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DERIVATION OF HEPATOCYTE LIKE CELLS FROM HUMAN PLURIPOTENT STEM CELLS

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Academic Dissertation

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Cover: Phase contrast microscopy of human pluripotent stem cell derived hepatocyte like cells. Immunofluorescence of hepatocyte like cells: Albumin (red)

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“Insanity is doing the same thing over and over again and expecting different results”

-Albert Einstein

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ABSTRACT

Human embryonic stem cells (hESC) and human induced pluripotent stem cells (hiPSC), collectively called human pluripotent stem cells (hPSC), represent an unlimited cell source of self-renewing cells for studying human developmental biology, for disease modeling and for regenerative medicine due to their capacity to differentiate in all cell types in human body. The revolutionary discovery of hiPSC technology, the method which can turn somatic cells back to pluripotent stage, may in the future enable generation of large quantities of autologous, patient derived disease-specific cells, such as parenchymal liver cells, hepatocytes. In addition to disease modeling, these cells would be valuable tools for drug discovery.

Liver diseases are a leading cause of death worldwide and to date the only treatment for end stage liver disease is organ transplantation. Human liver is the metabolic center of the body taking care of most of the xenobiotic metabolism and drug detoxification. Therefore, especially academic research and pharmacological industry is in urgent need for valid liver cell models for understanding disease pathogenesis, for drug discovery and toxicity evaluation. Successful hepatocyte differentiation from hPSCs has been described, however, a few fundamental issues need to be solved before these cells are usable in clinical approaches. Firstly, functional variation between different hPSC lines is complicating establishment of universally competent differentiation protocols. Secondly, so far described differentiation methods are unable to produce fully functional hepatocytes from hPSCs.

The first aim of the current work was to evaluate variability in differentiation capacity of different hPSC lines. The second aim was to elucidate the role of Acitivin/Nodal and Wnt signaling during endoderm differentiation and to study how these signals are affecting on the capability of DE-cell to differentiate in hepatocytes and pancreatic cells. The last aim was to study the effect of specific extracellular matrix (ECM) proteins, laminins, and various 3D culture environments on hepatic differentiation from hPSCs.

All hESCs and hiPSCs differentiated into hepatocyte like cells (HLC), however, individual cell lines tend to differentiate better than others. One hiPSC line showed poor differentiation capacity throughout the study. This hiPSC line revealed to be partially reprogrammed with residual transgenic *KLF4* expression. During more than 100 days long retinal pigmented epithelial cell (RPE) differentiation certain ectopic transgenes were reactivated in retrovirally derived hiPSC lines. RPE differentiation related reactivation of

transgenes raised a concern whether transgenes could also re-activate over time in other hiPSC derived cell types, such as hepatocytes. Our results have led us to use only non-integrating methods for production of iPSC- lines.

Definitive endoderm (DE) cells are precursors for both hepatic and pancreatic cells. DE-cells can be differentiated from hPSCs by activating Activin/Nodal and canonical Wnt signaling pathways. We showed that a short Wnt signaling activation in the beginning of DE differentiation is crucial for proper pancreas differentiation while longer Wnt activation favored hepatocyte differentiation. In addition, we showed that mixed hPSC/DE-cell population could be maintained in long-term cultures by activating Wnt signaling pathway.

Mature hepatocytes are not proliferating in the healthy liver while fetal hepatoblasts are constantly dividing. We showed that our “lab-made” laminin rich ECM, JAR-matrix, is supporting the differentiation of hPSC into hepatic progenitor cells, hepatoblasts. Hence, JAR-matrix could be potential ECM preparation for hepatoblasts expansion.

Two of the tested 3D approaches supported hPSC-derived HLCs viability and proliferation. ECM protein rich 3D environment enhanced Albumin secretion and *Albumin* gene expression in HLCs, whereas HLC aggregates in 3D environment without ECM proteins showed increased expression of metabolic genes. Our findings illustrated the importance of the cell culture environment for cellular identity of HLCs.

Taken together, this thesis work provides valuable biological and technical information for the optimization of hepatocyte differentiation from hPSCs.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications (I-III) and one submitted manuscript (IV), which are referred to in the text by their Roman numerals:

I Toivonen S, Ojala M, Hyysalo A, Ilmarinen T, Rajala K, Pekkanen-Mattila M, Äänismaa R, Lundin K, Plagi J, Weltner J, Trokovic R, Silvennoinen O, Skottman H, Narkilahti S, Aalto-Setälä K, Otonkoski T. *Comparative analysis of targeted differentiation of human induced pluripotent stem cells (hiPSCs) and human embryonic stem cells reveals variability associated with incomplete transgene silencing in retrovirally derived hiPSC lines*. Stem Cells Transl Med. 2013 Feb;2(2):83-93

II Toivonen S, Lundin K, Balboa D, Ustinov J, Tamminen K, Palgi J, Trokovic R, Tuuri T, Otonkoski T. *Acitivin A and Wnt-dependent specification of human definitive endoderm cells*. Exp Cell Res. 2013 Oct 15;319(17):2535-44

III Vuoristo S, Toivonen S, Weltner J, Mikkola M, Ustinov J, Trokovic R, Palgi J, Lund R, Tuuri T, Otonkoski T. *A novel feeder-free culture system for human pluripotent stem cell culture and induced pluripotent stem cell derivation.* PLoS One 2013 Oct2;8(10):e76205

IV Toivonen S, Malinen M, Kubleck J, Urtti A, Honkakoski P, Otonkoski T. *Comparison of 3D and 2D culture techniques for differentiation of human iPSC to hepatocytes.* Submitted manuscript.

In addition some unpublished data are presented.

ABBREVIATIONS

2D	Two dimensional
3D	Three dimensional
AFP	Alphafetoprotein
APC	Adenomatous Polyposis Coli
APOA-II	Apolipoprotein A-II
BSA	Bovine serum albumin
BSEP	Bile salt export pump
Cas	Clustered regularly interspaced short palindromic repeats associated protein
cDNA	Complementary deoxyribonucleic acid
CDX	Cadual type homeobox
c-Myc	V-myc myelocytomatosis viral oncogene
CK	Cytokeratin
CK	Casein kinase
CXCR	C-X-C chemokine receptor
CYP	Cytochrome P450
DAPI	4',6'-Diamidino-2-Phenylindole
DE	Definitive endoderm
Dkk	Dikkopf
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfo-oxide
DNA	Deoxyribonucleic acid

EB	Embryoid body
EC	Embryonic carcinoma
ECC	Embryonic carcinoma cell
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epithelia-mesenchymal transition
EpCAM	Epithelial cell adhesion molecule
EpiSC	Epiblats-derived stem cell
ESC	Embryonic stem cell
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FCS	Fetal calf serum
FDZ	Frizzled
FGF	Fibroblast growth factor
FOX	Forkhead box transcription factor
GATA	GATA binding factor
GSK	Glycogen synthase kinase
HBM	Hepatocyte basal medium
HCC	Hepatocellular carcinoma
HDAC	Histone deacetylase
hESC	Human embryonic stem cell
HGF	Hepatocyte growth factor

Hhex	Hematopoietically expressed homeobox
hiPSC	Human induced pluripotent stem cell
hiHep	Human induced hepatocyte
HLC	Hepatocyte like cell
HNF	Homeodomain transcription factor
hPSC	Human pluripotent stem cell
ICM	Inner cell mass
IL	Interleukin
iPSC	Induced pluripotent stem cell
IVF	*In vitro* fertilization
KLF	Krüppel like factor
KO-DMEM	KnockOut Dulbecco's Modified Eagle Medium
KO-SR	KnockOut serum replacement
LEF	Lymphoid enhancer-binding factor
LiCl	Lithium chloride
Lm	Laminin
LV	Lentivirus
NaB	Sodium Butyrate
NEAA	Non-Essential Amino Acids
NKX	NK homeobox
NFC	Nanofibrillar cellulose
NTCP	Na^+-Taurocholate cotransporting polypeptide

NR	Nuclear receptor
mEF	Mouse embryonic feeder
MET	MET proto-oncogen, receptor tyrosine kinase
EHS	Engelbterh-Holm-Swarm
MG	Matrigel
MDR	Multidrug transporter
MET	Mesenchymal-to-epithelial transition
MRP	Multidrug resistance-associated protein
MyoD	Myoblast determination protein
OATP	Organic anion transporting polypeptide
OCT	Octamer-binding transcription factor
OncM	Oncostatin M
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD	PetriDish®
PDX	Pancreatic and duodenal homeobox
PEG	Polyethylene glycol
PFA	Paraformaldehyde
PHH	Primary human hepatocyte
PI3K	Phosphatidylinositide 3-kinases
PP2A	Protein phosphatase 2A
Prox	Prospero homeobox

PSC	Pluripotent stem cell
rAAV	Recombinanat adeno associated virus
RAFT	Real Architecture For 3D Tissue
RNA	Ribonucleic acid
RPE	Retinal pigmented epithelial cell
RPMI	Roswell park memorial institute
RT	Room temperature
RT-qPCR	Real time quantitative polymerase chain reaction
SCNT	Somatic cell nuclear transfer
SEM	Standard error of mean
SeV	Sendai virus
shRNA	Short hairpin RNA
SNCT	Somatic cell nuclear transfer
SOX	SRY (sex determining region Y)-box
SSEA	Stage specific embryonic antigen
STM	Septum transversum mesenchyme
TGF-β	Transforming growth factor beta
TF	Transcription factor
TRA	Tumor related antigen
UGT1A1	UDP glucuronosyltransferase 1 family, polypeptide A
Wnt	Wingless signaling pathway

INTRODUCTION

There are two types of human pluripotent stem cells (hPSC). Human embryonic stem cells (hESC) are derived from the inner cell mass of developing embryos and human induced pluripotent stem cells (hiPSC) are reprogrammed from somatic cells (Takahashi et al, 2007; Thomson et al, 1998). For almost two decades hESCs have been valuable tools for studying human developmental biology. The discovery of hiPSC technology nine years ago has revolutionized the area of stem cell research once and for all. hiPSC technology enables production of patient specific cell lines which can be used in personalized drug development and are future promise for autologous cell transplants.

The liver is the largest inner organ in the human body and has an important role in many essential metabolic functions as well as detoxification of drugs and other xenobiotic. Liver diseases are a leading cause of death worldwide. The healthy liver has particular propensity to regenerate, however, in the case of severe liver diseases the liver is unable to restore its functions. Currently the only treatment for end stage liver disease is organ transplantation (Horton, 2012). The lack of suitable organ donors hampers transplantation therapies. In addition, current liver cell models used for distinguishing liver disease pathogenesis, drug discovery and toxicology testing have limitations due to interspecies variations and the lack of full functionality (Hackam & Redelmeier, 2006). Thus, hPSC derived hepatocytes represent an alternative cell source for hepatic cell modeling.

Hepatocyte differentiation from hPSCs has been described (Cayo et al, 2012; Shan et al, 2013; Si-Tayeb et al, 2010b; Yusa et al, 2011). However, so far published protocols are unable to derive fully functional hepatocytes (Baxter et al, 2015). Specific extracellular matrix (ECM) proteins like laminins and collagens (LeCluyse et al, 1994; Tanimizu et al, 2004) as well as three dimensional (3D) (Gunness et al, 2013) culture environments have been used in hepatocyte cultures to improve functionality and longevity of the cells. In the current thesis work I evaluate the differences between multiple hPSC lines to differentiate into hepatocytes. I also examine the biological cues driving hepatocyte differentiation *in vitro* and investigate the effect of ECM proteins and 3D culture environment on hepatocyte differentiation.

REVIEW OF LITERATURE

1. Historical perspective of stem cell research

Over the past decade the research in the area of pluripotent stem cells has gone through unparalleled progress. However, the findings described today in stem cell science are firmly grounded in research that has been going on for centuries. The term “stem cell” was first mentioned in the literature already in 1868 when the German biologist Ernst Haeckel used the phrase to describe the fertilized egg that becomes an organism (Droscher, 2014). The property of cell pluripotency was first described in 1891, when Hans Driesch observed that two separated cells of early sea urchin blastocyst can individually give rise to complete sea urchins (Robinton & Daley, 2012). Later, in 1909 the Russian scientist Alexander Maximow introduced the idea of blood stem cells, which are multipotent with the ability to differentiate into several types of cells (Ramalho-Santos & Willenbring, 2007).

The real beginning of stem cell research can be considered to have taken place in 1954, when Leroy Stevens described a mouse strain 129 that showed an incidence of testicular teratoma of about 1% (Stevens & Little, 1954). Teratomas, random cell clusters containing teeth, pieces of bone, muscles, skin and hair, have fascinated researchers for a long time. However, they are extremely rare in commonly used laboratory animals. With this new animal model, Stevens and colleagues were able to establish immortal pluripotent cell lines from teratocarcinomas. These cell lines were called embryonal carcinoma cell (ECC) lines (Kleinsmith & Pierce, 1964).

The studies of teratocarcinomas continued extensively until late 1970’s by which time multiple ECC lines had been established from both mouse and human origin. In 1981, researchers found that mouse ECCs can also be derived from teratocarcinomas that were experimentally induced by transplantation of implantation-stage mouse embryo. This motivated researches to investigate the possibility to isolate ESCs straight from mouse embryos leading to the establishment of the first mouse ESC lines in 1981 (Evans & Kaufman, 1981; Martin, 1981).

Establishment of the first human ESC line by James Thompson in 1998 lagged far behind their mouse counterparts (Thomson et al, 1998). The lack of human embryonic material and also legal and ethical problems are possible explanations for this delay. Ever since the development of research in the area of human pluripotent stem cells has been

extremely fast finally leading to the discovery of hiPSCs in 2007 (Takahashi et al, 2007). hiPSC technology enables derivation of patient specific iPSC lines, disease modeling and personalized drug testing *in vitro*, and thus has revolutionized the field of stem cell research.

2. Human embryonic stem cells

A structure called blastocyst is generated by multiple mitotic cell divisions during the embryogenesis soon after fertilization of an egg. The inner cell mass (ICM) of the blastocyst develops into the embryo while the outer cells, called trophoblasts, forms extra-embryonic tissue including the placenta, chorion, and the umbilical cord (Hardy et al, 1989). hESC lines are derived from the ICM of embryos that are produced by *in vitro* fertilization (IVF) for clinical purposes. To date hundreds of hESC lines have been generated from donated embryos (http://www.iscr-admin.com; http//hpscreg.eu/).

2.1. Characterization of hESC

Characteristic hESCs have high nucleus to cytoplasmic ration. They grow in tightly packed colonies with defined borders at the periphery. hESC are also characterized by presence of pluripotency specific surface markers like stage-specific embryonic antigen-4 (SSEA-4), tumor related antigens (TRA- antigens), OCT3/4 and NANOG (Thomson et al, 1998). Gene expression and protein synthesis of pluripotency markers are downregulated when the cells start to differentiate.

Telomerase is a ribonucleoprotein that adds telomere repeats to chromosome ends and is involved in maintaining telomere length (Harley, 1991; Harley et al, 1992). The self-renewal capacity of hESC is often evaluated by telomerase activity. Because of constant telomerase activity, hESC have an unlimited lifespan.

hESCs are prone to genetic instability (International Stem Cell et al, 2011). When ICM cells are transferred to the culture dish, the cell environment is drastically changed and hESCs are subjected to selective pressure from their new environment (Baker et al, 2007). Certain mutations, which are affecting apoptotic pathways, differentiation control or cell cycle, can provide growth advantages. These karyotypically abnormal cells could then easily take over the cell cultures in several passages. This is called ‘culture adaptation’ (Enver et al, 2005). Karyotypic abnormalities may reduce the differentiation capacity of hESC and increase the risk of tumorigenic effects *in vivo* (Baker et al, 2007; Draper et al,

2004; Mitalipova et al, 2005). Therefore hESCs are frequently karyotyped, the karyotype should be normal 46,XX or 46,XY.

The most important functional property of hESCs is their capability to differentiate. The pluripotent nature of hESCs is traditionally examined either *in vitro* with embryoid body (EB) assay, in which the cells form aggregates in suspension cultures, or *in vivo* with teratoma assays, in which hESCs are transplanted into immunodeficient mice (Mikkola et al, 2006; Yirme et al, 2008). Upon both assays the cells should be able to give rise to all three different germ layers; ectoderm, mesoderm and endoderm.

3. Induced pluripotency

"Pluripotency" in cell biology indicates a cell that has the potential to differentiate into any of the three germ layers (ectoderm, mesoderm, and endoderm) formed upon gastrulation. The term pluripotency is derived from the Latin words *plurimus* meaning "very many" and *potens* meaning "having power". "Induced pluripotency" indicates a biological situation where a cell that is not pluripotent is forced to become pluripotent.

4. Cellular reprogramming and human induced pluripotent stem cells

Finding of hPSC has been brilliantly described as a synthesis of long known scientific principles with newly described technologies (Stadtfeld & Hochedlinger, 2010). Already in the 1950s John Gurdon with colleagues reprogrammed somatic cells by transferring the nucleus of a somatic cell into an enucleated oocyte (somatic cell nuclear transfer, SCNT) leading to successful development of cloned *Xenopus Laevis* (Gurdon et al, 1958). Later the cloning of Dolly the sheep (Campbell et al, 1996) and other animals like mice (Eggan et al, 2004; Hochedlinger & Jaenisch, 2002) showed that the genome of even fully differentiated cells remains genetically totipotent and so can support the development of an entire organism.

The discovery of induced pluripotency was influenced by the observation that specific transcription factors (TF) are driving the cell type specific gene expression and thereby affecting cellular differentiation and maintenance of cellular identity (Stadtfeld & Hochedlinger, 2010). In addition to driving the expression of cell-type specific genes, TFs can suppress lineage-unsuitable genes. The first evidence of the power of TFs was discover already in 1987 when researchers found that transfection of skeletal muscle factor myoblast determination protein (MyoD) cDNA in fibroblasts caused the formation of myofibres from the transfected cells (Davis et al, 1987). Later T and B cells were

successfully converted into macrophages (Xie et al, 2004). These early trans-differentiation experiments provided evidence of somatic cell plasticity.

Taken together, the knowledge of SCNT, power of TFs and the evidences of somatic cell plasticity led to the breakthrough finding by Takahashi & Yamanaka, who first demonstrated direct reprogramming of adult mouse fibroblasts into pluripotent state (Takahashi & Yamanaka, 2006). This "first generation" iPSCs were derived by overexpressing four genes, *Octamer-binding transcription factor 3/4* (*OCT3/4*, O), *Sex determining region Y-box 2* (*SOX2*, S), *Krüppel-like factor 4* (*KLF4*, K) and *v-myc avian myelocytomatosis viral oncogene* (*C-MYC,* M), referred later in literature as "Yamanaka Factors, OSKM". Soon after the same group reported successful iPSC induction from adult human fibroblasts (Takahashi et al, 2007). Interestingly, shortly after Yamanaka's discovery Thomson's team reported successful reprogramming of human fibroblasts into pluripotent stage with a different set of reprogramming factors (Yu et al, 2007). Instead of using OSKM, they reprogrammed the cells with *OCT3/4*, *SOX2*, *NANOG* and *LIN28*. Professor Shinya Yamanaka and Sir John B. Gurdon were awarded with the Nobel Prize in Physiological Medicine in 2012 for their outstanding work, which led to discovery of iPSC technology.

hiPSC technology has revolutionized the field of stem cell research, since these cells enable patient specific disease modeling and cell based therapies. Rapid development of genome editing tools has made it possible to correct or cause specific mutations in the genome (Merkle et al, 2015; Ramalingam et al, 2014; Yusa et al, 2011). Therefore hiPSC based autologous (the cell donor and recipient are the same person) cell transplants are very likely a part of the regenerative medicine in the future. Derivation of hESC and hiPSC and their applications are described in Figure 1.

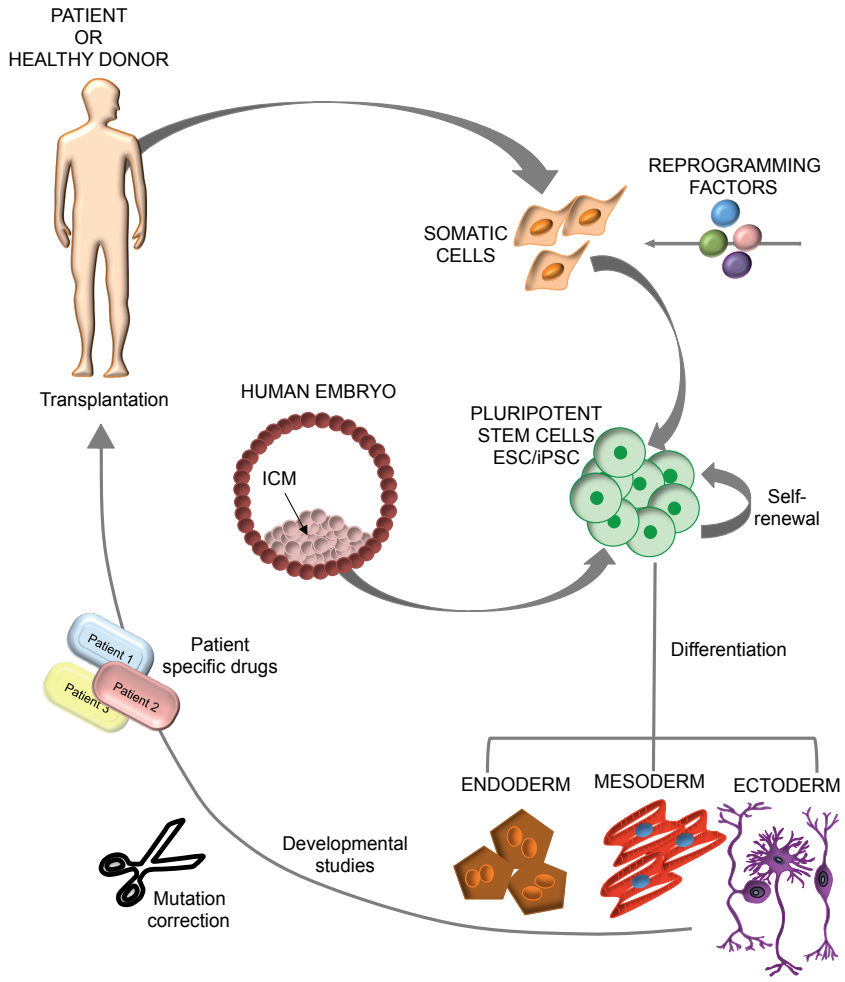

Figure 1. hPSCs can be derived either from the ICM of a human embryo or from human somatic cells by reprogramming them back to pluripotent stage. hPSCs have a potential to self-renew and differentiate into all three embryonic germ layers (endoderm, mesoderm, ectoderm) and their derivatives like hepatocytes (brown), cardiomyocytes (red) and neurons (purple). Cell differentiated from hPSCs can be used in disease modeling and in developmental biology studies. Cells differentiated from hiPSCs can additionally be used in personalized therapies. With novel genome editing tools specific mutations can be corrected in patient specific hiPSCs and in the future the corrected cells could be used as autologous cell transplants.

4.1. Characterization of hiPSC

hiPSCs are traditionally described as highly similar to their embryonic counterparts (Takahashi et al, 2007). They are morphologically similar to hESCs with the presence of pluripotency markers such as OCT4 and NANOG and they show high telomerase activity (Figure 2). hiPSCs can proliferate unlimitedly while maintaining their normal karyotype and they can differentiate similarly than hESCs. TFs used for reprogramming a somatic cell to pluripotent stage must be silenced in newly derived cells. How fast exogenous gene expression is silenced depends on the delivery method used (*Review of Literature, Section 5.*) (Hu, 2014). With Sendai-viral (SeV) system, transgenes should be silenced when the cells have been passaged approximately ten times (Chen et al, 2013).

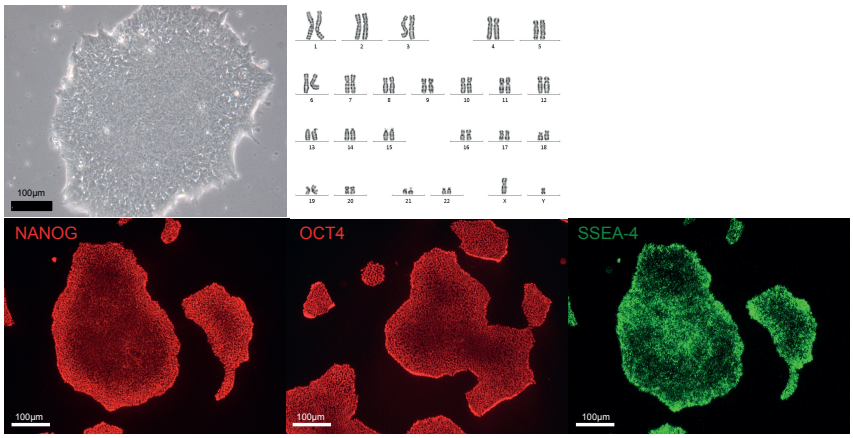

Figure 2. Characteristic morphology, karyotype and pluripotency marker expression of hPSCs. The cell line in the figure is human iPSC line Hel47.2 (Trokovic et al, 2015). Upper panel: Typical morphology of hPSC colony and normal karyotype 46,XY. Lower Panel: hPSC colonies stained positive for pluripotency markers NANOG (red), OCT-4 (red) and SSEA-4 (green).

5. Delivery methods for generating hiPSC lines

The first hiPSC lines were derived by mouse gamma retrovirus-based transgene expression system (Takahashi et al, 2007; Takahashi & Yamanaka, 2006). Another retrovirus-based vector that is commonly used for iPSC derivation is derived from Lentiviruses (LV) (Yu et al, 2007). Retroviral-based methods are genome integrating, which always involves a risk of insertional mutagenesis. In addition, another concern of integrating systems is residual expression of reprogramming factors and their potential reactivation since all of the reprogramming factors are tumorigenic if they are reactivated after transplantation. These issues prevent clinical use of iPSCs derived with the methods in which reprogramming factors are integrating in the genome (Nakagawa et al, 2010). Alternative approaches have been established for iPSC reprogramming. For instance, polycistrons are generated for reducing the number of integrations (Chang et al, 2009) and systems to excise the transgene from the genome after reprogramming have been established (Chakraborty et al, 2013). Protein transduction is a technology to delivery exogenous proteins into cells in culture (Matsushita & Matsui, 2005) and used for hiPSC reprogramming. However, this method is relatively inefficient (0.001%) and slow (Kim et al, 2009).

Somatic cells can also be forced to express pluripotency genes with RNA based approaches. RNA reprogramming can be achieved with RNA viruses like Sendai-viruses (SeV) (Ban et al, 2011), RNA replicons (Warren et al, 2012), miRNAs or synthetic mRNAs (Miyoshi et al, 2011).

Epstein Barr virus-based self-replicating episomal vectors have also been successfully used for reprogramming hiPSC from fibroblasts (Chen et al, 2011; Yu et al, 2009), blood cells (Su et al, 2014) and keratinocytes (Piao et al, 2014). hiPSC derived with episomal system may have advantages over hiPSCs generated with viral vectors since they have been shown to display lower immunogenicity (Zhao et al, 2011). In addition, small molecules can be used together with other reprogramming methods to achieve better reprogramming efficiency (Shimada et al, 2012).

Two reprogramming methods, Retroviral vectors and SeV vectors, will be discussed below in more detail.

5.1. Reprogramming with Retroviral vector

Retroviral transduction is the most widely used method for transgene delivery and gene therapy in cell and animal models (Hu, 2014). Retroviral vectors transduce effectively murine cells but not human cells, wherefore human fibroblasts have to be sensitized with murine viral receptor, mCAT1, which can be delivered into the cell with LV (Takahashi et al, 2007). Retrovirus can also be modified to transduce human cells. These pseudotypes of retroviruses are either amphotropic retroviral vectors or pantropic retroviral vectors (Hu, 2014). Amphotropic retroviral vectors have low reprogramming efficiency (16-28%), which is also depending on the cell type (Aasen et al, 2008; Oda et al, 2010). Pantropic retroviral vectors transduce human cells more efficiently and have been used for reprogramming multiple cell types (Brown et al, 2010; Zhou et al, 2012).

The silencing process of retroviruses in the cells starts already approximately after 4 days of culturing, however, silencing is not completed until the cells have been in culture for a longer period, usually at least 10 to 20 passages (Stadtfeld et al, 2008). The silencing also depends on possible epigenetic regulators like DNA methylation and histone modifications (methylation and deacetylation). Cytosine methylation of DNA implicates retroviral silencing since silent retroviruses are heavily methylated at CpG sites. Acetylation of histones leads to open euchromatin (lightly packed form of chromatin) formation and actively expressed retroviruses. Histone deacetylases (HDAC) (Cherry et al, 2000) catalyze the deacetylation of histones and participates retroviral silencing. Methylation of histone tails can be either repressive or active marks, depending on specific methylation sites (Hotta & Ellis, 2008).

The concern of re-activation and incomplete silencing of transgenes compromises the value of retrovirally derived hiPSCs. Moreover, retrovirally generated iPSCs are more immunogenic than those made with non-integrating methods (Zhao et al, 2011).

5.2. Reprogramming with SeV vectors

SeVs have been successfully used for hiPSC reprogramming of multiple human cell types (Ferrari et al, 2004; Fusaki et al, 2009; Seki et al, 2010). SeVs are DNA free RNA viruses that do not integrate into the host genome. Reprogrammed cells lose all their viral genomes upon proliferation. Hence, SeV vectors are powerful tools for basic research as well as molecular therapy and in regenerative medicine approaches (Nakanishi & Otsu, 2012). Also temperature sensitive SeVs have been generated (Ban et al, 2011). In that system, short temperature changes can remove viral genome from reprogrammed cells faster than from normal SeV reprogrammed cells. Since SeVs do not have any DNA phase in their life cycle, transgenes delivered with SeV vectors cannot be silenced by epigenetic modification. SeV is not pathogenic as such, however, it can infect the airway epithelium (Ferrari et al, 2004). Therefore, in handling of SeVs strict safety regulations must be followed, especially after oncogenes like *KLF-4* or *c-Myc* are installed in the vector.

6. hESCs and hiPSC, identical counterparts?

Superficially hiPSCs are identical with their embryonic counterparts. However, extensive exploration has shown that even individual hESC lines have more tendency to differentiate into a certain cell lineage or cell type than to another (Mikkola et al, 2006; Osafune et al, 2008). Many comparative studies have shown that hESC lines, as a group, differ from hiPSC lines. It has been long thought the cellular origin of ESCs and iPSCs could lead to significant differences between these two pluripotent cell types (Chin et al, 2009; Ghosh et al, 2010; Polo et al, 2010). Also the age of hiPSC lines impact on them; Late-passage hiPSCs resemble more hESCs in their gene expression signature than early-passage hiPSCs. In addition, a comparative genome-wide study between mouse iPSCs and ESCs has shown iPSCs to retain a unique gene expression profile defining them from ESCs (Chin et al, 2009).

Histone modifications and DNA-methylations are epigenetic marks that affect gene expression independently of the DNA sequence. The epigenetic status of the cell is cell-type specific. iPSCs derived from different somatic cell types have been shown to have residual epigenetic memory of their origin, which can affect their further differentiation

potential (Hu et al, 2010; Kim et al, 2011). Thus, successful reprogramming process requires complete erasure of the existing somatic epigenetic memory before the cell can be considered as pluripotent.

More recent studies, however, suggest that the transcriptional variation and differences in differentiation propensities between various hPSC lines is rather donor than cell-type dependent (Kajiwara et al, 2012; Rouhani et al, 2014). Moreover, analysis of genetically matched hESC and hiPSC lines has proven that these lines really are transcriptionally and epigenetically highly similar (Choi et al, 2015b).

The equivalence of hiPSCs and hESCs remains controversial, however, in the light of the recent findings it seems that transcriptional and epigenetic variation originating from genetic background dominates over variation due to cellular origin.

6.1. Establishing the naïve pluripotency

hPSCs differ from mouse PSCs in their differentiation potential, morphology and mechanisms that control their pluripotency (Fonseca et al, 2015). Mouse ESCs are derived from pre-implanted embryos (Evans & Kaufman, 1981). Subsequently mouse stem cell lines are also derived from post-implanted embryo. These cells are called epiblast stem cells (EpiSCs) (Brons et al, 2007). EpiSCs are more similar to hESCs in their morphology and epigenetic status than mouse ESCs (Tesar et al, 2007). EpiSCs represent a later stage in mouse development than mouse ESCs. Mouse ESC lines are more homogenous and more frequently give rise to chimeric embryos than EpiSCs (Huang et al, 2014a). These findings have raised the question whether hESCs are already at a more advanced differentiation stage than the mouse counterparts and whether it is possible to turn hESC into more primitive pluripotent stage. To date it has been shown that hESC conversion into a more pluripotent stage really is possible (Gafni et al, 2013; Takashima et al, 2014). These cells are called naïve or ground state hESCs. Numerous different methods to induce ground state pluripotency are described (Dodsworth et al, 2015). If the ground state pluripotency is true and effective method for derivation of naïve hPSCs can be established, these cells could be a solution to the heterogeneity problem of hPCSs.

7. Liver

Liver is the largest inner organ found in the human body having many essential functions. It synthesizes and stores amino acids, proteins, vitamins and fats and it plays an

important role in glycogen storage. The liver takes care of the blood filtration when the portal vein brings low oxygenated blood from the gastrointestinal tract. Liver is also responsible for cholesterol synthesis and transport, urea metabolism and bile production.

7.1. Liver architecture

The liver has a complex and unique architecture. Traditionally the liver is divided in hexagonally shaped functional units called lobules (Ishibashi et al, 2009). The liver contains approximately one million lobules. Parenchymal liver cells, hepatocytes, are organized in the lobule as cords lined by sinusoidal capillaries. Hepatocytes are mainly responsible for liver functions and occupy approximately 80% of the liver volume (Blouin et al, 1977). Cholangiocytes represents the other parenchymal cell type of the liver. Cholangiocytes are epithelial cells of the bile duct. Hepatocytes and cholangiocytes maintain liver functions by collaborating with endothelial cells, sinusoidal endothelial cells, Kuppfer cells, Pit cells and hepatic stellate cells. Pit- and Kuppfer cells are liver specific cells of the immune system (Si-Tayeb et al, 2010a). Stellate cells are perisinusoidal cells that maintain ECM, store Vitamin A, contributes to the regenerative response to injury and secrete cytokines (Taub, 2004).

Three vessels, a portal vein, bile duct and hepatic artery, are located in each periportal corner of the lobule. These three vessels are forming a structure called the portal triad. The portal vein is bringing low oxygenated blood from the gastrointestinal tract and spleen into the liver for hepatocyte filtration whereas the hepatic artery is bringing oxygenated blood from the heart into the liver. Blood from both sources is mixed in the liver sinusoids and flows toward the central vein that drains blood out from the liver. The central vein is located in the pericentral area of the lobule (Godoy et al, 2013; Si-Tayeb et al, 2010a). The architecture of a liver lobule is illustrated in Figure 3.

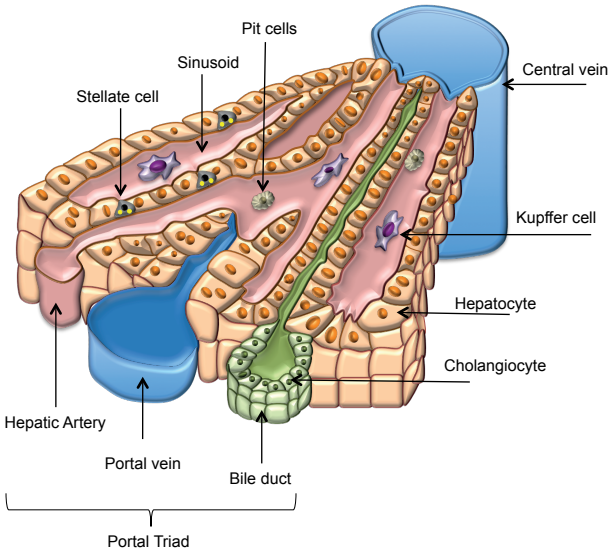

Figure 3. The liver lobule is the functional unit of the liver. Hepatocytes (orange) are organized as cords lined by the sinusoidal capillaries. Blood from the portal vein and hepatic artery flows through sinusoids towards the central vein. Bile acids, synthetized by hepatocytes, are secreted in bile canaliculi. The bile flows in the opposite direction than blood towards the bile ducts. Bile duct is formed from another parenchymal cell type, cholangiocytes (green).

7.2. Hepatocyte polarization, drug metabolism and transporter activity

Hepatocytes are polarized in a very unique way with apical and sinusoidal cell surface (Treyer & Musch, 2013). Sinusoidal (also known as basolateral) surface is faced toward sinusoidal endothelial cells. Endocrine secretion from the hepatocytes into the blood happen through the sinusoidal membrane. Bile canaliculi are formed on the apical surface between two hepatocytes and are surrounded by tight junctions (Godoy et al, 2013). Bile acids and bile salts are secreted into canaliculi from which the bile flows towards bile duct, in the opposite direction than blood (Gissen & Arias, 2015).

Liver is the major organ responsible for elimination of drugs and other xenobiotics. The greatest accumulation of drug metabolizing enzymes is in the liver, although other tissues also show some capacity to metabolize drugs, especially intestine, lungs and kidneys (Orhan, 2015).

Drugs enter into hepatocytes either with passive diffusion or via active transporters, such as solute carrier organic anion transporter family members OATP1B1 or NTCP, which are located on the sinusoidal membranes of hepatocytes (Faber et al, 2003). For many drugs, metabolism occurs in two phases, phase I and phase II (Moscovitz & Aleksunes, 2013). In the cytoplasm phase I enzymes introduce reactive and polar groups to the drug via oxidation, reduction or hydrolysis reaction. Then Phase II enzymes catalyze conjugation

of activated metabolites (phase I processed drug) with endogenous substances, like glucuronic acid. Drug metabolites are then excreted into bile or back to the blood either passively or via specific efflux transporters, like MRPs, MDRs or BSEPs (Faber et al, 2003). Taken together, drug metabolites are usually more hydrophilic than the parent drugs facilitating the excretion of metabolites through urine out of the body.

Correct organization and polarization of hepatocytes is crucial for their metabolic functions. Polarity can be divided into two categories, structural and functional. Structural polarity includes correct formation of the apical membrane with one or more canaliculi sealed by tight junctions, and the plasma membrane with microvilli. Functional polarity includes correct flow of various proteins across canalicular and sinusoidal membrane (Gissen & Arias, 2015). The protein flow is possible when transporters are correctly localized on the cell membranes. Defects in hepatocyte polarization can lead to major pathophysiological consequences (Gissen & Arias, 2015). The polarization of hepatocytes, drug metabolism and localization of transporters are illustrated in Figure 4.

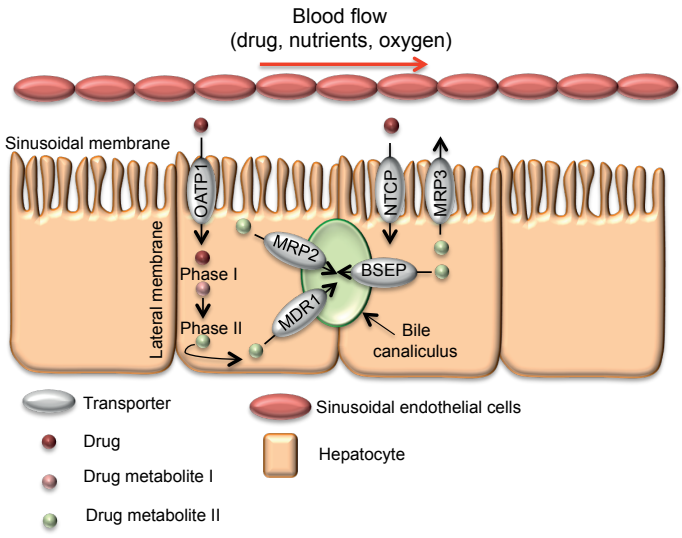

Figure 4. Simplified illustration of hepatocyte polarization and drug metabolism. The sinusoidal (or basolateral) membrane faces towards blood. Drugs are taken into the cells either passively or via influx transporters through sinusoidal membrane. Drug metabolism phase I and II enzymes are processing drugs in the cytoplasm. Drug metabolites are transferred either into bile canaliculi or blood via efflux transporters.

7.3. Liver zonation and heterogeneity of hepatocytes

Hepatocytes are localized in three different zones in the liver lobule along the porto-central axis. The periportal area of the lobule is Zone 1 (periportal) whereas Zone 3 (pericentral) is located close to the central vein. Zone 2 (midzonal) is located between zone 1 and 3 (Jungermann & Katz, 1989).

Hepatocytes in the liver are not identical in their function. Different metabolic functions are enriched in hepatocytes depending on their localization in the liver (Jungermann, 1995). Activities of metabolic enzymes can be zonated either in gradients or in compartments. If a metabolic enzyme is present in all hepatocytes, but in different amounts of activities, is it assigned to the gradient type of zonation, whereas an enzyme, which is present only in one zone is assigned to the compartment type (Schleicher et al, 2015). Also certain proteins are synthesized in hepatocytes in gradientally manner. For instance, all hepatocytes are secreting Albumin, however, the secretion is stronger in periportal than in pericentral hepatocytes. In contrast, plasma protein transthyretin and transferrin transporters are present in all hepatocytes in a zone-independent manner (Gascon-Barre et al, 1989).

Zonation patterns can be additionally described either as ”stable” or “dynamic”. A zonation pattern that does not change in different nutritional conditions represents stable pattern, while changes in nutritional or hormonal conditions are affecting on dynamic pattern. For instance, glutamine synthetase is active only in the pericentral area of the liver and represents stable enzyme. Most of the metabolic enzymes, however, are sensitive for nutritional changes and thus represent dynamic zonation pattern (Schleicher et al, 2015). Hepatocytes in each zone are more or less contributing in drug metabolism but they differ in the activity of various phase I and phase II enzymes (Godoy et al, 2013).

The liver zonation is regulated by the level of oxygen and nutrients in the blood and also by Wnt/β-catenin signaling activity (Gebhardt & Hovhannisyan, 2010; Gebhardt & Matz-Soja, 2014). Periportal hepatocytes are located close to the hepatic artery and are thus rich in oxygen and nutrients. On the contrary, the activity of Wnt/β-catenin signaling is gradientally increasing from periportal to pericentral direction (Gebhardt & Hovhannisyan, 2010; Gebhardt & Matz-Soja, 2014). ECM in adult liver is also very different in periportal than pericentral areas and thus affecting on liver zonation. The periportal area is rich in laminins, and collagen type II and IV while fibronectin and type I collagen are accumulated in the pericentral site (McClelland et al, 2008). Periportal hepatocytes are active in glucose uptake, β-oxidation, cholesterol synthesis and urogenesis (Kietzmann & Jungermann, 1997). In contrast, pericentral hepatocytes located in the zone 3 perform glucose uptake and glycogen synthesis from glucose. They are also active in synthesis of fatty- and bile acids and heme. Most of the enzymes involved in drug metabolism are accumulated in pericentral hepatocytes (Godoy et al, 2013). Liver zonation is schematically represented in Figure 5.

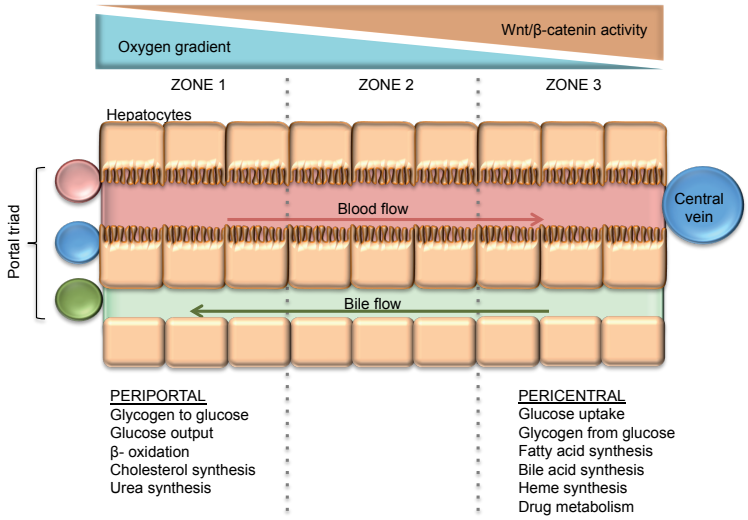

Figure 5. Schematic illustration of “metabolic zonation” of the liver. Blood flows from the periportal area towards central vein. The concentration of oxygen is decreasing from periportal to pericentral site, while Wnt/β-catenin activity is increasing in the opposite direction.

8. Overview of liver development

Mammalian embryonic development has been extensively studied with mouse models. During embryogenesis the inner cell mass of the developing embryo goes through gastrulation forming three germ layers, ectoderm, mesoderm and endoderm. Endoderm is a single cell thick layer, which forms the primitive gut tube when the embryo rotates along the anterior-posterior axis. Primitive gut tube is patterned into three progenitor domains, foregut, midgut and hindgut (Tremblay & Zaret, 2005). The foregut endoderm germ layer gives rise to the liver along with the ventral pancreas, lungs, thyroid and gastrointestinal tract by inductive signals from surrounding tissues (Wells & Melton, 1999). Liver development initiates when the ventral domain of the foregut thickens and forms the liver diverticulum. Subsequently, the diverticulum thickens and the cells go through morphological transitions turning into cuboidal hepatoblasts, a common progenitor for hepatocytes and cholangiocytes (Bort et al, 2006). Proliferating hepatoblasts delaminate into septum transversum mesenchyme (STM) forming a structure called the liver bud. The liver bud continues growing as it is vascularized and colonized by hematopoietic cells. Final maturation of the liver continues into the postnatal period.

Growth factors and signals from surrounding tissues regulate liver development in time and concentration dependent manner. Numerous efforts have been invested to study the transcriptional control of liver development, mainly with animal models and recently also with hPSCs (Gordillo et al, 2015; Zaret, 2002). The signaling pathways and transcriptional

regulation of liver development will be discussed in more detailed in the following chapters.

8.1. Wnt/β-catenin in early endoderm development

β-catenin is a protein playing a dual role in cells. It is involved in cell adhesion processes but also acts as a signaling effector (Lade & Monga, 2011). β-catenin signaling is regulated post-translationally since it is constitutively present in cytoplasm and the protein activity is regulated by phosphorylation events (Aberle et al, 1997). Cytoplasmic β-catenin is phosphorylated by a specific destruction complex, which consists of five components; AXIN, Adenomatous Polyposis Coli (APC), protein phosphatase 2A (PP2A), glycogen synthase kinase 3 (GKS3) and casein kinase 1 (CK1). The phosphorylated β-catenin goes through ubiquitination, which then allows its degradation. Nevertheless, secreted Wnt protein can induce the stabilization of β-catenin by binding its transmembrane receptor Frizzled (FZD) (Kikuchi, 2000). Active β-catenin is then translocated into the nucleus where it can bind to genome binding TFs and in this way regulates its target gene expression. Also some growth factors, like hepatocyte growth factor (HGF), can activate β-catenin signaling by binding to its tyrosine kinase receptors (Purcell et al, 2011). The Wnt/β-catenin signaling pathway is schematically represented in Figure 6.

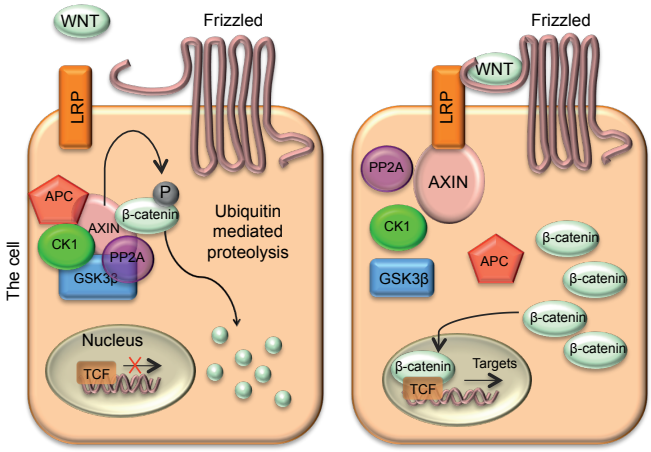

Figure 6. Wnt/β- catenin signaling. β-catenin is constitutively expressed in the cell. In absence of activating signals (shown on the left), like Wnt, a destruction complex phosphorylates β-catenin, which then is degraded via ubiquitin mediated proteolysis. If Wnt is present (shown on the right), its binds to transmembrane protein Frizzled, which forms a dimer with low density lipoprotein receptor-related protein (LRP) receptor. LRP binds to intracellular AXIN disturbing the formation of the destruction complex. This leads to dephosphorylation of β-catenin and translocation into the nucleus. β-catenin binds to T-cell factor (TCF) and lymphoid enhancer-binding protein (LEF)-family transcription factor (not in figure) and activates the expression of target genes.

Wnt signaling contribution to embryonic development is complex and still not fully understood. Nevertheless, Wnt signaling has a crucial role in primitive streak and endoderm patterning (Lade & Monga, 2011). Wnt/β-catenin signaling together with fibroblast growth factors (FGF) and bone morphogenic proteins (BMP) activate Nodal (*Review of Literature, Section 8.2*), which then initiates the gastrulation in the posterior epiblast (Haramoto et al, 2004; Onuma et al). After definitive endoderm formation Wnt signaling acts more actively in the posterior part of the endoderm repressing expression of *Hhex,* an important TF regulating hepatic development (Bort et al, 2006). Thus, repression of Wnt/β-catenin signaling in the anterior part of the definitive endoderm is required for *Hhex a*ctivation and proper hepatic commitment.

8.2. Nodal, member of TGFβ- superfamily

Nodal, three types of activins and different BMPs are members of the transforming growth factor β (TGFβ) superfamily (Kingsley, 1994; Shen, 2007). Nodal is a crucial factor for the initiation of gastrulation and also in mesoderm and endoderm segregation from the bipotential mesendoderm; high Nodal activity promotes endoderm development while lower Nodal activity guides mesodermal development (Vincent et al, 2003). Activin A is almost universally used as substituent to Nodal *in vitro*, as it binds to the same cell surface receptor as Nodal and activates the same intracellular effector proteins (D'Amour et al, 2005; Moustakas & Heldin, 2009). In general, TGFβ-ligands bind to heterotetrameric complex of different kinds of serine/threonine kinase receptors, known as type I and type II receptors (Kingsley, 1994; Massague, 1998). Activin/Nodal can bind to two types of type II receptors, which induce phosphorylation of type I receptors subsequently leading to phosphorylation of cytoplasmic Smad2/3. Phosphorylated Smad2/3 forms a complex with Smad4, which enters into the nucleus and regulates the expression of its downstream target genes (Figure 7).

TGFβ-signaling is affecting a diversity of cells and their gene expression and is relatively complex due to the large amount of different ligands and receptors (Kingsley, 1994). Moreover, the same ligands of the TGFβ-family are able to activate different genes in a cell-type- dependent manner. More recent findings suggest that cell type specific master TFs are responsible for directing the genomic targeting of SMADS in hESCs and hence determining the cell-type-specific effects of TGFβ-signaling (Mullen et al, 2011).

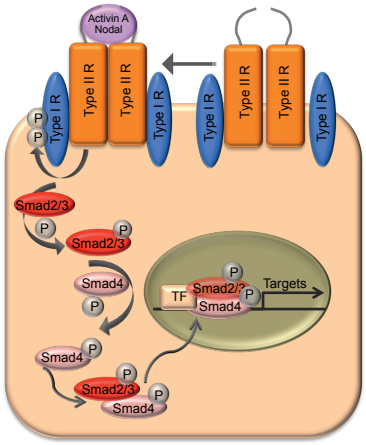

Figure 7. Nodal and its agonist Activin A are both binding to serine/threonine TGF-β type II receptor dimer kinase (Goumans & Mummery, 2000). Type II receptor dimer and the ligand then forms heterotetrameric complex with type I receptor. Type II receptors catalyzes the phosphorylation of Type I receptor, which induces the phosphorylation of Smad2 and Smad3 in cytoplasm. Phosphorylated Samd2/3 forms complex with Smad4, which accumulates in the nucleus and interacts with cell specific TFs to regulate the expression of target genes (Tian & Meng, 2006).

8.3. Hepatic specification from definitive endoderm

Hepatic development has been extensively studied with animal models. The first sign of liver development is seen when the cells in three distinct domains in ventral foregut endoderm start to express *Alphafetoprotein* (*Afp*) (Tremblay & Zaret, 2005). Proper initiation of hepatic induction requires BMPs secreted from STM and FGFs secreted from early cardiac mesoderm (Jung et al, 1999; Rossi et al, 2001). It is important to understand that the same signaling pathways are regulating also the development of other endodermal organs. For instance, FGF-mediated hepatic specification is highly concentration dependent. During the embryonic development, FGF levels are adjusted by controlling the position of liver endoderm relative to the developing heart. During embryogenesis cardiac mesoderm moves away from the hepatic endoderm thereby keeping the FGF concentration at low level (Calmont et al, 2006). Low concentration of FGFs induces liver specification while high FGF levels activate lung specific genes (Serls et al, 2005). FGF- mediated activation of hepatic genes is controlled through the activation of phosphatidylinositide 3-kinase (PI3K) independent MAPK pathway (Calmont et al, 2006). BMPs has been shown to be important for activation of *Albumin* gene expression (Wandzioch & Zaret, 2009) and studies in mice have indicated that BMPs also activate the expression *Gata4* (*Review of Literature, section 8.6*) and is that way associated with the specification of hepatic endoderm (Rossi et al, 2001).

As the ventral foregut endoderm cells commit to hepatic fate, Wnt signaling is actively repressed and BMP promotes *Hhex* (*Review of Literature, section 8.6*) gene expression (Pilcher & Krieg, 2002). In contrary, highly active Wnt signaling together with FGFs promotes the midgut and hindgut specific gene expression and suppresses the expression of hepatic genes in a dose dependent manner (Dessimoz et al, 2006).

8.4. Liver bud formation

The newly committed hepatic endoderm cells start to proliferate and form the thickened structure called liver diverticulum. The liver diverticulum is surrounded by a laminin-rich basal membrane, which breaks down and allows the hepatoblasts to delaminate into the STM and start liver bud formation (Margagliotti et al, 2008; Si-Tayeb et al, 2010a). The vascular endothelial growth factor (VEGF) secreted from endothelial precursors located between hepatic epithelium and STM is guiding hepatoblast delamination (Matsumoto et al, 2001). FGFs secreted from the cardiac mesoderm and BMPs from STM are promoting hepatocyte migration as well as the growth of the hepatic bud (Berg et al, 2007; Calmont et al, 2006). HGF is supporting both, hepatoblast proliferation and migration (Medico et al, 2001) and active Wnt/β-catenin signaling promotes hepatic growth (Monga, 2014). In addition, developmental studies performed with mouse models has shown HGF and FGFs to stimulate the activity of β-catenin and that way controlling the cell survival during liver bud formation (Berg et al, 2007; Monga et al, 2003). Liver development is schematically presented in Figure 8.

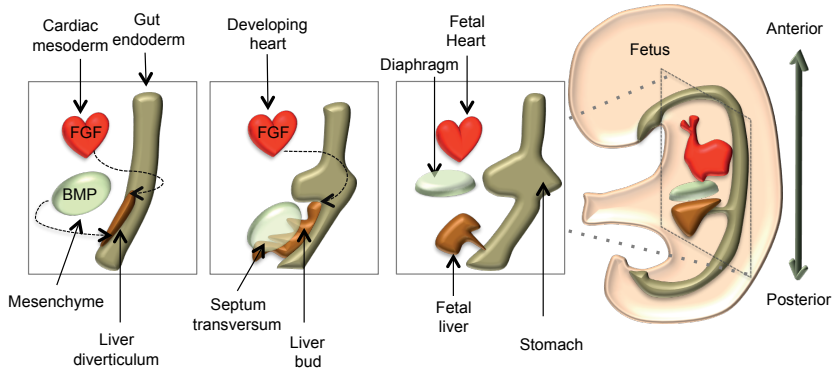

Figure 8. Liver development. Hepatic endoderm cells are located in the ventral part of anterior foregut endoderm. FGF secreted from the developing heart (red) and BMP from septum transversum mesenchyme (STM) (light green) are driving the formation of hepatic diverticulum (brown). Developing liver is moving away from the developing heart keeping the FGF concentration low. Hepatic bud is formed when proliferating hepatoblasts migrate into STM finally leading to the formation of fetal liver. Combination of numerous growth factors released from the surrounding cells in addition to complex transcriptional network controls the fetal liver maturation. Final liver maturation occurs after birth.

8.5. Hepatocyte and cholangiocyte maturation

Fetal liver contains three distinct hepatic cell populations; i) Hepatoblasts, which express AFP, Albumin and Cytokeratin-19 (CK-19) and are able to differentiate into cholangiocytes or hepatocytes, ii) AFP/CK19 positive cholangiocytes and iii) Albumin/CK18 positive hepatocytes (Gualdi et al, 1996). Many signals from surrounding tissues are guiding either hepatocyte or cholangiocyte maturation from hepatoblasts.

Initiation of cholangiocyte differentiation occurs around the portal veins (Couvelard et al, 1998). Differentiating cholangiocytes are first forming a monolayer and then a bilayer of cuboidal cells. Finally the bilayer gets surrounded by portal mesenchyme and undergo tubulogenesis forming intrahepatic bile ducts (Lemaigre, 2003). Epidermal growth factor (EGF) activates the Notch pathway in cholangiocytes and supports their maturation (Kitade et al, 2013; Zong et al, 2009). In addition, Wnt/β-catenin signaling also stimulates the expression of EGF (Tan et al, 2005), which along with HGF has been shown to induce hepatic gene expression in *in vitro* hepatocyte cultures (Michalopoulos et al, 2003).

Hepatoblasts, which are not facing towards portal veins start to differentiate into hepatocytes. Once hepatocytes are committed from hepatoblasts, they start to arrange into cords lined by sinusoidal epithelial cells and bile canaliculi. Hepatocytes gradually acquire more mature hepatocyte function and start to polarize. Maturation of hepatocytes is coordinated by Wnt/β-catenin and HGF signaling together with Oncostatin M (OncM) (Nejak-Bowen & Monga, 2008). HGF is binding to MET receptor activating hepatic gene expression (Kitade et al, 2013). OncM secreted by hematopoietic cells is driving hepatocyte specification together with interleukins (IL) such as IL-6. (Kamiya & Gonzalez, 2004).

ECM proteins are known to regulate various cellular functions, including cell differentiation. Therefore also dynamically changing composition of ECM plays an essential role in the cell fate determination of hepatoblasts (Couvelard et al, 1998; McClelland et al, 2008).

8.6. Transcriptional regulation of liver development

To date, the TFs that control the liver development are extensively studied (Gordillo et al, 2015; Si-Tayeb et al, 2010a). Albumin is the best characterized marker for early hepatic cells (Casio and Zaret, 1991). Before the initiation of *Albumin* gene expression Forkhead box (Fox) A and GATA binding factor (Gata) -4 are binding to the *Albumin* enhancer region (Bossard & Zaret, 1998; Gualdi et al, 1996). Binding of these factors opens the

chromatin structure and helps additional TFs to access to the promoter site, which can then activate the expression of specific genes. In other words, FoxA proteins make the cells developmentally “competent” (Kaestner, 2005). In addition to FoxA proteins and Gata-4, homeodomain TF Hnf1b is a critical inducer of liver development. In the absence of Hnf1b the mesenchymal portion of the fetal liver is normal, but cells in the liver bud fail to express hepatic specific genes (Lokmane et al, 2008).

Several TFs are controlling the formation of the liver bud and hepatocyte maturation. *Hhex* encodes a homeodomain protein that has multiple roles in liver development (Bort et al, 2004; Keng et al, 2000; Martinez Barbera et al, 2000). Initially, Hhex regulates hepatic endoderm cell proliferation and positioning of the ventral endoderm within cardiac mesoderm (Bort et al, 2004). Later Hhex is essential to complete the liver bud morphogenesis. Hhex is also needed for hepatocyte maturation from hepatoblasts (Bort et al, 2004; Hunter et al, 2007; Martinez Barbera et al, 2000). Other important factors for hepatoblast migration and liver bud formation are the homeodomain factor Hnf6 and Prospero-related TF Prox1 (Margagliotti et al, 2007; Sosa-Pineda et al, 2000). Hnf6 and Prox1 are differentially required for specifying hepatic (*Prox1*) or cholangiocyte (*Hnf6*) cell fate (Coffinier et al, 2002; Seth et al, 2014). Mutant mouse studies have shown that T Box transcription factor Tbx3 may act upstream of *Prox1* and is though to be important for promoting hepatocyte cell fate and repressing cholangiocyte fate (Ludtke et al, 2009).

Hepatocyte nuclear factor 4 alpha (HNF4a) has been intensively studied with mouse models and its importance has been shown also in hepatocyte differentiation from hESCs (DeLaForest et al, 2011; Li et al, 2000). Analysis of mutant mouse embryos has shown Hnf4a to be important for morphological and functional differentiation of hepatocytes and for the generation of hepatic epithelium (Hall et al, 1995; Li et al, 2000; Parviz et al, 2003). Loss of Hnf4a is severely affecting hepatic architecture and hepatocellular polarity since Hnf4a controls expression of several proteins involved in cell junction assembly (Battle et al, 2006). In addition, Hnf4a is a predominant regulator of mesenchymal-to-epithelial transition (MET) and also in that way controls the generation of correct liver architecture (Santangelo et al, 2011). HNF4a is also shown to be essential for specification of hepatic progenitor cells from hPSCs (DeLaForest et al, 2011).

Adult hepatocytes are very heterogeneous in their gene expression profile and function, mostly depending on their zonational location in the liver lobule. Liver zonation during organogenesis has been extensively studied and most of the results suggest that the

main regulator of zonation is Wnt/β-catenin signaling and its target gene expressions (Gebhardt & Matz-Soja, 2014; Lade & Monga, 2011). Wnt/β-catenin activity increases from the periportal towards the pericentral zones in the liver lobule (Benhamouche et al, 2006; Gebhardt & Hovhannisyan, 2010). Wnt downstream factor, lymphoid enhancer-binding factor 1 (LEF1), is in direct contact with HNF4a and is enhancing its expression and guiding pericentral hepatic differentiation (Colletti et al, 2009).

Although much is know about the transcriptional regulation of the specification and development of the early fetal liver, much less is known about the changes in gene regulation during the postnatal period. The extremely fast switch in gene expression from fetal-to-adult program is biologically interesting since the process is often reversed when hepatic cells become cancerous. Hepatocytes are highly proliferative in the fetal period but become quiescent after the birth and many cell cycle regulated genes become silenced during the perinatal period (Spera et al, 2006). Hematopoiesis is the main function of the fetal liver, whereas adult liver is active in the regulation of metabolism and detoxification processes. Hence many of the genes enriched in the fetal liver are silenced at birth while many other genes are induced (Timens & Kamps, 1997).

Afp represents the best-studied example of perinatal gene silencing (Spear, 1999; Spear et al, 2006). *Afp* is dramatically repressed at birth and become silenced in healthy adult liver but is induced again in the case of hepatocellular carcinoma (HCC). Two factors, Zinc-finger and homeobox 2 (Zhx2) and Zinc-finger and BTB domain containing 20 (Zbt20), have been found to control postnatal *Afp* repression (Belayew & Tilghman, 1982; Pachnis et al, 1984; Perincheri et al, 2005). Interestingly, screening of specific methylation sequences in HCC tissue samples has revealed hypermethylation of the *ZHX2* promoter. Hypermethylation of *ZHX2* promoter leads to reduced expression of *ZHX2*. This indicates that the silencing of *ZHX2* may take part in the progression of HCC (Lv et al, 2006). The understanding of AFP regulation in the adult liver could help to elucidate the basis of changes in gene expression in hepatocellular carcinoma and could also help identify new biomarkers of HCC that would be of value for better prodiagnostics (Peterson et al, 2011).

9. Liver cell models

Liver diseases are an important clinical problem and today the only treatment for severe liver disease is orthotropic liver transplantation (Starzl & Fung, 2010). In the future, cell transplantation could be an alternative for whole organ transplantation. In addition,

pharmaceutical industry needs liver cell cultures to predict and estimate drug metabolism and toxicity in the human liver (Godoy et al, 2013).

Liver and hepatocyte models used so far have their limitations. Interspecies variation in hepatocytes diminishes the value of animal studies. Limited metabolic activity of immortalized hepatic cell lines, like HepG2, hampers their clinical use (Gerets et al, 2012). Primary human hepatocytes (PHH) are theoretically perfect liver cell models, however, their poor availability and loss of function in cell cultures limits their use (Vacanti & Kulig, 2014). Therefore hPSCs may provide an excellent cell source for hepatocyte modeling. hiPSCs also hold a great promise for patient specific drug testing, studying host-pathogen interactions and modeling human diseases (Shlomai et al, 2014; Takayama et al, 2012).

10. Hepatocyte differentiation from hPSCs

Numerous studies on liver development have provided knowledge about the key signaling pathways and transcriptional regulators, which are orchestrating hepatocyte differentiation during mammalian embryogenesis and yielded insight into how hepatocytes could be derived from hPSCs. Upon *in vitro* differentiation hPSCs are guided into hepatic program by systematically following developmental events occurring *in vivo*. The progress of *in vitro* differentiation can be monitored by analyzing the developmental stage specific gene expression at mRNA and protein level.

The breakthrough in endodermal organ differentiation from hESCs can be dated in 2005, when D'Amour and co-workers described a high concentration of Activin A to effectively differentiate hESCs into definitive endoderm (DE) cells (D'Amour et al, 2005). Wnt/ β-catenin signaling activation, either by adding Wnt3a, or other signaling activators such as GSK3-β inhibitor CHIR99021, into medium in the very beginning of DE-differentiation aids endodermal differentiation (D'Amour et al, 2005; Touboul et al, 2010). However, like *in vivo*, repression of Wnt activation has to take place very soon, usually after 24 hour from the initiation of the differentiation. Endoderm formation takes approximately three to five days. DE-cells are characterized by their co-expression of TFs such as FOXA2, Hhex and SOX17 in protein and mRNA level (D'Amour et al, 2005).

DE-cells are then guided to differentiate into hepatoblasts with FGF and BMP supplementation in the culture medium. This step mimics hepatic diverticulum formation *in vivo*, and at this point the cells should express fetal hepatic markers AFP and HNF4a at protein and mRNA level. Further maturation is then carried out with hepatocyte growth

factor (HGF) treatment for several days. Thereafter hepatic maturation is driven with OncM, HGF, glucocorticoids, insulin and other factors known to be important for hepatic development *in vivo*. Finally, the *in vitro* differentiated cells display numerous hepatocyte features like *Albumin* expression and secretion, urea secretion, low-density lipoprotein (LDL) uptake, glycogen storage capability and cytochrome (CYP) P450 activity (Cayo et al, 2012; Touboul et al, 2010). The whole differentiation protocol takes approximately 20 days and is illustrated in the Figure 8.

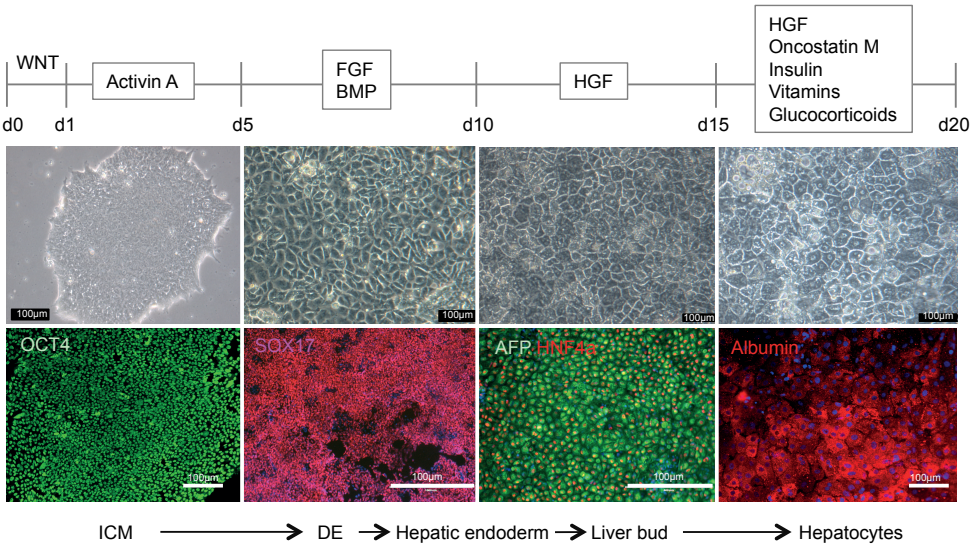

Figure 8. Representative example of hepatocyte differentiation from hPSCs. The timeline of the differentiation is seen in the upper panel. Molecules used for guiding the differentiation, are marked on the time line. The phase contrast microscopy below the time line is describing typical morphological changes of the cells during the differentiation. hPSC are growing as colonies. During the DE induction (d0-d5) colonies disappear and monolayer of cells fills the culture. Between day 10 and 15 the cells gain a polygonal shape. In the end of the differentiation, some of the cells are binuclear and contain lipid droplets. Quality of the differentiation is followed by analyzing the expression of developmental stage specific proteins. Undifferentiated pluripotent cells are expressing OCT4 (green). When the cells reach DE stage, OCT4 expression is lost and the cells express SOX17 (red). Subsequently, expression of AFP (green) / HNF4a (red) indicates hepatoblasts formation. At day 20 of differentiation hepatocyte like cells express Albumin (red). Corresponding developmental stages *in vivo* are shown at the bottom.

Over the recent years many different protocols for hepatocyte derivation from hPSCs have been described (Cayo et al, 2012; Hay et al, 2008; Touboul et al, 2010). Most of them are based on sequential addition of the growth factors described above, though the specific timing and concentrations vary slightly between the protocols.

11. Direct differentiation of somatic cells into hepatocyte like cells

Mouse fibroblasts have been directly converted to hepatic lineage by overexpressing defined TFs (Huang et al, 2011; Sekiya & Suzuki, 2011). More recently, Huang and coworkers demonstrated successful trans-differentiation of human fibroblasts into hepatocyte-like cells by overexpression of *FOXA3*, *HNF1A* and *HNF4a* (Huang et al, 2014b). These human induced hepatocytes (hiHeps) captured hepatic characteristics and were less tumorigenic than the cells derived from hPSCs. Somatic cell reprogramming directly into hepatic cells without a pluripotent step in between, could speed up hepatocyte production. However, these cells still need to be carefully characterized to prove their true hepatocytic identity.

12. Importance of ECM in hepatocyte differentiation and maintenance

ECM proteins, such as laminins, collagens, fibronectin and elastin, have an important role in hepatocyte differentiation and functional maintenance (Aszodi et al, 2006; Couvelard et al, 1998; McClelland et al, 2008). Biological cues from ECM proteins are guiding hepatic cell differentiation and liver zonation together with soluble signaling molecules secreted from the surrounding cells. Compared to other organs, the adult liver is relatively poor in ECM. Intrahepatic biliary cells (cholangiocytes) are surrounded by a typical collagen and laminin rich basement membrane (BM)(Desmet, 1985). In contrast, hepatocytes in the adult liver are in contact with the sinusoidal matrix, which is lacking laminins (Biagini & Ballardini, 1989).

Laminins are playing an important role in liver development. Laminins are heterotrimeric proteins consisting of α, β and γ subunits. Expression of specific laminins is firmly regulated and depends on the adjacent cell and tissue type (Virtanen et al, 2000). Integrins are heterodimeric cell surface receptors, which bind specifically to various ECM proteins, such as laminins (Hynes, 2002). During human liver development, hepatoblast are expressing a broad set of integrins. The integrin expression profile of hepatoblasts is dramatically changing when the hepatoblast is maturing either into hepatocyte or cholangiocyte (Couvelard et al, 1998). This suggests that highly dynamic interactions between fetal liver epithelial cells and mesenchyme is driving hepatocyte and cholangiocyte differentiation from hepatoblasts (Couvelard et al, 1998).

In the adult liver parenchyma, different ECM proteins are accumulated in the specific areas of the liver. Laminins, type III collagen and type IV collagen are found in the

periportal area while type I collagen and fibronectin are accumulated in the pericentral site of the liver (McClelland et al, 2008).

The liver has an amazing capacity to regenerate and most of the studies suggest that the proliferating liver cells are located periportally and ECM proteins along with the signals from the endothelial cells are promoting proliferation of these cells (Font-Burgada et al, 2015; Miyajima et al, 2014). Tanimizu et al. demonstrated the significance of ECM in the proliferation and differentiation of mouse hepatoblasts (Tanimizu et al, 2004). They showed that mouse hepatoblasts were able to proliferate on laminin-coated plates. If the laminin was changed to Matrigel (*Review of Literature, Section 12.1.*), or if the cells were embedded in 3D conditions they start to differentiate into hepatocyte or cholangiocytes, respectively. Similarly, human hepatoblasts have been shown to proliferate and maintain hepatoblast characteristics if they are cultured on laminin, type III collagen or on type IV collagen, all of which are periportal ECM proteins. Like their murine counterparts, human hepatoblasts are differentiating into hepatocytes when cultured on pericentral type I collagen (McClelland et al, 2008). Based on these studies the importance of ECM proteins on liver cell proliferation and maintenance is clear.

12.1. ECM in hepatocyte differentiation from hPSCs

The dynamics of the ECM during liver development causes challenges for hepatocyte differentiation from hPSCs. hPSCs have been traditionally been cultured on mitotically inactivated mouse embryonic fibroblasts (mEF) feeder cells. mEFs secrete growth factors that support self-renewal of undifferentiated hESCs (Thomson et al, 1998). Another commonly used cell culture matrix for hPSCs is Matrigel. Matrigel is a commercially available ECM preparation derived from mouse Engelbreth-Holm-Swarm (EHS) sarcoma. It is rich in type IV collagen and Laminin-111 (Kleinman et al, 1986). Many published hepatocyte differentiation protocols are carried out on mEFs or on Matrigel (Cai et al, 2007; Cheng et al, 2012; Si-Tayeb et al, 2010b). A culture environment used mainly for supporting pluripotency might hamper hepatic maturation during differentiation and thus other ECMs and co-culture systems have been studied for hepatocyte differentiation from hPSCs. Endothelial cells are known to be important for liver bud development. A co-culture system with human umbilical vein endothelial cells and mesenchymal cells has been shown to be beneficial for hepatic differentiation from hPSCs (Takebe et al, 2013). Type I collagen, which is mainly found in the pericentral are of the liver, has been successfully applied for hepatocyte differentiation from hESCs (Agarwal et al, 2008). In addition, Takayama et al. described Lamini-111 rich cell culture platform in which hPSC

derived hepatoblasts were successfully maintained more than 3 months. The cells also maintained their bi-potential differentiation capacity this whole time (Takayama et al, 2013b). More recently, Laminin-521 alone or in combination with Lamini-111 has shown to significantly improve hESC-derived hepatocytes cell function and phenotype (Cameron et al, 2015).

13. Three dimensional culture methods

It is generally accepted that three dimensional (3D) culture environment is necessary for the correct organotypic cell differentiation *in vitro* (Godoy et al, 2013). Cells growing in 3D are different in their morphology and organization compared to cells growing on two dimensional (2D) surface. 3D environment is particularly important for hepatocytes, because their proper functionality is highly dependent on correct cell polarization (Gissen & Arias, 2015). 3D cell culture systems have been studied extensively with murine cells and human hepatoma cell lines.

13.1. 3D culture systems with ECM proteins

3D sandwich cultures, in which cells are growing between ECM hydrogels such as Matrigel or type I Collagen, have been shown to enhance long term functionality, drug metabolism activity and improve formation of canalicular networks in rat hepatocytes (Dunn et al, 1991; LeCluyse et al, 1994; Swift et al, 2010). 3D Matrigel system has also been shown to be beneficial for studying hepatitis C virus cycle within human hepatic carcinoma cells (Molina-Jimenez et al, 2012). More recently, Gieseck et al. described an alternative 3D system to traditional collagen sandwich cultures. They encapsulated hiPSCs derived hepatocytes in neutralized collagen, which resemble physiological collagen density (The Real Architecture for 3D Tissue, RAFT). They showed better polarization, bile canaliculi formation and extended functional lifetime of the cells in 3D compared to 2D cultures (Gieseck et al, 2014).

Several studies have reported decellularized animal livers to be excellent 3D scaffolds for *in vitro* growth of multiple liver cell types (Kajbafzadeh et al, 2013; Soto-Gutierrez et al, 2011; Wang et al, 2011). Mazza and colleagues recently reported the very first study in which decellularized human livers were used as *in vitro* scaffold for human liver cells (Mazza et al, 2015). In this study, single lobes of whole human liver were decellularized and repopulated with different types of human cells, including human hepatic stellate cells, endothelial cells and HepG2 cells. In the future, this kind of approaches could provide alternative use for human livers found to be unsuitable for transplantation. In the wildest

vision, this kind of scaffold could be used to grow a whole new liver for patients suffering from end-stage liver disease. In this case the hepatic cell source could be hiPSCs reprogrammed from somatic cells of the patient.

13.2. 3D hydrogels without cell signaling domains

Natural hydrogels such as alginate, nanofibrillar cellulose (NFC) and polyethylene glycol (PEG) have been used in 3D hepatocyte cultures as such or in mixture with ECM proteins (Cho et al, 2009; Kojima et al, 2009; Malinen et al, 2014; Rebelo et al, 2015). Alginate is derived from marine algae sources and PEG is a synthetic polymer that can be cross-linked to form hydrogel. Crosslinking methods depend on the chain ends of PEG macromers. The NFC (commercially available as GrowDex™) is derived from bacteria or wood and consists of linear chains of glucose that form hydrogels in aqueous environment. NFC has been shown to support spheroid formation of HepG2 and HepaRG cells (Bhattacharya et al, 2012; Malinen et al, 2014). NFC is a particularly interesting biomaterial due to its defined single component structure and good availability.

13.3. Non-adherent systems for 3D hepatic spheroid formation

Multicellular spheroids can form when the cells are able to self-assemble. In spheroid structures cell-cell contacts are maximized. Tight cell-cell- contacts are important especially for hepatocytes because the formation of correct bile canaliculi structures is depended on tight junctions between the apical surfaces of hepatocytes (Vellonen et al, 2014). Various methods for spheroid formation have been reported (Godoy et al, 2013).

Bioreactors are dynamic 3D culture systems, in which porcine, rat and human liver cells have been shown to form tissue-like structures with increased metabolic enzyme activities (Darnell et al, 2011; Domansky et al, 2010; Schmelzer et al, 2009; Zeilinger et al, 2004). 3D bioreactors are also promising platforms for hepatic differentiation from hPSCs. However, bioreactor systems are usually complicated and expensive to set up, making them unsuitable for many researchers.

More practical and non-adherent 3D culture systems are commercially available, however, very few reports have described the use of these systems in hepatocyte differentiation from hPSCs. Special commercial hanging-drop system, GravityPLUS™, has been shown to improve liver-specific functions in HepG2 and HepaRG cells, when compared to 2D cultures (Gunness et al, 2013; Mueller et al, 2014). In addition, hESC derived endodermal cells have been differentiated into hepatocytes in non-adhered sponge like scaffolds

(AlgiMatrix®). The cells displayed better hepatic characteristics in Algimatrix than in 2D (Ramasamy et al, 2013).

14. Challenges in hepatic differentiation from hPSCs

A lot of improvements have been achieved in hepatocyte differentiation from hPSCs during last decade. However, there are still challenges to overcome. A number of different methods have been established for hepatocyte differentiation from hPSCs but none of them are able to produce fully mature hepatocytes (Baxter et al, 2015). In addition, hiPSC lines established in different laboratories from different donors act variably (Kim et al, 2011; Rouhani et al, 2014). Meta-analysis of the genetic homogeneity of different hPSCs from various laboratories has reveled that hPSC lines may have “lab-specific” gene expression patterns, which can affect on their differentiation capacity (Newman & Cooper, 2010). Also, the criteria for the characterization of hPSC-derived hepatocytes, such as Albumin, urea and fibronectin synthesis, phase I and II metabolic enzyme activity and induction of drug metabolism enzyme and transporter, need to be standardized (Hengstler et al, 2005; Sancho-Bru et al, 2009; Snykers et al, 2009).

Methods used to evaluate the hepatocyte functions are very different (Gerbal-Chaloin et al, 2014). For instance, hepatocyte specific function, Albumin secretion, is measured from the culture medium. The result, however, can be presented in many different ways: as quantity per volume or day or cell number or total protein. Results from the same assay described with different units are not comparative. The hepatic phenotype of hPSC-derived hepatocytes is often compared to PHHs, however, the quality of PHHs vary based on the culture conditions and duration. Also the age of the donor and the pathophysiologic status of the liver from which PHHs are isolated affect on the quality (Godoy et al, 2013). This complicates the analysis of hPSC-derived hepatocytes.

The methods used for hepatocyte differentiation from hPSCs are relatively expensive and time consuming. One high throughput system has been described for screening the effect of different signaling molecules on hepatocyte differentiation from hPSCs (Shan et al, 2013). However, low-cost high-productive differentiation system for hepatocytes has not been described.

The presence of remaining hPSCs in the cultures after hepatocyte differentiation poses a risk of tumor formation in case of cell transplantation. One possible way to achieve more homogenous cell populations that lacks undesirable cell types is cell sorting (Goldman et

al, 2013). However, lack of specific cell surface marker for hepatocytes hamper this technique. Epithelial cell adhesion molecule (EpCAM) positive cells isolated from the human liver are shown to be able to proliferate and differentiate into hepatocytes and cholangiocytes *in vitro* (Schmelzer et al, 2007). However, EpCAM is not a specific cell surface marker for hepatocytes, which hinders its use as a marker molecule for hPSCs derived hepatoblasts. Yang et al. described a novel integration-deficient LV-based strategy to purify hepatic cells derived from hPSCs (Yang et al, 2013). They transfected hPSCs with LV-vectors encoding green fluorescent protein (GFP) driven by the hepatocyte-specific apolipoprotein A-II (APOA-II) promoter. APO-II/GFP positive cells were sorted from the cultures and the cells were able to mature into a pure hepatocyte population. Some studies also suggest that specific culture substrates, such as certain type of laminins, automatically selects only hepatoblasts from the differentiation cultures and this way produce pure differentiation outcome (Takayama et al, 2013b).

AIMS OF THE STUDY

Hepatocytes derived from hPSCs are valuable new cell source as a liver cell model. However, differentiation protocols published so far are incapable to produce fully functional, mature hepatocytes. The differentiation efficiency depends on the biological cues provided to the cells and on the propensity of specific hPSC line to differentiate.

The specific aims of this thesis were:

1. To study the capability of various hPSC lines to differentiate into functional hepatocytes. hPSC lines were derived from human embryos or reprogrammed from adult or fetal human fibroblasts with either integrating retroviral or non-integrating SeV-based methods.

2. To investigate how the length of Wnt and Activin A signaling activation during DE differentiation affects the specification of hepatic and pancreatic lineages.

3. To compare the impact of different ECMs and 3D culture environments on the hepatocyte differentiation from hPSCs.

MATERIALS AND METHODS

This chapter describes all materials and methods used in this thesis work except the methods used for cardiomyocyte (I), retinal pigmented epithelium cell (I) and neuronal differentiation (I), which were carried out in BioMediTech (University of Tampere).

1. Ethical consideration

The generation of hESC lines and their use in Biomedicum Stem Cell Center Helsinki (I, II, III, IV) was approved by the Ethics Committee of the Helsinki University Central Hospital (statement nr. 143/E8/01, 18.12.2003). The generation and use (I, II, III,) of hiPSC lines in Biomedicum Stem Cell Center Helsinki was approved by Coordinating Ethics Committee, Helsinki and Uusimaa Hospital District (decision 423/13/00/08, 17.3.2009; and 54/2009, 9.7.2009).

Human embryonic stem cell line 08/023 (hESC3) (I) and human iPSC lines UTA.00112.hFF (hiPSC2) (I) and UTA.01006.WT (hiPSC4) (II) were generated at the University of Tampere with the permission of Ethical Committee of Pirkanmaa Hospital District.

2. Cell lines

Altogether 7 hiPSC and four hESC- lines were used in this thesis work. All the hPSC lines used are listed in Table 1. In addition, other cell lines were cultured; Hepatoma cell line (HepG2) as for a control for hPSC derived hepatic cells (*Materials and Methods, section 2.4*.), JAR choriocarcinoma cells for producing JAR-matrix preparation (*Materials and Mehods, section 2.5 and section 3*), mouse embryonic fibroblasts (mEFs) for the production mEF conditioned medium (*Materials and Methods, section 2.7*) and L-cells for the production of Wnt3a conditioned medium (*Materials and Methods, section 2.8*). All the cells lines were maintained at +37 °C in an atmosphere containing 5% CO_2. All the hPSC lines were routinely tested for a normal karyotype and mycoplasma negativity.

Table 1. hPSC lines used in this thesis.

Cell line	Origin	Induction method	Place established	Publication
H7	Embryo	-	WiCell*	I
H9	Embryo	-	WiCell*	III
FES29	Embryo	-	University of Helsinki	I,II,III
08/023	Embryo	-	University of Tampere	I
UTA.00112.hFF	Foreskin fibroblasts (CRL-2429, ATCC)	Retrovirus	University of Tampere	I
UTA.01006.WT	Healthy, 36-year old male dermal fibroblasts	Retrovirus	University of Tampere	I
A116	Healthy, 48-year old female dermal fibroblasts	Retrovirus	University of helsinki	I
FiPS5-7	Foreskin fibroblast (CRL-2429, ATCC)	Retrovirus	University of helsinki	I, II
HEL11-4	Healthy, 83-year old male dermal fibroblasts	Retrovirus	University of helsinki	III
HEL24.3	Foreskin fibroblasts (CRL-2429, ATCC)	Sendai virus	University of helsinki	I
HEL47.2	Healthy, 83-year old male dermal fibroblasts	Sendai virus	University of helsinki	IV

* WiCell Research Insititute, Madison, WI, USA, http://www.wicell.org

2.1. Generation and characterization of hESC lines (I, II, III, IV)

hESC lines H9 (I, IV) and H7 (I) were obtained from the WiCell Research Institute (Thomson et al, 1998). The FES29 (I, II) line has been derived in Biomedicum Stem Cell Center from *in vitro* excess human embryo, that were donated after an informed consent of the respective couple (Mikkola et al, 2006). hESC line 08/023 (I) has been derived in BioMediTech (formerly Regea) at University of Tampere from surplus, bad quality embryo that could not be used for infertility treatment (Skottman, 2010).

2.2. Generation and characterization of hiPSC lines (I, II, III, IV)

hiPSC lines FiPSC5-7 (I), A116 (I) and Hel11-4 (III) were derived in Biomedicum Stem Cell Center by using pMXs-cDNA vectors (*Oct4*, *Sox2*, *Klf4* and *cMyc*) along with FugeneHD (Roche Diagnostics GmbH, Mannheim, Germany). In both lines the

transgenes were transcribed from the retroviral promoter contained the 5' LTR of the vector (Kitamura et al, 2003; Takahashi et al, 2007). hiPSC lines UTA.00112.HFF (I) and UTA.01006.WT (I) were reprogrammed from adult human fibroblasts with mouse origin retroviruses in BioMediTech as described elsewhere (Lahti et al, 2011). hiPSC line Hel24.3 (I) and Hel47.2 (IV)(Trokovic et al, 2015) were derived in Biomedicum Stem Cell Center with non integrating method by using CytoTuneTM-iPS Sendai Reprogramming Kit (Life Technologies, Carlsbad, CA, USA) according to manufactures instructions (Fusaki et al, 2009).

2.3. Culture of hPSC (I, II, III, IV)

hPSC lines were cultured either on Mitomycin C treated mouse fibroblasts (mEFs) in hES (*Materials and methods, section 2.6.*) medium or on Matrigel in StemPro (Life Technologies) (I, II, III), in mEF conditioned medium supplemented with 12 ng/ml bFGF (I, II) or in Essential 8 (E8) (Life Technologies) (IV) medium. Cells cultured on mEFs or on in StemPro were passaged with 1 mg/ml Collagenase IV (Life Technologies) whereas cells growing in E8 medium were passaged with EDTA. All the cells were passaged approximately twice a week.

2.4. Culture of HepG2 cells (IV)

Human hepatoma HepG2 (CRL-2302; American Type Culture Collection (ATCC), Manassas, VA, http://www.attc.org) cells were cultured on plastic in Dubelcco's modified Eagle's medium (DMEM) with high glucose 4.5 g/l (Life Technologies), 10 % heat inactivated fetal bovine serum (FBS) (Life technologies) and Pen/Strep 100 µg/ml.

2.5. Culture of JAR choriocarcinoma cells (III)

Human choriocarcinoma cells, JAR (HTB-144;ATCC) were culture on standard tissue culture plates in RPMI1640 medium with L-Glutamine supplemented with 10 % FBS. The cells were passaged every three days with Trypsin-EDTA (Life technologies).

2.6. Human pluripotent stem cell culture medium (I, II)

hPSC culture medium was prepared as basal medium for mEF-CM medium (*Materials and Method, section 2.7*). KnockOut (KO)-DMEM (Life Technologies) was supplemented with 20% KnockOut Serum Replacement (KO-SR), 0.1 mM 2-Mercaptoethanol (β-MeOH), 100U/ml Penicillin, 100 µg/ml streptomycin, 2 mM L-Glutamine, 1% nonessential amino acids (NEAA) (all from Life Technologies), 1% Insulin Transferrin Selenium liquid media

supplement (ITS) and 6 ng/ml Basic Fibroblasts Growth Factor (FGF2) (Both from Sigma, St Louis, MO, USA).

2.7. Culture of mouse embryonic fibroblasts and production of conditioned medium (I, II)

Mouse embryonic fibroblasts (mEF) cells were isolated from 12.5 ICR fetuses and cultured in DMEM supplemented with 10 % FBS, 100 U/ml Penicillin, 100 μg/ml Streptomycin (Life Technologies). The cells were passaged every four days. For production of mEF-conditioned medium (mEF-CM) the mEFs were mitotically inactivated using Mitomycin C- treatment (10 μg/mL for 3 hours) (Sigma) as previously described (Mikkola et al, 2006). Then hPSC culture medium was incubated on mEFs for 24 hours. CM- medium was collected every fourth days, pooled and supplemented with 12 ng/ml of FGF2.

2.8. Culture of L-Cells and production of Wnt3a (II)

L-cells (CRL-2648;ATCC) with Wnt3a construct were established as described elsewhere (Shibamoto et al, 1998) and used for Wnt3a production. Frozen L-cells were thawed in high glucose DMEM supplemented with 10 % fetal calf serum (FCS) and Pen/Strep 100 µg/ml. Selection for transfected cells were done by culturing the cells in the presence of G418 until the culture plate was confluent. After selection the cells were cultured in DMEM with 10 % FCS and without G418 for 24 hours which after the collection medium were changed for the cells. Collection medium was otherwise the same as previous medium but the FCS was substituted with knockout serum replacement (KO-SR). During 7 days cell culture the collection medium was changed once and collected after first three days and in the end of cell culture period. Collected media was pooled and filtered. Wnt3a was concentrated with Amico Ultra-15 Centrifugal Filter Units according to the manufacture's instructions (Millipore). Concentrated Wnt3a was divided in aliquots and stored in -20 °C until use.

3. JAR-Matrix preparation (III)

JAR cells were plated on 0.1% gelatin (Sigma) pre-coated tissue culture dishes (1 x 10^4 cells/cm^2). JAR-Cells were culture approximately 48 hours, subsequently washed with 1 x PBS and extracted by incubating the cells in 1 mM NH_3 for 30 min in room temperature. The detached cell debris were washed with 1xPBS. The plates were balanced with DMEM/F-12+GlutaMax (Life Technologie) over night in standard cell culture conditions.

The plates were either used immediately or stored at + 4 °C in 1 x PBS. The plates could be used at least for up to four months.

4. Differentiation methods

During this thesis work hPSCs were differentiated into hepatocyte like cells (HLCs) with two different protocols. In study II, the cells were also differentiated into pancreatic progenitors and hindgut cells. Neuronal (I), cardiomyocyte (I) and RPE (I) differentiations were performed in BioMediTech at University of Tampere and hence not described here. These differentiation protocols can be found in the "Supplementary material and methods" part in the original study I.

4.1. Differentiation of hPSCs into DE (I, II, III, IV)

When hPSCs had reached 80-90 % confluence the cells were washed twice with PBS and cultured following 24 hours in RPMI1640+Glutamax medium supplemented with 100 ng/ml Activin A (provided by Marko Hyvönen, University of Cambridge, UK), 2 % B27 (Life technologies), 75 ng/ml Wnt3a and 1 mM sodium butyrate (NaB) (Sigma). 24 hours after the onset of the differentiation the NaB concentration was decreased to 0.5 mM and cells were culture further 2, 4 or 6 days in this medium. In the experiments where the effect of the duration of Wnt3a treatment was studied, Wnt3a was either removed from the medium after 24 hours from the onset of the differentiation or kept in medium during the whole DE induction time.

4.2. Hepatocyte differentiation from hPSCs (I, II)

After DE-differentiation the cells were guided to commit into hepatic cell fate as described earlier (Hay et al, 2008). Shortly, the cells were washed with PBS and cultured for seven days in DMEM supplemented with 20 % KO-SR, 1 % NEAA, 0.1 mM β-MeOH, 1 % dimethyl sulfoxide (DMSO) (all from Life Technologies) and 0.1 mM Glutamine. The cells were further matured into hepatic cells for additional seven days by culturing them in Leibovitz's L-15 medium supplemented with 8.3 % FCS (PromoCell GmbH, Heidelberg, Germany), 8.3 % Trypthose phosphate broth, 10 µM Hydrocortisone 21- Hemisuccinate (both from Sigma), 1 mM Insulin (Roche), 2mM Glutamine, 10 ng/ml HGF and 20 ng/ml OncM (both from R&D Systems, Minneapolis, MN, USA). During the differentiation the medium was refreshed daily.

4.3. Hepatocyte differentiation from hPSCs (III, IV)

In the latter part of the thesis, the cells were guided into hepatocyte like cells according to a published protocol (Si-Tayeb et al, 2010b) with slight variations. After DE-differentiation the cells were washed with PBS and cultured for 5 days in RPMI1640+Glutamax medium supplemented with 2 % B27, 20 ng/ml BMP4 and 10 ng/ml FGF2. Then the cells were washed with PBS and cultured further 5 days in RPMI1640+Glutamax medium supplemented with 2 % B27 and 20 ng/ml HGF. Lastly, the cells were maturated into hepatocyte like cells by culturing the cells in Hepatocyte Basal Medium (HBM) (Lonza, Walkersville, MD, USA) supplemented with SingleQuot (without EGF) (Lonza), 50 ng/ml HGF and 20 ng/ml Oncotatin M (both from Peprotech, Stockholm, Sweden). During the differentiation the medium was refreshed daily.

4.4. Culture of hPSCs derived HLCs in 3D MG (IV)

HLCs were differentiated from hPSCs with 20 days protocol (*Materials and Methods, section 3.3.*). HLCs were detached from the 2D cultures with Gentle Cell Dissociation Reagent (STEMCELL Technologies Inc, Grenoble, France) and 1.2 x 10^6 HLCs were mixed with 200 µl of ice-cold MG. MG-HLC- mix was divided in 48-well plates in 50 µl droplets. MG-HLC mixes were let to solidify in + 37°C for 30 min which after 400 µl of HBM supplemented with SingleQuot (without EGF), 50 ng/ml HGF and 20 ng/ml OncM was added in each 48-wells. The culture medium was refreshed every third day.

4.5. Culture of hPSCs derived HLCs in 3D NFC (IV)

HLCs were differentiated from hPSCs with 20 days protocol (*Materials and Methods, section 3.3.*). HLCs were detached with Gentle Cell Dissociation Reagent and 3.1 x 10^6 HLCs were seeded in 1 ml of NFC (Growdex, UPM Co, Helsinki, Finland) to establish 3D culture environment. HLC-NFC mix was divided in 65 µl droplets in ultra-low attachement 96-well plates (Corning, NY, USA). After 30 min incubation at +37°C 100 µl of HBM supplemented with SingleQuot (without EGF), 50 ng/ml HGF and 20 ng/ml OncM was added in each 96-wells. The culture medium was refreshed every third day.

4.6. Culture of hPSCs derived HLCs in agarose patterned micro compartments (Petri Dish ®) (IV)

HLCs were differentiated from hPSCs with 20 days protocol (*Materials and Methods, section 3.3.*). HLCs were detached from the 2D cultures with Gentle Cell Dissociation Reagent and 0.5 million HLCs were mixed with 1 ml of HBM supplemented with SingleQuot (without EGF), 50 ng/ml HGF and 20 ng/ml OncM. 75µl of the cell suspension

was then pipetted into the 3D PetriDish ® (3DPD) microwells. Microwells were formed beforehand by distributing warm and sterile agarose into micro-molds (3D Petri Dish ®, Microtissue, Sigma). After the agarose was solidified the microwell casts were placed into 24-well plate and 500 µl of PBS was added into the wells. The plates were stored in +4°C until the use.

Cell suspension was allowed to settle into the microwells for 15 to 20 min at +37°C before 500 µl of HBM supplemented with SingleQuot (without EGF), 50 ng/ml HGF and 20 ng/ml OncM was added to the wells. The culture medium was refreshed every second day.

4.7. Pancreatic differentiation (II)

DE-cells derived from hPSCs were differentiated into pancreatic direction by culturing the cells for 4 days in RPMI1640-Glutamax medium supplemented with 2% B27, 0.25 µM KAAD-Cyclopamine (Stemgent, San Diego, CA, USA), 2 µM all trans-Retinoic Acid (Sigma), 100 ng/ml Noggin (R&D Systems) and 50 ng/ml FGF10 (Peprotech). After 4 days the cells were washed with PBS and the medium was changed to DMEM supplemented with GlutaMax, 1% B27, 0.25 µM KAAD-Cyclopamine, 2 µM all trans retinoic acid, 100 ng/ml Noggin and 50 ng/ml FGF10. For the last 4 days the cells were cultured in DMEM supplemented with 2 mM GlutaMax, 1% B27 and 100 ng/ml Noggin.

4.8. Hindgut differentiation (II)

DE-cells derived from hPSCs were guided into hindgut direction by culturing the cells for four days in DME/F12+Glutamax medium supplemented with 2 % dFBS (Hy-Clone, Utah, USA), 500 ng/ml FGF4 and 500 ng/ml Wnta3a (both from R&D Systems).

5. Analyzing methods

5.1. Flow cytometry (I, II, III, IV)

The DE-differentiation efficiency was analyzed with flow cytometry. After DE-differentiation the cells were detached with TrypLE (Life Technologies) treatment followed a washing step with PBS. The cell pellet was suspended in FACS-buffer (5 % FCS in PBS) and the cells were count. Counted cells were introduced to PE Mouse Anti-Human CXCR4 antibody (BD Biosciences, San Jose, CA, USA) and incubated in room temperature for 30 min followed by three PBS washes. Cells were fixed with ice cold 4% PFA and suspended in FACS buffer. Samples were run with FACS Calibur (BD

Biosciences) and analyzed with CellQuestPro (BD Biosciences) or with FlowJo (Tree Star Inc., Ashland, OR, USA) software (I, II, III, IV).

5.2. RNA isolation and reverse transcriptase reaction (I, II, III, IV)

All RNA isolations were done with either NucleoSpin® RNA Clean-up kit or NucleoSpin® RNA Clean-up XS kit (both from Machery-Nagel, Duren, Germany). DNase treatment was performed separately with RQ1 RNase-free DNase (Promega, Madison, WI, USA). Otherwise all RNA isolations were done according to manufacture's instruction. After DNase treatment the samples were purified with NucleoSpin Clean-up Kit. For reverse transcriptase reaction 2 µg (I, II), 1 µg (III) or 1.5 µg (IV) of total RNA was taken into 20 µl reverse transcriptase reaction containing Oligo(dT)15 primers, Random Hexamers, M-MLV reverse transcriptase (all from Pormega), dNTP mix and RiboLock RNAse inhibitor (Thermo Fisher Scientific, Walthman, MA, USA). Prior to mixing the samples with reaction buffer double stranded RNA structures were broke with heat treatment for 1 min at +65 °C. Reverse transcriptase reactions were done by incubating reactions at +37 °C for 90 min followed by enzyme inactivation for 5 min at +95 °C.

5.3. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) (I, II, III, IV)

In study II, 20 µl polymerase chain reactions (PCR) were prepared by mixing 2 µl 10 x PCR Buffer (Applied Biosystems), 2 µl $MgCl_2$ 25 mM stock, 1.6 µl dNTP mix 2.5 mM each (Promega), 1.6 µl DMSO 50% stock, 5 µl mix of F/R primers (both from 2uM in mix) and 1µl RT reaction. In study I, III and IV 20 µl multiplication reactions were prepared by mixing 4 µl 5 x HOT FIREPol® EvaGreen® qPCR Mix Plus (Solid BioDyne, Tartu, Estonia), 10 µl nuclease free H_2O and 1 µl RT reaction. All qRT-PCR runs were prepared by using automated Corbett CAS-1200 liquid handling system and run with Corbett Rotor-Gene 6000 (Corbett Life Science, Sydney, Australia). Primer sequences are presented in Table 2.

Table 2. List of primers used for qPCR

Primer name	Forward sequence	Reverse sequence
Albumin	GAAAAGTGGGCAGCAAATGT	GGTTCAGGACCACGGATAGA
AFP	CGCTGCAAACGATGAAGCAG	AATCTGCAATGACAGCCTCAAG
Brachyury T	GCATGATCACCAGCCACTG	TTAAGAGCTGTGATCTCCTC
BSEP	ACGCATTGCTATTGCTCGGG	GAGCAACCTGCACCGTCTTT
CDX2	CCAGCGGCGGAACCTGTG	GTCTTTCGTCCTGGTTTTCAC
CYP1A2	TGGAGACCTTCCGACACTCCT	CGTTGTGTCCCTTGTTGTGC
CYP2C9	TGGAAAACACTGCAGTTGACTTGT	GACTTTAGCTGTTGACCTCTGGGT
CYP3A4	AAACCGGAGGCCTTTTGGTC	TGGTGAAGGTTGGAGACAGC
CyclophilinG	TCTTGTCAATGGCCAACAGAG	GGAAAAGTGGGCAGCAAATGT
FOXA2	AAGACCTACAGGCGCAGCT	CATCTTGTTGGGGCTCTGC
HNF4a	GGGCTTCTTGGACAACCTTTTCA	CGTATGGACACCCGGCTCAT
KLF4 (SeV)	TTCCTGCATGCCAGAGGAGCCC	AATGTATCGAAGGTGCTCAA
KLF4 (pMX)	TCGGACCACCTCGCCTTACA	TTATCGTCGACCACTGTGCTG
Lin28 (pMX)	AGAAATCCACAGCCCTACCC	TTATCGTCGACCACTGTGCTG
Nkx6.1	TATTCGTTGGGGATGACAGAG	TGGCCATCTCGGCAGCGTG
MDR1	ACGCATTGCCATAGCTCGTG	GGGCTTCTTGGACAACCTTTTCA
MRP2	GGCTGCCGGTGGTCAGATTA	GAACAGGATGGGGTCCTGGG
OATP1B1	TGGGCTTCAATACCGCTGAT	CAAGCCCAAGTAGACCCTTGAAAA
OCT4	TTGGGCTCGAGAAGGATGTG	TCCTCTCGTTGTGCATAGTCG
OCT4 (pMX)	CCTGTCTCCGTCACCACTCT	TTATCGTCGACCACTGTGCTG
OCT4 (SeV)	CCCGAAAGAGAAAGCGAACCAG	AATGTATCGAAGGTGCTCAA
SOX7	GTCTCCATGATGTCCCCTGT	TGGAGTGGAGTGGGTGGTAG
SOX17	CCGAGTTGAGCAAGATGCTG	TGCATGTGCTGCACGCGCA
SOX2	GCCCTGCAGTACAACTCCAT	TGCCCTGCTGCGAGTAGGA
SOX2 (pMX)	ACACTGCCCCTCTCACACAT	TTATCGTCGACCACTGTGCTG
SOX2 (SeV)	ATGCACCGCTACGACGTGAGCGC	AATGTATCGAAGGTGCTCAA
SOX17	CCGAGTTGAGCAAGATGCTG	TGCATGTGCTGCACGCGCA
PDX1	AAGTCTACCAAAGCTCACGCG	CGTAGGCGCCGCCTGC
UGT1A1	CTAGGCCCATCATGCCCAAT	AGGCTTCAAATTCCTGGGATAGT

5.4. Indirect immunonofluorescence (I, II, III, IV)

Cells were washed with PBS and fixed for 20 min in room temperature with 4 % PFA. Fixed cells were treated for 8 min with Ultra V Block (Thermo Fisher Scientific) prior to overnight primary antibody incubation in +4 °C. All primary antibodies used in this thesis are listed in Table 2. After primary antibody incubation the cells were washed several times with PBS and secondary antibodies diluted in 0.1 % Tween20 in PBS were added on the cells. Secondary antibodies are listed in the Table 3. Cells were incubated with secondary antibodies (1:500 dilution) for 30 min in room temperature for followed by several washes with PBS. Stained cells were mounted with Vectashield with DAPI (Vector laboratories, Burlingame, CA, USA).

Table 3. List of primary antibodies

Antigen	Species raised in	Dilution used	Manufacturer, catalog #
Albumin	Mouse	1:300	R&D Systems, MAB188835
AFP	Rabbit	1:500	Dako, A0008
HNF4a	Goat	1:800	Santa Cruz Biosciences, sc-6556
OCT4	Mouse	1:500	Santa Cruz Biosciences, sc-365509
SOX17	Goat	1:500	R&D Systems, AF1924
FOXA2	Goat	1:500	Santa Cruz Biosciences, sc-271103
Ki67	Rabbit	1:500	Novocastra, ACK02
CK19	Mouse	1:500	Dako, M0888
PDX1	Goat	1:500	R&D Systems, AF2419
NKX6.1	Mouse	1:500	BCBC Antibody core unit, Novo Dordisk, AB2024
SSEA-4	Mouse	1:500	Thermo Scientific, MA-021
NANOG	Mouse	1:500	Thermo Scientific, PA1-41577
TRA-1-60	Mouse	1:500	Thermo Scientific, MA1-023
CDX2	Mouse	1:500	Biogenex, AM392

5.5. Immunocytochemistry for paraffin sections (III)

The cell spheroids from 3D Matrigel and 3D PetriDish were recovered from the materials, pelleted and fixed with 4 % PFA at room temperature for 20 min. Fixed cells were embedded in 1 % paraffin and sectioned into 4 µm sections on glass slides. The sections were deparaffinized with 3 x 10 min xylene treatment and rehydrated with stepwise ethanol treatment; first absolute ethanol, following 96 % ethanol, 75 % ethanol and lastly H_2O (4 min, 4 min, 2 min and 2 min respectively). Antigens were retrieved with 3 min 900 W and the microwaves for 5 min 300 W following cooling down at RT. The sections were blocked with Ultra V Block at RT for 10 min following overnight primary antibody incubation at +4°C (Table 2). Next day the sections were washed with PBS and incubated with secondary antibodies at RT for 30 min which after the sections were mounted with Vectashield with DAPI.

5.6. ELISA analysis for Albumin secretion (I, II, III, IV)

The Albumin secretion from the hPSCs derived hepatic cells was analyzed with Human Albumin ELISA Quantitation Kit (Bethyl Laboratories, Montgomery, TX, USA) according to manufacture's instructions. In short, medium samples were collected from the cells and stored at -20 °C until analysis. Analysis was performed with ELISA reaction and well plates were read at 450 nm using SpectraMax 190 Absorbance Microplate reader (Molecular Devises). The concentration of human Albumin was measured from the medium and normalized to the amount of genomic DNA in the sample.

5.7. Quantification of Genomic DNA (I, II, IV)

In study I and II the amount of genomic DNA was determined using the FluoroReport Blue Fluorometric dsDNA Quantification Kit F-2962 (Life Technologies) by following the manufacture's instructions. In study IV, genomic DNA was measured from the cell lysate collected in RA1-cell lysate buffer (Macherey-Nagel). Aliquots of each lysate samples were diluted in water (1:400) and treated with a fluorescent DNA-binding dye from Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies). The fluorescence intensity was measured with FLUOstar Omega microplate reader with excitation and emission wavelength 485 nm and 520 nm, respectively.

6. Statistical analysis (I, II, III, IV)

Statistical analysis between 2 groups was performed with an unpaired Student *t*- test (I, II) or Mann-Whitney U test (I) and analysis between more than 2 groups was performed with

one-way ANOVA with SPSS software. *p*-value <0.05 was considered statistically significant.

RESULTS AND DISCUSSIONS

1. Differentiation capacity of multiple hPSC lines (I)

The differentiation capacity of different hiPSC cell lines is not necessarily identical. Previous studies have shown, that the origin of iPSCs might have an impact on their differentiation capacity. For instance, iPSC reprogrammed from retinal cells have been shown to have a tendency to spontaneously re-differentiate back to retinal cells and hepatoblasts-derived hiPSCs tend to differentiate more efficiently into hepatocytes than fibroblasts-derived iPSCs (Hu et al, 2010; Lee et al, 2012). The differences in the differentiation potential of various hiPSC and hESC lines were investigated in Study I.

1.1. hiPSC and hESC lines differentiated equally well into hepatocytes (I)

hiPSCs share the key characteristics and potential with hESC lines and allow the generation of patient-specific cell lines (Mallon et al, 2014). Multiple studies have compared gene expression and methylation profiles of ESCs and iPSC (Bock et al, 2011; Chin et al, 2009). Some results suggested that generation of hiPSCs can induce abnormalities at both epigenetic and genetic levels (Gore et al, 2011; Hussein et al, 2011). In addition, it is likely that iPSCs retain some epigenetic marks of the donor cell types (Kim et al, 2010).

We studied the capacity of four different hESC lines and five different hiPSC lines to differentiate into functional hepatocytes simultaneously with other differentiation directions (cardiomyocytes, RPE cells, neurons). Several studies suggest that some cell lines have a better potential to differentiate into ectodermal direction while other lines tend to differentiate into mesendodermal direction (Lappalainen et al, 2010; Osafune et al, 2008). In our experimental setup, RPE and neuronal cells represented ectodermal differentiation, while cardiomyocytes and hepatocytes represent mesendodermal differentiation.

hiPSC lines were generated in two different laboratories with two different sets of transgenes (*NANOG*, *OCT4*, *SOX2* and *LIN28* or *OCT4*, *SOX2*, *KLF4* and *c-MYC*). Four of the cells lines were derived with genome integrating retroviral system and one line with non-integrating SeV based method. The hiPSC lines were generated either from adult or fetal fibroblasts. All lines were adapted to similar culture conditions before onset of the differentiations in order to minimize lab-specific variations (Newman & Cooper, 2010).

All the lines were differentiated into hepatocytes with the protocol previously described by Hay et al (I, Fig.2 A) (Hay et al, 2008). The differentiation protocol is composed of three

different steps; DE differentiation, hepatocyte commitment and hepatocyte maturation. Progress of the differentiation was followed by analyzing developmental specific gene and protein expression in the cells between the differentiation steps. All tested cell lines differentiated efficiently into DE cells (I, Fig.S2) and no differences in the differentiation efficiency was observed between hESC and hiPSC lines (I, Fig.2 E). qPCR analysis showed marked upregulation of DE-marker genes *SOX17* and *HHEX* (I, Fig.2 B-C) and the cells expressed FOXA2 at protein level (I, Fig.S2). Pluripotency marker *OCT4* gene expression decreased during DE differentiation, however, this progress was somewhat slower in some hiPSCs than hESCs (I, Fig.2 D). A recent study by Choi et al. implies that transcriptional and epigenetic variation from genetic background dominates over variation due to cellular origin of hPSCs (Choi et al, 2015a). Hence slower decreasing of *OCT4* gene expression in certain hPSC lines is probably due to the genetic background of the cell line.

Subsequently, after hepatic commitment step no *OCT4* expression was detected and the cells expressed strongly *AFP* and *Albumin* at gene and protein level (I, Fig.2 B, F and G; I, Fig.S4 A). When the differentiation potentials were compared as a group (hESC vs. hiPSC) no significant differences were seen (I, Fig.2 F). However, hPSC lines showed individual differences in their hepatic differentiation propensities; hiPSC2 and hiPSC5 produced hepatic cells with highest Albumin secretion capability, while hiPSC3 and hiPSC4 were clearly more immature stage than any other hESC or hiPSC line in the end of the differentiation (I, Fig.2 F and G; I, Fig.S4 A). On the contrary, all three hESC lines differentiated along the hepatic program with approximately the same efficiency (I, Fig.2. F; I, Fig.S4 A).

1.2. hiPSC and hESC lines did not show systematic differences in their differentiation potential (I)

Many previous studies have shown systematic differences between transcriptional and epigenetic profiles of hESCs and hiPSCs (Chin et al, 2009; Ghosh et al, 2010), however, several reasons may inflate these differences such as limited number of hPSC lines used, hiPSCs were derived only a single donor or hESC and hiPSC lines used in analysis were of opposite sex (Loewer et al, 2010; Phanstiel et al, 2011; Teichroeb et al, 2011). We observed more variability in the hepatocyte differentiation efficiency between hiPSC lines than between hESC lines. However, the variability is most likely due to the different genetic background of hPSC lines than any other reason. Kajiwara et al. reported a

similar study in which they differentiated 28 hiPSC lines from two different cell origins, peripherial blood cells and dermal fibroblasts, derived with three various reprogramming methods. They conclude that the variations in hepatic differentiation were largely attributable to donor differences rather than to the types of the original cell (Kajiwara et al, 2012). More recent data by Rouhani and co-workers suggests that difference between individual cell donors is the major cause of transcriptional variation between hPSC lines (Rouhani et al, 2014). They compared RNA-seq data from iPSCs derived from a panel of tissues isolated in parallel from several different donors with the corresponding adult somatic cells and ES-cells. Despite the very limited number of cell lines used in our experiments, the results are in line with findings by others, since the propensity of the cells to differentiate was independent of the cell origin or the reprogramming method used. In addition, our results did not point out systematic differences in the differentiation efficiency between hiPSC and hESC lines toward mesendodermal (hepatocyte, cardiomyocyte) or ectodermal (neurons, RPE) cell lineages (I, Table 1.).

1.3. Defective differentiation capacity might be due to the incomplete transgene silencing (I)

Successful reprogramming requires complete transgene silencing and erasure of somatic cell memory (Nashun et al, 2015). Retroviruses integrate in the somatic cell genome upon hiPSC reprogramming posing a risk of reactivation or residual expression of transgenic reprogramming factors in the host genome. Therefore we analyzed transgene expression in all five hiPSC lines.

The result revealed constant exogenous *KLF4* expression in hiPSC4 line indicating that this line was only partially reprogrammed (I, Fig.1 A). Residual transgene expression had an obvious negative effect on cell differentiation throughout the study. hiPSC4 line differentiated into DE cells, however, after hepatocyte differentiation hiPSC4 secreted Albumin only in low level (I, Fig.2 F) and also *Albumin* gene expression was lower than in hepatocytes differentiated from other hPSCs (I, Fig.S4 A). Neither did the cells of this line captured cuboidal hepatocyte like shape like other hPSC lines (I, Fig.S5 C). hiPSC4 propensity to differentiate also into other cell types was limited. It had a tendency to form cystic structures with lowest amount of beating areas upon cardiac differentiation (I, Fig.3 D-E), the growth of neurospheres derived from hiPSC4 was weaker than that in other lines (I, Fig.4 C) and upon RPE differentiation this line showed remarkable variability between separate experiments (I, Fig.5). Residual expression of the integrating viral

transgenes in reprogrammed cells has been shown to affect their biological properties both *in vivo* and *in vitro* (Sommer et al, 2010; Yu et al, 2007). In addition, incomplete transgene silencing has been shown to influence epigenetic signature associated with full pluripotency (Sommer et al, 2012).

1.4. Transgene reactivation is a potential problem in hiPSC lines derived with genome integrating methods (I)

The other concern related to hiPSC lines generated with genome integrating retroviruses is possible reactivation of the integrated reprogramming factors. Therefore we analyzed exogenous transgene expression in all hiPSC lines before and after of the four differentiation protocols used.

No reactivation of transgenes was detected upon hepatocyte differentiation. Similarly, all transgenes remained silenced during cardiac and neuronal program (I, Fig.1B). Surprisingly, the level of transgenic *OCT4* expression increased significantly upon RPE differentiation (I, Fig.1B). OCT4 was also detected at protein level on day 82 of RPE differentiation by immunocytochemistry (I, Fig.S3). Interestingly, transgenic *LIN28* and *NANOG* expressions were also markedly increased during RPE differentiation. This was detected only in hiPSC1 line, the only line that was reprogrammed by using *LIN28* and *NANOG.*

It remains unclear why transgenes became reactivated specifically during RPE differentiation but not during hepatocyte differentiation. One possible reason might be the duration of the differentiation; the RPE protocol is almost three months longer than hepatocyte differentiation. This raises the concern of possible transgene reactivation also in other cell types differentiated from retrovirally derived hiPSCs over time, for instance in the case of transplantation. Potential reactivation of transgenes is one of the reasons for the oncogenicity of hiPSCs (Okita et al, 2007). *Oct4* and other pluripotency-associated genes have reported to be actively expressed in germ-cell cancers (Cheng et al, 2004; Clark et al, 2004; Gidekel et al, 2003; Jones et al, 2004a; Jones et al, 2004b) and ectopic expression of *Oct4* has been shown to cause dysplasia in mouse epithelial tissues (Foster et al, 2005; Hochedlinger et al, 2005). Retroviral integration also itself causes insertional mutagenesis and may alter the expression pattern of multiple genes (Nair, 2008).

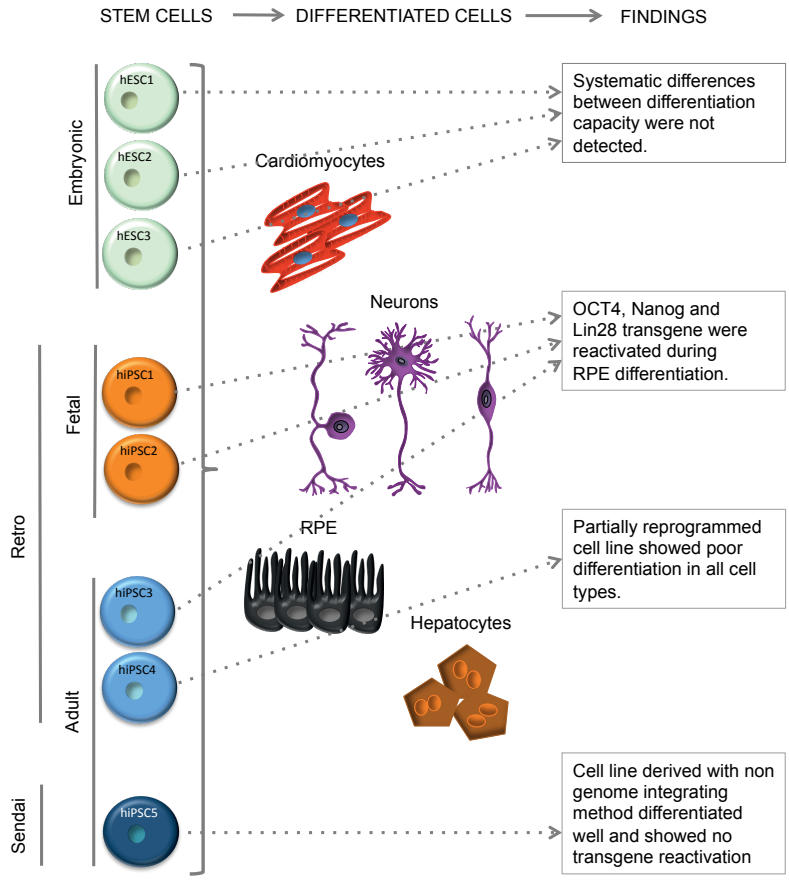

Figure 9. hPSCs from different origin, either reprogrammed from adult or fetal fibroblasts or derived from human embryo, did not show systematic differences in their propensity to differentiate into hepatocytes or other cell types (cardiomyocytes, neuronal, RPE cells). All the hiPSC lines reprogrammed with retroviral system revealed transgene reactivation during RPE differentiation and residual transgene expression was found in hiPSC4. Residual transgene expression in hiPSC4 had an obvious negative effect on the cell differentiation. hiPSC5 derived with non-integrating SeV-based system differentiated well into hepatocytes and RPE cells. hiPSC5 showed no residual expression or reaction of transgenes.

1.5. hiPSC line reprogrammed with SeV showed no transgene reactivation (I)

Since we found clear reactivation of transgenic *OCT4* in all hiPSCs reprogrammed with retrovirus based method during RPE differentiation, we asked whether similar upregulation were seen in hiPSC line derived with SeV vectors. As expected, hiPSC5 line derived with non-integrating SeV-method did not show any sign of reactivation of *OCT4* or other transgenes during hepatocyte or RPE differentiation (I, Fig.S3). Results from Study I are schematically presented in Figure 9.

The finding of transgene reactivation potential has raised a concern about the safety of those hiPSCs with integrated transgenes in their genome (Okita et al, 2011). Up to date,

plenty of different non-integrating methods have been established for hiPSC production (Hu, 2014). We have also tested various methods in our laboratory. For instance, we have established iPSC lines reprogrammed with recombinant adeno-associated virus (rAAV), which is episomal and should be lost from proliferating cells upon time. However, rAAV vector-mediated reprogramming led to frequent genomic integration of vector sequences during the reprogramming process suggesting that rAAV vectors are not compatible with the derivation of integration-free iPSCs (Weltner et al, 2012). More recently, our laboratory reported a system to replace transgenic OCT4 in human cell reprogramming by using catalytically inactive Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats associated protein 9) to induce *OCT4* transcription activation (Balboa et al, 2015). Currently, we are routinely using Sendai-viruses and episomal plasmid vectors for reprogramming. The episomal plasmid vectors used in our laboratory were first described by Okita and co-workers. (Okita et al, 2011).

2. Role of Activin A and Wnt3a in the regional specification of DE (II)

In study I of this thesis work we showed that multiple hiPSC lines are capable to differentiate into hepatocyte like cells (HLC). However, those HLCs are quite immature, since their hepatocyte specific gene expression levels were far from those of adult human liver (unpublished data). Hepatocyte differentiation from hPSC is based on a stepwise protocol, in which hPSCs are first guided into DE-stage (Step 1), then committed to hepatocyte program (Step 2) and finally maturated into hepatocytes (Step 3) (Mallanna & Duncan, 2013). Step 1 of the differentiation is especially important; if hPSCs fail to form proper DE-cells, they are also unable to differentiate further into endodermal organs, such as liver.

The segregation of endoderm germ layer occurs during gastrulation and is one of the first cell fate decisions that are made in development. After gastrulation a series of morphogenic movements transforms the endoderm into a primitive gut tube surrounded by mesoderm. The gut tube is regionalized along the dorso-ventral and anterior-posterior axes and subdivided into foregut, midgut and hindgut regions. Liver and ventral pancreas arises from the anterior portion of the ventral foregut endoderm. The use of Activin A, as a substitute of Nodal, has been extensively shown to be an efficient strategy to obtain DE-cells from hPSC *in vitro* (Brown et al, 2011; D'Amour et al, 2005). In addition, Wnt signaling is known to play an important role in the regional specification of endoderm (Jiang et al, 2013; Sherwood et al, 2011).

In Study II, my aim was to define how Acitivin A and Wnt3a signaling are interplaying during DE differentiation and how the activation of these signaling pathways affects the patterning of endoderm for further hepatic and pancreatic competence. The importance of Wnt3a and Activin A in the DE- induction before hepatocyte differentiation from hESCs has been earlier described (Hay et al, 2008). Additionally, Wnt3a has been shown to promote liver specific functions, such as Albumin secretion, of hESC-derived hepatocytes (Hay et al, 2008).

2.1. Extended Activin A and Wnt3a treatment produced more DE-cell (II)

hPSCs were differentiated into DE-cells for 3, 5 or 7 days (d3DE, d5DE and d7DE respectively) with high concentration of Activin A and Wnt3a treatment (II, Fig. 1 A). The amount of DE-cells systematically increased with extended differentiation time; three days DE differentiation produced 73.7% of C-X-C chemokine receptor type 5 (CXCR4) positive DE-cells, five days 83.6% and seven days differentiation 94.4% CXCR4+ DE-cells (II, Fig.1 B and C). Immunochemical analysis revealed remaining OCT4 positive cells in d3DE cultures. In contrast d7DE cell cultures contained very few if any OCT4+ cells and the cells strongly expressed FOXA2 and SOX17 DE-marker proteins (II, Fig.1 D) The expression of DE marker genes *HHEX*, *SOX17* and *FOXA2* increased upon differentiation while *SOX7* gene expression did not (II, Fig1 E). *SOX7* is expressed in primitive-, pariental- and visceral endoderm but not in DE while *HHEX*, *SOX17* and, *FOXA2* are expressed in all endoderm types (D'Amour et al, 2005; Kanai-Azuma et al, 2002). Hence, the absence of *SOX7* indicates successful DE-cell differentiation. Taken together, longer DE differentiation clearly produced the purest DE-cell population while after shorter, 3 days long differentiation, the cultures still contained both pluripotent stem cells and DE-cells.

2.2. Extended Activin A and Wnt3a treatment favored hepatic differentiation (II)

Propensity of d3DE, d5DE and d7DE cells to differentiate into hepatic and pancreatic progenitors was studied (II, Fig. 1 A). d5DE cells showed significantly higher *Albumin* expression after hepatocyte differentiation compared d3DE or d7DE cells. In immunocytochemical analysis d5DE and d7DE cells stained strongly positive for Albumin and the cells secreted Albumin (II, Fig.2). Albumin positive cells were also detected in d3DE cultures after hepatic differentiation. However, in these cells *Albumin* gene expression and Albumin secretion both were in low level (II, Fig.2). This suggests that the

longer DE induction favors hepatic differentiation. Previous study by Hay et al. also underlined the importance of Activin A and Wnt3a in hepatic differentiation (Hay et al, 2008). In their experiments Wnt3a supplementation during DE differentiation clearly supported hepatic differentiation of hESC derived DE-cells. Our results also correlate with the study of Toubol et al., who showed that inhibition of Activin A and Wnt3a pathways decreased hepatic differentiation from hESCs (Touboul et al, 2010). Multiple developmental studies done with animal models suggest that Wnt signaling activation is important in many phases of liver development including liver bud formation, hepatoblast proliferation and hepatocyte maturation (Lade & Monga, 2011).

In contrary, longer than 3 days DE differentiation did not support pancreatic commitment. After pancreas differentiation, cells co-expressing pancreatic and duodenal homeobox protein 1 (PDX1) with NK6 homeobox 1 (NKX6.1) protein were found only in d3DE cultures, whereas d5DE and d7DE cultures produced only PDX1+/NKX6.1- cells (II, Fig 2D). In addition, after pancreas differentiation d5DE and d7DE cultures expressed strongly intestinal marker gene *Caudal type homeobox 2* (*CDX2),* which together with strong *PDX1* expression indicates more posterior endoderm than pancreatic differentiation (II, Fig.2 C).

Taken together, long treatment with Activin A and Wnt3a inhibited pancreatic differentiation but promoted hepatic commitment (II, Fig.2). Developmental studies with mouse and Xenopus embryos have shown that repression of Wnt signaling after mesendoderm formation is crucial for correct endoderm development (Finley et al, 2003; Pilcher & Krieg, 2002). Based on our results we hypothesized that in human cells Wnt repression after mesendoderm formation might be more important for pancreas than for liver development.

2.3. Wnt3a treatment influences further differentiation capacity of DE-cells (II)

While Activin A is known to be essential for DE differentiation *in vitro* the effect of Wnt signaling in DE-differentiation from hPSC is controversial. To date many protocols for DE differentiation from hPSCs have been published, in all of them Activin A is the dominant signaling molecule driving the differentiation. Nevertheless, the activation of Wnt signaling varies between the protocols. In some protocols Wnt signaling is activated only for the first 24 hours of the differentiation (Touboul et al, 2010), in others Wnt-signaling is not

activated at all (Rashid et al, 2010) and in some protocols Wnt is activated throughout the DE differentiation (Gounaris et al, 2008; Hay et al, 2008). Comprehensive developmental studies in mice are supporting the protocols in which Wnt is activated only in the beginning of the DE-differentiation (Lade & Monga, 2011). However, signaling pathways, which are driving organ development might have certain differences between mice and human.

Therefore we asked whether Wnt3a is impairing the d5DE-cells commitment to the pancreatic program. For this we compared two different conditions for DE differentiation; i) Five days Activin A treatment with Wnt3a (d5Wnt) and ii) five days Activin A treatment with only 24 hours Wnt3a (d1Wnt). After d5Wnt and d1Wnt differentiations the cells were subsequently differentiated to hepatic and pancreatic cells. Interestingly, we found a clear difference in hepatic and pancreatic differentiation efficiency. d5Wnt cells differentiated into hepatic cells with high *Albumin* gene and Albumin protein expression, while these parameters were significantly lower in d1Wnt derived hepatic cells (II, Fig.3). This is logical, since Wnt signaling is known to have an important role in hepatocyte maturation and regeneration from bi-potential hepatoblasts (Boulter et al, 2012; Lade & Monga, 2011; So et al, 2013). In contrast, after pancreas differentiation d1Wnt cells showed abundant clusters of PDX1+/NKX6.1+ pancreatic progenitors while d5Wnt cell derived pancreatic cells remained positive only for PDX1 (II, Fig.3 B). Additionally, after pancreatic differentiation d5Wnt cells expressed high amounts of *CDX2* (II, Fig.3 A*)*, indicating that the cells represent a more posterior, hindgut-like phenotype (Sherwood et al, 2011). Hindgut commitment from hPSCs has been shown to be dependent on active Wnt/β-catenin signaling pathway (Tamminen et al, 2015)

Our results suggest that only short Wnt3a stimulation generates DE-cells which have propensity to differentiate into pancreatic direction. Previous study by Jiang et al. also suggests that the only a short activation of Wnt signaling in the beginning of DE differentiation from hPSCs is crucial for achieving proper differentiation outcome. They showed that specific demethylases (KDM6A/B) are activating Wnt signaling pathway in the early step of the differentiation and in later phase the same demethylases are repressing Wnt by activating the expression of Wnt antagonist *Dickoppf* (*DKK1*) (Jiang et al, 2013). However, they did not differentiate DE-cells into hepatocytes or pancreatic directions and thus their study does not reveal how the action of KDM6A/B is affecting the further differentiation potential of DE-cells. In our hands, d1Wnt DE-cells were also able to differentiated into hepatic directions but with significantly lower efficiency than d5Wnt cells.

In summary, there are clear differences in the regulatory pathways in early cell commitment between pancreas and hepatic phenotype. Activation of Wnt and Activin A signaling pathways are crucial for the DE differentiation from hPSCs and timing of these cues have clear impact on the further differentiation propensities of DE-cells. Results this far from the Wnt study are collected as schematic representation in the Figure 10.

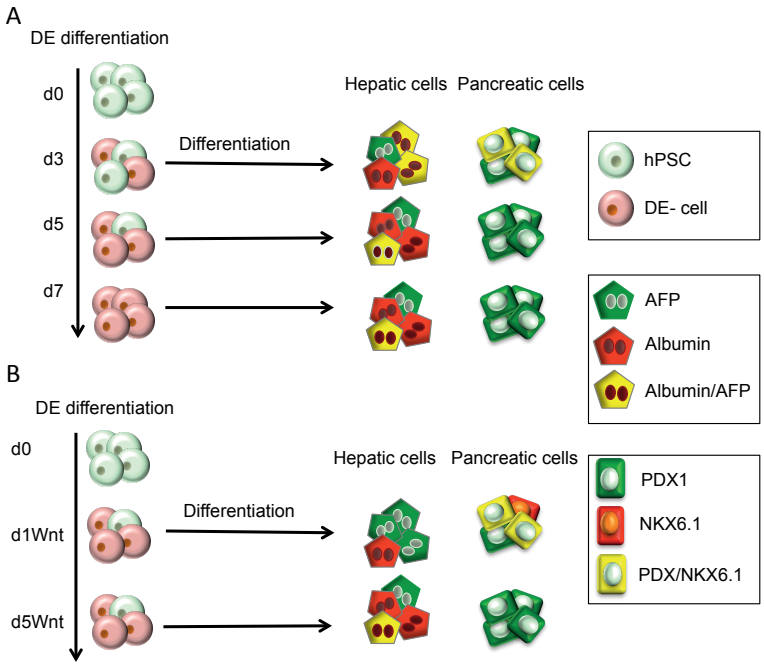

Figure 10. Schematic illustration of the role of Wnt signaling during hPSC differentiation into DE-cells and DE-cell differentiation into hepatic and pancreatic progenitor cells. (A) When Wnt is constantly present the cells are able to give rise to pancreas progenitors only after three days of DE differentiation. Five and seven days of DE differentiation produce pure DE-cell population with good hepatic differentiation capacity but restricted pancreas differentiation potential. (B) hPSCs are able to give rise to pancreas progenitors with longer DE differentiation, if Wnt is activated only for 24 hour from the initiation of the differentiation. Hepatic differentiation is less dependent on the duration of Wnt signaling.

2.4. Long-term maintenance of DE-cells is dependent on Wnt3a (II)

Hepatocyte and pancreas differentiation from hPSC is relatively slow and costly. It would be desirable to generate DE-progenitor cell lines, which would be able to proliferate *in vitro* and to differentiate into mature hepatocytes and pancreatic cells. These cells would have more restricted differentiation potential than hPSCs since they are “closer” to desired mature cell type and in this way differentiation could be more efficient. Therefore we asked whether DE-cells could be maintained and expanded in long-term culture. In this way the hepatic or pancreatic differentiation could be initiated directly from DE-cells.

In order to identify conditions supporting DE-cell maintenance, we tested various medium supplements and their different combinations, such as Activin A, FGF10, LiCl, NaB, B27 and FCS. Activin A is crucial for DE differentiation from hPSCs (D'Amour et al, 2005). FGF signaling is known to be important for endoderm development, normal growth and branching of pancreatic epithelium and is also essential to maintain proper gene expression profile of pancreatic bud (Bhushan et al, 2001; Jacquemin et al, 2006). FGF10 exerts its effect trough the same FGF receptor isoform as FGF7, which is shown to be important for liver progenitor cell proliferation and differentiation (Takase et al, 2013). NaB is an inhibitor of histone deacetylases and has been shown to improve hepatocyte differentiation (Kaneko et al, 1990; Wadee et al, 1994). Wnt/β-catenin is known to be one of the most important signaling pathways affecting hepatoblasts proliferation and maturation into hepatocytes (Monga, 2014; Wang et al, 2015). LiCl also activates canonical Wnt signaling pathway and together with Activin A promotes induction of DE-cell differentiation (Li et al, 2011). Based on cell proliferation capacity and viability (unpublished data) a medium supplemented with B27, Activin A and Wnt3a was chosen for long-term culturing of DE-cells.

DE-cells derived through the 3-day differentiation were able to maintain the expression of DE-marker genes, such as *FOXA2* and *SOX17* (II, Fig.4 A). However, when d7DE-cells were cultured in the same medium the cells survived only for one passage (II, Fig.4 E). We hypothesized that the almost 100% homogenous population of d7DE-cells were unable to proliferate in our culture conditions. This was proven with analyzing the identity of the proliferating cell types by monitoring their Ki-67 protein expression. Ki-67 is a cell-cycle dependent protein that is present in the nuclei of the G1, S, and G2 phases of the cell cycle as well as mitosis. Quiescent or resting cells in G0 phase do not express Ki-67 (Scholzen & Gerdes, 2000). In d3DE cultures 15-20% of the FOXA2+ DE-cells were co-expressing Ki-67 while 60-80% of OCT4+ pluripotent cells showed Ki-67 positivity (II, Fig.4 C and F). Among d7DE-cells only very few cells were FOXA2+/Ki-67+ (II, Fig.4 E).

Since only d3DE cells cultures, which contained both DE-cells and pluripotent cells (II, Fig 1D) were able to survive in long-term cultures we conclude that the pluripotent cells continuously proliferated and differentiated further into DE-cells (Figure 11).

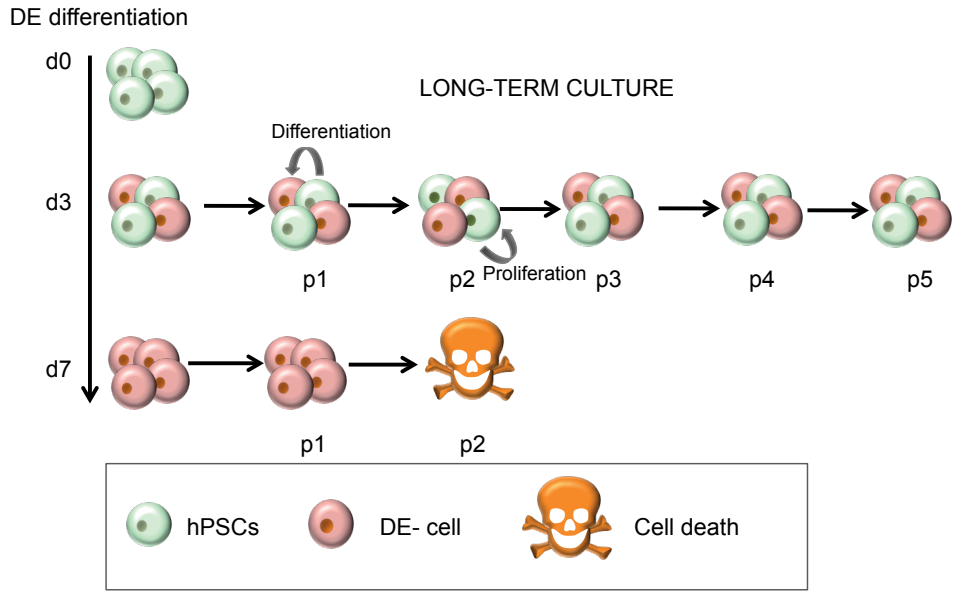

Figure 11. Schematic representation of long-term DE-cell cultures. d3DE-cells were able to maintain DE-specific gene expression at least for five passages. However, it is likely that remaining hPSCs among d3DE-cells are continuously proliferating and differentiating while DE-cells proliferation capacity is restricted. d7DE-cells were able to maintain only for one passage which after the cell died.

Wnt signaling is known to have a dual role; it is maintaining the pluripotency of mouse ESCs (Sokol, 2011) and also driving the cell differentiation during early development (Tam & Loebel, 2009). When we removed Wnt3a from the cell culture medium or blocked Wnt signaling with Dkk-1, d3DE-cells lost their DE characteristics and turned back into pluripotent stage (II, Fig.4 B). Similar results have been reported with mouse cells (Bakre et al, 2007). A very recent study from Ying et al. demonstrated that in hESCs, *OCT4* gene expression level is actually increasing during the first 24 hours of DE-differentiation. Furthermore, OCT4 was shown to promote DE-differentiation by removing the repressing methylation mark from the *SOX17* promoter site and Wnt stimulation was required for the enhanced OCT4 occupancy on SOX17 promoter (Ying et al, 2015). This data is supporting our hypothesis, since continuously Wnt3a supplementation was required for maintenance of *SOX17* gene expression in long-term d3DE-cell cultures. d7DE-cells, with the absence of constant *OCT4* gene expression, could not survive despite Wnt3a supplementation.

As a conclusion, we were unable to establish proliferating DE-progenitor cell line. Almost synchronous with our study Cheng et al. published a report in which they described successful derivation of endodermal progenitor cell line from hPSCs (Cheng et al, 2012).

However, in their system the maintenance of DE-cells was dependent on feeder cells (Cheng et al, 2012). Thus, specific signaling pathways for the proper DE-cell maintenance remains unknown.

3. Laminin rich ECM supports hepatoblast differentiation from hPSCs (III)

Growth factors and cytokines are known to affect the growth and differentiation of embryonic hepatic progenitors. Furthermore, cell-cell contacts and ECM also affect the survival, proliferation and differentiation of hepatic progenitors. Mouse fetal liver cells have been shown to be able to proliferate for several months on laminin coated cell culture plates (Tanimizu et al, 2004). A more recent studies demonstrated that also hPSC-derived hepatoblasts are able to proliferate and maintain their phenotype on human Laminin-111 coated dishes (Takayama et al, 2013b).

Our laboratory has extensively studied the interactions between hPSCs and ECM. We have found that undifferentiated hESCs can be maintained on purified human Laminin-511 (Lm-511) in defined culture environment (Vuoristo et al, 2009). However, purification and production of human laminins is laborious and expensive, which hamper their wider use. Therefore, we established a cost-effective cell culture matrix, JAR-matrix, which is derived from human choriocarsinoma cell line JAR. JAR-matrix is especially rich in Lm-111 and Lm-511. Since JAR-matrix turned out to be extremely suitable for hPSC cultures (III, Fig. 2), we asked whether it could also support hepatocyte differentiation. We cultured one hiPSC and one hESC line on JAR-matrix for at least five passages before the initiation of hepatic differentiation. Cells were differentiated in parallel on Matrigel. Matrigel contains many ECM proteins, including Lm-111 and collagens but no Lm-511 (III, Fig. 1).

hPCS lines differentiated equally well into DE-cells on JAR-matrix and Matrigel based on their DE-specific marker gene and protein expression (unpublished data). When DE-cells were further differentiated towards hepatocyte like cells (HLC), *AFP* gene expression in the cells cultured on JAR-matrix were significantly higher (5200-fold increase) than in the cells cultured on Matrigel (2700-fold increase) (III, Fig.4 A). On contrary, *Albumin* gene expression was higher in the cells cultured on Matrigel with 8500-fold increase than in the cells cultured on JAR-matrix (7400-fold increase) (III, Fig.4 A). Flow cytometry analysis confirmed the presence of Albumin positive cells in both culture systems. The Albumin positive cell population was, however, more homogeneous in the cells cultured on Matrigel (III, Fig.4 C). Wider population of Albumin+ cells was found from JAR-matrix

cultures, indicating the presence of two distinct Albumin+ cell population, Albumin+/AFP+ and Albumin+/ AFP-. These results suggest that hPSCs cultured on Matrigel had propensity to differentiate more efficiently into hepatocytes (Albumin+/ AFP-) than hPSCs cultured on JAR-matrix. hPSCs on JAR-matrix differentiated into hepatoblasts (Albumin+/ AFP+) but had restricted tendency to mature further into hepatocytes.

Integrins are cell surface receptors that specifically bind to certain ECM proteins, such as laminins and collagens. Hepatoblasts, hepatocytes and cholangiocytes all have a specific integrin expression pattern, which is dynamically changing over human liver development (Couvelard et al, 1998). The hepatoblasts are expressing a wide range of different integrins including α6β1, which specifically binds to Lm-511 and α1β1 receptor that bins to Lm-111. The expression of α6β1 and α1β1 integrins is, however, decreasing upon hepatocyte maturation. Eventually, mature hepatocytes are expressing only low levels of α1β1 and no α6β1 (Couvelard et al, 1998). Therefore it is likely that JAR-matrix, which is rich in Lm-511, mainly supports hepatoblast phenotype. Interestingly, we noticed clear detachment of hPSCs derived hepatocytes from JAR-matrix in the end of the differentiation (unpublished data). The cells probably started disengaging from the JAR-matrix when they reached hepatocyte phenotype and lost hepatoblasts-specific integrin expression pattern.

Laminins support hepatoblasts also *in vivo*. Rodent hepatic stem cells, called oval cells, reside around the hepatic portal area (Clement et al, 1988). Laminins are accumulated around these oval cells while laminin is not present around quiescent mature hepatocytes in the liver parenchyma (Paku et al, 2001). *In vitro* the laminins have been shown to be useful in sustaining rodent hepatoblasts (Tanimizu et al, 2004). In addition, Takayama and co-workers demonstrated that hPSC derived hepatoblasts can be cultured for at least 3 months on Lm-111 with maintaining their bi-potential differentiation capacity either into hepatocytes or cholangiocytes (Takayama et al, 2013b).

Taken together, instead of purified laminins, JAR-matrix is a potent and cost-effective culture substrate for hPSCs and hepatoblasts derived from hPSCs. Further studies are needed to find out whether this matrix could be used for effective hepatoblast expansion.

4. 3D cell culture systems for hepatocyte differentiation from hPSCs (IV)

Organotypic cell culture systems, in which cells are growing in 3D environment, mimic the *in vivo* situation better than traditional 2D cell cultures. In the liver, correct polarization of the hepatocytes is crucial for their functionality (Takayama et al, 2013a). Consequently, 3D environment could provide the cells a more natural environment for better polarization and in this manner enable enhanced transporter and metabolic activity of hepatocytes. In 3D cultures cells also achieve better cell-cell contacts, which are important for the formation of canalicular structures between hepatocytes (Malinen et al, 2012; Vellonen et al, 2014). 3D cultures could also extend functional lifetime of hepatocytes *in vitro* (Gieseck et al, 2014).

4.1. Cell viability and spheroid formation in 3D systems (IV)

We evaluated the suitability of three different 3D cell culture systems, 3D micro-compartment (PetriDish®, 3DPD), 3D nanofibrillar cellulose (NFC) hydrogel and 3D Matrigel (3DMG) for hepatic differentiation from hPSCs. hPSCs were first differentiated HLCs for 20 days on 2D Matrigel and then transferred to 3D environments (IV, Fig.1 D). Gene expression data were compared with HepG2 gene expression levels. HepG2 is human liver carcinoma cell line that is extensively used as *in vitro* model system for the study of human hepatocytes (Donato et al, 2008).

In NFC HLCs formed spheroids with very low efficiency (IV, Fig.3 C). In addition, cells did not proliferate in this material (IV, Fig2 B). Restricted proliferation of liver cells is typical in 3D hydrogels and has been reported also by others (Malinen et al, 2014). In contrast, recent studies done with HepaRG (hepatocarcinoma cell line (Gripon et al, 2002)) and HepG2 cells have shown proper spheroid formation in NFC (Bhattacharya et al, 2012; Malinen et al, 2014). We were unable to reproduce those results with hPSC-derived HLCs.

On the contrary, the cells cultured in 3DPD and 3DMG formed smooth surface spheroids (IV, Fig.3 A and B) and the cells were proliferating (IV, Fig2. B). Spheroids in 3DPD were significantly larger than in 3DMG and they also grew with time (IV, Fig.3, D). As a conclusion, 3DMG and 3DPD supported hPSCs aggregation and viability.

4.2. Expression of hepatic marker genes in HLCs cultured in 2D, 3DMG and 3DPD (IV)

Gene expression of HLCs cultured in 3DMG, 3DPD and regular 2D cultures were analyzed. The genes were divided in three categories: i) general hepatocyte markers (*HNF4a, AFP, Albumin*), ii) drug metabolism (*CYP2C9, CYP1A2, CYP3A4, UGT1A1*) and iii) transporters (*OATP1B1, NTCP, BSEP, MRP2, MDR1*) (IV, Fig 4).

In general, HLCs cultured in 3D showed higher expression of metabolic genes compared to 2D conditions (IV, Fig.4). Also xenobiotic influx and efflux transporter gene expressions were higher in HLCs cultured in 3D conditions than in 2D (IV, Fig.4). These gene expression levels were also higher than in HepG2 cells. In human liver CYP enzyme activities are mainly accumulated in the pericentral hepatocytes (Godoy et al, 2013) and thus 3D systems here appeared to promote the differentiation of more pericentral type of hepatocytes.

Despite the high metabolic gene expression pattern in HLCs cultured in 3DPD-cells, *Albumin* gene expression levels were minimal and markedly lower than in HepG2 cells and *AFP* gene expression showed an increasing trend over time (IV, Fig.4). Mature human hepatocytes in healthy liver are quiescent and not expressing *AFP* (Wang et al, 2015). Therefore our data suggest that 3DPD-cells are representing immature hepatocyte phenotype. 3D and 2D culture conditions have been compared also with HepG2 cells. Chang et al. found ECM protein free, rotating 3D culture environment to support both metabolic and synthetic functions of HepG2 cells. However, also in their experiments Albumin secretion decreased with time while metabolic activities remained markedly higher than in 2D (Chang & Hughes-Fulford, 2009).

3DMG-cells showed high *Albumin* and *AFP* expression along with relatively high metabolic gene expression levels, all of which were higher than in HLCs cultured on 2D or in HepG2 cells. A study by Kinasiewicz et al. described HepG2 spheroid culture in 3DMG. They reported enhanced synthetic and metabolic functions in 3DMG compared to 2D (Kinasiewicz et al, 2009). In addition to HepG2 cells, also another hepatocarcinoma cell line, Huh-7, has been shown to form aggregates when embedded in 3DMG. Huh-7 cell aggregates adopted hepatocyte polarization features and developed tight junction delimited bile canaliculi structures (Molina-Jimenez et al, 2012). However, HepG2 and Huh-7 are poor model for healthy hepatocytes because they are of hepatocellular

carcinoma origin (Thomas et al, 2014) and therefore might respond differently on 3DMG than hPSCs derived HLCs.

It is interesting, how genes of metabolically important hepatocyte specific enzymes can be highly expressed when, at the same time, *Albumin* gene expression is minimal in the cells. In adult human liver hepatocytes are localized in three different zones based on their metabolic functions (Gebhardt & Matz-Soja, 2014). Some of the functions are expressed only in a specific compartment of the liver while other metabolic and synthetic functions are expressed gradientally in all three zones (Schleicher et al, 2015). How liver zonation is regulated is not fully understood, however, at least oxygen and nutrient levels in the flowing blood, Wnt/β-catenin signaling activity and the composition of surrounding ECM are regulating the phenotype of the hepatocyte (Gebhardt & Matz-Soja, 2014; Jungermann & Kietzmann, 1997). For instance, Albumin is expressed in all hepatocytes but stronger in periportal hepatocytes while CYP activity is accumulated in pericentral hepatocytes (Godoy et al, 2013; Schleicher et al, 2015). On our study, 3DPD system supported CYP activity (pericentral) but not Albumin (periportal) synthesis, whereas 3DMG supported *Albumin* expression levels. 3DMG is rich in different ECM proteins, like laminins. Laminins are found in the periportal are of the liver and might be one reason for stronger Albumin expression in 3DMG than 3DPD cells. On the other hand, laminins have been shown to support hepatoblast instead of mature hepatocyte phenotype *in vitro*, which might hamper 3DMG cell metabolic activity (Takayama et al, 2013b; Tanimizu et al, 2004).

Taken together, it is possible that the culture conditions that support activity of metabolic enzymes may be different from those that support Albumin protein synthesis in hepatocytes.

4.3. Functionality of hiPSC derived HLCs (IV)

In order to confirm the gene expression data, we measured Albumin secretion and CYP3A4 functionality in hPCSs derived hepatic cells cultured in 3DMG, 3DPD and 2D conditions. Our result revealed that Albumin secretion was significantly higher in 2D than in 3D conditions (IV, Fig.6 A). In all culture systems tested for hPSC derived hepatic cells Albumin secretion levels stayed far from that of HepG2 cells (IV, Fig.6 A).

Albumin secretion was increasing in 3DPD-cells over time, and was significantly higher in the end of differentiation than in earlier time points (IV, Fig.6 A). The results are in line with gene expression data (IV, Fig.4), even though in both assays the Albumin levels were very low in 3DPD-cells.

3DMG showed lower Albumin secretion than in 2D, which argues against the PCR data (*Result and Discussion, Section 4.2.*). This might be due to technical issues, since part of the secreted Albumin might have got trapped in 3DMG structure. However, in 3DMG-cells Albumin secretion was significantly higher at day 30 of differentiation compared to other time points in the same condition. Albumin secretion was constantly decreasing in 2D and started to decrease in 3DMG after 30-day time point. In this aspect Albumin secretion data correlates with PCR results.

CYP3A4 activity levels were also in line with PCR data showing significantly higher activity in 3DPD cells compared to either HepG2 cells, 3DMG cells, or cells in 2D conditions (IV, Fig.4 B).

Taken together, the results from the functional analysis of the cells were correlating with gene expression data. Results from the 3D experiments are illustrated in the Figure 12.

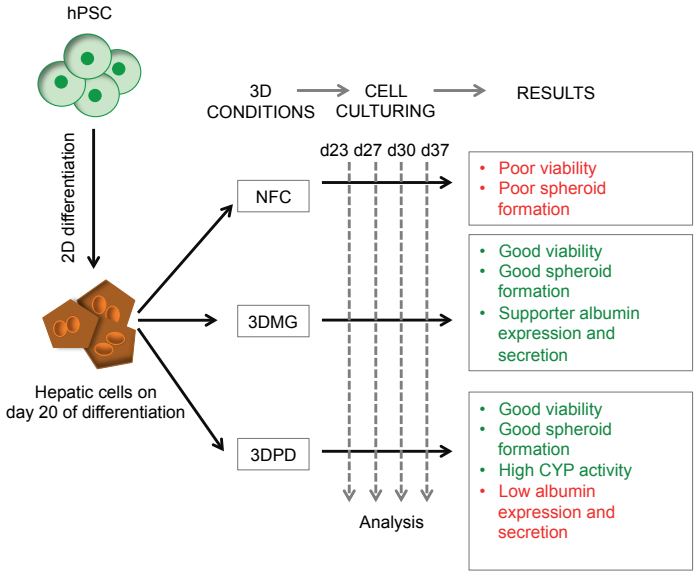

Figure 12. The experimental setup and results from testing 3D materials as platform for hPSCs derived HLC cultures. NFC supports neither the cell viability nor spheroid formation. Of all 3D conditions, hPSC derived HLCs cultured in 3DMG showed the highest Albumin expression and secretion. CYP- enzyme gene expressions were higher in 3DMG HLCs than in HepG2 cells. 3DPD showed highest metabolic gene expression, however, cells failed in Albumin secretion and *Albumin* gene expression.

4.4. Technical properties of NFC, 3DMG and 3DPD (IV)

We also evaluated the usability of the 3D techniques used in this study.

NFC was technically the most challenging. The difficulties were mainly related to the high viscosity of the material and problems in recovering the cells from the cultures for analysis. Also cell monitoring with phase contrast microscope was difficult, since NFC is not fully transparent. Nevertheless, NFC is a very interesting material due to the defined single component structure and good availability. Further optimization is needed before it is suitable for hPSC-derived HLCs cultures.

3DMG was easier to use than NFC. However, the cells have to go through temperature changes when they are seeded in ice-cold hydrogel. In addition, MG needs approximately half an hour to solidify. During this time the cells have to stay in the MG without medium. These issues can disturb the cell phenotype and could have negative effect on further maturation of the cells.

3DPD was relatively easy to handle and low-priced compared to hydrogels. Cells were easy to recover from micro-wells and there was no need to alter temperature nor is there need for starvation in any point of culturing.

CONCLUSIONS AND FUTURE PERSPECTIVES

The beauty of hPSCs is in their ability to self-renew and differentiate to almost any cell type in the adult human body. This unique feature is not present in any other cell type. Especially in the early days, the use of hESCs was controversial due to ethical, political and religious reasons, however, the discovery of hiPSCs partially overcame these problems. To date, both hESC and hiPSC are widely used in research.

Hepatocytes are valuable for academic research and pharmaceutical industry since they provide an excellent platform for studying pathogenetic mechanisms and also for drug screening. In the future, hPSC-derived hepatocytes could also be used for hepatocyte replacement therapy of liver failure. Even though the methods of hPSC differentiation into hepatocytes have progressed enormously over the last decade, more research is needed before clinical use can be considered.

Our data clearly showed that multiple hESC and hiPSC lines are able to differentiate into HLCs. All the studied hPSC lines differentiated into hepatocyte direction equally well, except one hiPSC line. This line was reprogrammed with a genome integrating retrovirus system and further analysis revealed incomplete silencing of *KLF4* transgene. Our data also revealed reactivation of transgenes during RPE differentiation in retrovirally derived hiPSC lines, which raised concerns of possible transgene reactivation over time also in hepatocytes derived from retrovirally generated hiPSCs. As expected, no transgenic reactivation was seen in a hiPSC line derived via non-integrating method. Today, the field has moved essentially into integration free methods, such as SeV- and episomal vectors, for hiPSC production.

The signals that regulate liver development in mammals are quite well understood. Although the early events in embryogenesis are well conserved, there are many examples of differences in the development of individual organs between man and other mammals. hPSCs have already brought a lot of new information about biological cues regulating human embryonic development. Here we focused on the interplay between Activin/Nodal and Wnt signaling in the early endoderm patterning. These two signaling pathways are extremely interesting due to their dual role; they are important factors for maintaining stemness but they also strongly regulate cellular differentiation. Our data suggest that short Wnt activation in the very beginning of DE differentiation is crucial for the pancreatic lineage while longer Wnt activation together with Active/Nodal signaling

supported hepatic differentiation. We also showed that a mixed population of hPSCs and DE-cells could be maintained in long-term culture. Cell-proliferation as well as DE-specific gene- and protein expression were dependent on OCT4 positive hPSCs and active Wnt signaling. Our results demonstrated that the interplay between Wnt- and Activin/Nodal signaling is crucial for the appearance of endodermal organ-specific progenitor cells and that the differentiation protocols used to guide hPSCs have to be optimized by correct timing of these cues.

In addition to soluble signaling factors like Wnt, also ECM proteins, such as collagens and laminins, are guiding hepatocyte differentiation. Differentiating hepatic cells are surrounded by dynamically changing ECM. Laminins have been shown to support mouse and human hepatoblasts isolated from the fetal liver as well as hepatoblasts differentiated from hPSCs. We established a cost-effective and user-friendly cell culture matrix produced by a tumor cell line rich in Lm-511 and Lm-111. Our data showed that the JAR-matrix supported growth of hPSCs and their differentiation into hepatic progenitor cells. JAR-matrix could be promising ECM preparation for hepatoblasts differentiation and expansion, since especially Lm-111 is shown to support hepatoblasts proliferation and preventing the cell differentiation into mature hepatocytes.

3D environment is particularly important for hepatocytes because it allows correct polarization of the cells. We found two different 3D-culture environments to support the viability and proliferation of hPSC-derived HLCs. In the adult liver, hepatocytes are localized in different metabolic zones and the zonation is at least partially regulated by ECM. We showed that hPSCs derived HLCs differently expressed metabolically important genes when cultured in ECM protein rich 3D environment or in 3D condition without ECM proteins. Our results suggest that both the physical 3D environment and the protein composition of ECM are regulating the gene expression of HLCs. This is an important aspect to take into consideration in the development of new 3D culture environments for hepatocyte differentiation and maturation.

Taken together, this thesis work is a comprehensive study on hPSC as a source of hepatic cells. Currently, the specification of hPSCs into HLCs has been well established. However, complete *in vitro* maturation of the HLCs has not yet been achieved. Further studies are needed to understand the molecular basis of the hepatocyte maturation process, and the signaling cues regulating it, before hPSC derived hepatocytes can be

fully exploited industrially and clinically. Every piece of information is taking us closer to that goal.

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REFERENCES

Aasen T, Raya A, Barrero MJ, Garreta E, Consiglio A, Gonzalez F, Vassena R, Bilic J, Pekarik V, Tiscornia G, Edel M, Boue S, Izpisua Belmonte JC (2008) Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes. *Nature biotechnology* **26:** 1276-1284

Aberle H, Bauer A, Stappert J, Kispert A, Kemler R (1997) beta-catenin is a target for the ubiquitin-proteasome pathway. *The EMBO journal* **16:** 3797-3804

Agarwal S, Holton KL, Lanza R (2008) Efficient differentiation of functional hepatocytes from human embryonic stem cells. *Stem Cells* **26:** 1117-1127

Aszodi A, Legate KR, Nakchbandi I, Fassler R (2006) What mouse mutants teach us about extracellular matrix function. *Annual review of cell and developmental biology* **22:** 591-621

Baker DE, Harrison NJ, Maltby E, Smith K, Moore HD, Shaw PJ, Heath PR, Holden H, Andrews PW (2007) Adaptation to culture of human embryonic stem cells and oncogenesis in vivo. *Nature biotechnology* **25:** 207-215

Bakre MM, Hoi A, Mong JC, Koh YY, Wong KY, Stanton LW (2007) Generation of multipotential mesendodermal progenitors from mouse embryonic stem cells via sustained Wnt pathway activation. *The Journal of biological chemistry* **282:** 31703-31712

Balboa D, Weltner J, Eurola S, Trokovic R, Wartiovaara K, Otonkoski T (2015) Conditionally Stabilized dCas9 Activator for Controlling Gene Expression in Human Cell Reprogramming and Differentiation. *Stem cell reports* **5:** 448-459

Ban H, Nishishita N, Fusaki N, Tabata T, Saeki K, Shikamura M, Takada N, Inoue M, Hasegawa M, Kawamata S, Nishikawa S (2011) Efficient generation of transgene-free human induced pluripotent stem cells (iPSCs) by temperature-sensitive Sendai virus vectors. *Proceedings of the National Academy of Sciences of the United States of America* **108:** 14234-14239

Battle MA, Konopka G, Parviz F, Gaggl AL, Yang C, Sladek FM, Duncan SA (2006) Hepatocyte nuclear factor 4alpha orchestrates expression of cell adhesion proteins during the epithelial transformation of the developing liver. *Proceedings of the National Academy of Sciences of the United States of America* **103:** 8419-8424

Baxter M, Withey S, Harrison S, Segeritz CP, Zhang F, Atkinson-Dell R, Rowe C, Gerrard DT, Sison-Young R, Jenkins R, Henry J, Berry AA, Mohamet L, Best M, Fenwick SW, Malik H, Kitteringham NR, Goldring CE, Piper Hanley K, Vallier L, Hanley NA (2015) Phenotypic and functional analyses show stem cell-derived hepatocyte-like cells better mimic fetal rather than adult hepatocytes. *Journal of hepatology* **62:** 581-589

Belayew A, Tilghman SM (1982) Genetic analysis of alpha-fetoprotein synthesis in mice. *Molecular and cellular biology* **2:** 1427-1435

Benhamouche S, Decaens T, Godard C, Chambrey R, Rickman DS, Moinard C, Vasseur-Cognet M, Kuo CJ, Kahn A, Perret C, Colnot S (2006) Apc tumor suppressor gene is the "zonation-keeper" of mouse liver. *Developmental cell* **10:** 759-770

Berg T, Rountree CB, Lee L, Estrada J, Sala FG, Choe A, Veltmaat JM, De Langhe S, Lee R, Tsukamoto H, Crooks GM, Bellusci S, Wang KS (2007) Fibroblast growth factor 10 is critical for liver growth during embryogenesis and controls hepatoblast survival via beta-catenin activation. *Hepatology* **46:** 1187-1197

Bhattacharya M, Malinen MM, Lauren P, Lou YR, Kuisma SW, Kanninen L, Lille M, Corlu A, GuGuen-Guillouzo C, Ikkala O, Laukkanen A, Urtti A, Yliperttula M (2012) Nanofibrillar cellulose hydrogel promotes three-dimensional liver cell culture. *Journal of controlled release : official journal of the Controlled Release Society* **164:** 291-298

Bhushan A, Itoh N, Kato S, Thiery JP, Czernichow P, Bellusci S, Scharfmann R (2001) Fgf10 is essential for maintaining the proliferative capacity of epithelial progenitor cells during early pancreatic organogenesis. *Development* **128:** 5109-5117

Biagini G, Ballardini G (1989) Liver fibrosis and extracellular matrix. *Journal of hepatology* **8:** 115-124

Blouin A, Bolender RP, Weibel ER (1977) Distribution of organelles and membranes between hepatocytes and nonhepatocytes in the rat liver parenchyma. A stereological study. *The Journal of cell biology* **72:** 441-455

Bock C, Kiskinis E, Verstappen G, Gu H, Boulting G, Smith ZD, Ziller M, Croft GF, Amoroso MW, Oakley DH, Gnirke A, Eggan K, Meissner A (2011) Reference Maps of human ES and iPS cell variation enable high-throughput characterization of pluripotent cell lines. *Cell* **144:** 439-452

Bort R, Martinez-Barbera JP, Beddington RS, Zaret KS (2004) Hex homeobox gene-dependent tissue positioning is required for organogenesis of the ventral pancreas. *Development* **131:** 797-806

Bort R, Signore M, Tremblay K, Martinez Barbera JP, Zaret KS (2006) Hex homeobox gene controls the transition of the endoderm to a pseudostratified, cell emergent epithelium for liver bud development. *Developmental biology* **290:** 44-56

Bossard P, Zaret KS (1998) GATA transcription factors as potentiators of gut endoderm differentiation. *Development* **125:** 4909-4917

Boulter L, Govaere O, Bird TG, Radulescu S, Ramachandran P, Pellicoro A, Ridgway RA, Seo SS, Spee B, Van Rooijen N, Sansom OJ, Iredale JP, Lowell S, Roskams T, Forbes SJ (2012) Macrophage-derived Wnt opposes Notch signaling to specify hepatic progenitor cell fate in chronic liver disease. *Nature medicine* **18:** 572-579

Brons IG, Smithers LE, Trotter MW, Rugg-Gunn P, Sun B, Chuva de Sousa Lopes SM, Howlett SK, Clarkson A, Ahrlund-Richter L, Pedersen RA, Vallier L (2007) Derivation of pluripotent epiblast stem cells from mammalian embryos. *Nature* **448:** 191-195

Brown ME, Rondon E, Rajesh D, Mack A, Lewis R, Feng X, Zitur LJ, Learish RD, Nuwaysir EF (2010) Derivation of induced pluripotent stem cells from human peripheral blood T lymphocytes. *PloS one* **5:** e11373

Brown S, Teo A, Pauklin S, Hannan N, Cho CH, Lim B, Vardy L, Dunn NR, Trotter M, Pedersen R, Vallier L (2011) Activin/Nodal signaling controls divergent transcriptional

networks in human embryonic stem cells and in endoderm progenitors. *Stem Cells* **29:** 1176-1185

Cai J, Zhao Y, Liu Y, Ye F, Song Z, Qin H, Meng S, Chen Y, Zhou R, Song X, Guo Y, Ding M, Deng H (2007) Directed differentiation of human embryonic stem cells into functional hepatic cells. *Hepatology* **45:** 1229-1239

Calmont A, Wandzioch E, Tremblay KD, Minowada G, Kaestner KH, Martin GR, Zaret KS (2006) An FGF response pathway that mediates hepatic gene induction in embryonic endoderm cells. *Developmental cell* **11:** 339-348

Cameron K, Tan R, Schmidt-Heck W, Campos G, Lyall MJ, Wang Y, Lucendo-Villarin B, Szkolnicka D, Bates N, Kimber SJ, Hengstler JG, Godoy P, Forbes SJ, Hay DC (2015) Recombinant Laminins Drive the Differentiation and Self-Organization of hESC-Derived Hepatocytes. *Stem cell reports*

Campbell KH, McWhir J, Ritchie WA, Wilmut I (1996) Sheep cloned by nuclear transfer from a cultured cell line. *Nature* **380:** 64-66

Cayo MA, Cai J, DeLaForest A, Noto FK, Nagaoka M, Clark BS, Collery RF, Si-Tayeb K, Duncan SA (2012) JD induced pluripotent stem cell-derived hepatocytes faithfully recapitulate the pathophysiology of familial hypercholesterolemia. *Hepatology* **56:** 2163-2171

Chakraborty S, Christoforou N, Fattahi A, Herzog RW, Leong KW (2013) A robust strategy for negative selection of Cre-loxP recombination-based excision of transgenes in induced pluripotent stem cells. *PloS one* **8:** e64342

Chang CW, Lai YS, Pawlik KM, Liu K, Sun CW, Li C, Schoeb TR, Townes TM (2009) Polycistronic lentiviral vector for "hit and run" reprogramming of adult skin fibroblasts to induced pluripotent stem cells. *Stem Cells* **27:** 1042-1049

Chang TT, Hughes-Fulford M (2009) Monolayer and spheroid culture of human liver hepatocellular carcinoma cell line cells demonstrate distinct global gene expression patterns and functional phenotypes. *Tissue engineering Part A* **15:** 559-567

Chen G, Gulbranson DR, Hou Z, Bolin JM, Ruotti V, Probasco MD, Smuga-Otto K, Howden SE, Diol NR, Propson NE, Wagner R, Lee GO, Antosiewicz-Bourget J, Teng JM, Thomson JA (2011) Chemically defined conditions for human iPSC derivation and culture. *Nature methods* **8:** 424-429

Chen IP, Fukuda K, Fusaki N, Iida A, Hasegawa M, Lichtler A, Reichenberger EJ (2013) Induced pluripotent stem cell reprogramming by integration-free Sendai virus vectors from peripheral blood of patients with craniometaphyseal dysplasia. *Cellular reprogramming* **15:** 503-513

Cheng L, Thomas A, Roth LM, Zheng W, Michael H, Karim FW (2004) OCT4: a novel biomarker for dysgerminoma of the ovary. *The American journal of surgical pathology* **28:** 1341-1346

Cheng X, Ying L, Lu L, Galvao AM, Mills JA, Lin HC, Kotton DN, Shen SS, Nostro MC, Choi JK, Weiss MJ, French DL, Gadue P (2012) Self-renewing endodermal progenitor lines generated from human pluripotent stem cells. *Cell stem cell* **10:** 371-384

Cherry SR, Biniszkiewicz D, van Parijs L, Baltimore D, Jaenisch R (2000) Retroviral expression in embryonic stem cells and hematopoietic stem cells. *Molecular and cellular biology* **20:** 7419-7426

Chin MH, Mason MJ, Xie W, Volinia S, Singer M, Peterson C, Ambartsumyan G, Aimiuwu O, Richter L, Zhang J, Khvorostov I, Ott V, Grunstein M, Lavon N, Benvenisty N, Croce CM, Clark AT, Baxter T, Pyle AD, Teitell MA, Pelegrini M, Plath K, Lowry WE (2009) Induced pluripotent stem cells and embryonic stem cells are distinguished by gene expression signatures. *Cell stem cell* **5:** 111-123

Cho NJ, Elazar M, Xiong A, Lee W, Chiao E, Baker J, Frank CW, Glenn JS (2009) Viral infection of human progenitor and liver-derived cells encapsulated in three-dimensional PEG-based hydrogel. *Biomedical materials* **4:** 011001

Choi J, Lee S, Mallard W, Clement K, Tagliazucchi GM, Lim H, Choi IY, Ferrari F, Tsankov AM, Pop R, Lee G, Rinn JL, Meissner A, Park PJ, Hochedlinger K (2015a) A comparison of genetically matched cell lines reveals the equivalence of human iPSCs and ESCs. *Nature biotechnology*

Choi J, Lee S, Mallard W, Clement K, Tagliazucchi GM, Lim H, Choi IY, Ferrari F, Tsankov AM, Pop R, Lee G, Rinn JL, Meissner A, Park PJ, Hochedlinger K (2015b) A comparison of genetically matched cell lines reveals the equivalence of human iPSCs and ESCs. *Nat Biotechnol* **33:** 1173-1181

Clark AT, Rodriguez RT, Bodnar MS, Abeyta MJ, Cedars MI, Turek PJ, Firpo MT, Reijo Pera RA (2004) Human STELLAR, NANOG, and GDF3 genes are expressed in pluripotent cells and map to chromosome 12p13, a hotspot for teratocarcinoma. *Stem Cells* **22:** 169-179

Clement B, Rescan PY, Baffet G, Loreal O, Lehry D, Campion JP, Guillouzo A (1988) Hepatocytes may produce laminin in fibrotic liver and in primary culture. *Hepatology* **8:** 794-803

Coffinier C, Gresh L, Fiette L, Tronche F, Schutz G, Babinet C, Pontoglio M, Yaniv M, Barra J (2002) Bile system morphogenesis defects and liver dysfunction upon targeted deletion of HNF1beta. *Development* **129:** 1829-1838

Colletti M, Cicchini C, Conigliaro A, Santangelo L, Alonzi T, Pasquini E, Tripodi M, Amicone L (2009) Convergence of Wnt signaling on the HNF4alpha-driven transcription in controlling liver zonation. *Gastroenterology* **137:** 660-672

Couvelard A, Bringuier AF, Dauge MC, Nejjari M, Darai E, Benifla JL, Feldmann G, Henin D, Scoazec JY (1998) Expression of integrins during liver organogenesis in humans. *Hepatology* **27:** 839-847

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:** 1534-1541

Darnell M, Schreiter T, Zeilinger K, Urbaniak T, Soderdahl T, Rossberg I, Dillner B, Berg AL, Gerlach JC, Andersson TB (2011) Cytochrome P450-dependent metabolism in HepaRG cells cultured in a dynamic three-dimensional bioreactor. *Drug metabolism and disposition: the biological fate of chemicals* **39:** 1131-1138

Davis RL, Weintraub H, Lassar AB (1987) Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell* **51:** 987-1000

DeLaForest A, Nagaoka M, Si-Tayeb K, Noto FK, Konopka G, Battle MA, Duncan SA (2011) HNF4A is essential for specification of hepatic progenitors from human pluripotent stem cells. *Development* **138:** 4143-4153

Desmet VJ (1985) Intrahepatic bile ducts under the lens. *Journal of hepatology* **1:** 545-559

Dessimoz J, Opoka R, Kordich JJ, Grapin-Botton A, Wells JM (2006) FGF signaling is necessary for establishing gut tube domains along the anterior-posterior axis in vivo. *Mechanisms of development* **123:** 42-55

Dodsworth BT, Flynn R, Cowley SA (2015) The Current State of Naive Human Pluripotency. *Stem Cells*

Domansky K, Inman W, Serdy J, Dash A, Lim MH, Griffith LG (2010) Perfused multiwell plate for 3D liver tissue engineering. *Lab on a chip* **10:** 51-58

Donato MT, Lahoz A, Castell JV, Gomez-Lechon MJ (2008) Cell lines: a tool for in vitro drug metabolism studies. *Current drug metabolism* **9:** 1-11

Dorey K, Hill CS (2006) A novel Cripto-related protein reveals an essential role for EGF-CFCs in Nodal signalling in Xenopus embryos. *Developmental biology* **292:** 303-316

Draper JS, Smith K, Gokhale P, Moore HD, Maltby E, Johnson J, Meisner L, Zwaka TP, Thomson JA, Andrews PW (2004) Recurrent gain of chromosomes 17q and 12 in cultured human embryonic stem cells. *Nature biotechnology* **22:** 53-54

Droscher A (2014) Images of cell trees, cell lines, and cell fates: the legacy of Ernst Haeckel and August Weismann in stem cell research. 36 Thesis, Hist Philos Life Sci,

Dunn JC, Tompkins RG, Yarmush ML (1991) Long-term in vitro function of adult hepatocytes in a collagen sandwich configuration. *Biotechnology progress* **7:** 237-245

Eggan K, Baldwin K, Tackett M, Osborne J, Gogos J, Chess A, Axel R, Jaenisch R (2004) Mice cloned from olfactory sensory neurons. *Nature* **428:** 44-49

Enver T, Soneji S, Joshi C, Brown J, Iborra F, Orntoft T, Thykjaer T, Maltby E, Smith K, Abu Dawud R, Jones M, Matin M, Gokhale P, Draper J, Andrews PW (2005) Cellular differentiation hierarchies in normal and culture-adapted human embryonic stem cells. *Human molecular genetics* **14:** 3129-3140

Evans MJ, Kaufman MH (1981) Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292:** 154-156

Faber KN, Muller M, Jansen PL (2003) Drug transport proteins in the liver. *Advanced drug delivery reviews* **55:** 107-124

Ferrari S, Griesenbach U, Shiraki-Iida T, Shu T, Hironaka T, Hou X, Williams J, Zhu J, Jeffery PK, Geddes DM, Hasegawa M, Alton EW (2004) A defective nontransmissible

recombinant Sendai virus mediates efficient gene transfer to airway epithelium in vivo. *Gene therapy* **11:** 1659-1664

Finley KR, Tennessen J, Shawlot W (2003) The mouse secreted frizzled-related protein 5 gene is expressed in the anterior visceral endoderm and foregut endoderm during early post-implantation development. *Gene expression patterns : GEP* **3:** 681-684

Fonseca SA, Costas RM, Pereira LV (2015) Searching for naive human pluripotent stem cells. *World journal of stem cells* **7:** 649-656

Font-Burgada J, Shalapour S, Ramaswamy S, Hsueh B, Rossell D, Umemura A, Taniguchi K, Nakagawa H, Valasek MA, Ye L, Kopp JL, Sander M, Carter H, Deisseroth K, Verma IM, Karin M (2015) Hybrid Periportal Hepatocytes Regenerate the Injured Liver without Giving Rise to Cancer. *Cell* **162:** 766-779

Foster KW, Liu Z, Nail CD, Li X, Fitzgerald TJ, Bailey SK, Frost AR, Louro ID, Townes TM, Paterson AJ, Kudlow JE, Lobo-Ruppert SM, Ruppert JM (2005) Induction of KLF4 in basal keratinocytes blocks the proliferation-differentiation switch and initiates squamous epithelial dysplasia. *Oncogene* **24:** 1491-1500

Fusaki N, Ban H, Nishiyama A, Saeki K, Hasegawa M (2009) Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. *Proceedings of the Japan Academy Series B, Physical and biological sciences* **85:** 348-362

Gafni O, Weinberger L, Mansour AA, Manor YS, Chomsky E, Ben-Yosef D, Kalma Y, Viukov S, Maza I, Zviran A, Rais Y, Shipony Z, Mukamel Z, Krupalnik V, Zerbib M, Geula S, Caspi I, Schneir D, Shwartz T, Gilad S, Amann-Zalcenstein D, Benjamin S, Amit I, Tanay A, Massarwa R, Novershtern N, Hanna JH (2013) Derivation of novel human ground state naive pluripotent stem cells. *Nature* **504:** 282-286

Gascon-Barre M, Benbrahim N, Tremblay C (1989) Hepatic zonation of drug metabolizing enzymes. Studies on hepatocytes isolated from the periportal or perivenous region of the liver acinus. *Canadian journal of physiology and pharmacology* **67:** 1015-1022

Gebhardt R, Hovhannisyan A (2010) Organ patterning in the adult stage: the role of Wnt/beta-catenin signaling in liver zonation and beyond. *Developmental dynamics : an official publication of the American Association of Anatomists* **239:** 45-55

Gebhardt R, Matz-Soja M (2014) Liver zonation: Novel aspects of its regulation and its impact on homeostasis. *World J Gastroenterol* **20:** 8491-8504

Gerbal-Chaloin S, Funakoshi N, Caillaud A, Gondeau C, Champon B, Si-Tayeb K (2014) Human induced pluripotent stem cells in hepatology: beyond the proof of concept. *The American journal of pathology* **184:** 332-347

Gerets HH, Tilmant K, Gerin B, Chanteux H, Depelchin BO, Dhalluin S, Atienzar FA (2012) Characterization of primary human hepatocytes, HepG2 cells, and HepaRG cells at the mRNA level and CYP activity in response to inducers and their predictivity for the detection of human hepatotoxins. *Cell biology and toxicology* **28:** 69-87

Ghosh Z, Wilson KD, Wu Y, Hu S, Quertermous T, Wu JC (2010) Persistent donor cell gene expression among human induced pluripotent stem cells contributes to differences with human embryonic stem cells. *PloS one* **5:** e8975

Gidekel S, Pizov G, Bergman Y, Pikarsky E (2003) Oct-3/4 is a dose-dependent oncogenic fate determinant. *Cancer cell* **4:** 361-370

Gieseck RL, 3rd, Hannan NR, Bort R, Hanley NA, Drake RA, Cameron GW, Wynn TA, Vallier L (2014) Maturation of induced pluripotent stem cell derived hepatocytes by 3D-culture. *PloS one* **9:** e86372

Gissen P, Arias IM (2015) Structural and functional hepatocyte polarity and liver disease. *Journal of hepatology*

Godoy P, Hewitt NJ, Albrecht U, Andersen ME, Ansari N, Bhattacharya S, Bode JG, Bolleyn J, Borner C, Bottger J, Braeuning A, Budinsky RA, Burkhardt B, Cameron NR, Camussi G, Cho CS, Choi YJ, Craig Rowlands J, Dahmen U, Damm G, Dirsch O, Donato MT, Dong J, Dooley S, Drasdo D, Eakins R, Ferreira KS, Fonsato V, Fraczek J, Gebhardt R, Gibson A, Glanemann M, Goldring CE, Gomez-Lechon MJ, Groothuis GM, Gustavsson L, Guyot C, Hallifax D, Hammad S, Hayward A, Haussinger D, Hellerbrand C, Hewitt P, Hoehme S, Holzhutter HG, Houston JB, Hrach J, Ito K, Jaeschke H, Keitel V, Kelm JM, Kevin Park B, Kordes C, Kullak-Ublick GA, LeCluyse EL, Lu P, Luebke-Wheeler J, Lutz A, Maltman DJ, Matz-Soja M, McMullen P, Merfort I, Messner S, Meyer C, Mwinyi J, Naisbitt DJ, Nussler AK, Olinga P, Pampaloni F, Pi J, Pluta L, Przyborski SA, Ramachandran A, Rogiers V, Rowe C, Schelcher C, Schmich K, Schwarz M, Singh B, Stelzer EH, Stieger B, Stober R, Sugiyama Y, Tetta C, Thasler WE, Vanhaecke T, Vinken M, Weiss TS, Widera A, Woods CG, Xu JJ, Yarborough KM, Hengstler JG (2013) Recent advances in 2D and 3D in vitro systems using primary hepatocytes, alternative hepatocyte sources and non-parenchymal liver cells and their use in investigating mechanisms of hepatotoxicity, cell signaling and ADME. *Archives of toxicology* **87:** 1315-1530

Goldman O, Han S, Sourrisseau M, Dziedzic N, Hamou W, Corneo B, D'Souza S, Sato T, Kotton DN, Bissig KD, Kalir T, Jacobs A, Evans T, Evans MJ, Gouon-Evans V (2013) KDR Identifies a Conserved Human and Murine Hepatic Progenitor and Instructs Early Liver Development. *Cell stem cell* **12:** 748-760

Gordillo M, Evans T, Gouon-Evans V (2015) Orchestrating liver development. *Development* **142:** 2094-2108

Gore A, Li Z, Fung HL, Young JE, Agarwal S, Antosiewicz-Bourget J, Canto I, Giorgetti A, Israel MA, Kiskinis E, Lee JH, Loh YH, Manos PD, Montserrat N, Panopoulos AD, Ruiz S, Wilbert ML, Yu J, Kirkness EF, Izpisua Belmonte JC, Rossi DJ, Thomson JA, Eggan K, Daley GQ, Goldstein LS, Zhang K (2011) Somatic coding mutations in human induced pluripotent stem cells. *Nature* **471:** 63-67

Goumans MJ, Mummery C (2000) Functional analysis of the TGFbeta receptor/Smad pathway through gene ablation in mice. *The International journal of developmental biology* **44:** 253-265

Gounaris E, Tung CH, Restaino C, Maehr R, Kohler R, Joyce JA, Ploegh HL, Barrett TA, Weissleder R, Khazaie K (2008) Live imaging of cysteine-cathepsin activity reveals dynamics of focal inflammation, angiogenesis, and polyp growth. *PloS one* **3:** e2916

Gripon P, Rumin S, Urban S, Le Seyec J, Glaise D, Cannie I, Guyomard C, Lucas J, Trepo C, Guguen-Guillouzo C (2002) Infection of a human hepatoma cell line by hepatitis B virus. *Proceedings of the National Academy of Sciences of the United States of America* **99:** 15655-15660

Gualdi R, Bossard P, Zheng M, Hamada Y, Coleman JR, Zaret KS (1996) Hepatic specification of the gut endoderm in vitro: cell signaling and transcriptional control. *Genes & development* **10:** 1670-1682

Gunness P, Mueller D, Shevchenko V, Heinzle E, Ingelman-Sundberg M, Noor F (2013) 3D organotypic cultures of human HepaRG cells: a tool for in vitro toxicity studies. *Toxicological sciences : an official journal of the Society of Toxicology* **133:** 67-78

Gurdon JB, Elsdale TR, Fischberg M (1958) Sexually mature individuals of Xenopus laevis from the transplantation of single somatic nuclei. *Nature* **182:** 64-65

Hackam DG, Redelmeier DA (2006) Translation of research evidence from animals to humans. *Jama* **296:** 1731-1732

Hall GP, Redgrave TG, Yeoh GC (1995) The ontogeny of apolipoprotein expression in rat liver. mRNA levels in developing liver and cultured fetal rat hepatocytes. *European journal of biochemistry / FEBS* **228:** 332-336

Haramoto Y, Tanegashima K, Onuma Y, Takahashi S, Sekizaki H, Asashima M (2004) Xenopus tropicalis nodal-related gene 3 regulates BMP signaling: an essential role for the pro-region. *Developmental biology* **265:** 155-168

Hardy K, Handyside AH, Winston RM (1989) The human blastocyst: cell number, death and allocation during late preimplantation development in vitro. *Development* **107:** 597-604

Harley CB (1991) Telomere loss: mitotic clock or genetic time bomb? *Mutation research* **256:** 271-282

Harley CB, Vaziri H, Counter CM, Allsopp RC (1992) The telomere hypothesis of cellular aging. *Experimental gerontology* **27:** 375-382

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 (2008) 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:** 12301-12306

Hengstler JG, Brulport M, Schormann W, Bauer A, Hermes M, Nussler AK, Fandrich F, Ruhnke M, Ungefroren H, Griffin L, Bockamp E, Oesch F, von Mach MA (2005) Generation of human hepatocytes by stem cell technology: definition of the hepatocyte. *Expert opinion on drug metabolism & toxicology* **1:** 61-74

Hochedlinger K, Jaenisch R (2002) Monoclonal mice generated by nuclear transfer from mature B and T donor cells. *Nature* **415:** 1035-1038

Hochedlinger K, Yamada Y, Beard C, Jaenisch R (2005) Ectopic expression of Oct-4 blocks progenitor-cell differentiation and causes dysplasia in epithelial tissues. *Cell* **121:** 465-477

Horton R (2012) GBD 2010: understanding disease, injury, and risk. *Lancet* **380:** 2053-2054

Hotta A, Ellis J (2008) Retroviral vector silencing during iPS cell induction: an epigenetic beacon that signals distinct pluripotent states. *Journal of cellular biochemistry* **105:** 940-948

Hu K (2014) All roads lead to induced pluripotent stem cells: the technologies of iPSC generation. *Stem cells and development* **23:** 1285-1300

Hu Q, Friedrich AM, Johnson LV, Clegg DO (2010) Memory in induced pluripotent stem cells: reprogrammed human retinal-pigmented epithelial cells show tendency for spontaneous redifferentiation. *Stem Cells* **28:** 1981-1991

Huang K, Maruyama T, Fan G (2014a) The naive state of human pluripotent stem cells: a synthesis of stem cell and preimplantation embryo transcriptome analyses. *Cell stem cell* **15:** 410-415

Huang P, He Z, Ji S, Sun H, Xiang D, Liu C, Hu Y, Wang X, Hui L (2011) Induction of functional hepatocyte-like cells from mouse fibroblasts by defined factors. *Nature* **475:** 386-389

Huang P, Zhang L, Gao Y, He Z, Yao D, Wu Z, Cen J, Chen X, Liu C, Hu Y, Lai D, Hu Z, Chen L, Zhang Y, Cheng X, Ma X, Pan G, Wang X, Hui L (2014b) Direct reprogramming of human fibroblasts to functional and expandable hepatocytes. *Cell stem cell* **14:** 370-384

Hunter MP, Wilson CM, Jiang X, Cong R, Vasavada H, Kaestner KH, Bogue CW (2007) The homeobox gene Hhex is essential for proper hepatoblast differentiation and bile duct morphogenesis. *Developmental biology* **308:** 355-367

Hussein SM, Batada NN, Vuoristo S, Ching RW, Autio R, Narva E, Ng S, Sourour M, Hamalainen R, Olsson C, Lundin K, Mikkola M, Trokovic R, Peitz M, Brustle O, Bazett-Jones DP, Alitalo K, Lahesmaa R, Nagy A, Otonkoski T (2011) Copy number variation and selection during reprogramming to pluripotency. *Nature* **471:** 58-62

Hynes RO (2002) Integrins: bidirectional, allosteric signaling machines. *Cell* **110:** 673-687

International Stem Cell I, Amps K, Andrews PW, Anyfantis G, Armstrong L, Avery S, Baharvand H, Baker J, Baker D, Munoz MB, Beil S, Benvenisty N, Ben-Yosef D, Biancotti JC, Bosman A, Brena RM, Brison D, Caisander G, Camarasa MV, Chen J, Chiao E, Choi YM, Choo AB, Collins D, Colman A, Crook JM, Daley GQ, Dalton A, De Sousa PA, Denning C, Downie J, Dvorak P, Montgomery KD, Feki A, Ford A, Fox V, Fraga AM, Frumkin T, Ge L, Gokhale PJ, Golan-Lev T, Gourabi H, Gropp M, Lu G, Hampl A, Harron K, Healy L, Herath W, Holm F, Hovatta O, Hyllner J, Inamdar MS, Irwanto AK, Ishii T, Jaconi M, Jin Y, Kimber S, Kiselev S, Knowles BB, Kopper O, Kukharenko V, Kuliev A, Lagarkova MA, Laird PW, Lako M, Laslett AL, Lavon N, Lee DR, Lee JE, Li C, Lim LS, Ludwig TE, Ma Y, Maltby E, Mateizel I, Mayshar Y,

Mileikovsky M, Minger SL, Miyazaki T, Moon SY, Moore H, Mummery C, Nagy A, Nakatsuji N, Narwani K, Oh SK, Oh SK, Olson C, Otonkoski T, Pan F, Park IH, Pells S, Pera MF, Pereira LV, Qi O, Raj GS, Reubinoff B, Robins A, Robson P, Rossant J, Salekdeh GH, Schulz TC, Sermon K, Sheik Mohamed J, Shen H, Sherrer E, Sidhu K, Sivarajah S, Skottman H, Spits C, Stacey GN, Strehl R, Strelchenko N, Suemori H, Sun B, Suuronen R, Takahashi K, Tuuri T, Venu P, Verlinsky Y, Ward-van Oostwaard D, Weisenberger DJ, Wu Y, Yamanaka S, Young L, Zhou Q (2011) Screening ethnically diverse human embryonic stem cells identifies a chromosome 20 minimal amplicon conferring growth advantage. *Nature biotechnology* **29:** 1132-1144

Ishibashi H, Nakamura M, Komori A, Migita K, Shimoda S (2009) Liver architecture, cell function, and disease. *Seminars in immunopathology* **31:** 399-409

Jacquemin P, Yoshitomi H, Kashima Y, Rousseau GG, Lemaigre FP, Zaret KS (2006) An endothelial-mesenchymal relay pathway regulates early phases of pancreas development. *Developmental biology* **290:** 189-199

Jiang W, Wang J, Zhang Y (2013) Histone H3K27me3 demethylases KDM6A and KDM6B modulate definitive endoderm differentiation from human ESCs by regulating WNT signaling pathway. *Cell research* **23:** 122-130

Jones TD, Ulbright TM, Eble JN, Baldridge LA, Cheng L (2004a) OCT4 staining in testicular tumors: a sensitive and specific marker for seminoma and embryonal carcinoma. *The American journal of surgical pathology* **28:** 935-940

Jones TD, Ulbright TM, Eble JN, Cheng L (2004b) OCT4: A sensitive and specific biomarker for intratubular germ cell neoplasia of the testis. *Clinical cancer research : an official journal of the American Association for Cancer Research* **10:** 8544-8547

Jung J, Zheng M, Goldfarb M, Zaret KS (1999) Initiation of mammalian liver development from endoderm by fibroblast growth factors. *Science* **284:** 1998-2003

Jungermann K (1995) Zonation of metabolism and gene expression in liver. *Histochemistry and cell biology* **103:** 81-91

Jungermann K, Katz N (1989) Functional specialization of different hepatocyte populations. *Physiological reviews* **69:** 708-764

Jungermann K, Kietzmann T (1997) Role of oxygen in the zonation of carbohydrate metabolism and gene expression in liver. *Kidney international* **51:** 402-412

Kaestner KH (2005) The making of the liver: developmental competence in foregut endoderm and induction of the hepatogenic program. *Cell Cycle* **4:** 1146-1148

Kajbafzadeh AM, Javan-Farazmand N, Monajemzadeh M, Baghayee A (2013) Determining the optimal decellularization and sterilization protocol for preparing a tissue scaffold of a human-sized liver tissue. *Tissue engineering Part C, Methods* **19:** 642-651

Kajiwara M, Aoi T, Okita K, Takahashi R, Inoue H, Takayama N, Endo H, Eto K, Toguchida J, Uemoto S, Yamanaka S (2012) Donor-dependent variations in hepatic differentiation from human-induced pluripotent stem cells. *Proceedings of the National Academy of Sciences of the United States of America* **109:** 12538-12543

Kamiya A, Gonzalez FJ (2004) TNF-alpha regulates mouse fetal hepatic maturation induced by oncostatin M and extracellular matrices. *Hepatology* **40:** 527-536

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:** 2367-2379

Kaneko Y, Nakayama T, Tsukamoto A, Kurokawa K (1990) Alteration of differentiation state of human hepatocytes cultured with novobiocin and butyrate. *Cancer research* **50:** 3101-3105

Keng VW, Yagi H, Ikawa M, Nagano T, Myint Z, Yamada K, Tanaka T, Sato A, Muramatsu I, Okabe M, Sato M, Noguchi T (2000) Homeobox gene Hex is essential for onset of mouse embryonic liver development and differentiation of the monocyte lineage. *Biochemical and biophysical research communications* **276:** 1155-1161

Kietzmann T, Jungermann K (1997) Modulation by oxygen of zonal gene expression in liver studied in primary rat hepatocyte cultures. *Cell biology and toxicology* **13:** 243-255

Kikuchi A (2000) Regulation of beta-catenin signaling in the Wnt pathway. *Biochemical and biophysical research communications* **268:** 243-248

Kim D, Kim CH, Moon JI, Chung YG, Chang MY, Han BS, Ko S, Yang E, Cha KY, Lanza R, Kim KS (2009) Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins. *Cell stem cell* **4:** 472-476

Kim K, Doi A, Wen B, Ng K, Zhao R, Cahan P, Kim J, Aryee MJ, Ji H, Ehrlich LI, Yabuuchi A, Takeuchi A, Cunniff KC, Hongguang H, McKinney-Freeman S, Naveiras O, Yoon TJ, Irizarry RA, Jung N, Seita J, Hanna J, Murakami P, Jaenisch R, Weissleder R, Orkin SH, Weissman IL, Feinberg AP, Daley GQ (2010) Epigenetic memory in induced pluripotent stem cells. *Nature* **467:** 285-290

Kim K, Zhao R, Doi A, Ng K, Unternaehrer J, Cahan P, Huo H, Loh YH, Aryee MJ, Lensch MW, Li H, Collins JJ, Feinberg AP, Daley GQ (2011) Donor cell type can influence the epigenome and differentiation potential of human induced pluripotent stem cells. *Nature biotechnology* **29:** 1117-1119

Kinasiewicz A, Kawiaki J, Weynski A. (2006). 3D Matrigel Improves Differentiatited Functions of HepG2 Cells *in vitro*. *Biocybernetics adn Biomedical Engineering* **26**: 47-55.

Kingsley DM (1994) The TGF-beta superfamily: new members, new receptors, and new genetic tests of function in different organisms. *Genes & development* **8:** 133-146

Kitade M, Factor VM, Andersen JB, Tomokuni A, Kaji K, Akita H, Holczbauer A, Seo D, Marquardt JU, Conner EA, Lee SB, Lee YH, Thorgeirsson SS (2013) Specific fate decisions in adult hepatic progenitor cells driven by MET and EGFR signaling. *Genes & development* **27:** 1706-1717

Kitamura T, Koshino Y, Shibata F, Oki T, Nakajima H, Nosaka T, Kumagai H (2003) Retrovirus-mediated gene transfer and expression cloning: powerful tools in functional genomics. *Experimental hematology* **31:** 1007-1014

Kleinman HK, McGarvey ML, Hassell JR, Star VL, Cannon FB, Laurie GW, Martin GR (1986) Basement membrane complexes with biological activity. *Biochemistry* **25:** 312-318

Kleinsmith LJ, Pierce GB, Jr. (1964) Multipotentiality of Single Embryonal Carcinoma Cells. *Cancer research* **24:** 1544-1551

Kojima R, Yoshimoto K, Takahashi E, Ichino M, Miyoshi H, Nagasaki Y (2009) Spheroid array of fetal mouse liver cells constructed on a PEG-gel micropatterned surface: upregulation of hepatic functions by co-culture with nonparenchymal liver cells. *Lab on a chip* **9:** 1991-1993

Lade AG, Monga SP (2011) Beta-catenin signaling in hepatic development and progenitors: which way does the WNT blow? *Developmental dynamics : an official publication of the American Association of Anatomists* **240:** 486-500

Lahti AL, Kujala VJ, Chapman H, Koivisto AP, Pekkanen-Mattila M, Kerkela E, Hyttinen J, Kontula K, Swan H, Conklin BR, Yamanaka S, Silvennoinen O, Aalto-Setala K (2011) Model for long QT syndrome type 2 using human iPS cells demonstrates arrhythmogenic characteristics in cell culture. *Disease models & mechanisms*

Lappalainen RS, Salomaki M, Yla-Outinen L, Heikkila TJ, Hyttinen JA, Pihlajamaki H, Suuronen R, Skottman H, Narkilahti S (2010) Similarly derived and cultured hESC lines show variation in their developmental potential towards neuronal cells in long-term culture. *Regenerative medicine* **5:** 749-762

LeCluyse EL, Audus KL, Hochman JH (1994) Formation of extensive canalicular networks by rat hepatocytes cultured in collagen-sandwich configuration. *The American journal of physiology* **266:** C1764-1774

Lee SB, Seo D, Choi D, Park KY, Holczbauer A, Marquardt JU, Conner EA, Factor VM, Thorgeirsson SS (2012) Contribution of hepatic lineage stage-specific donor memory to the differential potential of induced mouse pluripotent stem cells. *Stem Cells* **30:** 997-1007

Lemaigre FP (2003) Development of the biliary tract. *Mechanisms of development* **120:** 81-87

Li F, He Z, Li Y, Liu P, Chen F, Wang M, Zhu H, Ding X, Wangensteen KJ, Hu Y, Wang X (2011) Combined activin A/LiCl/Noggin treatment improves production of mouse embryonic stem cell-derived definitive endoderm cells. *Journal of cellular biochemistry* **112:** 1022-1034

Li J, Ning G, Duncan SA (2000) Mammalian hepatocyte differentiation requires the transcription factor HNF-4alpha. *Genes & development* **14:** 464-474

Loewer S, Cabili MN, Guttman M, Loh YH, Thomas K, Park IH, Garber M, Curran M, Onder T, Agarwal S, Manos PD, Datta S, Lander ES, Schlaeger TM, Daley GQ, Rinn JL (2010) Large intergenic non-coding RNA-RoR modulates reprogramming of human induced pluripotent stem cells. *Nature genetics* **42:** 1113-1117

Lokmane L, Haumaitre C, Garcia-Villalba P, Anselme I, Schneider-Maunoury S, Cereghini S (2008) Crucial role of vHNF1 in vertebrate hepatic specification. *Development* **135:** 2777-2786

Ludtke TH, Christoffels VM, Petry M, Kispert A (2009) Tbx3 promotes liver bud expansion during mouse development by suppression of cholangiocyte differentiation. *Hepatology* **49:** 969-978

Lv Z, Zhang M, Bi J, Xu F, Hu S, Wen J (2006) Promoter hypermethylation of a novel gene, ZHX2, in hepatocellular carcinoma. *American journal of clinical pathology* **125:** 740-746

Malinen MM, Kanninen LK, Corlu A, Isoniemi HM, Lou YR, Yliperttula ML, Urtti AO (2014) Differentiation of liver progenitor cell line to functional organotypic cultures in 3D nanofibrillar cellulose and hyaluronan-gelatin hydrogels. *Biomaterials* **35:** 5110-5121

Malinen MM, Palokangas H, Yliperttula M, Urtti A (2012) Peptide nanofiber hydrogel induces formation of bile canaliculi structures in three-dimensional hepatic cell culture. *Tissue engineering Part A* **18:** 2418-2425

Mallanna SK, Duncan SA (2013) Differentiation of hepatocytes from pluripotent stem cells. *Current protocols in stem cell biology* **26:** Unit 1G 4

Mallon BS, Hamilton RS, Kozhich OA, Johnson KR, Fann YC, Rao MS, Robey PG (2014) Comparison of the molecular profiles of human embryonic and induced pluripotent stem cells of isogenic origin. *Stem cell research* **12:** 376-386

Margagliotti S, Clotman F, Pierreux CE, Beaudry JB, Jacquemin P, Rousseau GG, Lemaigre FP (2007) The Onecut transcription factors HNF-6/OC-1 and OC-2 regulate early liver expansion by controlling hepatoblast migration. *Developmental biology* **311:** 579-589

Margagliotti S, Clotman F, Pierreux CE, Lemoine P, Rousseau GG, Henriet P, Lemaigre FP (2008) Role of metalloproteinases at the onset of liver development. *Development, growth & differentiation* **50:** 331-338

Martin GR (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proceedings of the National Academy of Sciences of the United States of America* **78:** 7634-7638

Martinez Barbera JP, Clements M, Thomas P, Rodriguez T, Meloy D, Kioussis D, Beddington RS (2000) The homeobox gene Hex is required in definitive endodermal tissues for normal forebrain, liver and thyroid formation. *Development* **127:** 2433-2445

Massague J (1998) TGF-beta signal transduction. *Annual review of biochemistry* **67:** 753-791

Matsumoto K, Yoshitomi H, Rossant J, Zaret KS (2001) Liver organogenesis promoted by endothelial cells prior to vascular function. *Science* **294:** 559-563

Matsushita M, Matsui H (2005) Protein transduction technology. *Journal of molecular medicine* **83:** 324-328

Mazza G, Rombouts K, Rennie Hall A, Urbani L, Vinh Luong T, Al-Akkad W, Longato L, Brown D, Maghsoudlou P, Dhillon AP, Fuller B, Davidson B, Moore K, Dhar D, De Coppi P, Malago M, Pinzani M (2015) Decellularized human liver as a natural 3D-scaffold for liver bioengineering and transplantation. *Scientific reports* **5:** 13079

McClelland R, Wauthier E, Uronis J, Reid L (2008) Gradients in the liver's extracellular matrix chemistry from periportal to pericentral zones: influence on human hepatic progenitors. *Tissue engineering Part A* **14:** 59-70

Medico E, Gentile A, Lo Celso C, Williams TA, Gambarotta G, Trusolino L, Comoglio PM (2001) Osteopontin is an autocrine mediator of hepatocyte growth factor-induced invasive growth. *Cancer research* **61:** 5861-5868

Merkle FT, Neuhausser WM, Santos D, Valen E, Gagnon JA, Maas K, Sandoe J, Schier AF, Eggan K (2015) Efficient CRISPR-Cas9-mediated generation of knockin human pluripotent stem cells lacking undesired mutations at the targeted locus. *Cell reports* **11:** 875-883

Michalopoulos GK, Bowen WC, Mule K, Luo J (2003) HGF-, EGF-, and dexamethasone-induced gene expression patterns during formation of tissue in hepatic organoid cultures. *Gene expression* **11:** 55-75

Mikkola M, Olsson C, Palgi J, Ustinov J, Palomaki T, Horelli-Kuitunen N, Knuutila S, Lundin K, Otonkoski T, Tuuri T (2006) Distinct differentiation characteristics of individual human embryonic stem cell lines. *BMC developmental biology* **6:** 40

Mitalipova MM, Rao RR, Hoyer DM, Johnson JA, Meisner LF, Jones KL, Dalton S, Stice SL (2005) Preserving the genetic integrity of human embryonic stem cells. *Nature biotechnology* **23:** 19-20

Miyajima A, Tanaka M, Itoh T (2014) Stem/progenitor cells in liver development, homeostasis, regeneration, and reprogramming. *Cell stem cell* **14:** 561-574

Miyoshi N, Ishii H, Nagano H, Haraguchi N, Dewi DL, Kano Y, Nishikawa S, Tanemura M, Mimori K, Tanaka F, Saito T, Nishimura J, Takemasa I, Mizushima T, Ikeda M, Yamamoto H, Sekimoto M, Doki Y, Mori M (2011) Reprogramming of mouse and human cells to pluripotency using mature microRNAs. *Cell stem cell* **8:** 633-638

Molina-Jimenez F, Benedicto I, Dao Thi VL, Gondar V, Lavillette D, Marin JJ, Briz O, Moreno-Otero R, Aldabe R, Baumert TF, Cosset FL, Lopez-Cabrera M, Majano PL (2012) Matrigel-embedded 3D culture of Huh-7 cells as a hepatocyte-like polarized system to study hepatitis C virus cycle. *Virology* **425:** 31-39

Monga SP (2014) Role and regulation of beta-catenin signaling during physiological liver growth. *Gene expression* **16:** 51-62

Monga SP, Monga HK, Tan X, Mule K, Pediaditakis P, Michalopoulos GK (2003) Beta-catenin antisense studies in embryonic liver cultures: role in proliferation, apoptosis, and lineage specification. *Gastroenterology* **124:** 202-216

Moscovitz JE, Aleksunes LM (2013) Establishment of metabolism and transport pathways in the rodent and human fetal liver. *International journal of molecular sciences* **14:** 23801-23827

Moustakas A, Heldin CH (2009) The regulation of TGFbeta signal transduction. *Development* **136:** 3699-3714

Mueller D, Kramer L, Hoffmann E, Klein S, Noor F (2014) 3D organotypic HepaRG cultures as in vitro model for acute and repeated dose toxicity studies. *Toxicology in vitro : an international journal published in association with BIBRA* **28:** 104-112

Mullen AC, Orlando DA, Newman JJ, Loven J, Kumar RM, Bilodeau S, Reddy J, Guenther MG, DeKoter RP, Young RA (2011) Master transcription factors determine cell-type-specific responses to TGF-beta signaling. *Cell* **147:** 565-576

Nair V (2008) Retrovirus-induced oncogenesis and safety of retroviral vectors. *Current opinion in molecular therapeutics* **10:** 431-438

Nakagawa M, Takizawa N, Narita M, Ichisaka T, Yamanaka S (2010) Promotion of direct reprogramming by transformation-deficient Myc. *Proceedings of the National Academy of Sciences of the United States of America* **107:** 14152-14157

Nakanishi M, Otsu M (2012) Development of Sendai virus vectors and their potential applications in gene therapy and regenerative medicine. *Current gene therapy* **12:** 410-416

Nashun B, Hill PW, Hajkova P (2015) Reprogramming of cell fate: epigenetic memory and the erasure of memories past. *The EMBO journal* **34:** 1296-1308

Newman AM, Cooper JB (2010) Lab-specific gene expression signatures in pluripotent stem cells. *Cell stem cell* **7:** 258-262

Oda Y, Yoshimura Y, Ohnishi H, Tadokoro M, Katsube Y, Sasao M, Kubo Y, Hattori K, Saito S, Horimoto K, Yuba S, Ohgushi H (2010) Induction of pluripotent stem cells from human third molar mesenchymal stromal cells. *The Journal of biological chemistry* **285:** 29270-29278

Okita K, Ichisaka T, Yamanaka S (2007) Generation of germline-competent induced pluripotent stem cells. *Nature* **448:** 313-317

Okita K, Matsumura Y, Sato Y, Okada A, Morizane A, Okamoto S, Hong H, Nakagawa M, Tanabe K, Tezuka K, Shibata T, Kunisada T, Takahashi M, Takahashi J, Saji H, Yamanaka S (2011) A more efficient method to generate integration-free human iPS cells. *Nature methods* **8:** 409-412

Onuma Y, Yeo CY, Whitman M (2006) XCR2, one of three Xenopus EGF-CFC genes, has a distinct role in the regulation of left-right patterning. *Development* **133:** 237-250

Orhan H (2015) Extrahepatic targets and cellular reactivity of drug metabolites. *Current medicinal chemistry* **22:** 408-437

Osafune K, Caron L, Borowiak M, Martinez RJ, Fitz-Gerald CS, Sato Y, Cowan CA, Chien KR, Melton DA (2008) Marked differences in differentiation propensity among human embryonic stem cell lines. *Nature biotechnology* **26:** 313-315

Pachnis V, Belayew A, Tilghman SM (1984) Locus unlinked to alpha-fetoprotein under the control of the murine raf and Rif genes. *Proceedings of the National Academy of Sciences of the United States of America* **81:** 5523-5527

Paku S, Schnur J, Nagy P, Thorgeirsson SS (2001) Origin and structural evolution of the early proliferating oval cells in rat liver. *The American journal of pathology* **158:** 1313-1323

Parviz F, Matullo C, Garrison WD, Savatski L, Adamson JW, Ning G, Kaestner KH, Rossi JM, Zaret KS, Duncan SA (2003) Hepatocyte nuclear factor 4alpha controls the development of a hepatic epithelium and liver morphogenesis. *Nature genetics* **34:** 292-296

Perincheri S, Dingle RW, Peterson ML, Spear BT (2005) Hereditary persistence of alpha-fetoprotein and H19 expression in liver of BALB/cJ mice is due to a retrovirus insertion in the Zhx2 gene. *Proc Natl Acad Sci U S A* **102:** 396-401

Phanstiel DH, Brumbaugh J, Wenger CD, Tian S, Probasco MD, Bailey DJ, Swaney DL, Tervo MA, Bolin JM, Ruotti V, Stewart R, Thomson JA, Coon JJ (2011) Proteomic and phosphoproteomic comparison of human ES and iPS cells. *Nature methods* **8:** 821-827

Piao Y, Hung SS, Lim SY, Wong RC, Ko MS (2014) Efficient generation of integration-free human induced pluripotent stem cells from keratinocytes by simple transfection of episomal vectors. *Stem cells translational medicine* **3:** 787-791

Pilcher KE, Krieg PA (2002) Expression of the Wnt inhibitor, sFRP5, in the gut endoderm of Xenopus. *Gene expression patterns : GEP* **2:** 369-372

Polo JM, Liu S, Figueroa ME, Kulalert W, Eminli S, Tan KY, Apostolou E, Stadtfeld M, Li Y, Shioda T, Natesan S, Wagers AJ, Melnick A, Evans T, Hochedlinger K (2010) Cell type of origin influences the molecular and functional properties of mouse induced pluripotent stem cells. *Nature biotechnology* **28:** 848-855

Purcell R, Childs M, Maibach R, Miles C, Turner C, Zimmermann A, Sullivan M (2011) HGF/c-Met related activation of beta-catenin in hepatoblastoma. *Journal of experimental & clinical cancer research : CR* **30:** 96

Ramalho-Santos M, Willenbring H (2007) On the origin of the term "stem cell". *Cell stem cell* **1:** 35-38

Ramalingam S, Annaluru N, Kandavelou K, Chandrasegaran S (2014) TALEN-mediated generation and genetic correction of disease-specific human induced pluripotent stem cells. *Current gene therapy* **14:** 461-472

Ramasamy TS, Yu JS, Selden C, Hodgson H, Cui W (2013) Application of three-dimensional culture conditions to human embryonic stem cell-derived definitive endoderm cells enhances hepatocyte differentiation and functionality. *Tissue engineering Part A* **19:** 360-367

Rashid ST, Corbineau S, Hannan N, Marciniak SJ, Miranda E, Alexander G, Huang-Doran I, Griffin J, Ahrlund-Richter L, Skepper J, Semple R, Weber A, Lomas DA, Vallier L (2010) Modeling inherited metabolic disorders of the liver using human induced pluripotent stem cells. *The Journal of clinical investigation* **120:** 3127-3136

Rebelo SP, Costa R, Estrada M, Shevchenko V, Brito C, Alves PM (2015) HepaRG microencapsulated spheroids in DMSO-free culture: novel culturing approaches for

enhanced xenobiotic and biosynthetic metabolism. *Archives of toxicology* **89:** 1347-1358

Robinton DA, Daley GQ (2012) The promise of induced pluripotent stem cells in research and therapy. *Nature* **481:** 295-305

Rossi JM, Dunn NR, Hogan BL, Zaret KS (2001) Distinct mesodermal signals, including BMPs from the septum transversum mesenchyme, are required in combination for hepatogenesis from the endoderm. *Genes & development* **15:** 1998-2009

Rouhani F, Kumasaka N, de Brito MC, Bradley A, Vallier L, Gaffney D (2014) Genetic background drives transcriptional variation in human induced pluripotent stem cells. *PLoS genetics* **10:** e1004432

Sancho-Bru P, Najimi M, Caruso M, Pauwelyn K, Cantz T, Forbes S, Roskams T, Ott M, Gehling U, Sokal E, Verfaillie CM, Muraca M (2009) Stem and progenitor cells for liver repopulation: can we standardise the process from bench to bedside? *Gut* **58:** 594-603

Santangelo L, Marchetti A, Cicchini C, Conigliaro A, Conti B, Mancone C, Bonzo JA, Gonzalez FJ, Alonzi T, Amicone L, Tripodi M (2011) The stable repression of mesenchymal program is required for hepatocyte identity: a novel role for hepatocyte nuclear factor 4alpha. *Hepatology* **53:** 2063-2074

Schleicher J, Tokarski C, Marbach E, Matz-Soja M, Zellmer S, Gebhardt R, Schuster S (2015) Zonation of hepatic fatty acid metabolism - The diversity of its regulation and the benefit of modeling. *Biochimica et biophysica acta* **1851:** 641-656

Schmelzer E, Mutig K, Schrade P, Bachmann S, Gerlach JC, Zeilinger K (2009) Effect of human patient plasma ex vivo treatment on gene expression and progenitor cell activation of primary human liver cells in multi-compartment 3D perfusion bioreactors for extra-corporeal liver support. *Biotechnology and bioengineering* **103:** 817-827

Schmelzer E, Zhang L, Bruce A, Wauthier E, Ludlow J, Yao HL, Moss N, Melhem A, McClelland R, Turner W, Kulik M, Sherwood S, Tallheden T, Cheng N, Furth ME, Reid LM (2007) Human hepatic stem cells from fetal and postnatal donors. *The Journal of experimental medicine* **204:** 1973-1987

Scholzen T, Gerdes J (2000) The Ki-67 protein: from the known and the unknown. *Journal of cellular physiology* **182:** 311-322

Seki T, Yuasa S, Oda M, Egashira T, Yae K, Kusumoto D, Nakata H, Tohyama S, Hashimoto H, Kodaira M, Okada Y, Seimiya H, Fusaki N, Hasegawa M, Fukuda K (2010) Generation of induced pluripotent stem cells from human terminally differentiated circulating T cells. *Cell stem cell* **7:** 11-14

Sekiya S, Suzuki A (2011) Direct conversion of mouse fibroblasts to hepatocyte-like cells by defined factors. *Nature* **475:** 390-393

Serls AE, Doherty S, Parvatiyar P, Wells JM, Deutsch GH (2005) Different thresholds of fibroblast growth factors pattern the ventral foregut into liver and lung. *Development* **132:** 35-47

Seth A, Ye J, Yu N, Guez F, Bedford DC, Neale GA, Cordi S, Brindle PK, Lemaigre FP, Kaestner KH, Sosa-Pineda B (2014) Prox1 ablation in hepatic progenitors causes defective hepatocyte specification and increases biliary cell commitment. *Development* **141:** 538-547

Shan J, Schwartz RE, Ross NT, Logan DJ, Thomas D, Duncan SA, North TE, Goessling W, Carpenter AE, Bhatia SN (2013) Identification of small molecules for human hepatocyte expansion and iPS differentiation. *Nature chemical biology* **9:** 514-520

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

Sherwood RI, Maehr R, Mazzoni EO, Melton DA (2011) Wnt signaling specifies and patterns intestinal endoderm. *Mechanisms of development* **128:** 387-400

Shibamoto S, Higano K, Takada R, Ito F, Takeichi M, Takada S (1998) Cytoskeletal reorganization by soluble Wnt-3a protein signalling. *Genes to cells : devoted to molecular & cellular mechanisms* **3:** 659-670

Shimada H, Hashimoto Y, Nakada A, Shigeno K, Nakamura T (2012) Accelerated generation of human induced pluripotent stem cells with retroviral transduction and chemical inhibitors under physiological hypoxia. *Biochemical and biophysical research communications* **417:** 659-664

Shlomai A, Schwartz RE, Ramanan V, Bhatta A, de Jong YP, Bhatia SN, Rice CM (2014) Modeling host interactions with hepatitis B virus using primary and induced pluripotent stem cell-derived hepatocellular systems. *Proceedings of the National Academy of Sciences of the United States of America* **111:** 12193-12198

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

Si-Tayeb K, Noto FK, Nagaoka M, Li J, Battle MA, Duris C, North PE, Dalton S, Duncan SA (2010b) Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells. *Hepatology* **51:** 297-305

Skottman H (2010) Derivation and characterization of three new human embryonic stem cell lines in Finland. *In vitro cellular & developmental biology Animal* **46:** 206-209

Snykers S, De Kock J, Rogiers V, Vanhaecke T (2009) In vitro differentiation of embryonic and adult stem cells into hepatocytes: state of the art. *Stem Cells* **27:** 577-605

So J, Martin BL, Kimelman D, Shin D (2013) Wnt/beta-catenin signaling cell-autonomously converts non-hepatic endodermal cells to a liver fate. *Biology open* **2:** 30-36

Sokol SY (2011) Maintaining embryonic stem cell pluripotency with Wnt signaling. *Development* **138:** 4341-4350

Sommer CA, Christodoulou C, Gianotti-Sommer A, Shen SS, Sailaja BS, Hezroni H, Spira A, Meshorer E, Kotton DN, Mostoslavsky G (2012) Residual expression of reprogramming factors affects the transcriptional program and epigenetic signatures of induced pluripotent stem cells. *PloS one* **7:** e51711

Sommer CA, Sommer AG, Longmire TA, Christodoulou C, Thomas DD, Gostissa M, Alt FW, Murphy GJ, Kotton DN, Mostoslavsky G (2010) Excision of reprogramming transgenes improves the differentiation potential of iPS cells generated with a single excisable vector. *Stem Cells* **28:** 64-74

Sosa-Pineda B, Wigle JT, Oliver G (2000) Hepatocyte migration during liver development requires Prox1. *Nature genetics* **25:** 254-255

Soto-Gutierrez A, Zhang L, Medberry C, Fukumitsu K, Faulk D, Jiang H, Reing J, Gramignoli R, Komori J, Ross M, Nagaya M, Lagasse E, Stolz D, Strom SC, Fox IJ, Badylak SF (2011) A whole-organ regenerative medicine approach for liver replacement. *Tissue engineering Part C, Methods* **17:** 677-686

Spear BT (1999) Alpha-fetoprotein gene regulation: lessons from transgenic mice. *Seminars in cancer biology* **9:** 109-116

Spear BT, Jin L, Ramasamy S, Dobierzewska A (2006) Transcriptional control in the mammalian liver: liver development, perinatal repression, and zonal gene regulation. *Cellular and molecular life sciences : CMLS* **63:** 2922-2938

Stadtfeld M, Hochedlinger K (2010) Induced pluripotency: history, mechanisms, and applications. *Genes & development* **24:** 2239-2263

Stadtfeld M, Maherali N, Breault DT, Hochedlinger K (2008) Defining molecular cornerstones during fibroblast to iPS cell reprogramming in mouse. *Cell stem cell* **2:** 230-240

Starzl TE, Fung JJ (2010) Themes of liver transplantation. *Hepatology* **51:** 1869-1884

Stevens LC, Little CC (1954) Spontaneous Testicular Teratomas in an Inbred Strain of Mice. *Proceedings of the National Academy of Sciences of the United States of America* **40:** 1080-1087

Su RJ, Neises A, Zhang XB (2014) Generation of iPS Cells from Human Peripheral Blood Mononuclear Cells Using Episomal Vectors. *Methods Mol Biol*

Swift B, Pfeifer ND, Brouwer KL (2010) Sandwich-cultured hepatocytes: an in vitro model to evaluate hepatobiliary transporter-based drug interactions and hepatotoxicity. *Drug metabolism reviews* **42:** 446-471

Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131:** 861-872

Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126:** 663-676

Takase HM, Itoh T, Ino S, Wang T, Koji T, Akira S, Takikawa Y, Miyajima A (2013) FGF7 is a functional niche signal required for stimulation of adult liver progenitor cells that support liver regeneration. *Genes & development* **27:** 169-181

Takashima Y, Guo G, Loos R, Nichols J, Ficz G, Krueger F, Oxley D, Santos F, Clarke J, Mansfield W, Reik W, Bertone P, Smith A (2014) Resetting transcription factor control circuitry toward ground-state pluripotency in human. *Cell* **158:** 1254-1269

Takayama K, Inamura M, Kawabata K, Katayama K, Higuchi M, Tashiro K, Nonaka A, Sakurai F, Hayakawa T, Furue MK, Mizuguchi H (2012) Efficient generation of functional hepatocytes from human embryonic stem cells and induced pluripotent stem cells by HNF4alpha transduction. *Molecular therapy : the journal of the American Society of Gene Therapy* **20:** 127-137

Takayama K, Kawabata K, Nagamoto Y, Kishimoto K, Tashiro K, Sakurai F, Tachibana M, Kanda K, Hayakawa T, Furue MK, Mizuguchi H (2013a) 3D spheroid culture of hESC/hiPSC-derived hepatocyte-like cells for drug toxicity testing. *Biomaterials* **34:** 1781-1789

Takayama K, Nagamoto Y, Mimura N, Tashiro K, Sakurai F, Tachibana M, Hayakawa T, Kawabata K, Mizuguchi H (2013b) Long-term self-renewal of human ES/iPS-derived hepatoblast-like cells on human laminin 111-coated dishes. *Stem cell reports* **1:** 322-335

Takebe T, Sekine K, Enomura M, Koike H, Kimura M, Ogaeri T, Zhang RR, Ueno Y, Zheng YW, Koike N, Aoyama S, Adachi Y, Taniguchi H (2013) Vascularized and functional human liver from an iPSC-derived organ bud transplant. *Nature* **499:** 481-484

Tam PP, Loebel DA (2009) Specifying mouse embryonic germ cells. *Cell* **137:** 398-400

Tamminen K, Balboa D, Toivonen S, Pakarinen MP, Wiener Z, Alitalo K, Otonkoski T (2015) Intestinal Commitment and Maturation of Human Pluripotent Stem Cells Is Independent of Exogenous FGF4 and R-spondin1. *PloS one* **10:** e0134551

Tan X, Apte U, Micsenyi A, Kotsagrelos E, Luo JH, Ranganathan S, Monga DK, Bell A, Michalopoulos GK, Monga SP (2005) Epidermal growth factor receptor: a novel target of the Wnt/beta-catenin pathway in liver. *Gastroenterology* **129:** 285-302

Tanimizu N, Saito H, Mostov K, Miyajima A (2004) Long-term culture of hepatic progenitors derived from mouse Dlk+ hepatoblasts. *Journal of cell science* **117:** 6425-6434

Taub R (2004) Liver regeneration: from myth to mechanism. *Nature reviews Molecular cell biology* **5:** 836-847

Teichroeb JH, Betts DH, Vaziri H (2011) Suppression of the imprinted gene NNAT and X-chromosome gene activation in isogenic human iPS cells. *PloS one* **6:** e23436

Tesar PJ, Chenoweth JG, Brook FA, Davies TJ, Evans EP, Mack DL, Gardner RL, McKay RD (2007) New cell lines from mouse epiblast share defining features with human embryonic stem cells. *Nature* **448:** 196-199

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM (1998) Embryonic stem cell lines derived from human blastocysts. *Science* **282:** 1145-1147

Tian T, Meng AM (2006) Nodal signals pattern vertebrate embryos. *Cellular and molecular life sciences : CMLS* **63:** 672-685

Timens W, Kamps WA (1997) Hemopoiesis in human fetal and embryonic liver. *Microsc Res Tech* **39:** 387-397

Touboul T, Hannan NR, Corbineau S, Martinez A, Martinet C, Branchereau S, Mainot S, Strick-Marchand H, Pedersen R, Di Santo J, Weber A, Vallier L (2010) Generation of functional hepatocytes from human embryonic stem cells under chemically defined conditions that recapitulate liver development. *Hepatology* **51:** 1754-1765

Tremblay KD, Zaret KS (2005) Distinct populations of endoderm cells converge to generate the embryonic liver bud and ventral foregut tissues. *Developmental biology* **280:** 87-99

Treyer A, Musch A (2013) Hepatocyte polarity. *Comprehensive Physiology* **3:** 243-287

Trokovic R, Weltner J, Otonkoski T (2015) Generation of iPSC line HEL47.2 from healthy human adult fibroblasts. *Stem cell research* **15:** 263-265

Vacanti JP, Kulig KM (2014) Liver cell therapy and tissue engineering for transplantation. *Seminars in pediatric surgery* **23:** 150-155

Vellonen KS, Malinen M, Mannermaa E, Subrizi A, Toropainen E, Lou YR, Kidron H, Yliperttula M, Urtti A (2014) A critical assessment of in vitro tissue models for ADME and drug delivery. *Journal of controlled release : official journal of the Controlled Release Society* **190:** 94-114

Vincent SD, Dunn NR, Hayashi S, Norris DP, Robertson EJ (2003) Cell fate decisions within the mouse organizer are governed by graded Nodal signals. *Genes & development* **17:** 1646-1662

Virtanen I, Gullberg D, Rissanen J, Kivilaakso E, Kiviluoto T, Laitinen LA, Lehto VP, Ekblom P (2000) Laminin alpha1-chain shows a restricted distribution in epithelial basement membranes of fetal and adult human tissues. *Experimental cell research* **257:** 298-309

Vuoristo S, Virtanen I, Takkunen M, Palgi J, Kikkawa Y, Rousselle P, Sekiguchi K, Tuuri T, Otonkoski T (2009) Laminin isoforms in human embryonic stem cells: synthesis, receptor usage and growth support. *J Cell Mol Med* **13:** 2622-2633

Wadee AA, Paterson A, Coplan KA, Reddy SG (1994) HLA expression in hepatocellular carcinoma cell lines. *Clinical and experimental immunology* **97:** 328-333

Wandzioch E, Zaret KS (2009) Dynamic signaling network for the specification of embryonic pancreas and liver progenitors. *Science* **324:** 1707-1710

Wang B, Zhao L, Fish M, Logan CY, Nusse R (2015) Self-renewing diploid Axin2(+) cells fuel homeostatic renewal of the liver. *Nature* **524:** 180-185

Wang Y, Cui CB, Yamauchi M, Miguez P, Roach M, Malavarca R, Costello MJ, Cardinale V, Wauthier E, Barbier C, Gerber DA, Alvaro D, Reid LM (2011) Lineage restriction of human hepatic stem cells to mature fates is made efficient by tissue-specific biomatrix scaffolds. *Hepatology* **53:** 293-305

Warren L, Ni Y, Wang J, Guo X (2012) Feeder-free derivation of human induced pluripotent stem cells with messenger RNA. *Scientific reports* **2:** 657

Wells JM, Melton DA (1999) Vertebrate endoderm development. *Annual review of cell and developmental biology* **15:** 393-410

Weltner J, Anisimov A, Alitalo K, Otonkoski T, Trokovic R (2012) Induced pluripotent stem cell clones reprogrammed via recombinant adeno-associated virus-mediated transduction contain integrated vector sequences. *Journal of virology* **86:** 4463-4467

Xie H, Ye M, Feng R, Graf T (2004) Stepwise reprogramming of B cells into macrophages. *Cell* **117:** 663-676

Yang G, Si-Tayeb K, Corbineau S, Vernet R, Gayon R, Dianat N, Martinet C, Clay D, Goulinet-Mainot S, Tachdjian G, Tachdjian G, Burks D, Vallier L, Bouille P, Dubart-Kupperschmitt A, Weber A (2013) Integration-deficient lentivectors: an effective strategy to purify and differentiate human embryonic stem cell-derived hepatic progenitors. *BMC biology* **11:** 86

Ying L, Mills JA, French DL, Gadue P (2015) OCT4 Coordinates with WNT Signaling to Pre-pattern Chromatin at the SOX17 Locus during Human ES Cell Differentiation into Definitive Endoderm. *Stem cell reports* **5:** 490-498

Yirme G, Amit M, Laevsky I, Osenberg S, Itskovitz-Eldor J (2008) Establishing a dynamic process for the formation, propagation, and differentiation of human embryoid bodies. *Stem cells and development* **17:** 1227-1241

Yu J, Hu K, Smuga-Otto K, Tian S, Stewart R, Slukvin, II, Thomson JA (2009) Human induced pluripotent stem cells free of vector and transgene sequences. *Science* **324:** 797-801

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:** 1917-1920

Yusa K, Rashid ST, Strick-Marchand H, Varela I, Liu PQ, Paschon DE, Miranda E, Ordonez A, Hannan NR, Rouhani FJ, Darche S, Alexander G, Marciniak SJ, Fusaki N, Hasegawa M, Holmes MC, Di Santo JP, Lomas DA, Bradley A, Vallier L (2011) Targeted gene correction of alpha1-antitrypsin deficiency in induced pluripotent stem cells. *Nature* **478:** 391-394

Zaret KS (2002) Regulatory phases of early liver development: paradigms of organogenesis. *Nature reviews Genetics* **3:** 499-512

Zeilinger K, Holland G, Sauer IM, Efimova E, Kardassis D, Obermayer N, Liu M, Neuhaus P, Gerlach JC (2004) Time course of primary liver cell reorganization in

three-dimensional high-density bioreactors for extracorporeal liver support: an immunohistochemical and ultrastructural study. *Tissue engineering* **10:** 1113-1124

Zhao T, Zhang ZN, Rong Z, Xu Y (2011) Immunogenicity of induced pluripotent stem cells. *Nature* **474:** 212-215

Zhou T, Benda C, Dunzinger S, Huang Y, Ho JC, Yang J, Wang Y, Zhang Y, Zhuang Q, Li Y, Bao X, Tse HF, Grillari J, Grillari-Voglauer R, Pei D, Esteban MA (2012) Generation of human induced pluripotent stem cells from urine samples. *Nature protocols* **7:** 2080-2089

Zong Y, Panikkar A, Xu J, Antoniou A, Raynaud P, Lemaigre F, Stanger BZ (2009) Notch signaling controls liver development by regulating biliary differentiation. *Development* **136:** 1727-1739