Journal of Cellular Physiology

Differences in definitive endoderm induction approaches using growth factors and small molecules

Journal:	*Journal of Cellular Physiology*
Manuscript ID	Draft
Wiley - Manuscript type:	Original Research Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Bogacheva , Mariia; University of Helsinki, Faculty of Pharmacy Khan, Sofia; National Institute of Advanced Industrial Science and Technology Kanninen, Liisa; University of Helsinki, Faculty of Pharmacy Yliperttula, Marjo ; University of Helsinki, Faculty of Pharmacy Leung, Alan; Yale University, Yale Stem Cell Center and Department of Genetics, Yale School of Medicine Lou, Yan-Ru; University of Helsinki, Faculty of Pharmacy
Key Words:	Human pluripotent stem cell, Definitive endoderm, Cell differentiation

Differences in definitive endoderm induction approaches using growth factors and small molecules

Mariia S. Bogacheva [1], Sofia Khan [2], Liisa K. Kanninen [1], Marjo Yliperttula [1], Alan W. Leung [3], Yan-Ru Lou [1,*]

[1] Drug Research Program, Division of Pharmaceutical Biosciences, Faculty of Pharmacy, University of Helsinki, Helsinki, Finland

[2] Computational Bio Big-Data Open Innovation Laboratory (CBBD-OIL), National Institute of Advanced Industrial Science and Technology (AIST), Tokyo, Japan

[3] Yale Stem Cell Center and Department of Genetics, Yale School of Medicine, Yale University, New Haven, Connecticut, United States

[*] Corresponding author: Yan-Ru Lou

Division of Pharmaceutical Biosciences, Faculty of Pharmacy, University of Helsinki, P.O. Box 56 (Viikinkaari 5 E), FI-00014 Helsinki, Finland

E-mail address: yan-ru.lou@helsinki.fi; Tel: +358 2941 59125

ABSTRACT

Definitive endoderm (DE) is the first stage of human pluripotent stem cell (hPSC) differentiation into hepatocyte-like cells. Developing human liver cell models for pharmaceutical applications is highly demanding. Due to the vast number of existing protocols to generate DE cells from hPSCs, we aimed to compare the specificity and efficiency of selected published differentiation conditions. We differentiated two hPSC lines (induced PSC and embryonic stem cell) to DE cells on Matrigel matrix using growth factors (Activin A and Wnt-3a) and small molecules (sodium butyrate and IDE 1) in different combinations. By studying dynamic changes during six days in cell morphology and the expression of markers for pluripotency, DE, and other germ layer lineages, we found that Activin A is essential for DE differentiation, while Wnt-3a and sodium butyrate are dispensable. Although sodium butyrate exerted rapid DE differentiation kinetics, it caused massive cell death and could not generate sufficient cells for further differentiation and applications. We further discover that IDE 1 could not induce DE as reported previously. Hereby, we compared different conditions for DE induction and found an effective six day-protocol to obtain DE cells for the further differentiation and applications.

KEYWORDS

Human pluripotent stem cell; Definitive endoderm; Cell differentiation

1 INTRODUCTION

Differentiation of human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), into hepatocyte-like cells *in vitro* is a promising approach to creating a human cell model for drug toxicity screening. Considering the fact that obtaining mature hepatocytes remains challenging (Schwartz et al., 2014), there is a necessity for detailed study of each step of hPSC differentiation. The purity, maturity, and functionality of hepatocyte-like cells highly depend on the efficacy of every step of the differentiation process. The first defined cell stage of differentiation is the formation of definitive endoderm (DE) characterized by the expression of transcription factors such as SRY-box 17 (SOX17), mix paired-like homeobox (MIXL1), hepatocyte nuclear factor 3β (HNF3B, also known as FOXA2) and receptors such as chemokine receptor type 4 (CXCR4). The DE contributes cell precursors to many organs including the liver, pancreas, thyroid, thymus, digestive tracts, to epithelial lining of the respiratory tracts and lungs. The DE stage defines further cell fate, depending, in part, on extracellular conditions. Controlled formation of DE *in vitro* and further hepatic lineage differentiation holds an enormous potential for therapeutic and pharmaceutical applications.

In vertebrates the Nodal (transforming growth factor β (TGFβ)-related ligand) signaling, mediated by Mixer homeoproteins, is required for the DE development in a dose-dependent manner (Shen, 2007). In this study, we examined the influence of different growth factors and small molecules, including Activin A (AA), Wnt-3a, Sodium Butyrate salt (NaB), IDE 1 and their combinations during the induction of DE differentiation. AA, similar to Nodal, is a member of the TGFβ superfamily which participates in regulation of several biological processes, including cell differentiation and proliferation. During normal embryonic development, AA plays an important role in endoderm/mesoderm formation and patterning in a concentration dependent manner and has been used at high concentration in generation of DE cells from hPSCs (D'Amour et al., 2005). Wnt-3a is a cysteine-rich glycoprotein and a ligand of Wnt-signaling, which participates in primitive

streak cell type formation from ESC (Gadue et al., 2006; Lindsley et al., 2006) and in human hepatic endoderm development (Hay et al., 2008a). NaB is a histone deacetylase inhibitor which has an anti-proliferative and differentiation-inducing activity in various normal cells (Boffa et al., 1978; Kruh, 1982) and has been used in differentiation of ESCs to DE cells (Hay et al., 2008b; Jiang et al., 2007) and early pancreatic progenitors (Goicoa et al., 2006). IDE 1 is a synthetic small cell-permeable molecule that can activate TGFβ signaling. It was shown to promote direct differentiation of ESCs into the endodermal lineage and was more potent than AA or Nodal in promoting DE induction (Borowiak et al., 2009).

In the present study, we identified an efficient and robust method for DE cell differentiation from hESCs and hiPSCs among four most frequently used protocols.

2 RESULTS

2.1 Cell morphological changes during differentiation

We earlier used the two hPSC lines WA07 and iPS(IMR90)-4 in hepatic differentiation studies by using M1 media for six days in generation of DE (Kanninen et al., 2016a; Kanninen et al., 2016b). We had also tested M5, but due to massive cell death we could not obtain enough DE cells for hepatic specification. To find a more effective DE induction method preferably shorter than six days and based on small molecules, we performed DE induction in both cell lines with six different compositions of differentiation media (Table 1). We purchased NaB from three manufactures to find a less toxic one if cell death was caused by chemical impurity. The concentrations of growth factors and small molecules used in the present study were chosen from the literature (Borowiak et al., 2009; D'Amour et al., 2005; Hay et al., 2008b; Tahamtani et al., 2013; Toivonen et al., 2013). Both WA07 and iPS(IMR90)-4 cells displayed typical stem cell morphology during culturing before differentiation procedure (Figures 1 and 2). We monitored cell morphology during differentiation experiments and observed gradual change of cell shape and reorganization of stem

cell colonies to single cells. The speed of morphological changes varied in both cell lines, depending on the differentiation media. Figures 1 and 2 show differences at three time points – days 1, 4, and 6. Treatment of WA07 cells with M1 and M2 led to gradual morphological changes starting after day 1 checkpoint: cells in colonies became loose and some cells acquired mesenchymal-like morphology (Figure 1). By day 6 the cells not only acquired DE morphology, but also grew and proliferated into a monolayer. We noticed that the fastest differentiation started upon the treatment with NaB-containing media; however, those cells had very low survival rate. After 24 hours of NaB treatment, cell number was decreased at least in half, and on day 6 of differentiation an extremely low number of cells survived. Additionally we tested NaB at lower concentrations: a) 0.8 mM for priming stage (first 24 hours), followed with 0.4 mM for induction (days 1-5); b) 0.5 mM for both priming and induction; c) 0.5 mM at priming stage followed with 0.1 mM for induction. We did not observe an improved cell survival with lower concentrations of NaB (data not shown). Among three studied NaB-containing media, NaB from Millipore showed the highest toxicity. After day 4 checkpoint there was the smallest number of attached cells in M3-treated wells that were not enough for the analysis of gene expression profile. Since WA07 cell line showed extremely low survival with this condition, we excluded M3 in the subsequent experiments. M6 media based on IDE 1 most likely did not affect cell morphology because no cells with DE morphology were observed during the differentiation and cells overgrew and their morphology became unclear by the end of the differentiation experiments. To know if IDE 1 at higher concentrations can induce DE differentiation, we tested IDE 1 at 250, 500, and 1000 nM but did not observe any morphological changes. We treated iPS(IMR90)-4 cells with five different media: M1, M2, M4, M5, and M6. Cells exhibited similar morphological changes as seen in WA07 culture with the only distinction that more cells survived after treatments with NaB-containing media (Figure 2).

2.2 Characterization of gene expression profiles in stem cells and their derivatives

To understand how treatments with different media conditions affect the DE differentiation, we studied gene expression profiles of the hPSCs and their derivatives during differentiation process at three time points (same as we observed morphology transformation) by qPCR. We compared gene expression levels of specific markers for pluripotency, DE, mesoderm, and ectoderm with those in undifferentiated stem cells to show the kinetics of up and downregulations. In WA07-derived DE cells, the mRNA expression levels of the pluripotency marker *OCT4* (encoding octamer-binding protein 4) significantly decreased by day 6 of differentiation in cells treated with all conditions comparing with undifferentiated cells (Figure 3a). The cells in M4 did not survive for 6 days. The loss of *OCT4* indicates the differentiation induced by soluble factors. The expression level of the second pluripotency marker we measured, *NANOG* (encoding Nanog homeobox transcription factor), did not significantly decrease in most cases except in the cells in M6 beginning from day 4 (Figure 3b). Comparing with undifferentiated cells, the mRNA expression level of DE marker *CER1* (Cerberus 1) increased significantly in most cases from day 4 except the cells in M6 (Figure 3c). M1 and M5 caused significant increase in *CER1* mRNA expression from day 4 to day 6. Comparing with undifferentiated cells, the expression level of *SOX17* increased in the cells in M2 from day 1 and in the others from day 4, but it did not change significantly in M6 (Figure 3d). Comparing with undifferentiated cells, *CXCR4* increased in the cells in M1, M2, M4, and M5 from day 4 but not in M6 (Figure 3e). M1 and M5 treatments induced further increase in *CXCR4* expression from day 4 to day 6, whereas M2 treatment caused slight decrease in *CXCR4* as well as in *SOX17*. Comparing with undifferentiated cells, *HNF3B* gene expression was significantly induced in the cells in M2 from day 1 and cells in M4 and M5 from day 4 of differentiation (Figure 3f). M1 also increased *HNF3B* gene expression to the similar level by day 6 as M2, but the increase was not statistically significant. M5 treatment significantly upregulated *HNF3B* expression from day 4 to day 6. *HNF4A* expression increased mostly on day 4 and day 6 in comparison with day 0, except for the cells in M6 which did not cause significant change (Figure 3g). M1 and M5

treatments further upregulated *HNF4A* expression from day 4 to day 6. Expression of hepatocyte nuclear factor 6 (*HNF6*) was unaltered in most conditions except in M6 on day 4 (Figure 3h). The level of *BRACHYURY* mRNA expression peaked on day 1 of differentiation in all the conditions except M6, afterwards it decreased to the level similar to that in stem cells (Figure 3i). The expression of ectoderm marker SRY-box 1 (*SOX1*) increased in the cells in M4 and M5 from day 4 (Figure 1j).

In iPS(IMR90)-4 cells *OCT4* mRNA expression was slightly upregulated in M1, M4, and M5 on day 1 and then downregulated in all the conditions from day 4 in comparison with day 0 (Figure 4a). *NANOG* gene expression was downregulated in M2 and M6 but was upregulated in other conditions (Figure 4b). *CER1* mRNA expression was upregulated during the differentiation in all the conditions except M6 (Figure 4c). Gene expression level of *SOX17* became significantly higher from day 1 of differentiation in M2 and from day 4 in M1, M4, and M5 (Figure 4d). *CXCR4* (Figure 4e), *HNF3B* (Figure 4f), and *HNF4A* (Figure 4g) mRNA expression significantly increased from day 4 in M1, M2, M4, and M5. *HNF6* mRNA expression level slightly increased from day 1 in the cells in M4 and M5 and from day 4 in M1, but it decreased in M6 (Figure 4h). *BRACHYURY* mRNA expression was first upregulated on day 1 in M1, M2, M4, and M5 and then decreased by day 4 (Figure 4i). *SOX1* gene expression slightly increased in M5 at all the time points, in M6 on day 1, and in M1 and M4 on day 4 (Figure 4j). In addition, we found significant increase in *CER1*, *CXCR4*, *HNF3B*, and *HNF4A* gene expression from day 4 to day 6 in conditions M1, M2, M4, and M5. However, *SOX17* expression was slightly decreased from day 4 to day 6 in M1 and M5.

2.3 Analysis of hierarchical cluster plots

By using hierarchical cluster analysis, we grouped medium conditions in clusters according to their influence on gene expression (Figure 5). Hierarchical analyses for both cell lines showed similarities between conditions M1 and M2 and between conditions M4 and M5. Condition M6 had dissimilarities with both groups.

2.4 Characterization of cell markers at the protein level

After gene expression profile analyses, we concluded that conditions M1 and M2 led to significant increase of DE gene markers by day 6 and provided satisfactory cell survivals. We analyzed protein expression of pluripotency and DE markers by immunofluorescence staining and further confocal microscope and fluorescent microscope imaging (Figures 6 and 7). The majority of DE cells from both cell lines lost OCT4 after 6-day treatment with M1 and M2 media conditions (Figures 6a and 7a). Fluorescent signals for NANOG in both cell lines after differentiation in M1 and M2 decreased, but did not disappear completely (Figures 6b and 7b) that is in agreement with qPCR results (Figures 3b and 4b). Similar to the upregulation of *SOX17* mRNA expression (Figures 3d and 4d), SOX17 protein expression was also increased in the cells in both conditions M1 and M2 as indicated by bright immunofluorescence (Figure 6 and 7). On the other hand, HNF3B, which significantly increased at the mRNA level in both cell lines in M2 and in iPS(IMR90)-4-derived cells in M1 (Figures 3f and 4f), showed weak positive immunofluorescence (Figures 6 and 7). The expression of HNF4A in the iPS(IMR90)-4-derived cells after treatment with M1 was weaker than that in the cells in M2. In WA07-derived cells the brightness of signals was similar for both treatment conditions. Alpha fetoprotein (AFP) is a specific hepatic marker and is absent from DE. In our experiments all the differentiated cells were AFP-negative.

3 DISCUSSION

This study presents efficient DE cell formation from hPSCs *in vitro* using soluble factors in serum-free condition. Main properties of hiPSCs and hESCs are indefinite proliferation *in vitro* and the ability to differentiate into a large number of cell types *in vitro* and *in vivo*. These features are maintained by the expression of pluripotent transcription factors, including OCT4 (Nichols et al., 1998) and NANOG (Chambers et al., 2003; Mitsui et al., 2003). Downregulation of OCT4 was accompanied by the loss of pluripotency properties and gain of specialization during the

differentiation experiments with current protocols. In most cases we did not observe changes in *NANOG* mRNA expression, but we observed decrease in its protein expression. Considering the fact of *NANOG* expression activation by TGFβ/Activin signaling (Xu et al., 2008), its stable mRNA expression during experiments does not indicate differentiation failure. An earlier study reported that each pluripotency marker had specific kinetics of downregulation during differentiation into three germ layers, OCT4 being lost by day 9 of differentiation whereas NANOG was still detectable on day 9 (Ramirez et al., 2011).

During gastrulation, DE and mesoderm emerge from a common precursor known as mesendoderm in the primitive streak via an epithelial-to-mesenchymal transition. In our studies, we observed morphological change of hPSCs towards mesenchymal-like cells. Bipotential BRACHYURY positive cells can give rise to DE and mesoderm cells (Kubo et al., 2004). BRACHYURY is also expressed in mesoderm and controls the organization of mesoderm (Wilkinson et al., 1990). We confirmed the transient expression of BRACHYURY on day 1 indicating the emergence of mesendoderm population, which is in concordance with previous research by Hay and co-authors, showing that in the first priming stage of differentiation, cells passed through mesendoderm to DE (Hay et al., 2008b). By day 6 of differentiation, the expression of BRACHYURY in most cases returned to a similar level to that in stem cells.

For identification of DE formation efficacy, we checked gene expression dynamics of four markers: *CER1, SOX17, CXCR4* and *HNF3B*. *CER1* is a DE marker, whose role is to inhibit NODAL signaling (Katoh and Katoh, 2006). SOX17 is an important protein for the development of DE (Kanai-Azuma et al., 2002) and for further foregut differentiation (Spence et al., 2009). *CXCR4* is expressed in large variety of cell types including DE (McGrath et al., 1999). HNF3B (FOXA2) is a transcription factor expressed by the primitive streak and continuously expressed by DE progenitors (Ang et al., 1993). It is involved in the development of multiple endoderm-derived organ systems such as the liver, pancreas, and lungs. In this study, we found that NaB-containing

conditions more effectively upregulated *SOX17*, *CXCR4*, and *HNF3B* mRNA expression than the other conditions. The difference is clearly observed in WA07-derived cells. Successful DE formation in conditions M1, M2, M4, and M5 was evidenced by the simultaneous upregulation of several DE markers. Six-day DE induction in conditions M1, M2, M4, and M5 is more efficient than four day-induction in terms of gene expression upregulation of DE markers. Our results show that IDE 1 did not lead to DE induction, which is in agreement with an earlier report showing no increase in the expression of SOX17, FOXA2, and CXCR4 (Tahamtani et al., 2013). Given the fact that pluripotency markers were downregulated, IDE 1 might have activated other lineage pathway(s). Downregulation of *HNF6* and the absence of DE markers after IDE 1 treatment additionally support our hypothesis of non-specific lineage commitment. Further investigation is required to identify the exact lineage(s). Parallel downregulation of pluripotency marker and simultaneous upregulation of DE markers enable us to conclude the effectiveness of M1, M2, M4, and M5 for DE formation. Effective DE differentiation includes morphological modification of cells, increased expression of DE gene markers, and decreased pluripotency gene expression. Two conditions, M1 and M2, demonstrate similar effects on both hESC and hiPSC differentiation to DE.

DE gives rise to multiple organs during embryo development. For our research interest, it is important to obtain hPSC-derived DE cells that are suitable for further hepatic specialization. HNF4A is a liver-enriched transcription factor that controls the expression of many hepatic genes (Bolotin et al., 2010). Its upregulation in AA-containing conditions confirms the potential of the cells to differentiate to hepatic lineage. We determined the expression of *HNF6*, which is involved in hepatogenesis (Si-Tayeb et al., 2010). The upregulation of *HNF6* expression in iPS(IMR90)-4-derived cells in M1, M4, and M5 may indicate hepatic commitment but needs to be investigated further.

To analyze whether current protocol may lead to ectoderm formation we measured the expression of *SOX1* in stem cells and derivatives. *SOX1* is an ectoderm marker, whose protein

expression emerges from 4-6 days of embryoid body formation (Pekkanen-Mattila et al., 2010). A slight increase in *SOX1* expression was seen in both cell lines treated with NaB-containing media and also in iPS(IMR90)-4 cells treated with AA or IDE 1.

Effective formation of DE cells includes morphological change of cells, increased expression of DE markers, and decreased expression of pluripotency genes. Six-day induction protocols are more effective in generation of DE cells than four-day protocols. Two conditions, M1 and M2, exhibited similar effects on both hESC and hiPSC differentiation. The AA alone condition might serve to exclude unwanted WNT-induced neural crest formation (Leung et al., 2016) and at the same time appears to be more cost effective. High cell viability and proliferation activity under conditions M1 and M2 for both cell lines allow for the generation of enough DE cells for further differentiation. Two compositions of AA and NaB from two different companies appeared to be similarly effective. Due to high cell mortality, which we observed in our experiments, we do not consider NaB as a suitable component for obtaining sufficient number of DE cells for downstream analyses and applications.

4 MATERIALS AND METHODS

4.1 Cell lines

The hESC line WA07 (RRID:CVCL_9772) and hiPSC line iPS(IMR90)-4 (RRID:CVCL_C437) were purchased from WiCell research institute Inc (Madison, USA) and cultured on Matrigel (BD Biosciences) with daily changes of mTeSR™1 medium (STEMCELL™ Technologies). Subculture was performed every 4-5 days using Versene 1:5000 (Invitrogen, 15040033) for cell detachment.

4.2 Stem cells differentiation to DE

Two days after passaging, DE induction was performed for six days. RPMI-1640 medium (Gibco, 31870-025), supplemented with 1x GlutaMAX™ (Gibco, 35050-038) and 1x B-27 (Gibco, 17504-044) was a base differentiation medium (DM). Six different mixtures of growth factors and/or small

molecules in DM were used (Table 1). Condition 1 (M1) contained 100 ng/ml AA (PeproTech, 120-14E) in DM. Condition 2 (M2) contained 100 ng/ml AA and 75 ng/ml Wnt-3a (R&D Systems, 5036-WN-010) in DM. Condition 3 (M3) was a mixture of 100 ng/ml AA and 1 mM sodium butyrate (NaB, Millipore, 19-137) in the first day, followed with 100 ng/ml AA and 0.5 mM NaB for the following five days. Condition 4 (M4) contained 100 ng/ml AA and 1 mM sodium butyrate (NaB, Abcam, UK) in the first day, followed with 100 ng/ml AA and 0.5 mM NaB for the following five days. Condition 5 (M5) contained 100 ng/ml AA and 1 mM sodium butyrate (NaB, Sigma Aldrich, B5887) in the first day, followed with 100 ng/ml AA and 0.5 mM NaB for the following five days. Condition 6 (M6) represents 100 nM IDE 1 (Tocris, 4015) in DM. Differentiation media were renewed daily. Differentiation experiments were performed three times and analyzed on differentiation days 0, 1, 4, and 6.

4.3 RNA isolation and cDNA conversion

At each check point day, cells were lysed using an RLT-buffer (Qiagen). Total RNAs were extracted using an RNeasy Mini kit (Qiagen, 74104) according to the instructions of the manufacturer. The RNA to cDNA conversion was performed using a High Capacity RNA-to-cDNA kit (Applied Biosystems, 4387406).

4.4 Quantitative PCR (qPCR) and hierarchical clustering analyses

qPCR reactions of the obtained cDNA samples were carried out on a StepOnePlus Real-Time PCR System (Applied Biosystems) using a Fast SYBR Green Master Mix (Applied Biosystems, 4385612) or TaqMan Universal Master Mix II (Applied Biosystems, 4440038). Ribosomal protein, large, P0 (*RPLP0*) was used as a housekeeping gene. All the used primers and TaqMan Gene Expression Assay mixes are listed in Table 2. All primers were designed by Primer Express v2.0 software (Applied Biosystems) (Kanninen et al., 2016a), except the primers for *OCT4* (Yu et al., 2007) and *HNF3B* (D'Amour et al., 2005), and they were synthesized by Oligomer Oy (Helsinki, Finland). The relative quantification of each target gene in comparison with the housekeeping gene

was made by a standard curve method based on a published mathematical model (Pfaffl, 2001). The relative gene expression was calculated with reference to the undifferentiated hPSCs on day 0.

In order to investigate the gene expression dynamics under different differentiations conditions, we performed a hierarchical clustering of the conditions using a complete agglomeration method with Euclidean distance measure using the heatmap.2 function in R package "gplots".

4.5 Immunofluorescent staining

Cell culturing for immunostaining was performed in either 8-well Lab-Tek® Chamber Slide™ systems (Nunc, 177445) or black 96-well µ-plates (ibidi, 89626). The duration of differentiation experiments was six days. The cells were fixed with 4% paraformaldehyde for 10 minutes and then permeabilized with either 0.1% Triton X-100 or 0.5% Saponin for 10 minutes, followed with blocking by 10% normal goat or donkey serum (Millipore) for one hour. Then the cells were stained with the primary antibodies (Table 3). Negative controls included omission of the primary antibodies and staining with nonimmunized normal rabbit IgG (Peprotech 500-P00), goat IgG (Santa Cruz Biotechnology sc-2018), and mouse IgG (Peprotech 500-M00) (Supplementary Figure S1). On the following day the cells were stained with the secondary antibody conjugated with Alexa Fluor 594 (Invitrogen, 1:400) for one hour. After that, cell nuclei were stained with DAPI (Sigma-Aldrich, D8417, 12.5 µg/ml in MilliQ water) for two minutes. The cells in Chamber Slides were mounted with a ProLong® Gold antifade reagent (Invitrogen, P36934). Samples in 96-well µ-plates were filled up with 1xPBS. The protein expression was visualized with two microscopes: a confocal microscope Leica TCS SP5II HCS A with aHCX PL APO 20x/0.7 Imm Cor (glycerol) objective and fluorescence wide field microscope Leica DM6000B with a 20x/0.7 HC PL APO CS wd=0.59 objective.

4.6 Statistical analyses

Statistical significance was determined by one-way analysis of variance followed by Bonferroni posttest (SigmaPlot 11.0, RRID:SCR_003210). Differences of $P < 0.05$ (*), $P < 0.01$ (**), and $P <$

0.001 (***) in relative gene expression between differentiated cells and undifferentiated hPSCs on day 0 were considered significant (Figures 3-4). For each differentiation condition, differences of $P < 0.05$ (*) and $P < 0.001$ (***) in relative gene expression of DE and hepatic markers between day 4 and day 6 were considered significant (Figures 3-4).

ACKNOWLEDGMENTS

This work was supported by grants from Academy of Finland (Nos. 294193 and 294194 to Y.-R.L.) and Finnish National Agency for Education (TM-15-9578 to Y.-R.L. for M.S.B.). M.S.B. acknowledges support from the Doctoral Programme in Drug Research in University of Helsinki. L.K.K. acknowledges support from the Doctoral Programme in Materials Research and Nanosciences in University of Helsinki. We would like to thank the Light Microscopy Unit at the Institute of Biotechnology for providing confocal microscopy, Dr. Tomi Mäkelä at the Research Programs Unit, Faculty of Medicine for providing StepOnePlus system, and Dr. Petri Auvinen at the Institute of Biotechnology for providing NanoDrop spectrophotometer.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Y.-R.L. designed the research; M.S.B., L.K.K., and Y.-R.L. performed the experiments; M.S.B., S.K., A.W.L., and Y.-R.L. analyzed the data; M.S.B., A.W.L., and Y.-R.L. wrote the paper. All authors read the manuscript.

FIGURE LEGENDS

FIGURE 1 Morphology of WA07 cells and their derivatives on day 1, day 4, and day 6 of differentiation. Scale bars = 100 μm. ND: no data.

FIGURE 2 Morphology of iPS(IMR90)-4 cells and their derivatives on day 1, day 4, and day 6 of differentiation. Scale bars = 100 μm.

FIGURE 3 Relative mRNA expression of the pluripotency (a, b), DE (c-f), hepatic (g), bile duct (h), mesendoderm (i), and ectoderm (j) markers in the WA07 cells during the differentiation. The mRNA expression was analyzed by real-time qPCR. Relative mRNA expression was normalized to the housekeeping gene *RPLP0*, and fold inductions were calculated with reference to the undifferentiated WA07 cells on day 0. N = 3 biological samples. Error bars are SD. * $P < 0.05$, *** $P < 0.01$, and *** $P < 0.001$ in comparison with day 0 are shown above bars. * $P < 0.05$ and *** $P < 0.001$ between day 4 and day 6 are shown above lines. M1-M6 are medium conditions according to Table 1. ND: no data.

FIGURE 4 Relative mRNA expression of the pluripotency (a, b), DE (c-f), hepatic (g), bile duct (h), mesendoderm (i), and ectoderm (j) markers in the iPS(IMR90)-4 cells during the differentiation. The mRNA expression was analyzed by real-time qPCR. Relative mRNA expression was normalized to the housekeeping gene *RPLP0*, and fold inductions were calculated with reference to the undifferentiated iPS(IMR90)-4 cells on day 0. N = 3 biological samples. Error bars are SD. * $P < 0.05$, *** $P < 0.01$, and *** $P < 0.001$ in comparison with day 0 are shown above bars. *** $P < 0.001$ between day 4 and day 6 are shown above lines. M1-M6 are medium conditions according to Table 1.

FIGURE 5 Heat map representing gene expression levels and hierarchical clustering of different treatments. The values are log2 transformed relative gene expressions. The dendrogram represents the distance (dissimilarity) between treatment clusters. The intensity of the red and blue color indicates high and low expression levels, respectively. Data analysis was performed with gplots package in R (version 3.3.1).

FIGURE 6 Immunostaining of the pluripotency, DE, and hepatic markers in the WA07 cells differentiated in conditions M1 and M2 for six days. Scale bars = 100 μm.

FIGURE 7 Immunostaining of the pluripotency, DE, and hepatic markers in the iPS(IMR90)-4 cells differentiated in conditions M1 and M2 for six days. Scale bars = 100 μm.

TABLE 1 Medium composition for the DE induction

TABLE 2 Primers and TaqMan® Gene Expression Assay mixes used in qPCR

TABLE 3 Antibodies used in immunofluorescent staining

SUPPORTING INFORMATION

Supplementary Figure S1 is found online in the supporting information tab for this article.

REFERENCES

Ang SL, Wierda A, Wong D, Stevens KA, Cascio S, Rossant J, Zaret KS. 1993. The formation and maintenance of the definitive endoderm lineage in the mouse: involvement of HNF3/forkhead proteins. Development 119(4):1301-1315.

Boffa LC, Vidali G, Mann RS, Allfrey VG. 1978. Suppression of histone deacetylation in vivo and in vitro by sodium butyrate. The Journal of biological chemistry 253(10):3364-3366.

Bolotin E, Liao H, Ta TC, Yang C, Hwang-Verslues W, Evans JR, Jiang T, Sladek FM. 2010. Integrated approach for the identification of human hepatocyte nuclear factor 4alpha target genes using protein binding microarrays. Hepatology 51(2):642-653.

Borowiak M, Maehr R, Chen S, Chen AE, Tang W, Fox JL, Schreiber SL, Melton DA. 2009. Small molecules efficiently direct endodermal differentiation of mouse and human embryonic stem cells. Cell stem cell 4(4):348-358.

Chambers I, Colby D, Robertson M, Nichols J, Lee S, Tweedie S, Smith A. 2003. Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell 113(5):643-655.

D'Amour KA, Agulnick AD, Eliazer S, Kelly OG, Kroon E, Baetge EE. 2005. Efficient differentiation of human embryonic stem cells to definitive endoderm. Nature biotechnology 23(12):1534-1541.

Gadue P, Huber TL, Paddison PJ, Keller GM. 2006. Wnt and TGF-beta signaling are required for the induction of an in vitro model of primitive streak formation using embryonic stem cells. Proceedings of the National Academy of Sciences of the United States of America 103(45):16806-16811.

Goicoa S, Alvarez S, Ricordi C, Inverardi L, Dominguez-Bendala J. 2006. Sodium butyrate activates genes of early pancreatic development in embryonic stem cells. Cloning and stem cells 8(3):140-149.

Hay DC, Fletcher J, Payne C, Terrace JD, Gallagher RC, Snoeys J, Black JR, Wojtacha D, Samuel K, Hannoun Z, Pryde A, Filippi C, Currie IS, Forbes SJ, Ross JA, Newsome PN, Iredale JP. 2008a. Highly efficient differentiation of hESCs to functional hepatic endoderm requires ActivinA and Wnt3a signaling. Proceedings of the National Academy of Sciences of the United States of America 105(34):12301-12306.

Hay DC, Zhao D, Fletcher J, Hewitt ZA, McLean D, Urruticoechea-Uriguen A, Black JR, Elcombe C, Ross JA, Wolf R, Cui W. 2008b. Efficient differentiation of hepatocytes from human embryonic stem cells exhibiting markers recapitulating liver development in vivo. Stem cells 26(4):894-902.

Jiang J, Au M, Lu K, Eshpeter A, Korbutt G, Fisk G, Majumdar AS. 2007. Generation of insulin-producing islet-like clusters from human embryonic stem cells. Stem cells 25(8):1940-1953.

Kanai-Azuma M, Kanai Y, Gad JM, Tajima Y, Taya C, Kurohmaru M, Sanai Y, Yonekawa H, Yazaki K, Tam PP, Hayashi Y. 2002. Depletion of definitive gut endoderm in Sox17-null mutant mice. Development 129(10):2367-2379.

Kanninen LK, Harjumaki R, Peltoniemi P, Bogacheva MS, Salmi T, Porola P, Niklander J, Smutny T, Urtti A, Yliperttula ML, Lou YR. 2016a. Laminin-511 and laminin-521-based matrices for efficient hepatic specification of human pluripotent stem cells. Biomaterials 103:86-100.

Kanninen LK, Porola P, Niklander J, Malinen MM, Corlu A, Guguen-Guillouzo C, Urtti A, Yliperttula ML, Lou YR. 2016b. Hepatic differentiation of human pluripotent stem cells on human liver progenitor HepaRG-derived acellular matrix. Experimental cell research 341(2):207-217.

Katoh M, Katoh M. 2006. CER1 is a common target of WNT and NODAL signaling pathways in human embryonic stem cells. International journal of molecular medicine 17(5):795-799.

Kruh J. 1982. Effects of sodium butyrate, a new pharmacological agent, on cells in culture. Molecular and cellular biochemistry 42(2):65-82.

Kubo A, Shinozaki K, Shannon JM, Kouskoff V, Kennedy M, Woo S, Fehling HJ, Keller G. 2004. Development of definitive endoderm from embryonic stem cells in culture. Development 131(7):1651-1662.

Leung AW, Murdoch B, Salem AF, Prasad MS, Gomez GA, Garcia-Castro MI. 2016. WNT/beta-catenin signaling mediates human neural crest induction via a pre-neural border intermediate. Development 143(3):398-410.

Lindsley RC, Gill JG, Kyba M, Murphy TL, Murphy KM. 2006. Canonical Wnt signaling is required for development of embryonic stem cell-derived mesoderm. Development 133(19):3787-3796.

McGrath KE, Koniski AD, Maltby KM, McGann JK, Palis J. 1999. Embryonic expression and function of the chemokine SDF-1 and its receptor, CXCR4. Developmental biology 213(2):442-456.

Mitsui K, Tokuzawa Y, Itoh H, Segawa K, Murakami M, Takahashi K, Maruyama M, Maeda M, Yamanaka S. 2003. The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell 113(5):631-642.

Nichols J, Zevnik B, Anastassiadis K, Niwa H, Klewe-Nebenius D, Chambers I, Scholer H, Smith A. 1998. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell 95(3):379-391.

Pekkanen-Mattila M, Pelto-Huikko M, Kujala V, Suuronen R, Skottman H, Aalto-Setala K, Kerkela E. 2010. Spatial and temporal expression pattern of germ layer markers during human embryonic stem cell differentiation in embryoid bodies. Histochemistry and cell biology 133(5):595-606.

Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic acids research 29(9):e45.

Ramirez JM, Gerbal-Chaloin S, Milhavet O, Qiang B, Becker F, Assou S, Lemaitre JM, Hamamah S, De Vos J. 2011. Brief report: benchmarking human pluripotent stem cell markers during differentiation into the three germ layers unveils a striking heterogeneity: all markers are not equal. Stem cells 29(9):1469-1474.

Schwartz RE, Fleming HE, Khetani SR, Bhatia SN. 2014. Pluripotent stem cell-derived hepatocyte-like cells. Biotechnology advances 32(2):504-513.

Shen MM. 2007. Nodal signaling: developmental roles and regulation. Development 134(6):1023-1034.

Si-Tayeb K, Lemaigre FP, Duncan SA. 2010. Organogenesis and development of the liver. Developmental cell 18(2):175-189.

Spence JR, Lange AW, Lin SC, Kaestner KH, Lowy AM, Kim I, Whitsett JA, Wells JM. 2009. Sox17 regulates organ lineage segregation of ventral foregut progenitor cells. Developmental cell 17(1):62-74.

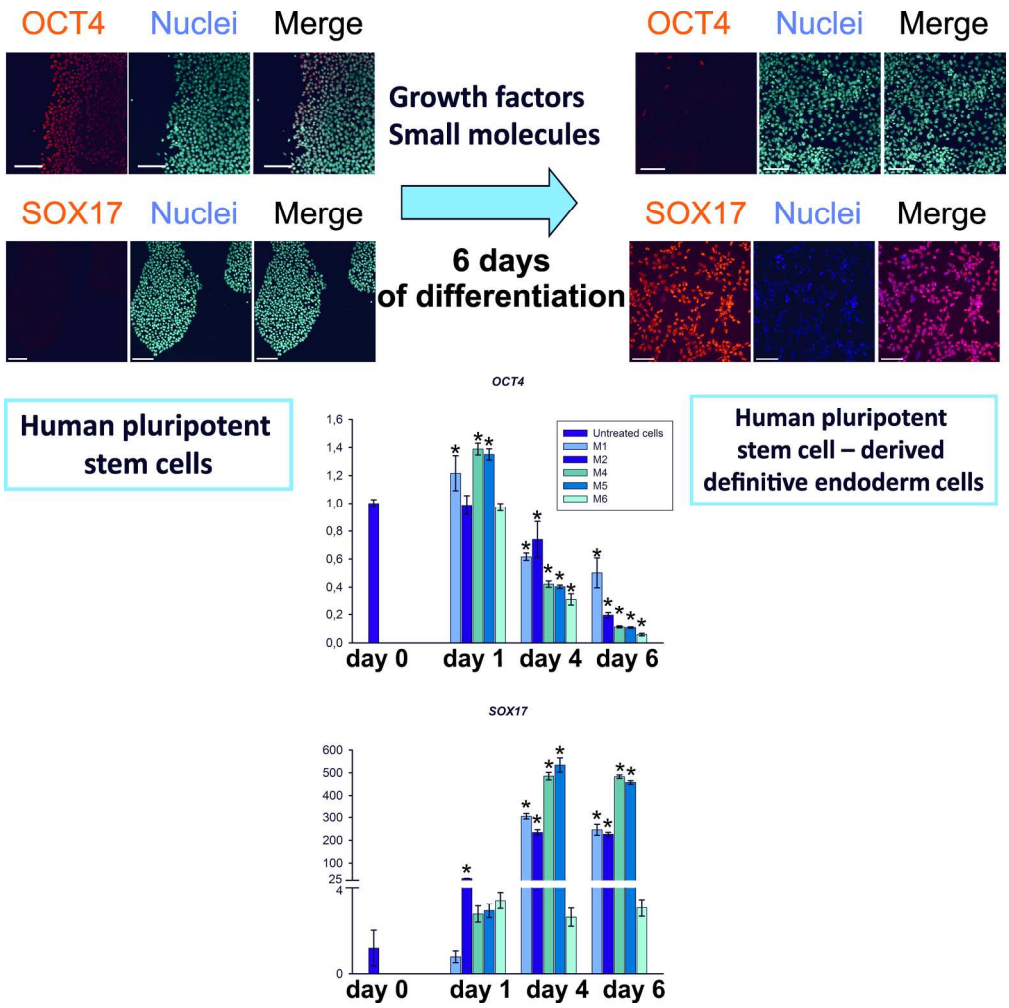
Tahamtani Y, Azarnia M, Farrokhi A, Sharifi-Zarchi A, Aghdami N, Baharvand H. 2013. Treatment of human embryonic stem cells with different combinations of priming and inducing factors toward definitive endoderm. Stem cells and development 22(9):1419-1432.

Toivonen S, Lundin K, Balboa D, Ustinov J, Tamminen K, Palgi J, Trokovic R, Tuuri T, Otonkoski T. 2013. Activin A and Wnt-dependent specification of human definitive endoderm cells. Experimental cell research 319(17):2535-2544.

Wilkinson DG, Bhatt S, Herrmann BG. 1990. Expression pattern of the mouse T gene and its role in mesoderm formation. Nature 343(6259):657-659.

Xu RH, Sampsell-Barron TL, Gu F, Root S, Peck RM, Pan G, Yu J, Antosiewicz-Bourget J, Tian S, Stewart R, Thomson JA. 2008. NANOG is a direct target of TGFbeta/activin-mediated SMAD signaling in human ESCs. Cell stem cell 3(2):196-206.

Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin, II, Thomson JA. 2007. Induced pluripotent stem cell lines derived from human somatic cells. Science 318(5858):1917-1920.



Graphical abstract

199x197mm (300 x 300 DPI)

TABLE 1 Medium composition for the DE induction

Condition	DE priming (day 0) medium composition	DE induction (days 1-5) medium composition
M1	DM, 100 ng/ml AA	DM, 100 ng/ml AA
M2	DM, 100 ng/ml AA, 75 ng/ml Wnt-3a	DM, 100 ng/ml AA, 75 ng/ml Wnt-3a
M3	DM, 100 ng/ml AA, 1 mM NaB (Millipore)	DM, 100 ng/ml AA, 0.5 mM NaB (Millipore)
M4	DM, 100 ng/ml AA, 1 mM NaB (Abcam)	DM, 100 ng/ml AA, 0.5 mM NaB (Abcam)
M5	DM, 100 ng/ml AA, 1 mM NaB (Sigma)	DM, 100 ng/ml AA, 0.5 mM NaB (Sigma)
M6	DM, 100 nM IDE 1	DM, 100 nM IDE 1

DM: RPMI-1640, 1 x GlutaMAX™, and 1 x B-27

TABLE 2 Primers and TaqMan® Gene Expression Assay mixes used in qPCR

Gene	Accession	Size (bp)	Sequence (5' to 3')
RPLP0	NM_001002.3 NM_053275.3	74	F: AATCTCCAGGGGCACCATT R: CGCTGGCTCCCACTTTGT
OCT4	NM_002701.4 NM_203289.4 NM_001173531.1	161	F: CAGTGCCCGAAACCCACAC R: GGAGACCCAGCAGCCTCAAA
NANOG	NM_024865.2	80	F: GCAGAAGGCCTCAGCACCTA R: GGTTCCCAGTCGGGTTCAC
HNF3B	NM_021784.4 NM_153675.2	89	F: GGGAGCGGTGAAGATGGA R: TCATGTTGCTCACGGAGGAGTA
BRACHYURY	NM_001270484.1 NM_003181.3	118	F: AGAACGGCAGGAGGATGTTTCC R: ACGTACTTCCAGCGGTGGTTGT

Gene	TaqMan® Gene Expression Assay ID
RPLP0	Hs99999902_m1
CER1	Hs00193796_m1
SOX17	Hs00751752_s1
CXCR4	Hs00607978_s1
HNF4A	Hs00230853_m1
HNF6	Hs00413554_m1
SOX1	Hs01057642_s1

TABLE 3 Antibodies used in immunofluorescent staining

Antibody	Manufacturer	RRID	Dilution ratio
rabbit anti-OCT4	Santa Cruz Biotechnology sc-9081	AB_2167703	1:500
rabbit-anti NANOG	Abcam ab21624	AB_446437	1:50
rabbit anti-HNF4A	Sigma-Aldrich HPA004712	AB_1079075	1:91
goat anti-NANOG	R&D Systems AF1997	AB_355097	1:40
goat anti-SOX17	R&D Systems AF1924	AB_355060	1:50
goat anti-HNF3B	Santa Cruz Biotechnology sc-6554	AB_2262810	1:50
mouse anti-AFP	Sigma-Aldrich A8452	AB_258392	1:500

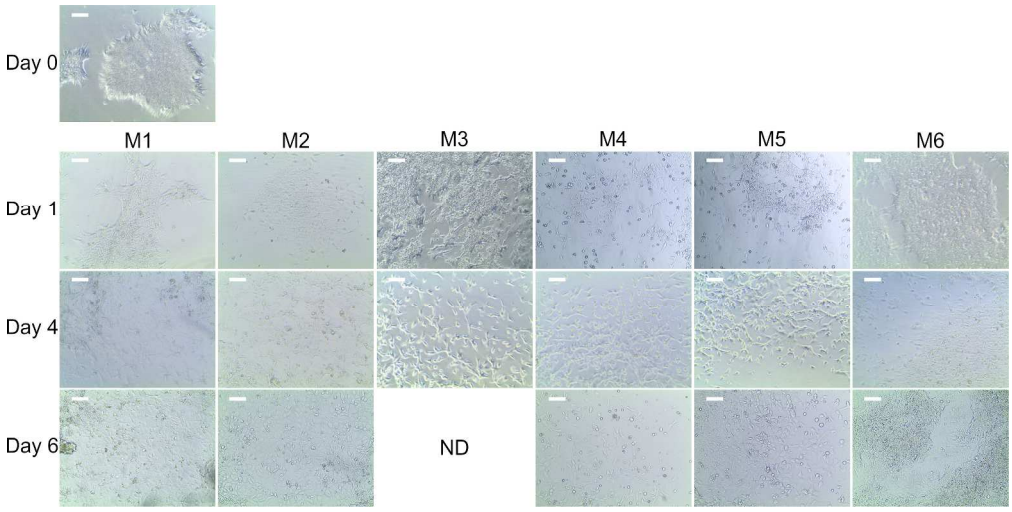


FIGURE 1 Morphology of WA07 cells and their derivatives on day 1, day 4, and day 6 of differentiation. Scale bars = 100 μm. ND: no data.

278x139mm (300 x 300 DPI)

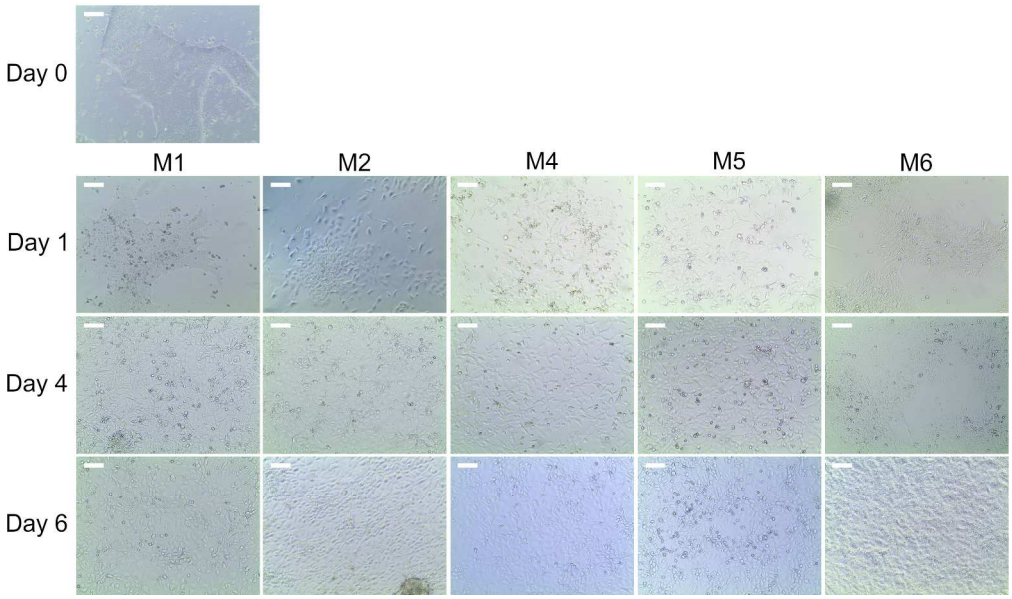


FIGURE 2 Morphology of iPS(IMR90)-4 cells and their derivatives on day 1, day 4, and day 6 of differentiation. Scale bars = 100 μm.

236x139mm (300 x 300 DPI)

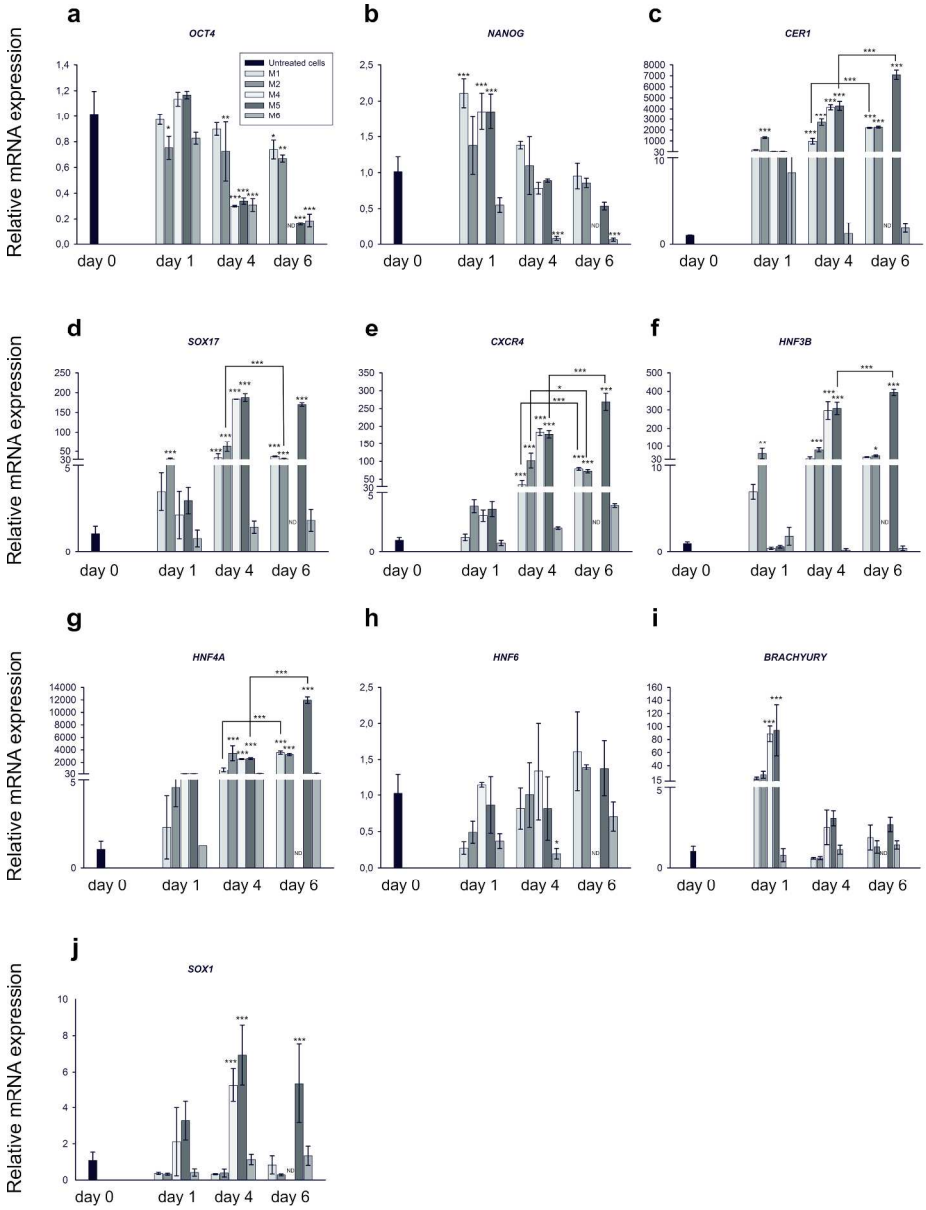


FIGURE 3 Relative mRNA expression of the pluripotency (a, b), DE (c-f), hepatic (g), bile duct (h), mesendoderm (i), and ectoderm (j) markers in the WA07 cells during the differentiation. The mRNA expression was analyzed by real-time qPCR. Relative mRNA expression was normalized to the housekeeping gene *RPLP0*, and fold inductions were calculated with reference to the undifferentiated WA07 cells on day 0. N = 3 biological samples. Error bars are SD. * $P < 0.05$, *** $P < 0.01$, and *** $P < 0.001$ in comparison with day 0 are shown above bars. * $P < 0.05$ and *** $P < 0.001$ between day 4 and day 6 are shown above lines. M1-M6 are medium conditions according to Table 1. ND: no data.

240x310mm (300 x 300 DPI)

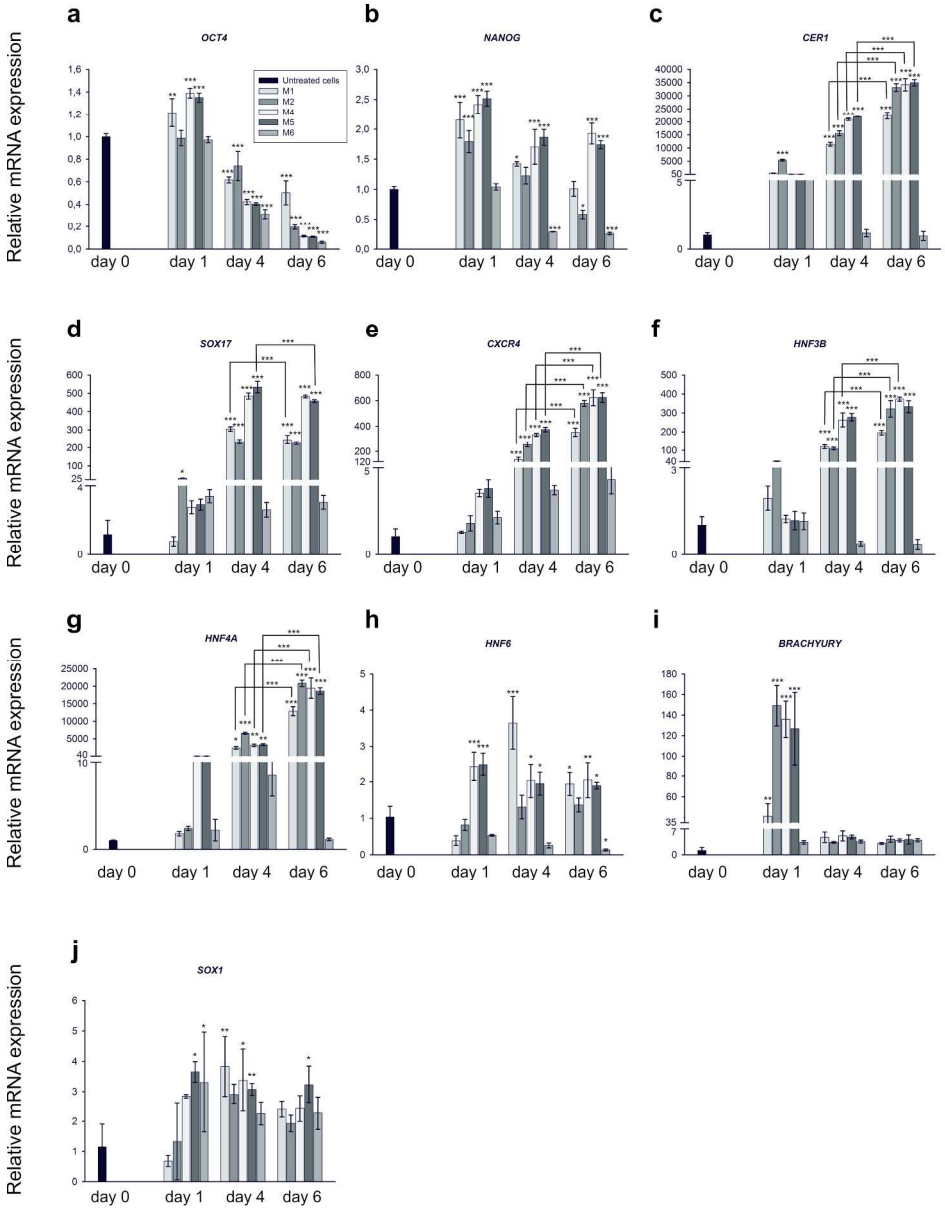


FIGURE 4 Relative mRNA expression of the pluripotency (a, b), DE (c-f), hepatic (g), bile duct (h), mesendoderm (i), and ectoderm (j) markers in the iPS(IMR90)-4 cells during the differentiation. The mRNA expression was analyzed by real-time qPCR. Relative mRNA expression was normalized to the housekeeping gene *RPLP0*, and fold inductions were calculated with reference to the undifferentiated iPS(IMR90)-4 cells on day 0. N = 3 biological samples. Error bars are SD. * $P < 0.05$, *** $P < 0.01$, and *** $P < 0.001$ in comparison with day 0 are shown above bars. *** $P < 0.001$ between day 4 and day 6 are shown above lines. M1-M6 are medium conditions according to Table 1.

239x303mm (300 x 300 DPI)

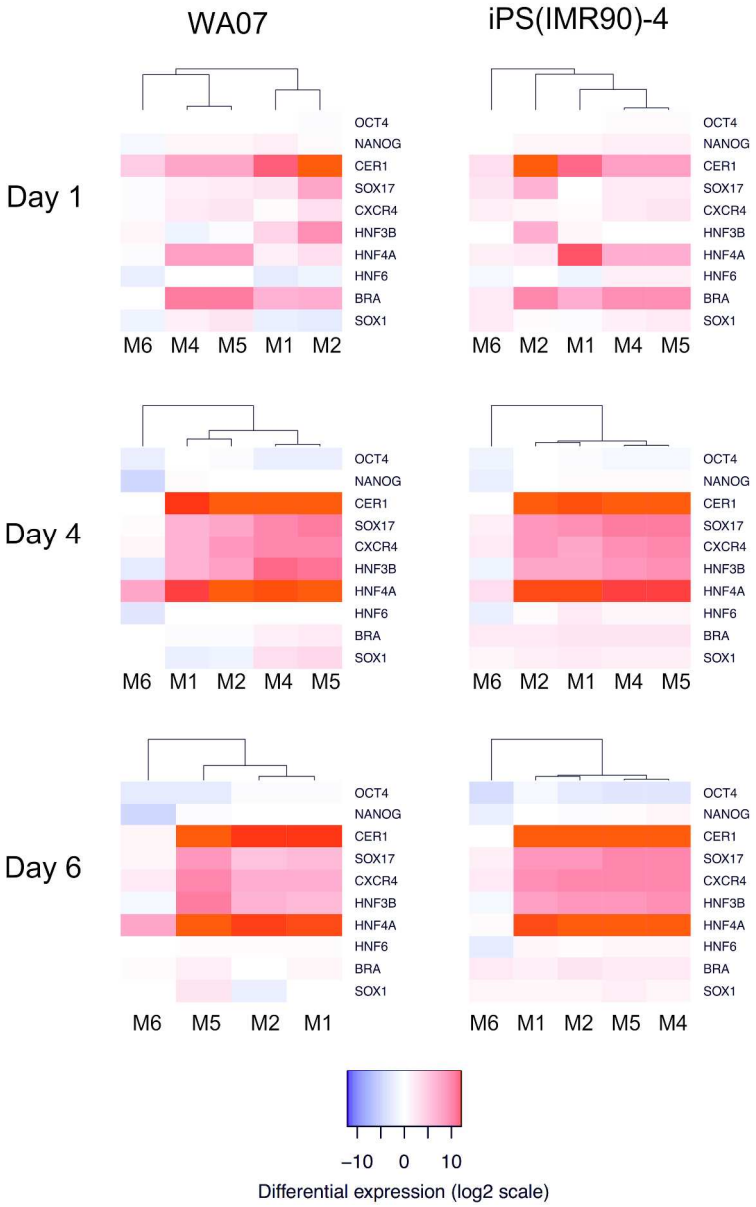


FIGURE 5 Heat map representing gene expression levels and hierarchical clustering of different treatments. The values are log2 transformed relative gene expressions. The dendrogram represents the distance (dissimilarity) between treatment clusters. The intensity of the red and blue color indicates high and low expression levels, respectively. Data analysis was performed with gplots package in R (version 3.3.1).

199x321mm (300 x 300 DPI)

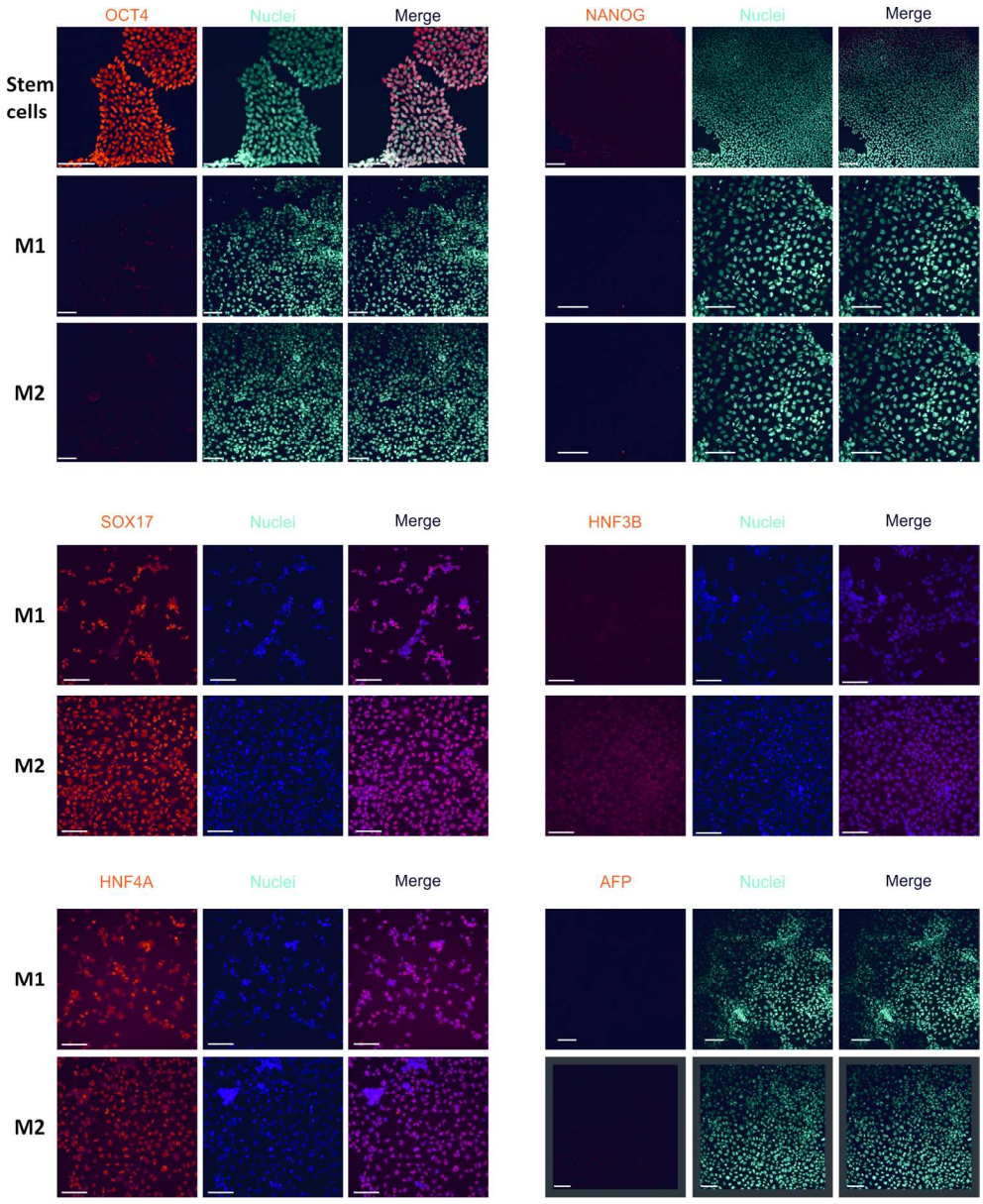


FIGURE 6 Immunostaining of the pluripotency, DE, and hepatic markers in the WA07 cells differentiated in conditions M1 and M2 for six days. Scale bars = 100 μm.

168x206mm (300 x 300 DPI)

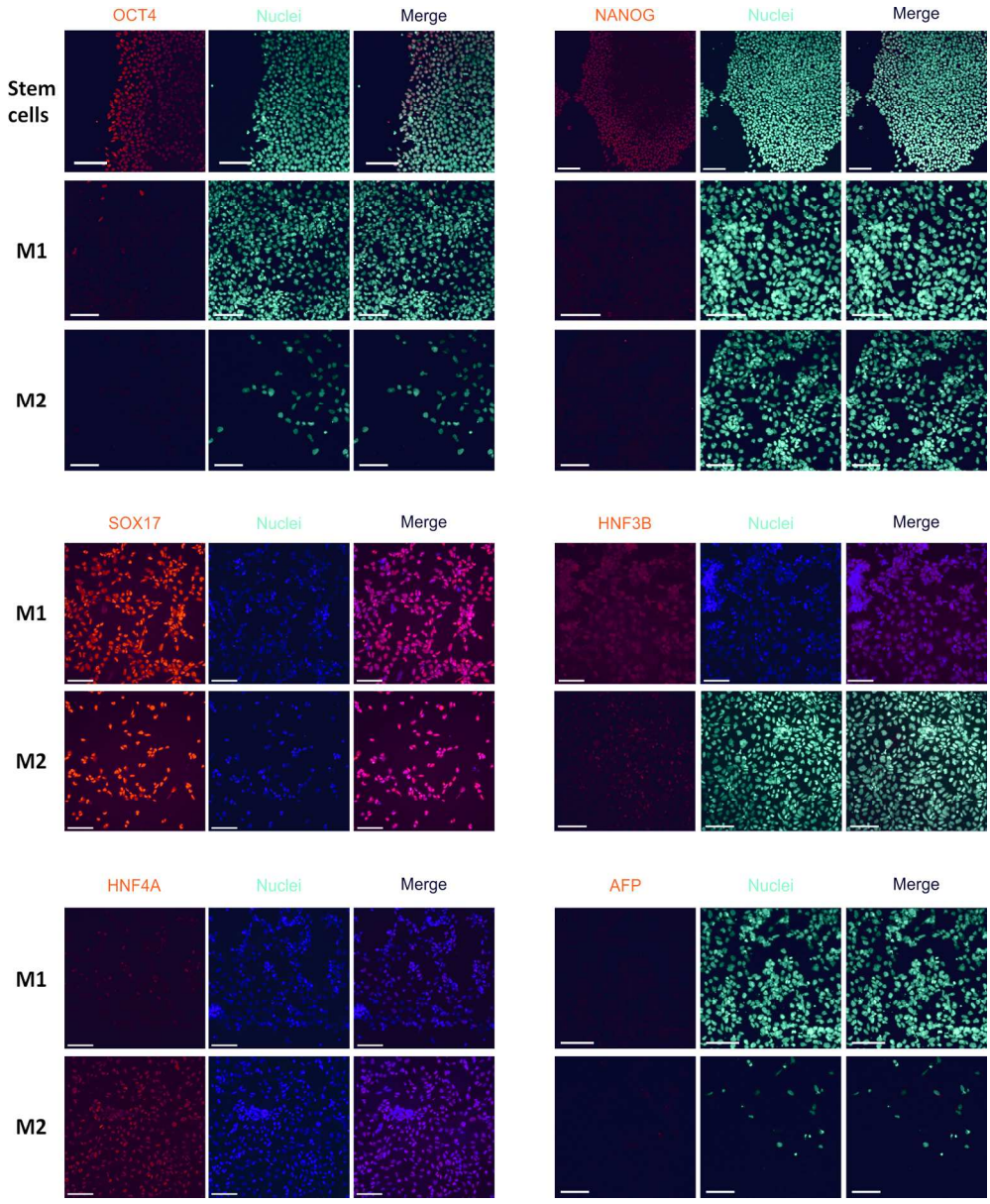


FIGURE 7 Immunostaining of the pluripotency, DE, and hepatic markers in the iPS(IMR90)-4 cells differentiated in conditions M1 and M2 for six days. Scale bars = 100 μm.

169x205mm (300 x 300 DPI)