Kit (Qiagen, UK) according to manufacturer's instructions. Total RNA was reverse transcribed using QuantiTect Reverse Transcription Kit (Qiagen), as per manufacturer's instructions (Qiagen). Quantitative PCR was carried out using Taqman Fast Advance Mastermix and appropriate primers (Applied Biosystems). The primers are listed in Table S1 (Supporting Information). The samples were analyzed using Roche LightCycler 480 Real-Time PCR System. Results were normalized to GAPDH and expressed as relative expression over the control sample. Quantitative PCRs were run in triplicate. Data analysis was performed using Roche LightCycler 480 Software (version 1.5).

PCR Array: Reverse transcription (RT) of RNA samples used on the PCR array was performed using RT^2 First Strand Kit, as per manufacturer's instructions (Qiagen). Real-time PCR reactions were performed using RT^2 Profiler PCR array (Qiagen) in a 384-well optical plates. Real-time reactions were conducted on an ABI 7900HT (Applied Biosystems). Results were PCR reactions performed in duplicate for each target gene. The gene expression was analyzed by RT^2 Profiler PCR array Data Analysis version 5.0 (Qiagen) in the form of cycle threshold (Ct) values.

Cell Transfection: hESC-derived HLCs were transfected for two consecutive days (day 13 and day 14) postreplating using the Silencer Select siRNAs (Life Technologies). siRNAs were used at a final concentration of 80×10^{-6} M diluted in Opti-MEM I containing Lipofectamine 2000 (Life Technologies). The siRNA are listed in Table S2 (Supporting Information). Cells were collected at day 15 postreplating for further analysis. Each knockdwon was perfromed in triplicate and scrambled RNA controls were used throughout.

Cytochrome P450 Functional Assay: CYP3A and CYP1A2 activity were measured using the pGlo kit from Promega and the assays were carried out as per the manufacturer's instructions (http://www.promega.com/tbs/tb325/tb325.pdf). CYP activities were expressed as relative light units (RLU), per mg^{-1} of protein per mL^{-1} of tissue culture medium.

Measurement of Secreted Albumin: hESC-derived HLCs were cultured in HepatoZYME-SFM. The supernatants were collected for analysis by ELISA (Alpha Diagnostic International) and the data was expressed as micrograms (μg) or nanograms (ng) of albumin or AFP per mL^{-1}, per 24 hours, per mg^{-1} of protein.

Immunofluorescence: Cell cultures at different points during cellular differentiation were fixed in 100% methanol (Sigma-Aldrich) at −20 °C for 30 min. Postfixation, cell monolayers were washed three times with PBS for 5 min at room temperature. After blocking with PBS/0.1%Tween/10% BSA for 1 h, the cells were incubated with primary antibodies diluted in PBS/0.1%Tween/1% BSA overnight at 4 °C. Following this the cells were washed three times with PBS/0.1%Tween/1% BSA and then incubated with the appropriate secondary antibody diluted in PBS/0.1%Tween/1% BSA for 1 h at room temperature. Following this the cells were washed twice with PBS/0.1%Tween/1% BSA and once with PBS. Fixed and stained monolayers were then stained with DAPI (Life Technologies) for 20 min at room temperature. Following this cultures were mounted with PermaFluor aqueous mounting medium (Thermo Scientific). The cells were analyzed using a Zeiss Axio Observed Z1 microscope and processing Zeiss Axiovison Rel 4.8 software. The percentage of positive cells and standard deviation was estimated from at least five random fields of view with a least 400 cells per field of view. The primary and secondary antibodies are listed in Table S3 (Supporting Information).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

[1] J. L. Sterneckert, P. Reinhardt, H. R. Schöler, *Nat. Rev. Genet.* **2014**, *15*, 625.

[2] D. Szkolnicka, W. Zhou, B. Lucendo-Villarin, D. C. Hay, *Annu. Rev. Pharmacol. Toxicol.* **2013**, *53*, 147.

[3] S. T. Rashid, S. Corbineau, N. Hannan, S. J. Marciniak, E. Miranda, G. Alexander, I. Huang-Doran, J. Griffin, L. Ahrlund-Richter, J. Skepper, R. Semple, A. Weber, D. A. Lomas, L. Vallier, *J. Clin. Invest.* **2010**, *120*, 3127.

[4] J. Cai, Y. Zhao, Y. Liu, F. Ye, Z. Song, H. Qin, S. Meng, Y. Chen, R. Zhou, X. Song, Y. Guo, M. Ding, H. Deng, *Hepatology* **2007**, *45*, 1229.

[5] D. C. Hay, J. Fletcher, C. Payne, J. D. Terrace, R. C. J. Gallagher, J. Snoeys, J. R. Black, D. Wojtacha, K. Samuel, Z. Hannoun, *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 12301.

[6] P. Roelandt, S. Obeid, J. Paeshuyse, J. Vanhove, A. Van Lommel, Y. Nahmias, F. Nevens, J. Neyts, C. M. Verfaillie, *J. Hepatol.* **2012**, *57*, 246.

ADVANCED
HEALTHCARE
MATERIALS
www.advhealthmat.de

Materials
Views
www.MaterialsViews.com

COMMUNICATION

[7] R. E. Schwartz, K. Trehan, L. Andrus, T. P. Sheahan, A. Ploss, S. A. Duncan, C. M. Rice, S. N. Bhatia, *Proc. Natl. Acad. Sci. U.S.A.* **2012**, *109*, 2544.

[8] A. Shlomai, R. E. Schwartz, V. Ramanan, A. Bhatta, Y. P. de Jong, S. N. Bhatia, C. M. Rice, *Proc. Natl. Acad. Sci. U.S.A.* **2014**, *111*, 12193.

[9] X. Wu, J. M. Robotham, E. Lee, S. Dalton, N. M. Kneteman, D. M. Gilbert, H. Tang, *PLoS Pathog.* **2012**, *8*, e1002617.

[10] X. Zhou, P. Sun, B. Lucendo-Villarin, A. G. N. Angus, D. Szkolnicka, K. Cameron, S. L. Farnworth, A. H. Patel, D. C. Hay, *Stem Cell Rep.* **2014**, *3*, 204.

[11] S. Ng, R. E. Schwartz, S. March, A. Galstian, N. Gural, J. Shan, M. Prabhu, M. M. Mota, S. N. Bhatia, *Stem Cell Rep.* **2015**, *4*, 348.

[12] C. N. Medine, B. Lucendo-Villarin, C. Storck, F. Wang, D. Szkolnicka, F. Khan, S. Pernagallo, J. R. Black, H. M. Marriage, J. A. Ross, M. Bradley, J. P. Iredale, O. Flint, D. C. Hay, *Stem Cells Transl. Med.* **2013**, *2*, 505.

[13] K. Takayama, Y. Morisaki, S. Kuno, Y. Nagamoto, K. Harada, N. Furukawa, M. Ohtaka, K. Nishimura, K. Imagawa, F. Sakurai, M. Tachibana, R. Sumazaki, E. Noguchi, M. Nakanishi, K. Hirata, K. Kawabata, H. Mizuguchi, *Proc. Natl. Acad. Sci. U.S.A.* **2014**, *111*, 16772.

[14] D. Szkolnicka, S. L. Farnworth, B. Lucendo-Villarin, C. Storck, W. Zhou, J. P. Iredale, O. Flint, D. C. Hay, *Stem Cells Transl. Med.* **2014**, *3*, 141.

[15] G. Holmgren, A.-K. Sjögren, I. Barragan, A. Sabirsh, P. Sartipy, J. Synnergren, P. Björquist, M. Ingelman-Sundberg, T. B. Andersson, J. Edsbagge, *Drug Metab. Dispos.* **2014**, *42*, 1401.

[16] D. Bader, A. Riskin, O. Vafsi, A. Tamir, B. Peskin, N. Israel, R. Merksamer, H. Dar, M. David, *Clin. Chim. Acta* **2004**, *349*, 15.

[17] K. Takayama, K. Kawabata, Y. Nagamoto, K. Kishimoto, K. Tashiro, F. Sakurai, M. Tachibana, K. Kanda, T. Hayakawa, M. K. Furue, H. Mizuguchi, *Biomaterials* **2013**, *34*, 1781.

[18] L. Sivertsson, J. Synnergren, J. Jensen, P. Björquist, M. Ingelman-Sundberg, *Stem Cells Dev.* **2013**, *22*, 581.

[19] M. Vosough, E. Omidinia, M. Kadivar, M.-A. Shokrgozar, B. Pournasr, N. Aghdami, H. Baharvand, *Stem Cells Dev.* **2013**, *22*, 2693.

[20] D. C. Hay, S. Pernagallo, J. J. Diaz-Mochon, C. N. Medine, S. Greenhough, Z. Hannoun, J. Schrader, J. R. Black, J. Fletcher, D. Dalgetty, A. I. Thompson, P. N. Newsome, S. J. Forbes, J. A. Ross, M. Bradley, J. P. Iredale, *Stem Cell Res.* **2011**, *6*, 92.

[21] J. P. Iredale, *J. Clin. Invest.* **2007**, *117*, 539.

[22] D. Muller, B. Quantin, M. C. Gesnel, R. Millon-Collard, J. Abecassis, R. Breathnach, *Biochem. J.* **1988**, *253*, 187.

[23] J. M. Freije, I. Díez-Itza, M. Balbín, L. M. Sánchez, R. Blasco, J. Tolivia, C. López-Otín, *J. Biol. Chem.* **1994**, *269*, 16766.

[24] O. Garcia-Irigoyen, S. Carotti, M. U. Latasa, I. Uriarte, M. G. Fernández-Barrena, M. Elizalde, R. Urtasun, U. Vespasiani-Gentilucci, S. Morini, J. M. Banales, W. C. Parks, J. A. Rodriguez, J. Orbe, J. Prieto, J. A. Páramo, C. Berasain, M. A. Ávila, *Liver Int.* **2014**, *34*, e257.

[25] A. Liu, P. Garg, S. Yang, P. Gong, M. A. Pallero, D. S. Annis, Y. Liu, A. Passaniti, D. Mann, D. F. Mosher, J. E. Murphy-Ullrich, S. E. Goldblum, *J. Biol. Chem.* **2009**, *284*, 6389.

[26] C. Ozaki, M. Yoshioka, S. Tominaga, Y. Osaka, S. Obata, S. T. Suzuki, *Cell Struct. Funct.* **2010**, *35*, 81.

[27] T. Ezaki, R.-J. Guo, H. Li, A. B. Reynolds, J. P. Lynch, *Am. J. Physiol.: Gastrointest. Liver Physiol.* **2007**, *293*, G54.

[28] A. Tsuchiya, W.-Y. Lu, B. Weinhold, L. Boulter, B. M. Stutchfield, M. J. Williams, R. V. Guest, S. E. Minnis-Lyons, A. C. MacKinnon, D. Schwarzer, T. Ichida, Y. Aoyagi, R. Gerardy-Schahn, S. J. Forbes, *Hepatology* **2014**, *60*, 1727.

[29] B. Lucendo-Villarin, K. Cameron, D. Szkolnicka, P. Travers, F. Khan, J. Walton, J. P. Iredale, M. Bradley, D. C. Hay, *J. Visualized Exp.* **2014**, *91*, 51723.