Differentiation of human Embryonic Stem Cells into hepatocytes as a tool to analyse dynamic regulatory events during hepatogenesis *in vitro*

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Contents

Abbreviations

Abbreviations

cRNA	complementary ribonucleic acid
APC	adenomatous polyposis coli
ATP	adenosine triphosphate
AVE	anterior visceral endoderm
BMP	bone morphogenic protein
BP	biological process
BSA	bovine serum albumin
CC	cellular compartment
cDNA	complementary deoxyribonucleic acid
CM	conditioned medium
Ct	threshold cycle
DE	definitive endoderm
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dpc	days post-coitum
DVE	distal visceral endoderm
E	embryonic day
EBs	embryoid bodies
EC	embryonic carcinoma
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ExEn	extraembryonic endoderm
FDA	Food and Drug Administration
FGF	fibroblast growth factor
GO	gene ontology
hESCs	human Embryonic Stem Cells
HGF	hepatocyte growth factor
iPSCs	induced Pluripotent Stem Cells

ITS	insulin-transferrin-sodium selenite
LETFs	liver enriched transcription factors
MAPK	mitogen-activated protein kinase
MEFs	mouse embryonic fibroblasts
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
NIH	National Institute of Health
OSM	oncostatin M
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PI3K	phosphatidylinosytol-3-kinase
RNA	ribonucleic acid
RT	room temperature
SDS	sodium dodecyl sulfate
SR	serum replacement
SSC	sodium chloride/sodium citrate
STM	septum transversum mesenchyme
TGFβ	transforming growth factor β
UM	unconditioned medium
VE	visceral endoderm

Semantics

°C	degrees Celsius
μg	micrograms
μl	microlitres
μM	micromol
g	grams / gravity
hr/hrs	hours
M	mol
mg	milligrams
min	minutes
ml	millilitres
mm	millimetres
mM	millimol
ng	nanograms
nm	nanometres
s	seconds
U	units
V	volt

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Abstract

The differentiation of human embryonic stem cells (hESCs) into functional hepatocytes provides a powerful *in vitro* model system for analysing the molecular mechanisms involved in liver development. Moreover, a well-characterized renewable source of hepatocytes differentiated from human ES cells could be used for *in vitro* assays of drug metabolism and toxicology. The mechanisms underlying the molecular embryological events leading to fetal liver development has been studied by genetic analysis in rodents and explants studies in the model organisms such as the chick embryo. Nowadays the pluripotent characteristics of human ES cells provide great promise for the study of early development in man. Especially because protocols leading to differentiation of human ES cells into cells possessing morphologic and molecular features typical for hepatocytes are available.

Genetic studies in the model organisms have unveiled the major inducers of hepatogenesis such as FGF4, BMP2, HGF, oncostatin M and dexamethasone. Currently the biggest challenge is to gain insight into target genes and associated signaling pathways regulated by extracellular ligands, which are normally sequentially supplemented into in vitro differentiation protocols. This knowledge will in turn enhance our understanding of the dynamic developmental events at the transcript and proteome level. This study was aimed at elucidating mechanisms of *in vitro* hepatogenesis, which mimics the *in vivo* process. In order to performed time-resolved analysis of the process of hepatogenesis, I used a reproducible *in vitro* system (two different protocols) and analysed the transcriptomes of the differentiating human ES cells in a step-wise manner (definitive endoderm induction, early hepatic cells, hepatic maturation) throughout the entire process. This *in vitro* analysis has revealed a broad spectrum of molecular cascades involving cell surface receptors, transcriptional regulators and associated signaling pathways that might be driving hepatogenesis *in vivo*. In addition, these experiments revealed that at the transcriptional level human ES cells-derived hepatocyte-like cells have some similarity to fetal liver, but in some aspects present an individual, exclusive transcription pattern.

In summary, the results suggest that an *in vitro* system for hepatic differentiation is a potent tool for analyzing molecular pathways involved at each stage of this process. However, further effort is required to obtain more mature cells, and the knowledge gained from the transcript analysis might aid in achieving this goal.

Abstract (German)

Die Differenzierung humaner embryonaler Stammzellen (hESC) in funktionelle Hepatozyten bietet ein effektives *in vitro* Modellsystem zur Untersuchung von molekularen Mechanismen, die an der Leberentwicklung beteiligt sind. Darüber hinaus kann die gut charakterisierte, erneuerbare, aus humanen ES-Zellen differenzierte Hepatozytenquelle für *in vitro* Tests verwendet werden, welche die Verstoffwechselung von Pharmaka untersuchen, sowie in der Toxikologie. Mechanismen der zugrunde liegenden molekularembryologischen Prozesse, die zu der fötalen Leberentwicklung führen, wurden in der Vergangenheit mittels genetischer Analysen in Nagetieren sowie durch Explantatstudien an Modellorganismen wie Hühnerembryonen untersucht. Die pluripotenten Eigenschaften der embryonalen Stammzellen eröffnen heutzutage diverse Möglichkeiten zur Erforschung der Frühentwicklung des Menschen. Unter anderem stehen Protokolle zur Verfügung die die Differenzierung von humanen ES-Zellen in Zellen mit typischen morphologischen und molekularen Hepatozytenmerkmalen ermöglichen.

Durch genetische Studien an Modellorganismen wurden die wichtigsten Induktoren der Hepatogenese wie FGF4, BMP2, HGF sowie Oncostatin M und Dexamethason aufgedeckt. Die derzeit größte Herausforderung ist es, deren leberspezifische Zielgene zu identifizieren und einen Einblick in den jeweiligen Induktionsprozess zu gewinnen. Solche Induktionsprozesse werden durch extrazelluläre Liganden reguliert, welche gewöhnlich während des *in vitro* Differenzierungsprozesses hinzugefügt werden. Dieses Wissen erweitert wiederum unser Verständnis der dynamischen Entwicklungsprozesse auf der Transkriptions- und Proteomebene. Das Ziel dieser Studie war es, *in vitro* Hepatogenesemechanismem aufzuklären, die den Prozess imitieren, welcher *in vivo* abläuft. Um die zeitliche Abfolge der differenziellen Genxpression im Prozess der Hepatogenese zu untersuchen, verwendete ich ein reproduzierbares *in vitro* System (zwei verschiedene Protokolle) und untersuchte schrittweise die Transkriptome verschiedener Differenzierungsstadien der humanen ES-Zellen: endgültig induziertes Entoderm, frühe Leberzellen, adulte Leberzellen.

Diese *in vitro* Analysen haben ein breites Spektrum molekularer Kaskaden aufgedeckt, in die Oberflächenrezeptoren, Transkriptionsregulatoren sowie intrazelluläre Systeme der Signalübertragung involviert sind und welche für die Induktion der Hepatogenese *in vivo* verantwortlich sein könnten. Darüber hinaus haben die Experimente gezeigt, dass

die Hepatozyten-ähnlichen Zellen, die aus undifferenzierten hES-Zellen generiert wurden, auf der Transkriptionsebene eine ähnliche Genaktivität aufweisen wie die Zellen der fötalen Leber. Jedoch präsentieren sie in einigen Aspekten ein individuelles und einzigartiges Transkriptionsmuster.

Zusammenfassend legen die Ergebnisse nahe, dass das *in vitro* System zur Differenzierung humaner embryonaler Stammzellen zu Hepatozyten eine effektive Methode zur Untersuchung von molekularen Prozessen aller Differenzierungsstadien der Hepatogenese ist. Jedoch sind weitere Anstrengungen erforderlich um weiterentwickelte Hepatozyten zu erhalten. Dazu könnten wiederum die neu gewonnenen Erkenntnisse aus der Transkriptomanalyse angewendet werden.

1

Introduction

1.1 Human Embryonic Stem Cells (hESCs) and induced Pluripotent Stem Cells (iPSCs) – history and applications

Quite a few discoveries during the last 30 years can be considered not only as key breakthroughs for cell and developmental biology, but also as essential events that have significantly influenced our view of life. The establishment of embryonic stem (ES) cell lines derived from mouse (Evans and Kaufman, 1981) and human embryos (Thomson et al., 1998) has been one of such events. Since then, cell therapy based on unrestricted, renewable source of cells has become an attractive concept of regenerative medicine. In 1981, two groups succeeded in cultivating pluripotent cell lines from mouse blastocysts. To culture stem cells, Evans and Kaufman applied a feeder layer of mouse embryonic fibroblasts (MEFs) (Evans and Kaufman, 1981), whereas Martin used EC cells-conditoned medium (Martin, 1981). Later, the techniques used to isolate and culture mouse ES cells were employed to generate human ES cell lines from preimplanted embryos produced by *in vitro* fertilization (Thomson et al., 1998). Currently more than 400 human ES cell lines are available but just some of them are well characterized and deposited in international stem cell banks (Jensen et al., 2009). Today 21 human ES cell lines, derived prior to August 2001, are listed on the US National Institutes of Health (NIH) Stem Cell Registry of the National Stem Cell Bank.

An inherent feature of human ES cells is the capability to differentiate into all types of cells in the adult human body. These cells can provide a defined and renewable source of human cells applicable for cell therapies and also for industrial in vitro tests (Ameen et al., 2008) The implementation of human ES cells into industrial procedures raises a big hope for future improved cell assays. Unfortunately, the directed and controlled derivation of lineage-restricted functional cell types still remains one of the most difficult issues. Immune rejection of transplanted human ES cell-derived tissues presents a serious challenge for regenerative medicine. If stem cell-derived tissues do not possess close major histocompatibility complex (MHC) match to the patient, life-long immune suppression would be required (Rao and Condic, 2008). Moreover it is difficult to calculate approximately the number of human ES cell lines necessary to provide a good match for specific patient populations. The required amount vary enormously, from several

hundred (Rao, 2006b,c) to several million (Rao, 2006a) lines. This number depends, of course, on the genetic diversity of the population under consideration and on the definition of acceptable degree of immune mismatch.

The immune concerns of human ES cell-derived tissues transplantation can be overcome by the development of patient-specific pluripotent stem cell lines. In contrast to human ES cells derived from embryos produced by fertilization, patient specific stem cell lines would offer compatibility at both major and minor antigenic sites. There have been various attempts over last 10 years to obtain patient-matched pluripotent stem cells by reprogramming adult cells to an embryonic state. Takahashi and Yamanaka (2006) first demonstrated that adult mouse skin cells can be reprogrammed into a state that is similar to an ES cell by expressing only four factors (Oct4, Klf4, Sox2, and c-Myc). Recently, many independent groups have produced human induced pluripotent stem cells (iPSCs) (Yu et al., 2007; Hanna et al., 2007; Nakagawa et al., 2008) making patient-specific cell lines a possibility for the first time. A huge advantage of this direct reprogramming is avoiding the ethical concerns over the use of human embryos for research.

Direct reprogramming technology represents a basic breakthrough that provides a new path for generating various differentiated cell types with an isogenic gene profile. This would be practically impossible to attain in therapeutic relevant numbers otherwise.

1.2 Endoderm development *in vivo*

The understanding of endoderm formation is difficult since the most internal endodermal tissue is less accessible than ectodermal and mesodermal tissue. The embryonic endoderm gives rise to the epithelial lining of the digestive and respiratory systems, and the associated organs like thyroid, lungs, liver, pancreas and gallbladder. The endoderm is surrounded by mesoderm, which develops into blood, kidneys, heart, muscle, and bones; and the outermost germ layer the ectoderm, which becomes the nervous system and skin. The primary germ layers are specified during gastrulation, when tissue movements create the three layers organization. Prior to gastrulation, cells that will give rise to the embryo are organized into a radially symmetric cup of epithelial cells (the epiblast) with no morphological A-P (anterior-posterior) axis. A layer of cells called the visceral endoderm (VE), which contributes to the extraembryonic tissues including the yolk sac, surrounds the epiblast. Shortly before gastrulation, a small subset of distally located visceral endoderm cells at E5.5 move interiorly to populate the anterior visceral endoderm (AVE) (Thomas and Beddington, 1996; Thomas et al., 1998). Prior to AVE formation (E5.5 in mice) Nodal is symmetrically expressed throughout the epiblast (Varlet et al., 1997). At this time, Nodal antagonists Lefty and Cer1 are expressed in AVE precursors at the distal tip of the embryo. Re-distribution of the distal AVE cells expressing Lefty1/Cer1 toward the anterior restricts Nodal signaling activity and expression to the posterior epiblast, which in turn determines the site of primitive

streak and endoderm formation (Figure 1.1). Definitive endoderm (DE) cells originate from the anterior primitive streak during gastrulation (E6-E7.5) in mice (Lawson and Pedersen, 1987).

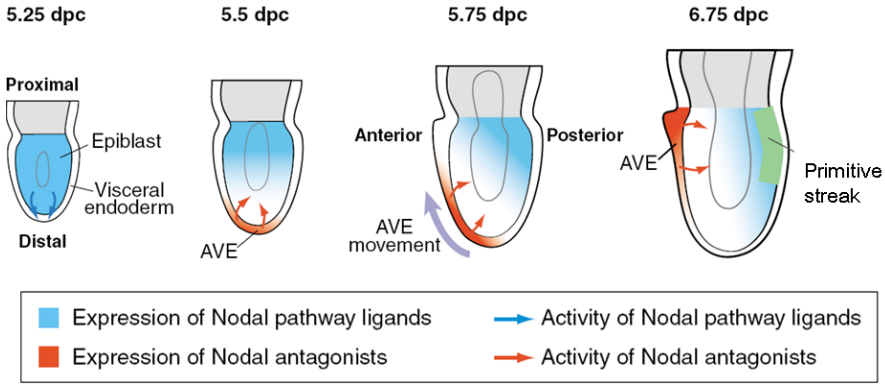

Figure 1.1: Model of anterior-posterior (A-P) patterning by Nodal signalling and antagonism in the mouse embryo. Following implantation Nodal is expressed throughout the epiblast [5.25 days post-coitum(dpc)], and induces development of the anterior visceral endoderm (AVE, red) at the distal end of egg cylinder at 5.5 dpc. Nodal signalling is also required for the movement of the AVE to the anterior side (5.75 dpc), where the expression of Nodal antagonists (Lefty1, Cer1) by the AVE is essential for the specification of primitive streak in the epiblast. Adapted from Shen (2007).

The first sight of gastrulation is *de novo* expression of *Fgf8*, *brachyury*, *Wnt3*, and *eomesodermin* genes in the posterior epiblast. Shortly after this the primitive streak is morphologically apparent as a furrow in the epiblast (Zaret, 2002).

At the early gastrula stage, definitive endoderm cells are first detected and by the late gastrula embryo they are believed to be determined so they are no longer able to form ectoderm or mesoderm.

1.2.1 Nodal signaling pathway – molecular mechanism of endoderm specification

Nodal involvement in early embryogenesis can be summarized in three steps. First, Nodal is required for the induction of the distal visceral endoderm (DVE), part of the extraembryonic tissue. Second, in response to Nodal, some cells of the visceral endoderm (VE) secrete Nodal antagonists, and inhibition of Nodal in the vicinity of these cells prevent Nodal-induced proliferation in a portion of the VE, hence providing asymmetry and positional cues for determining anterior versus posterior. Third, Nodal induces cells of the DVE to migrate toward the anterior (becoming the anterior visceral endoderm (AVE)), thus setting up the A-P axis (Wu and Hill, 2009).

The Nodal gene was originally identified in mice and was named because of its expression in the mouse gastrula embryonic organizer, the node (Zhou et al., 1993; Conlon et al., 1994). The Nodal-related proteins belong to the Activin-type subfamily of transforming growth factor β (TGFβ) – secreted signaling ligands. Secreted Nodal proteins are though to act as morphogens, eliciting distinct cells responses depending of the con-

centration and/or duration of exposure to the Nodal ligand. Like other TGFβ family members, Nodal proteins are translated as pre-proproteins and assemble into dimers in the secretory pathway. The production of mature Nodal ligands requires proteolytic processing by proprotein convertases Spc1/furin or Spc4/Pace4. Nodal ligands bind to heteromeric receptors complexes consisting of two type II (ActRIIA or ActRIIB) and two type I (Alk4 or Alk 7) transmembrane serine/threonine kinases and EGF-CFC coreceptor (Cripto). After ligand binding the activated type I receptor phosphorylates cytosolic proteins Smad2 and Smad3. Smad2 appears to be involved in endoderm specification since Smad3 is not expressed in the early endoderm precursors (Tremblay et al., 2000) (Figure 1.2). Phosphorylated Smad2 binds to Smad4 and this complex rapidly translocates to the nucleus, where it interacts with a variety of sequence-specific transcription factors to regulate gene expression. Many different DNA-binding transcription factors (Foxh1/Fast and Mix-like factors) may interact with activated Smads during endoderm development. This interaction with transcription factors is crucial because Smads bind DNA very weakly and with limited specificity.

The activity of Nodal signaling pathway is regulated at various levels prior to interaction with the receptor complex. Regulation of proteolytic processing of Nodal ligands is one of ways to regulate their biological activity *in vivo*. Moreover, secreted antagonists such as Lefty, can block Nodal signaling by binding to Nodal ligands and preventing their interaction with the EGF-CFC coreceptors (Figure 1.2). These levels of regulation impact the spatial, temporal and strength of Nodal signaling during vertebrate mesendoderm formation (Schier, 2003).

Nodal signaling acts in a dose-dependent manner, with high level of signaling necessary for definitive endoderm specification and lower levels are sufficient to specify mesoderm. Initiation of Nodal expression involves Wnt/β-catenin signaling, whereas the maintenance of its expression is regulated by Foxh1-/Smad2-dependent autoregulation (Norris et al., 2002). In the mouse embryo Nodal processing is mediated by the proprotein convertases SPC1/SPC4, which are expressed in the extraembryonic ectoderm and visceral endoderm (Constam and Robertson, 1999). Numerous downstream signaling components of the Nodal pathway play important roles in gastrulation and endoderm specification. Loss of function of Cripto, Smad2, FoxH1, eomesodermin and Foxa2, all results in gastrulation and/or endoderm specification defects. Embryos lacking Nodal arrest shortly before gastrulation and fail to establish a primitive streak (Zhou et al., 1993; Conlon et al., 1994).

1.2.1.1 Nodal downstream targets regulate endoderm development

Many core molecular mechanisms of vertebrate endoderm development are evolutionary conserved and Nodal signaling is a pivotal player at the top of the genetic hierarchy controlling the expression of key Mix-like, Sox, FoxA, and Gata transcription factors. Together these factors make up a “core” endoderm regulatory track. Many of these factors regulate each other’s expression. This feed-forward system allows the rapid

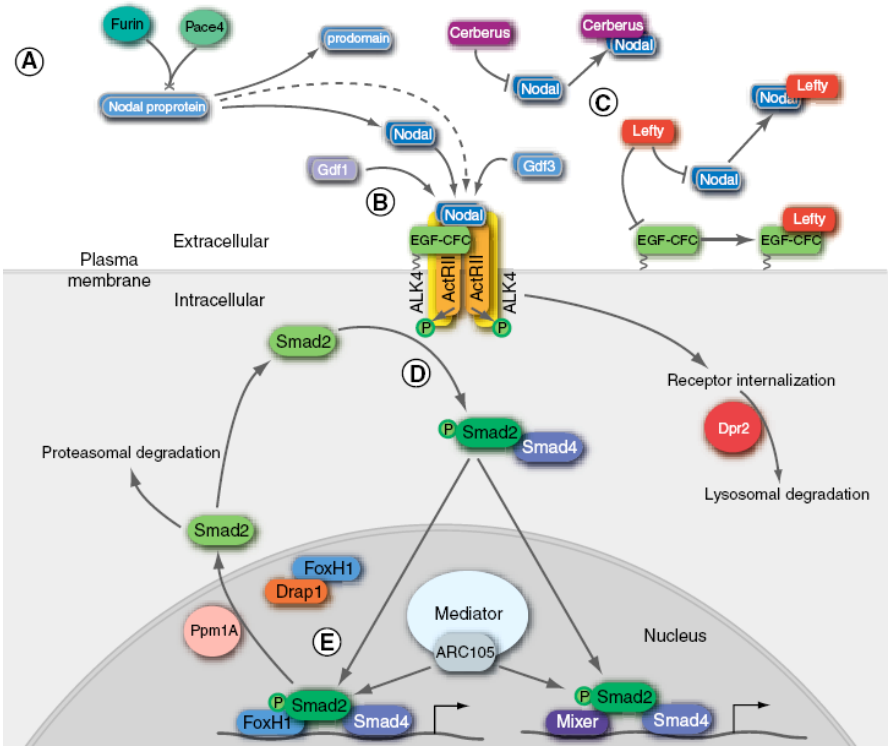

Figure 1.2: Schematic outline of the Nodal signalling pathway. (A) Nodal ligands are expressed as homodimeric proproteins and are cleaved extracellularly by the proprotein convertases Furin and Pace4. (B) Mature Nodal ligands, as well as Gdf1 and Gdf3, can bind to an EGF-CFC co-receptor complex with type I receptor (ALK4) and type II receptor (ActRII or ActRIIB) dimmers. (C) Cerberus (Cer1) and Lefty (Left) are soluble antagonists that can interact with Nodal ligands; Lefty proteins can also interact with EGF-CFC co-receptors and inhibit their function. (D) Receptor activation leads to the phosphorylation of the type I receptor by the type II kinase, as well as phosphorylation of Smad2 (or Smad3). Activated Smad2 or Smad3 associates with Smad4 and translocates to the nucleus, while the receptor complex undergoes internalization into endosomes and can be targeted by Dpr2 for lysosomal degradation. (E) Inside the nucleus, activated Smad2-Smad4 (or Smad3-Smad4) comlexes interact with the winged-helix transcription factor FoxH1 or Mixer homeoproteins on target promoters, leading to transcriptional activation through interactions with ARC105 and the Mediator complex. Pathway activity can be inhibited by the interaction of Drap1 with FoxH1 or by the Smad phosphatase Ppm1A, which promotes the nuclear export of Smad2 and probably targets it for proteosomal degradation. Adapted from Shen (2007).

establishment of an endoderm transcription profile in the hours between activation of zygotic transcription at the early blastula to the gastrula stage, when endodermal fate is specified.

Mix-like 1 (Mixl-1) is expressed in primitive streak at the time of endoderm formation. Its transcription is activated by Nodal signaling via Foxh1/Smad DNA-binding sites in its promoter (Hart et al., 2005). Mouse embryos lacking Mixl-1 have a thickened anterior primitive streak and do not possess obvious endoderm specification defects. So Mixl-1 may play a role late in gastrulation at the stage when posterior endoderm is already specified.

In mouse, Gata4 and Gata6 are involved in visceral endoderm specification while mice lacking Gata5 are viable (Kuo et al., 1997; Narita et al., 1997; Molkentin et al., 1997, 2000; Morrisey et al., 1998). Overexpression of both Gata4 and Gata6 in mouse ES cells promotes their differentiation into visceral but not definitive endoderm. Gata factors have a role in later stages of liver and lung development (Zhao and Duncan, 2005). Gata 4/5/6 can regulate the transcription of genes expressed in endodermal derived tissue such as intestinal *Ifabp* and liver *Hnf4* in tissue culture (Morrisey et al., 1998).

In mouse, Sox17 is first expressed in the distal visceral endoderm overlying the extraembryonic portion of the early gastrula embryo (E6). Sox17 transcripts are first observed at the mid gastrula stage (E7), when expression is transiently observed in cells adjacent to the anterior primitive streak. By E7.5, Sox 17 is no longer expressed in endoderm cells adjacent to the primitive streak but rather in anterior definitive endoderm and by E8 expression is restricted to the hindgut. Fate mapping experiments have identified definitive endoderm cells overlying the primitive streak of the early gastrula embryo (E6.5) (Lawson and Pedersen, 1987). The first observed phenotype in Sox17$^{-/-}$ embryos is in the posterior, where there is a reduction in the amount of definitive endoderm and an expansion of the domain of visceral endoderm. This suggests that Sox17 is involved in the specification of posterior, but not anterior definitive endoderm.

In mouse, three Fox factors are expressed during gastrulation, Foxa1 (Hnf3α), Foxa2 (Hnf3β), and Foxa3 (Hnf3γ) (Ang et al., 1993; Sasaki and Hogan, 1993). These factors have overlapping as well as distinct expression domains during gastrulation. Foxa2 is first expressed in the anterior primitive streak of the midgastrula stage embryo, and at the end of gastrulation is expressed in anterior endoderm, node, notochord, and ventral floor plate. Expression of Foxa1 initiates slightly later and overlaps with Foxa2 in the notochord, ventral floor plate, and endoderm. Foxa3 is not expressed until the late gastrula stage. Embryos lacking either Foxa1 or Foxa3 are viable demonstrating that initial endoderm specification does not require these factors. In other hand embryos lacking Foxa2 have defects in gastrulation. In Foxa2$^{-/-}$ embryos foregut and midgut development are severely disrupted, but hindgut development is less affected (Dufort et al., 1998). It has been shown that Foxa1 and Foxa2 have redundant functions so only lost of both genes results in a failure to initiate the liver development (Lee et al., 2005).

1.2.2 Role of Wnt signaling in endoderm development

Up to now, one Wnt ligand, Wnt3 has been shown to be necessary for initiation of gastrulation in the mouse (Liu et al., 1999). Role of Wnt3 in endoderm formation is difficult to investigate since $Wnt3^{-/-}$ embryo arrest early in development. Genetic ablation of β-catenin, the downstream effector of Wnt3 signaling, results in embryos that fail to gastrulate (Huelsken et al., 2000) and have defects in the AVE prior to gastrulation (absence of Hex1 expression and failure of the AVE marker Cer1 shift from the proximal to the anterior). β-catenin primarily functions in the epiblast to establish the AVE and initiate gastrulation. Gene expression profiling of β-catenin, Wnt3, and Cripto (a Nodal coreceptor) mutant embryos at E6.5, indicates that all these genes may act in a common pathway with Wnt/β-catenin regulating Cripto expression in the early gastrula embryo (Morkel et al., 2003). Embryos lacking β-catenin in the anterior primitive streak developed ectopic cardiac cells in the posterior (Lickert et al., 2002). Mouse ES cells lacking β-catenin less efficiently contribute to endoderm and often formed cardiac clusters in the endoderm germ layer. These results suggest that β-catenin is involved in an endoderm versus mesoderm cell fate decision. Marvin *et al.* discussed another interpretation, that appearance of cardiac cells could be due to a later mesendoderm patterning role of β-catenin, because repression of Wnt signaling in posterior mesoderm has been shown to result in ectopic cardiac specification (Marvin et al., 2001).

A gain-of-function approach has also provided evidence for the involvement of Wnt signaling in endoderm specification. Mouse ES cell line containing mutations in *APC* (*adenomatous polyposis coli*) gene were used to investigate the consequences of elevated Wnt signaling on ES cells differentiation. APC is necessary for targeting β-catenin protein for degradation and these ES cells have elevated levels of Wnt signaling. When injected into mice they formed teratomas, which express the defects to form neural and some mesoderm derivatives like bone and muscle. This observation confirmed Wnt signaling is involved in endoderm specification (Kielman et al., 2002).

1.2.3 Nodal and Wnt signaling in mesendoderm development - separating endoderm from mesoderm

In the mouse there is evidence of a common mesendoderm progenitor whose descendants give rise to both mesoderm and endoderm, but it is not yet clear if all endoderm derives from common mesendoderm progenitor or if some endoderm also derives directly from epiblast. Dose of Nodal signaling is critical, with high doses promoting endoderm and low doses promoting mesoderm. It is not yet known how different levels of Nodal signaling and hence different numbers of phosphorylated Smad molecules activates distinct transcriptional programs. In the endoderm lineage Nodal/Smad2/Hoxh1 activates and/or maintains the expression of critical transcription factors as well as transcription of Nodal ligands. Some Mix-like factors then form a complex with Smad2 to activate

downstream target genes, thus reinforcing Nodal-dependent endodermal fate in a feed-forward loop. Mix-like proteins appear to be involved in promoting endoderm fate while at the same time repressing mesoderm gene expression.

In all vertebrates the TGFβ/Nodal signaling pathway is at the top of molecular hierarchy and is absolutely required to initiate endoderm development. Nodal signaling is also necessary for mesoderm development and in cooperation with Wnt/β-catenin pathway also regulates early mesendoderm patterning. This fact supports the idea of a common mesendoderm progenitor (Yamamoto et al., 2004). Prior to gastrulation the AVE expresses several secreted Nodal and Wnt antagonists that limit the activity of these signaling pathways to the posterior. Active Nodal and Wnt signaling in the posterior is necessary for gastrulation and perturbations in either of these pathways results in gastrulation defects that often cause a failure of the definitive endoderm and mesoderm to form (Figure 1.1). Endoderm preferentially derives from progenitors located in the anterior primitive streak, whereas mesoderm progenitors are found throughout the primitive streak and in more lateral domains of the epiblast. A number of the endoderm and mesoderm cells derive from a common mesendoderm progenitor are found in the anterior primitive streak. In vertebrates the dose of Nodal signaling is involved in the cell fate decision between endoderm and mesoderm, in which high levels of Nodal signaling are required for the specification of the endoderm lineage. In mouse the canonical Wnt/β-catenin signaling pathway has also been implicated in the endoderm versus mesoderm decision. Loss of β-catenin in the anterior primitive streak results in the formation of ectopic cardiac mesoderm clusters in the endoderm germ layer, which could be due to impaired endoderm differentiation of a mesendoderm progenitor cell.

1.3 Endoderm development *in vitro* - creating endoderm from human ES cells

The number of diseases affecting endodermally derived cell types is growing. Studies of endoderm organ development in model organisms have identified the underlying mechanisms for numerous inherited disorders in humans. The field of regenerative medicine is now benefiting from studies of developmental biology by translating these findings into potential clinical applications through the directed differentiation of human ES cells into endoderm derivatives.

There have been several publications reporting culture conditions that enhance the *in vitro* differentiation of human ES cells into endoderm derivatives such as liver hepatocytes and pancreatic β-cells (Rambhatla et al., 2003; Lavon et al., 2004; Schwartz et al., 2005; Baharvand et al., 2006; Hay et al., 2007). However in these reports the overall efficiency of differentiation into these cell types was relatively low and still stochastic. One explanation for why these approaches were inefficient was that ES cells must also follow a developmental progression, first forming definitive endoderm before being competent to differentiate into organ-specific cell types. Since expressing pancreatic

regulatory transcription factors such as Pdx1 and Pax4 does not promote efficient pancreatic differentiation of naïve mouse ES cells (Blyszczuk et al., 2003; Miyazaki et al., 2004). Most data suggested that the step-wise differentiation, like that which occurs during endoderm organogenesis in the embryo, was required for efficient differentiation of human ES cells into specific cell types.

The understanding of the conserved molecular network that controls endoderm development in embryo allowed activating the same program in stem cells. Successfully directed differentiation of mouse (Kubo et al., 2004; Yasunaga et al., 2005) and human ES cells (D'Amour et al., 2005) into definitive endoderm cells in culture was attained by treating cells with Activin A (or Nodal). The effects of Activin A (and Nodal) on human ES cell differentiation are nearly identical to the activity of these ligands on naïve ectoderm cells in Xenopus (Hudson et al., 1997). The timing of differentiation and the molecular responses during endoderm specification *in vivo* versus in human ES cell culture are remarkably similar. *In vivo* gastrulation occurs within 4-5 days in humans. Treatment of ES cell cultures with Activin A initiates the expression of markers in a temporal fashion nearly identical to gastrulation; early gastrulation markers *FGF8*, *WNT3*, and *Eomesodermin*, are expressed after 1 day, the mid gastrulation markers *GSC*, *MIXL1*, and *Brachyury* after 2-3 days, and markers of definitive endoderm including *SOX17*, *FOXA2*, and *CXCR4* are expressed after 3-5 days. The use of Activin A to promote the efficient differentiation of human ES cells into definitive endoderm lineage demonstrates how critical studies of endoderm biology in model organisms are for directing the differentiation of human ES cells.

1.3.1 Expandable endodermal progenitors

Recently, Rossant and colleagues demonstrated generation of definitive endoderm cells by the expression of the *SOX17* gene in human ES cells, in the absence of Activin A.

To force differentiation of undifferentiated human ES cells into definitive and extraembryonic endodermal progenitors Rossant and colleagues (Seguin et al., 2008) constitutively expressed lineage-determining transcription factors, *SOX7* and *SOX17*. Constitutive expression of *SOX7*, which is normally expressed in extraembryonic endoderm (ExEn), but not in definitive endoderm (DE), gave rise to homogenous population of cells with flattened epitheloid phenotype. Expression of *SOX17*, which is expressed in both ExEn and DE, resulted in cells with human ES cells-like morphology. Both cell types shared expression of endodermal markers, including *GATA4*, *GATA6*, and *FOXA2*. While *SOX7* positive cells uniquely expressed ExEn markers, *LAMB1*, *SPARC*, *COLIV*. *SOX17* positive cells specifically expressed DE markers, including *CXCR4*, and *GSC*. These cells exhibited a stable ExEn and DE phenotype for 20 passages even in the absence of cytokine induction. In teratoma assays, *SOX7* limits the lineage potential of hESCs, while *SOX17* maintains multipotency for endodermal and mesodermal tissue. Both cell lines were unable to become neuroectoderm, whereas *SOX17* positive cells were capable of generating both hepatocytes and insulin-producing pancreatic cells

(D'Amour et al., 2006; Pankratz et al., 2007; Cai et al., 2007). This work provides evidence that constitutively expression of a single SOX transcription factor is sufficient to induce a stable expandable endodermal phenotype in human ES cells. These results provide a huge step forward in terms of generating endoderm-derived tissues, providing the possibility for the production of expandable progenitors. Furthermore, the production of defined population of specialized cells from hES cells generally takes several weeks, so it is crucial to isolate and maintain intermediate precursor cells that can be cryopreserved and sustain the capacity to proliferate and differentiate upon thawing.

1.4 Hepatogenesis *in vivo*. Regulatory phases of early liver development

How the endoderm is patterned to produce specific organ lineages, which can be applied to direct the differentiation of stem cells *in vitro* is another interesting topic in developmental biology.

Liver development is a chronological process of distinct biological events. Each step of differentiation is regulated by inherently planned mechanisms as well as by the extracellular signals. Over the last decade significant progress has been made in understanding of the molecular mechanisms that direct early aspects of mammalian liver development. Studies using tissue explant cultures and molecular biology techniques as well as the analysis of the transgenic and knockout mice have identified signaling molecules and transcription factors that are necessary for the beginning of hepatogenesis.

1.4.1 Specifying the hepatic lineage by cardiac mesoderm

A crucial question in the area of the gut organogenesis is how individual tissues are specified at different domains along the antero-posterior axis of the endoderm (Figure 1.3). Studies in model organisms have shown that endodermal domains are usually patterned by interactions with overlying mesodermal tissue (Douarin, 1975). The classical tissue transplantation studies performed by LeDouarin, using chick embryos, showed that cardiogenic mesoderm, which is transiently in the close vicinity of the prospective hepatic endoderm, provides a signal that is crucial for inducing liver progenitors in endoderm (Douarin, 1975). On the other side mesoderm from other areas of the chick embryo does not induce the liver. Interestingly, at an earlier stage, the endoderm itself is important for inducing cardiogenic mesoderm (Sugi and Lough, 1995). Fibroblast growth factors (Fgfs) signaling from the cardiac mesoderm induces the liver in the ventral foregut endoderm (Figure 1.4). At the time of hepatogenesis, the cardiogenic mesoderm express at least three out of the eighteen known Fgfs, and the ventral foregut endoderm expresses at least two of the four tyrosine kinase Fgf receptors (Jung et al., 1999). At the time of hepatic induction, the endoderm uniquely expresses Fgf receptor 4 (Fgfr4) (Stark et al., 1991) and both the endoderm and cardiac mesoderm express Fgf receptor

1 (Fgfr1) (Sugi and Lough, 1995). In situ immunohistiochemistry studies showed that Fgf1 and Fgf2 are both induced in the cardiac mesoderm at the 7-8 somite stages in mouse (Jung et al., 1999). Purified Fgf1 and Fgf2 were each found to efficiently induce early liver-specific genes within the ventral foregut endoderm, when the endoderm was isolated from 2-6 somite stages. The induction of serum albumin (Alb), α-fetoprotein (Afp), and transthyretin (Ttr), was as strong as that induced by cardiac mesoderm. In contrast, Fgf8 had only partial hepatogenic activity and it failed to induce hepatic development. However, it was found to contribute toward the morphogenic outgrowth of the hepatic tissue following specification. The early expression of Fgf8 or a related molecule appears to strengthen the morphogenetic activity of the emerging hepatic cells. It appears, that Fgf8 works together with a signal that has not been identified to stimulate cell outgrowth (Jung et al., 1999).

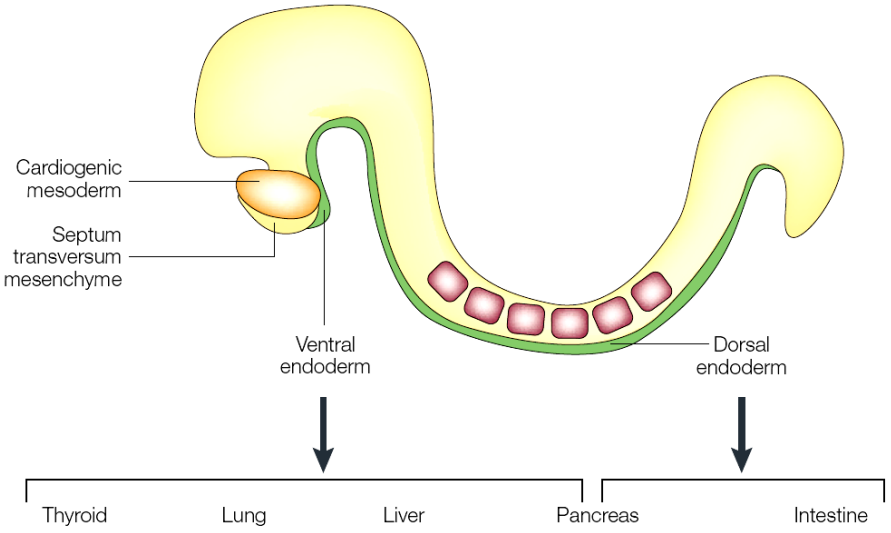

Figure 1.3: Patterning of the definitive endoderm in mouse. When definitive endoderm is formed during gastrulation, it initially appears as a single-cell thick, epithelial sheet that covers the bottom surface of the developing embryo. Invaginations at the anterior and posterior ends of the embryo begin to generate the foregut and hindgut. Shortly after that, but well before closure of the gut tube, the ventral domain of the foregut begins to develop into the liver, lung, thyroid and the ventral part of the pancreas. The entire process is very rapid; in mouse embryos, the future ventral tissue domains become patterned within two days after forming the definitive endoderm. Adapted from Zaret (2002).

The isolated ventral foregut endoderm does not remain undifferentiated when it is cultivated without cardiogenic mesoderm or Fgfs. Rather, it starts the expression of pancreatic genes, as a default lineage (Deutsch et al., 2001). Fgf or cardiogenic mesoderm suppress the pancreatic program in the endoderm and induces the liver program. So ventral foregut endoderm consist of a multipotential cell population that undergoes a cell-fate choice during spatial patterning. The ventral pancreatic bud derives from the most distal endoderm that extends away from the developing heart and is able to follow

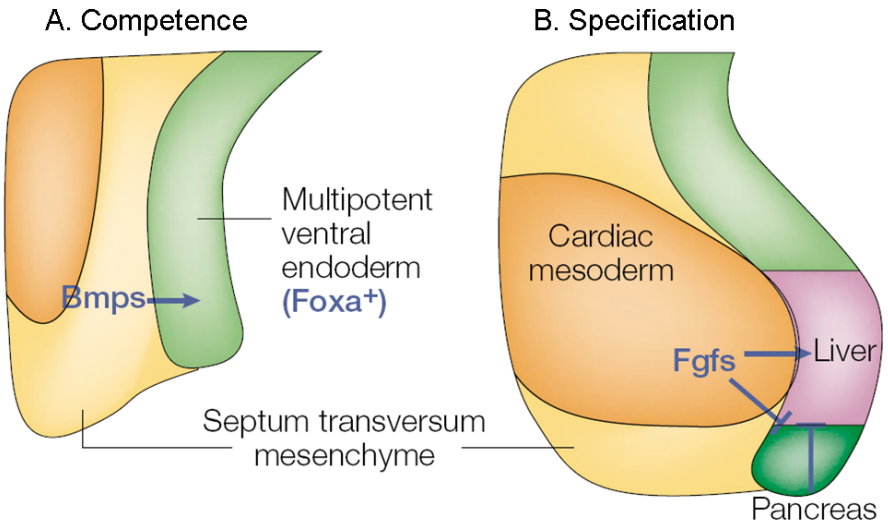

Figure 1.4: Signals and tissue interactions that pattern the hepatic domain. (A) The ventral foregut endoderm gains the competence to develop into various tissues as a result of the expression of transcription factors in the endoderm. These include Foxa proteins, as well as signals that affect the endoderm, including bone morphogenetic proteins (Bmps) secreted by the adjacent cells of septum transversum mesenchyme (STM). (B) During tissue specification, fibroblast growth factor (Fgf) signals from the cardiogenic mesoderm, together with Bmp signals from the STM, initiate the liver gene program in proximal endoderm, as well as blocking that for pancreas. Adapted from Zaret (2002).

the default pancreatic fate (Figure 1.4).

1.4.2 Specifying the hepatic lineage by septum transversum mesenchyme

The early chick studies of LeDouarin identified a second stage of hepatic induction, which appears when mesoderm derived cells in the septum transversum promote growth and further differentiation of the newly specified hepatic endoderm (Douarin, 1975; Fukuda-Taira, 1981) (Figure 1.4).

These additional hepatogenic signals originate from septum transversum mesenchyme (STM) cells. The septum transversum derives from lateral plate mesoderm and gives rise to the epicardium of the heart and also the diaphragm. Before hepatic induction, prospective septum transversum mesenchyme cells surround the developing cardiac region near the ventral foregut endoderm. Bone morphogenetic proteins 2 and 4 (Bmp2 and Bmp4) are strongly expressed in the STM, before and during hepatic induction (Figure 1.4). Rossi et al. (2001) confirmed that STM collaborate with developing cardiac tissue to control specification of the liver lineage. The role of Bmp4 signaling during the onset of hepatogenesis has been confirmed by experiments with Noggin (Bmp4 antagonist). This molecule was found to inhibit albumin mRNA expression in co-culture of cardiac tissue and 2-6 somite stage ventral endoderm. This result was contradictory with previous finding by Jung et al. in that FGF alone was sufficient to induce hepatogenesis within cultured ventral endoderm. However, the endoderm cultures con-

tained small numbers of Mrg1 positive cells, which is a marker of STM. The amount of STM cells was sufficient to supply a sufficient amount of Bmps to allow hepatic induction by exogenously added FGFs (Rossi et al., 2001). Additionally, for the induction of hepatogenesis, secretion of Bmps by STM appears to be critical for outgrowth of the budding hepatoblasts. The Fgfs and Bmps act in a concert manner on the ventral foregut endoderm to direct the onset of hepatogenesis.

The *Hlx* knockout embryos additionally confirmed the STM role in controlling developmental growth of the liver. *Hlx* gene encodes a homeobox transcription factor and its expression in developing liver is restricted to cells derived from STM. Mouse embryos lacking the *Hlx* gene start the liver development normally, however, by E15.5 the mutant livers had failed to expand and reached only 3% of the size of control livers (Hentsch et al., 1996). Although the targets of *Hlx* are not known, it must be required for expression of paracrine factors, from STM, that control hepatogenesis (such as hepatocyte growth factor (HGF)).

1.4.3 Hepatic gene induction in embryonic endoderm cells

The tissue interactions described the exact location and timing of hepatogenesis during embryonic development. At least three different kinds of mesoderm cells, including the cardiac mesoderm (Douarin, 1975; Gualdi et al., 1996), the septum transversum mesenchyme (STM) (Rossi et al., 2001), end endothelial cells (Cleaver and Melton, 2003), coordinately induce liver development in the endoderm, apparently by employing different signaling molecules (Zaret, 2002; Duncan, 2003). Nevertheless, the mechanisms by which the cells of the endoderm really follow and adopt a hepatic fate are best considered as intracellular responses to these signals. The intracellular network by which FGF signaling helps induce hepatic genes and stabilize emerging hepatic cells within the endodermal epithelium has just recently been elucidated.

FGF-mediated induction of hepatic genes function through the MAPK pathway and not the PI3K/AKT pathway. Although the PI3K/AKT pathway is activated in foregut endoderm cells, its inhibition does not block hepatic gene induction in explants; however it does block tissue growth. At the beginning of hepatogenesis, the FGF/MAPK and PI3K/AKT pathways are induced separately in the foregut endoderm and do not cross-regulate at the initial stages of tissue patterning (Calmont et al., 2006). The inherent ability of FGF to activate MAPK and not PI3K in the foregut endoderm helps define the intracellular network that give the endoderm cells the ability to induce liver and explain how a common signal such as FGF trigger a specific cellular response. There is a strong correlation between the expression of different FGF ligands and phospho-ERK activation during the period of hepatic specification. Although FGFs represent a proliferative signal for the endoderm shortly after tissue patterning (Bhushan et al., 2001) and MAPK signaling can stimulate cell proliferation (Lavine et al., 2005). Calmont *et al.* demonstrated that FGF/MAPK signaling initiates the hepatic differentiation and that it is distinct from the effects on cell proliferation and growth.

1.4.4 Growth of hepatic endoderm into the liver bud

The liver emerges from the definitive gut endoderm first as a thickening of the ventral endoderm epithelium and then as a bud of cells that proliferate and migrate into the surrounding septum transversum mesenchyme (Figure 1.5). The septum transversum is a collagen-rich environment colonized by loosely joined mesenchyme cells (Cascio and Zaret, 1991) which defines the area of the embryonic body cavity into which the hepatic bud grows. These pre-hepatic cells, which delaminate from the foregut and migrate into septum transversum are called hepatoblasts (Medlock and Haar, 1983). Their analyses in culture suggest that they are bipotential, capable of giving rise to both the hepatocyte and cholangiocyte cell lineages (Rogler, 1997). As the hepatoblasts migrate they closely associate with primitive sinusoidal endothelial cells that form capillary-like structure between the migrating hepatic strings (Enzan et al., 1997). The hepatoblasts have irregular shape, large nuclear to cytoplasmic ratio and relatively few organelles when compared to mature hepatocytes. The process of differentiation of hepatoblast to hepatocyte is gradual, taking several days during development of the rodent embryo.

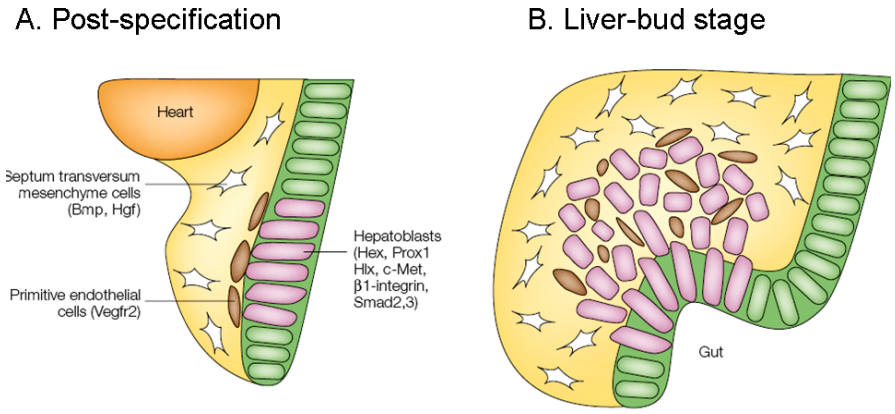

Figure 1.5: Development of the liver bud. (A) After the hepatic endoderm is specified it begins to extend toward the midgut. Cells such as STM cells and primitive endothelial cells, signalling molecules (such as Bmp, Hgf and Vegf2) and transcription factors (such as Hex, Prox1, Hlx and c-Met) are crucial to promote the morphogenesis of the liver bud itself. (B) Liver bud morphogenesis is marked by the remodelling of the extracellular matrix around the hepatoblasts and E-cadherin-based connections between the cells, and proliferation and migration into the surrounding STM. The hepatic endoderm (green) makes a transition from an epithelium to a non-polarized cell type during this period. Primitive endothelial cells, or angioblasts, appear near the hepatoblasts and also promote their outgrowth into the STM. Adapted from Zaret (2002).

After the liver bud is generated, it appears that other groups of growth signaling pathways are involved in further liver specification. These signals are, hepatocyte growth factor (HGF), oncostatin M (OSM) and glucocorticoids.

Hepatocyte growth factor (HGF) is a powerful mitogen originally discovered to play a role in the regeneration of the adult liver, after partial hepatectomy (Michalopoulos and DeFrances, 1997). The HGF/c-Met pathway mediates the interaction between

mesenchyme and epithelial cells during development since HGF is expressed in the STM that surrounds the developing liver bud, and c-met, the HGF receptor, is expressed on embryonic hepatocytes (Schmidt et al., 1995). Mice lacking HGF fail to complete development and die in uterus. The mutation affects the embryonic liver, which is reduced in size and shows extensive loss of parenchymal cells. In addition, development of the placenta, mainly of trophoblast cells, is impaired. Thus, HGF is essential for the development of several epithelial organs (Schmidt et al., 1995).

Hematopoiesis plays an important role in hepatic maturation. After the liver bud emerges from the gut tube, hematopoietic cells migrate there and propagate. The hematopoietic cells produce oncostatin M (OSM), a growth factor belonging to the interleukin-6 (IL-6) family (Zarling et al., 1986). In the developing liver, OSM is expressed by CD45+ hematopoietic cells, but not by hepatocytes. OSM stimulates the expression of hepatic differentiation markers and induces morphologic changes and multiple liver-specific functions like ammonia clearance, lipid synthesis, glycogen synthesis, detoxification, and cell adhesion. With the maturation of bone marrow and spleen around birth, hematopoiesis in the liver reduces and hematopoietic stem cells migrate from the liver to the organs responsible for adult-type hematopoiesis (Kinoshita and Miyajima, 2002). While OSM expression in the liver starts in mid gestation and decreases in postnatal stages, HGF is mainly expressed in the liver during the first few days after birth. OSM and HGF induce hepatic maturation through different signaling pathways. Hepatic maturation induced by OSM depends on STAT3 and HGF-induced differentiation is STAT3-independent. Like OSM, HGF in the presence of dexamethasone induced expression of glucose-6-phosphatase (G6P), tyrosine amino transferase (TAT), and accumulation of glycogen in fetal hepatic cells, but at a lower level than OSM. Both OSM and HGF induce production of albumin (ALB) but its secretion appears only in response to OSM (Kamiya et al., 2001).

Glucocorticoids are involved in hepatic maturation and alter the proliferation and function of adult hepatocytes. In the fetal liver, physiological concentration of dexamethasone (Dex), a synthetic glucocorticoid, suppress α-fetoprotein (AFP) production and DNA synthesis and promote albumin (ALB) production. OSM alone fails to induce differentiated liver phenotypes, implying the importance of glucocorticoids as triggers for hepatic maturation (Kinoshita and Miyajima, 2002).

Finally, endothelium-derived signals are also involved in hepatic development (Matsumoto et al., 2001). Endothelial-endoderm contacts can be observed before the onset of liver morphogenesis as angioblasts aggregate between the thickening hepatic epithelium and the septum transversum mesenchyme. These interactions signify liver budding from the ventral foregut endoderm. Liver cells can delaminate and drift into the septum transversum mesenchyme, mix together with angioblasts before the arrangement of functional blood vessels. The role of endothelial cells during liver development has been analyzed in mutant mice that lack the most endothelial cells. Hepatic endoderm was observed to thicken normally and liver gene expression was normal. Neverthe-

less, hepatic cells did not delaminate and proliferate into the septum transversum in Vegfr2 (Flk-1) mutant embryos. The results from liver buds from wild type and mutant embryos cultured *in vitro* clearly showed that hepatic differentiation and growth was impaired in mutant explants, underlying the meaning of endothelial signals during liver morphogenesis. Sustained presence of vascular tissues is required since inhibition of endothelial development also inhibited the expression of albumin. LeCouter et al. (2003) demonstrated that VEGF-A induces liver endothelial cells to control adjacent hepatocytes by promoting the secretion of growth and survival factors. Administration of circulating VEGF-A caused an extensive growth of the liver because of increased proliferation of hepatocytes and other liver cell types. This effect is a result of the secretion of HGF and IL-6 from the sinusoidal endothelium through activation of VEGFR1 (Flt-1). VEGFR1 (Flt-1)-specific ligand stimulates hepatocyte proliferation in the absence of endothelial proliferation, while VEGFR-2 (Flk-1)-specific ligand results in increased endothelial cells growth in the absence of hepatocytes proliferation.

Complete functional hepatic maturation ultimately takes place after birth upon HGF signaling produced by the surrounding non-parenchymal liver cells (sinusoidal, stellate and endothelial cells). Key signaling and molecular actions are hence patterned to occur in the right place at the right time. Simulation of these *in vivo* signaling patterns has contributed to the improvement of *in vitro* differentiation protocols.

1.4.5 Other molecules affecting hepatogenesis

Other signaling molecule affecting the developing hepatocytes is transforming growth factor β (TGFβ). Liver development was found to be severely disturbed in Smad2$^{+/-}$ and Smad3$^{+/-}$ mouse embryos at E14.5 (Weinstein et al., 2001) (Figure 1.5).

The presence and composition of extracellular matrix (ECM) has a significant effect on the gene expression profiles of cultured primary hepatocytes (Michalopoulos et al., 2001). A role of ECM signaling during the development of the liver emanate from studies of β-1 integrin. Chimeric mice generated by combining wild type embryos with β-1 integrin$^{-/-}$ ES cells showed that cells lacking β-1 integrin were unable to colonize the liver. Hence, there is a requirement for β-1 integrin in defining or maintaining the hepatocyte cell lineage (Fassler and Meyer, 1995).

1.5 Transcriptional regulation of hepatogenesis

1.5.1 Transcriptional regulation of early hepatogenesis

The creation of the liver occurs in a two-step process, beginning with the establishment of competence in the foregut endoderm to respond to signals from cardiac mesoderm, followed by the induction of liver-specific gene expression.

A main question in gut organogenesis is if there are present different domains of developmental competence in the endoderm that decided where different tissues could

arise. Such a domain competence could be due to differential expression of transcription factors or signal-transduction molecules along the antero-posterior axis in the endoderm. There is some evidence for a domain of competence for liver formation and there is significant importance of transcription factors in establishing the endodermal domain that gives rise to liver. Three highly related FoxA (forkhead box A) proteins, are expressed in the fetal and adult liver as well as other endoderm-derived tissues. The FoxA proteins regulate almost all liver-specific genes as well as genes in the lung and pancreas (Zaret, 1999). The FOXA (HNF3) proteins were first discovered by their ability to bind to the promoters of the genes encoding α-1-antytrypsin (A1AT) and transthyretin (TTR) (Kaestner et al., 1999). In the endoderm, the beginning of FoxA gene expression precedes the induction of the hepatic program by FGFs signals. Expression of FoxA2 (formerly Hnf3β) starts in the primitive streak during gastrulation. FoxA1 (Hnf3α) (Sasaki and Hogan, 1993) expression initiates in the gut endoderm at E7-E8, before organogenesis, whereas FoxA3 (Hnf3γ) expression begins in the gut endoderm at E8-E9 but is restricted to the midgut and hindgut regions (Ang et al., 1993; Monaghan et al., 1993). FoxA1 and FoxA3 genes are also expressed in early neural tissues (ectoderm) and in the notochord (mesoderm), but all FoxA genes are restricted to the endoderm-derived organs in adults (Lai et al., 1991).

Among the transcription factors found to be expressed in the definitive endoderm, the transcription factors Hnf3β and Gata4 have each been found to act as mediators of competence in the foregut endoderm (Kaestner et al., 1998), and both of these factors are important for hepatic gene expression (Ang and Rossant, 1994; Kuo et al., 1997; Molkentin et al., 1997). *In vivo* footprinting analyses of the albumin enhancer in E9.5 mouse liver buds revealed that several binding sites, including Hnf3β, Gata4 and Nf-1 sites, were occupied. In contrast, extracts from the gut endoderm, a tissue capable to follow a hepatic fate but uncommitted and not expressing albumin, showed in this same assay that only Hnf3β and Gata4 sites were occupied (Gualdi et al., 1996; Bossard and Zaret, 1998). Hnf3β and Gata4 are able to bind silent hepatic enhancers and mark them as possessing the potential to be expressed following induction. These two transcription factors have a capacity to recognize their binding sites in the compacted chromatin (Cirillo et al., 1998; Chaya et al., 2001; Cirillo et al., 2002). The binding of either Hnf3β or Gata4 induce opening of compacted chromatin without recruitment of histone acetylases.

Other transcription factors involved in early stages of liver development are Hex and Prox1. Hex encodes a homeobox transcription factor; which is essential for very early aspects of hepatic development and which is amongst the earliest markers of developing liver. Hex mRNA at E8.5 is restricted to two distinct regions within the ventral endoderm, the future sites of the liver and thyroid (Keng et al., 1998; Thomas et al., 1998). $Hex^{-/-}$ midgestation embryos display a dramatic loss of the fetal liver parenchyma and this phenotype is the earliest disorder of hepatogenesis so far described. Hex mutants failed to form liver bud (Keng et al., 2000; Martinez Barbera et al., 2000).

Knockout experiments have also revealed a role of the second homeobox transcription factor called Prox1 (Figure 1.5) during early stages of hepatic development (Oliver et al., 1993). The livers of Prox1$^{-/-}$ embryos at E14.5 were drastically smaller than those of controls. Prox1$^{-/-}$ hepatoblasts failed to expand and migrate, but they were able to express albumin (Alb) and α-fetoprotein (Afp) entailing that the cells have been specified to follow a hepatic fate (Sosa-Pineda et al., 2000).

1.5.2 Transcriptional regulation of hepatic maturation

During the 1980s a huge effort by many labs allowed the identification of transcription factors that bound transcriptional regulatory elements of genes that are predominantly expressed in the liver (Lai and Darnell, 1991; Sladek and Darnell, 1992). Among liver-specific transcription factors possessing various structural motives are homeodomain proteins HNF1α and HNF1β, the winged helix proteins FOXA1, FOXA2, and FOXA3, the leucine zipper proteins C/EBPα and β, the orphan nuclear receptor HNF4α and the onecut protein HNF6. Whereas none of these factors is solely expressed in liver, it is suggested that the stringency of hepatic gene expression is completed by the unified action of tissue-specific and hormone-dependent transcription factors (Lemaigre et al., 1996; Samadani and Costa, 1996). Knockout mice have been created for all of the genes encoding these factors and the result was that loss of a single factor has in general negligible effect on hepatocyte differentiation. For example disruption of either Hnf3α, β or gamma in the liver does not influence hepatocyte differentiation, even though modest changes in expression of a subset of hepatocyte genes have been described (Kaestner et al., 1998, 1999; Shih et al., 1999; Sund et al., 2000). Disturbance of some genes does have serious consequences on liver function. For example, though early liver development is normal in c/ebp$\alpha^{-/-}$ embryos the neonates die from hypoglycemia as a result of failure of the liver to accumulate glycogen (Wang et al., 1995). Additionally, although fetal livers develop normally, hepatocyte proliferation was decreased in newborn c/ebp$\alpha^{-/-}$ livers (Timchenko et al., 1997). Since c/EBP is inhibiting cell proliferation by stabilizing the cyclin dependent kinase inhibitor p21 and by regulating S-phase-specific E2F-p107 complexes (Harris et al., 2001). Transcription factors cooperate to coordinate gene expression so many mutations do not disrupt hepatocyte differentiation. The majority of promoters are bound by multiple factors and, therefore, it seems that loss of a specific factor can be compensated for by other transcription factors present within the cell. As this model may be generally applicable it has been confirmed that during development of the fetal liver the nuclear hormone receptor, Hnf4α, is crucial for expression of a large array of genes that define hepatocyte function (Li et al., 2000). Hnf4α acts as an essential regulator of hepatocyte differentiation. Hnf1α transcriptional regulatory elements possess the Hnf4α binding site. Hnf4α controls hepatocyte differentiation through the activation of a cascade of transcription factors that eventually define the gene expression profile of the mature hepatocytes. Hnf4$\alpha^{-/-}$ embryos arrest during gastrulation, prior the start of hepatogenesis, because of defects in visceral endoderm function (Duncan

et al., 1997). The combined application of molecular genetics, molecular biology and embryology led us to slowly understand of the mechanisms that control hepatogenesis.

1.6 Hepatogenesis *in vitro* - differentiation of human ES cells into hepatocyte-like cells

The microenvironment of developing hepatocytes is a continuously altering process of successively occurring biological events (Zhao and Duncan, 2005). Every step of cell growth and differentiation is strongly regulated by intra-and extracellular contact, as well as cell independent mechanisms. Liver development is controlled by several distinct paracrine factors. The crucial role was assigned to transcription factors and cytokines, which have been documented as important molecules during the major steps of the liver development. Nodal (Activin A) (Figure 1.2), FGFs, BMP (Figure 1.4), HGF and OSM (Figure1.5) are herein the most essential extracellular signals (Zhao and Duncan, 2005; Kinoshita and Miyajima, 2002; Lemaigre and Zaret, 2004; Clotman and Lemaigre, 2006). At the intracellular level, the liver enriched transcription factors hepatocyte nuclear factor ((HNF)3α, β), HNF4α, HNF1α, β, HNF6, and CCAAT enhancer binding protein ((C/EBP)α, β) act at specific developmental stages in order to regulate liver-specific gene expression (Kyrmizi et al., 2006).

In principle, experimental conditions that have been applied to trigger cultured pluripotent human ES cells into functional hepatocytes, are based on reconstructing the *in vivo* microenvironment. They employed the addition of soluble medium factors and reconstruction of cell-matrix and cell-cell interactions. Overexpression of liver-enriched transcription factors (LETF) genes is also an option, but has the limitations.

1.6.1 Induction of hepatic cell fate via addition of soluble factors (cytokines, growth factors, hormones)

The use of growth factors and cytokines is essential for human ES cells differentiation into hepatocyte-like cell *in vitro*. Hormones and corticosteroids rather play a supporting role.

The development of endoderm represents an important step in initiating the early stages of liver development. Two conditions are required to induce approximately 70-80% of definitive endoderm from human ES cells: signaling by Activin A/Nodal ligands and release from inhibitory signals generated by PI3K through insulin/IGF (D'Amour et al., 2005, 2006). Essentially, Activin A enriches human ES cell culture for definitive endoderm. FGFs and BMPs, are effective in mediating early hepatic differentiation. HGF supports mid-late hepatic phenotype (e.g. ALB expression) (Kumashiro et al., 2005; Zhou et al., 2007) but do not induce functional maturation (Chinzei et al., 2002). Stepwise addition of FGF, HGF and a combination of insulin-transferrin-sodium selenite (ITS), dexamethasone and OSM was successful. Additional alterations of the final

sequential approach even result in 70-80% purity of ES-derived hepatocytes within the culture system (Agarwal et al., 2008; Shiraki et al., 2008). Inseparable component of most differentiation protocols is the co-exposure to serum, which contains hormones, growth factors and other undefined substances that might influence the stochastic differentiation of pluripotent ES cells (Jochheim et al., 2004). Recently many efforts have been made to work under serum-free conditions (Hay et al., 2008a,b).

1.6.2 Induction of hepatic cell fate via genetic alterations

HNF3β (FOXA2) functions as a crucial regulator of initial intracellular signaling pathway in the liver developement (Kinoshita and Miyajima, 2002; Duncan, 2000). In addition it can act as a driving force of ES differentiation along the hepatic lineage. HNF3β-transfected ES cells obtain a hepatic phenotype, more efficiently and earlier than their untransfected counterparts (Ishizaka et al., 2002; Kanda et al., 2003). Using this approach ES cells differentiation in culture is in fact driven by the same transcriptional events as in early liver organogenesis *in vivo*. HNF3β not only influenced the hepatic gene expression but also hepatocytes functions. A major obstacle of the constitutive overexpression of transcription factors is the risk of unpredictable gene upregulation *in vitro*.

1.6.3 Human ES cells differentiation into hepatocyte-like cells – protocols - overview

Development of hepatocytes from human ES cells represents the final of a difficult developmental plan. Cells with hepatocyte-like morphology and function have been derived from human ES cells and described in many studies. The early studies report spontaneous differentiation of human ES cells with no specific efforts to enrich for hepatocyte-like cell population (Schuldiner et al., 2000). These observations were soon followed by more directed differentiation strategies and modified culture conditions that supported hepatic differentiation of human ES cells (Rambhatla et al., 2003; Lavon et al., 2004; Schwartz et al., 2005; Baharvand et al., 2006; Hay et al., 2007). The cells displayed suitable morphology and expressed some hepatocyte-associated markers, like albumin (ALB), α-1-antitrypsin (A1AT), cytokeratin 8 and 18 (CK8 and CK18). Additionally, the hepatocyte-like cells expressed hepatic transcription factors, such as FOXA2, HNF1, and GATA4. Functional studies of the cells indicated glycogen accumulation, inducible cytochrom P450 (CYP) activity, production of urea and albumin, and uptake of indocyanine green (ICG).

Initially, differentiation protocols used the fact that human ES cells can spontaneously differentiate into cell types of three germ layers, including hepatocytes, upon removal of FGF2 when cultured as embryoid bodies (EBs), and then plated on gelatin (Odorico et al., 2001; Lavon and Benvenisty, 2005). Even if EBs are able to form functional and specialized cell types, including hepatocytes, the differentiation effectiveness in number of lineage-specific cell types gained is relatively low (Rambhatla et al., 2003;

Lavon et al., 2004). Improvement in EB technology was their culture in presence of differentiation inducing factors, conditioned medium, and growth factors to enrich for specific cell population (Lavon and Benvenisty, 2005). In 2003 the first article was published showing the generation of hepatocyte-like cells derived from human ES cells (Rambhatla et al., 2003). Then a number of approaches have been used to differentiate and to obtain enriched populations of hepatocyte-like cells. One study (Lavon et al., 2004) used gene manipulation to select the cells through an albumin (ALB) promoter. Nevertheless, the cells expressing a hepatic phenotype were isolated from EBs, as a consequence only a few cells were produced and the functionality of the cells was not examined. In one of the protocols, combined treatment with insulin, Dex and collagen type I followed by sodium butyrate, led to an improved number of mature hepatic gene-expressing cells (10-15%) (Rambhatla et al., 2003) The lack of success of these early attempts at differentiating human ES cells into functional hepatocytes put attention on the basics of normal embryonic development. This knowledge was crucial for enhanced understanding the early stages of definitive endoderm development. The most significant input is a protocol in which the use of Activin A in serum-free conditions, resulted in enrichment of human ES cells in definitive endoderm cells (up to 80%) (D'Amour et al., 2005). Using a modification of this protocol and a combination of protocols previously reported using mouse ES cells, Cai et al. (2007) reported that the addition of FGF, BMP, and HGF can stimulate hepatic fate, and that the later addition of OSM and Dex to the cell culture induced even more differentiated hepatocyte-like cells in 18 days of protocol duration. It was the first published differentiation protocol mimicking hepatogenesis, including definitive endoderm formation step. Afterwards, several different protocols have been established directing the cells through the definitive endoderm and hepatic development (Hay et al., 2008a; Agarwal et al., 2008).

Three differentiation protocols (Cai et al., 2007; Hay et al., 2008a; Agarwal et al., 2008) have been investigated in details and performed by me applying both H1 and H9 cell lines. Differentiation protocols published between 2003-2008 with human ES cells are listed in Table 1.1.

Table 1.1: Strategies for *in vitro* differentiation of human ES cells into hepatocytes-like cells

Cell line	**Hepatic differentiation conditions**	**Hepatic features**	**References**
H1, H9	EBs differentiation: matrigel, 20%FBS, 5mM NaB; direct differentiation: 4D: 1%DMSO, D6-7:2.5mM NaB, D8-11: HCM, 2.5mM NaB, 2.5ng/ml HGF	Negative for AFP, positive for ALB, A1AT, CK8, CK18, glycogen storage, inducible CYP1A2 activity, 10-15% yield	Rambhatla *et al.*

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Table 1.1 – continued from previous page

H9	hESCs stable transfected with ALB-eGFP, CM from culture of primary hepatocytes and aFGF; 20 days old EBs were dissociated and plated as DES for 10 days -6% of cells were eGFP+, these cells were further isolated into a homogenous population (=95%), grown for 2 weeks, frozen and thawed	D30 (20 days EBs+10 days DES): FGG, FGB, ALB, APOH, APOB, APOA4, FGA, AFP; co-localization of cardiac and hepatic cells in teratomas	Lavon *et al.*
H1, H9	EBs, Collagen type1, FGF4, HGF	HNF3β, GATA4, HNF1, CK18, urea and Albumin production, phenobarbital-induced CYP450, ICG uptake	Schwartz *et al.*
H1	EBs into 2D (collagen type1) and 3D (collagen type1 scaffold) culture systems, D9-12: 100ng/ml aFGF, D12-20: 20ng/ml HGF, D15-20: 10ng/ml OSM, 0.1μM Dex, 5mg/ml insulin, 5mg/ml transferrin, 5μg/ml ITS, cultured till D28	ALB, CK18, TTR, TDO2, TAT, A1AT, G6P, CYP7A1, ATP and ALB secretion, urea production, hepatocyte-like ultrastructure (glycogen granules, Golgi apparatus). In 3D:ALB and G6P detected earlier and higher levels of urea and AFP; ICG uptake, glycogen storage, HNF3β.	Baharvand *et al.*
H1and clonal sublines	Differentiation onset upon 70% confluency, D1-D7: UM, 1%DMSO; D8-D16: HCM, 10ng/ml HGF, D17-D20: 10ng/ml OSM, 10ng/ml HGF, HCM.	AFP, ALB, HNF4a, CYP3A4 activity, Albumin production, glycogen storage, ICG uptake, HePar1, TTR, A1AT, TDO2, C/EBPa.	Hay *et al.*
H1, H9	D0-D3:100ng/ml ActA, D4-D8: 30ng/ml FGF4, 20ng/ml BMP2, D9-D18: 20ng/ml HGF (5days), 10ng/ml OSM, 0.1μM Dex (5days)	70% of cells ALB positive, TAT, TDO2, PEPCK, CYP7A1, CYP3A4, CYP2B6, CK8, CK18, G6P, A1AT, albumin secretion, glycogen storage, ICG and low.density lipoprotein uptake, inducible CYP450 activity; integrate into CCl4 injured liver of SCID mice and expressed A1AT for 2 months, infected by HIV-HCV pseudotype viruses.	Cai *et al.*
SA001, SA002, SA002.5, AS034, SA121, SA167	Cells were allowed to differentiate for 18-30 days in VitroHES^{TM} with 4ng/ml FGF2, medium was replaced after 13 days and again after 18-25 days	A1AT, ALB, HNF3ß, AFP, CK18, LFABP, GSTA1-1, GSTM1-1, GSTP1-1, glycogen storage, GST catalytic activity	Söderdahl *et al.*

Continued on next page

Table 1.1 – continued from previous page

SA002, SA002.5, SA167	Cells were cultured on MEFs and allowed to differentiate for 18-30 days in VitroHES^{TM} with 4ng/ml FGF2, medium was changed every 7-10 days	inducible CYP450 activity and activity assays, CYP1A1, 1B1, 2A6/2A7/2A13, 2B6, 2C8, 2C9, 2D6, 2E1, 3A5, HNF1α, HNF3β, HNF4α, RXRβ, C/EBPα,β, ALB, G6P, APOE, UGTs.	Ek *et al.*
HSF6	EBs-D0-D9, D10 plated on collagen type I, D20-24 hESCs transduced with a lentiviral vector containing the GFP gene driven by A1AT	purification of GFP+ hESCs by LMPC, glycogen storage, ICG uptake, inducible CYP450 activity, albumin secretion, AFP, ALB, HNF4α, A1AT, CK18; D30 in LMPC GFP+hESCs: G6P, CYP2B6, CYP2B6, CYP2E1, CYP3A4, CYP1A1, D23: GATA4, C/EBPα,β, HNF3β, BMP4; injected into NOD-SCID mice and human ALB, A1AT, CYP1B1 were detected and evaluated with CCD camera	Duan *et al.*
H9, H1-GFP	Differentiation onset upon 80% confluency, D5:upon confluence 1:1 split, from D5: on collagen, D0-D5:100ng/ml ActA, D5-D11: 10ng/ml FGF4,10ng/ml HGF D11-D20:10ng/ml FGF4, 10ng/ml HGF, 10ng/ml OSM, Dex	D11-D20: HNF4α, AFP, ALB, DPPAIV, A1AT, D20: CYP7A1, CYP3A4, glycogen storage, ICG uptake, albumin secretion	Agarwal *et al.*
H1, H7	Differentiation onset upon 50-70% confluency, D3/5:1/2 split, matrigel, D0-D3/5-D10/12:SR, from D10/12 8.3% FBS; D0-D3/5: 100ng/ml ActA, from D10/12: insulin, hydrocortisone, 10ng/ml HGF, 20ng/ml OSM, D0-D1/2: 1mM NaB, D3/5-D10/12: 1% DMSO	D0-D3/5: CXCR4, HNF3β, SOX17, D3/5-D10/12: HNF4α, HNF1, TTR, AFP; from D 10/12: ALB, APOF, CAR, TO, TAT, CYP3A4/7, CYP2C9/19, glycogen storage, albumin/fibrinogen/fibronectin/A2M secretion, inducible CYP450 activity	Hay *et al.*
H9	D0-D2: EBs, D3-D5:ActA, D6-D10:co- culture with stably relesed FGF2 hFLSCs, D11-D16: hFLSCs-derived FGF2, 20ng/ml HGF, 10ng/ml OSM, 0.1μM Dex, HepatoZYME media,	AFP, ALB, CK18, glycogen storage, ICG uptake, albumin secretion, CYP1B1, urea production, hepatocyte-like ultrastructure,	Pei *et al.*

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Table 1.1 – continued from previous page

H1, H9	Differentiation onset upon 80% confluency, D5:upon confluence 1:1 split, from D5: on collagen, D0-D5:100ng/ml ActA, 50ng/ml WNT3a, D5-D11: 10ng/ml FGF4,10ng/ml HGF, D11-D20:10ng/ml FGF4, 10ng/ml HGF, 10ng/ml OSM, Dex	D11-D20: HNF4α, AFP, ALB, DPPAIV, A1AT, D20: CYP7A1, CYP3A4, CYP1A2, glycogen storage, ICG uptake, Albumin secretion injected into NOD-SCID mice and human ALB, CK18 expression, human albumin production,	Hay *et al.*
H5	D0-D5: undifferentiatrd hESCs, FGF2, 10ng/ml Noggin, D6-D12: 100ng/ml FGF1, 10ng/ml FGF4,D13-D19: 20ng/ml HGF, 10ng/ml FGF4, D20-D33: 20ng/ml HGF, 10μM Dex, 10ng/ml OSM, ITS,	ICG uptake, albumin secretion, glycogen storage, urea production, low-density lipoprotein uptake, hepatocyte-like ultrastructure, ALB, CK18, HepPar1, HNF3β, HNF4α, C/EBPα,β, CK8, CK18, TTR, AFP, A1AT, TDO2, G6P, CYP7A1, APOB,	Baharvand *et al.*
H9	D0-D8: EBs in DMEM with 20% FBS, D8: EBs plated on 0.25% gelatin in DMEM, 20%FBS, 100ng/ml FGF1, D13: cells transduced with ALB and AFP promoter driving GFP, lentiviral system	whole genome transcriptional profiling, albumin and AFP secretion,	Chiao *et al.*

D - day, ActA - Activin A, NaB - sodium butyrate, CM - conditioned medium, EBs - embryoid bodies, DES - differentiated ES cells, LFABP-liver fatty acid binding protein, G6P- glucose-6-phosphatase, UM - unconditioned medium, HCM - hepatocyte culture medium, MEFs - mouse embryonic fibroblasts, CCD - charged-coupled device camera, ALB- albumin, TAT-tyrosine aminotransferase, A2M-alpha-2-macroglobulin, GST-glutathione transferase, GSTA1-1 -glutathione transferase alpha , GSTM1-1 - glutatione transferase mu, GSTP1-1 - glutathione transferase pi, UGTs - UDP-glucuronosyltransferases, RXRβ - retinoid-X-receptor β, APO - apolipoprotein, LMPC - laser microdissection and pressure catapulting, Dex - dexamethasone, hFLSCs - human fetal liver stromal cells, ICG-indocyanine green, OSM - oncostatin M, HGF- hepatocyte growth factor, FGF1 - acidic fibroblast growth factor, FGF2 - basic fibroblast growth factor, CK8,18 - cytokeratin 8, 18, AFP - α-fetoprotein, TTR - transthyretin, TDO2 - tryptophan-2,3-dioxygenase, FBS - fetal bovine serum, A1AT - α-1-antitrypsin, HNF3β - hepatocyte nuclear factor 3β, HNF4α - hepatocyte nuclear factor 4α, C/EBPα,β - C enhancer binding protein α and β, DMSO - dimethyl sulfoxide, GFP - green fluorescent protein, CYP- cytochrome P450, HepPAR1 - hepatocyte paraffin 1, ITS - insulin/transfferin/selenium, HNF1α - hepatocyte nuclear factor 1α, NOD-SCID -non-obese diabetic-severe combined immunodeficiency mice.

1.6.4 Present characterization strategies of human ES-derived hepatocyte-like cells and their restrictions

Human ES cells-derived hepatocyte-like cells may be characterized *in vitro* at four levels: morphological, RNA, protein and functional activity levels. Usually, the analytical work is limited to the elucidation of (i) endodermal/hepatogenic RNA transcripts via (quantitative) RT-PCR, and (ii) proteins by immunofluorescence. Most endodermal markers studied include LETFs (HNF1α, β, HNF3β, HNF4α, C/EBPα,β), plasma proteins (AFP, ALB, TTR), and cytoskeletal proteins (CK8, CK18). A minority of studies examine the expression of CYPs and other late enzymes such as tryptophan

2,3-dioxygenase (TDO2) and tyrosine aminotransferase (TAT).

The differentiation of human ES cells towards the hepatocyte lineage often involves uncontrolled processes, resulting in a heterogeneous cell population. Genes such as TAT (Vogel et al., 1973), phosphoenol-pyruvate carboxykinase (Yanez et al., 2003), and LEFTs (Ihara et al., 2005; Martis et al., 2006; Ogasawara et al., 2007; Gu et al., 2007) are also expressed in other somatic cells such as lung, intestine, pancreas and kidney and thus cannot be considered as true hepatocyte markers. In addition, genes like AFT and TTR are both expressed in liver tissue and in the extra-embryonic yolk sac (Gulbis et al., 1998). For this reason, exclusive analysis of one of the latter markers cannot count as proof for a genuine hepatic phenotype.

The differentiation of hepatoblasts into hepatocytes is a stable developmental process. It is known that embryonic, fetal, and adult hepatocytes possess divergent molecular phenotypes (Schmelzer et al., 2006). Basically, hepatogenesis *in vivo* implies serial expression of early (HNF3β, AFP, TTR), midlate (HNF4α, HNF1α, ALB, CK18) and late (TDO2, TAT, C/EBPα, CYPs) marker genes (Kinoshita and Miyajima, 2002; Clotman and Lemaigre, 2006). AFP is expressed very early in embryonic development and during the fetal stages. Its expression gradually levels off with increasing development and disappears entirely in adult live (Cascio and Zaret, 1991). AFP represents thus a reliable marker to discriminate between distinct developmental stages. On the other hand, most, but not all, metabolic and detoxifying enzymes do not become functional before birth. Indeed, during the terminal step of liver organogenesis, the liver becomes a functional, metabolic organ: hepatocytes start both to control the levels of metabolites and serum proteins in the bloodstream and express numerous new genes and proteins related to specific functions of the adult liver (Duncan, 2000; Kmiec, 2001; Gomez-Lechon et al., 2004). Hence, to confirm the status of differentiation of hepatocyte-like cells, functional evaluation of enzymes levels must be performed. Most popular functional analysis is principally focused on glycogen storage, urea metabolism and albumin secretion. Just modest attention has been paid to other metabolic functions, including CYP450-dependent activity and sensitivity to model inducers such as phenobarbital (CYP2B6, CYP3A4), rifampicin (CYP3A4) and 3-methylcholantrene (CYP1A1/2). Inducible CYP-dependent activity is considered to be a key determinant of the functional hepatic phenotype (Hines and McCarver, 2002; McCarver and Hines, 2002), characterization must include the above mentioned functionality assays as well.

The ultimate proof of functional hepatic behavior *in vivo* is transplantation of *ex vivo* generated stem cells-derived hepatocyte-like cells in immunodeficient animal models suffering from liver injury (Imamura et al., 2004; Aurich et al., 2007). Examples of recipients permissive for engraftment of both allogeneic and xenogeneic cells are partially hepatectomized Pfp/Rag2$^{-/-}$ mice (Aurich et al., 2007), carbon tetrachloride (CCl4)-injured severe combined immunodeficient (SCID) mice, and urokinase-type plasminogen activator $^{+/+}$(uPA$^{+/+}$)/non-obese diabetic (NOD)-(SCID) mice (Brulport et al., 2007; Hengstler et al., 2005). Positive homing, engraftment, repopulation, and functional

maturation is principally explored by application of molecular imaging techniques, immunohistochemistry, *in situ* hybridization and serology (Luk et al., 2005; Brulport et al., 2007; Meuleman et al., 2005). Regardless of apparently convincing evidence that stem cells derived hepatocyte-like cells could contribute to liver reconstitution, there will always exist the possibility of false positive results because of use of imprecise labeling techniques (Pearson, 2006).

1.7 Human ES cells-derived hepatocyte-like cells – applications

1.7.1 Regenerative medicine

Stem cell differentiation into hepatocytes is of huge importance, while access to large number of these cells would allow their use instead of whole organ transplantation as a possible treatment for severe liver diseases.

Facing an increasing global population of hepatic patients whose care requires extensive economic and health care resources human ES cells are explored as a source for generating unlimited amounts of hepatocytes for transplantation. Several candidate cell types have been considered, but human ES cells are the most attractive because of their pluripotent nature and suitability for cell-replacement therapy. Therefore a precise understanding of the developmental processes that guide to a specific cell fate may help to repeat the events *in vitro* and engineer artificial cells and tissues to fight liver diseases (Nussler et al., 2006).

Many differentiation protocols have already been established to produce almost all cell types from human ES cells. In theory, human ES cells could be applied to a wide range of human diseases. The *in vitro* developmental potential and the success of ES cells in animal models reveal the principle of using human ES-derived cells as a regenerative source for transplantation therapies of human diseases.

Many experimental obstacles must be solved before specified cell types derived from human ES cells can be applied to humans. Important is the understanding of the genetic and epigenetic changes that take place during *in vitro* cultivation. It is going to be necessary to purify defined cell lineages that are appropriate for cell-based therapies. As well control of karyotypic changes during passaging. The cells once incorporated into the tissue must function in a normal physiological way. The guarantee of donor/recipient immunocompatibility and lack of tumor formation must be ensured. Previous to therapeutically applicable, any ES-based treatment must show limited toxicity potential, immunological rejection and tumor formation. Till now human ES cells research has not fulfilled these requirements.

Currently, no human ES cell-based therapies are performed on humans. Recently, 23rdof January 2009, Geron Corporation received U.S. Food and Drug Administration (FDA) clearance to begin world's first human clinical trials of ES cells-based therapy. Geron's first product manufactured from human ES cells, which enter clinical test-

ing is GRNOPC1 (oligodendrocyte progenitor cells). The ultimate goal for the use of GRNOPC1 in man is to achieve spinal cord repair by injecting these cells directly into the spinal cord lesions.

Till now only allogeneic or matched donor-derived adult stem cells have been employed in human cell-grafting therapies. The best example is bone marrow transplantations for the treatment of leukemia after myeloablative therapies.

1.7.2 Pharmacology (hepatotoxicity tests)

Hepatocytes are key cells in drug discovery procedure and they are applied for examinations of novel targets in metabolic diseases. Additionally, these cells have broad applications in studies of liver metabolism and pharmacokinetic properties of novel compounds, as well as in hepatotoxicity trials. The main funds of pharmaceutical companies are spent on to screens for metabolic properties early in drug discovery processes (Masimirembwa et al., 2001). The early metabolic testing relies on the accomplishment of metabolic degradation of the compound, mechanism of the metabolism, and induction or inhibition of any drug metabolizing enzymes. In drug metabolism studies hepatocytes are currently used as the gold standard (Cross and Bayliss, 2000).

There is a huge requirement for *in vitro* models of hepatocytes especially for toxicity studies of new drugs since unpredicted human metabolism is today one of the main causes of the withdrawal of potential new drugs from pharmaceutical projects. In the future the extensive use of human ES cell-derived hepatocytes is expected in toxicology while the leading cause of preclinical failure of new drugs is hepatotoxicity (Schuster et al., 2005). Therefore, novel enhanced models to evaluate toxic effects of new drugs early in the development stage are needed. It is very difficult to predict hepatotoxic properties of new compounds in humans and, in many cases toxicity is observed only in the late phases of the drug discovery procedure. Hence, the species differences and extensive use of animal models in drug toxicity trials is the main problem. For this purpose the standardization of the production of functional human hepatocytes is needed. Principally, any cell type existing today does not copy the complexity and function of the liver. The human models available today are utilizing cancer cell lines or primary cells isolated from tissue biopses, unfortunately these two cell types possess significant limitations. Available hepatic cell lines contain very low levels of metabolizing enzymes and possess substantially different levels of other important proteins in comparison to native hepatocytes (Wilkening et al., 2003). A prime example here is the commonly used human hepatocellular carcinoma cell line (HepG2), which is limited by the poor phenotypic and functional match to *in vivo* hepatocytes. The human primary isolated hepatocytes could be remedy in this situation. Even though, primary human liver cell are available, they quickly lose functional properties when maintained *in vitro* and a key limitation in this case is the requirement of repeated sourcing (Rodriguez-Antona et al., 2002). The variability and purity in these material results in practical constrain that limit its utility. As first hepatotoxicity trials have been performed using mouse

ES cell-derived hepatocytes (Kulkarni and Khanna, 2006). The similar model based on human cells is of prime importance. The challenge at this point is to adapt human ES cell-derived hepatocytes to a format for drug discovery trials.

Appropriate source of hepatocytes could significantly improve the development of new drug discovery strategies and give possibilities to perform *in vitro* metabolism studies and toxicity trials. The first study to describe the actual drug-metabolizing effects in human ES cell-derived hepatic cells was published by Soto-Gutierrez et al. (2006). The authors showed the cells significantly metabolized lidocaine. Published by Soderdahl et al. (2007) hepatocyte-like cells derived from human ES cells expressed several mature liver markers. Moreover, these cells express functional glutathione transferase activity at levels comparable to human hepatocytes. The presence of specific biotransforming enzymes is crucial for the future industrial use of stem cell-derived hepatocytes. The cytochromes and the enzymes involved in drug conjugation (e.g. UDP glucurunosyltransferases), are the most important drug metabolizing enzymes (Rendic and Di Carlo, 1997).

Since the human liver is an organ consisting of many cell types besides the hepatocytes and the other cell types (Kuppffer cells, stellate cells, cholangiocytes) are important for the complex structure of the liver. For that reason, to be able to completely understand and foresee the positive and negative effects of new drug *in vitro*, more complex models must be developed. This additionally highlights the potential for human ES cells as a source for human hepatotoxicity models, since principally any cell type can be generated from pluripotent stem cells. Human ES cell studies can give a hope to imitate simple liver tissue and further improve the chances to precisely predict human toxicity *in vitro*. It is probable that pharmaceutical companies that effectively incorporate stem cell technologies will have a competitive advantage.

1.8 Aim of this work

The main focus is to understand the molecular mechanisms involved in differentiation of human ES cells into endoderm and further into hepatocyte lineage.

The study was aimed at investigating the differentiation of human ES cells into hepatocytes. The main two goals of this study were:

- Efficient differentiation of human ES cells into hepatocyte-like cells.
- Expression profiling analysis to reveal regulatory events leading to hepatic differentiation *in vitro*.

To achieve these, the following investigations were carried out:

- Three hepatocyte differentiation protocols were performed and cells were analysed after each step of the protocol by immunofluorescence staining, Western blotting and real time RT-PCR.

- Step-wise transcriptional profiles of cells during differentiation process was investigated in order to find potentially new target genes which behave similar to already known markers during the time-course of differentiation.
- The microarray data was analysed for the regulation of single target genes, GO-annotated biological processes, cellular compartments, molecular functions and pathways.
- The changes in gene expression of selected genes were validated by Real-time RT-PCR.
- Hepatocyte functions of human ES cells-derived hepatocyte-like cells was determined by applying two independent assays.

2

Materials and Methods

2.1 Mouse Embryonic Fibroblasts Culture

For long-term culture human ES cells can be grown on a layer of mitotically inactivated mouse embryonic fibroblasts (MEFs) or under feeder-free conditions in CM (Conditioned Medium) on Matrigel®-coated plates to maintain their undifferentiated state. Success of both culture conditions fully depends on the quality of feeders, since they directly affect the growth of human ES cells.

2.1.1 Isolation of Mouse Embryonic Fibroblasts (MEFs)

The pregnant female mice (CF-1, Harlan, USA) were sacrificed at 13 or 14 d.p.c. (days post-coitum) by cervical dislocation. Uterine horns were dissected out, briefly rinsed in 70% (v/v) ethanol and placed into a falcon containing PBS without Ca^{2+} Mg^{2+} (Gibco, Invitrogen). Afterwards they were placed into a petri dish and each embryo was separated from its placenta and embryonic sac. Next head and red organs were dissected, washed in PBS and embryos were placed in a clean petri dish. Using sterile razor blade the tissue was finely minced until it become pipettable. Then 1ml of 0.05% trypsin/EDTA (Gibco, Invitrogen) and DNase I (USB, 100 Kunitz units per ml of trypsin) was added per an embryo. Afterwards the tissue was transferred into 50ml falcon tubes and incubated for 15 minutes at 37°C. After each 5 minutes of incubation cells were dissociated by pipetting up and down. Then trypsin was inactivated by adding about 1 volume of freshly prepared MEF medium (Appendix A.1). Remaining pieces of tissue were settled down to bottom of the tube and removed. After low-speed centrifugation of supernatant cell pellet was resuspended in warm MEF medium. Approximately 3-4 embryos were plated in each T150 (TPP) flask coated with 0.2% gelatine (Gelatine from bovine skin, Type B, Sigma) for 2 hours. The fibroblasts (P0, passage 0) were the only cells that have the ability to be attached to the gelatine-coated flasks. The next day cells were 80-90% confluent and at this stage a major part of P0 cells were frozen for future usage. The remaining one T150 flask of P0 was expanded (kept growing) till P3 or P4, then inactivated and used as feeders to thaw human ES cells or to produce conditioned medium (CM).

2.1.2 Cryopreservation (freezing) of MEFs

Culture media was removed from T150 flasks, cells were washed with PBS without $Ca^{2+}Mg^{2+}$ (Gibco, Invitrogen) and trypsinized for 5 minutes at 37°C. Trypsin was neutralized by adding 10ml of MEF medium (Appendix A.1). Then cells were centrifuged at low speed for 5min. Afterwards the supernatant was removed and the cells resuspended in cold freezing media (Appendix A.2). The cell suspension was transferred into cryovials (TPP) (1ml per vial). Usually 1 embryo equivalent was frozen per vial. Cryovials were placed inside pre-cooled freezing container (Nalgene) and transferred to -80°C freezer. This procedure enabled that cells freeze really slowly. The next day vials were transfer to the liquid nitrogen tank for long-term storage.

2.1.3 Thawing and maintaining of MEFs

The vial with frozen MEFs was removed from the liquid nitrogen and thawed in a warm water bath. When just a small crystal of ice remains then vial was sterilized with 70% (v/v) ethanol. Afterwards the cells suspension was pipetted dropwise into 10ml of warm MEF medium to reduce osmotic shock. To remove the DMSO present in freezing media, cells were pelleted by centrifugation (5min., low speed). Then the supernatant was removed and the cells, resuspended in 20ml of MEF medium and plated in T150 flask. The next day the media was replaced and after 2 days cells were about 90% confluent. The subconfluent cells can be split 1:2 or 1:3 with trypsin. Mouse Embryonic Fibroblasts should be only used till passage 5, to support human ES cells growth. After this time, they attain a senescence state and do not divide any more in a sufficient manner.

2.1.4 Inactivation and plating of MEFs (Feeders preparation)

T150 flasks were coated with 0.2% gelatine (Gelatine from bovine skin, Type B, Sigma) and incubated at RT for at least 2 hours. During this time mitomycin C (Roche) was diluted in PBS (1mg/ml) and filtered sterilized and media from MEFs was aspirated and cells were washed with PBS without $Ca^{2+}Mg^{2+}$ (Gibco, Invitrogen). Then 20ml of MEF media, containing 10μg/ml of mitomycin C was placed on MEFs. After 2h incubation at 37°C mitomycin C containing media was removed and cells were washed twice with PBS, trypsinized, centrifuged and resuspend in warm media. Afterwards the cells were counted and plated at 56.000 cells/cm^2 in T150 flasks to be used for CM production for next 6 days.

2.2 Human ES cells culture in feeder-free conditions

Human ES cell lines H1 and H9 (WiCell Research Institute, Madison, Wisconsin) from passage 39 to 66 were maintained under sterile conditions in a humidified incubator in a 5%CO_2-95% air atmosphere at 37°C (INNOVA CO-170 Incubator, New Brunswick

Scientific). To manipulate (cut or removed differentiated colonies) cells were placed under HERAguard® Clean Bench (Heraeus, Thermo Fischer Scientific Inc.) with Leica MZ 95 StereoMicroscop (Leica, Vashaw Scientific Inc.). Under these culture conditions, hESCs were confirmed to stain positive for OCT4, SSEA-4, TRA-1-60, and TRA-1-81 (ES Cell Characterization Kit, Chemicon).

2.2.1 The Human Basic Fibroblast Growth Factor preparation

Recombinant human basic fibroblast growth factor (bFGF, FGF2, Peprotech) (50μg) was dissolved in 5ml of PBS with 0.2% BSA (Bovine Serum Albumin, Fraction V, 99% purity, Sigma). FGF2 solution is very sticky and cannot be filtered so BSA solution was filtered (0.2μM Acrodisc® Syringe Filters, Pall Corporation). Stock aliquots were kept at -20°C (for long-term storage).

2.2.2 Conditioned medium preparation

Next day after plating inactivated MEFs at 56.000 cells/cm^2 density, MEF media was replaced with human ES medium (UM, unconditioned medium)(Appendix A.3) (0.5ml/cm^2) supplemented freshly with 4ng/ml of FGF2. CM was collected from feeder flasks after 24h incubation and fresh human ES medium containing 4ng/ml of FGF2 was added to the feeders. This procedure was repeated for the next 6 days. Each day, collected CM was placed at -20°C. After 6 days whole media was mixed together and filtered (Corning, 0.22μM, PAS). Then 50ml aliquots were made and stored at -80°C. CM was supplemented with additional 4ng/ml of FGF2 before adding to hESCs grown on Matrigel®.

2.2.3 Measurement of Activin A in Conditioned Medium

Enzyme-linked immunosorbent assay (ELISA) was carried out using products from R&D Systems. Before use all samples (UM and CM) and reagents were brought to room temperature. Capture antibody (Human/Mouse/Rat Activin A MAb, R&D Systems) was diluted in PBS with 1% BSA, added to microplate (100μl/well) and incubated overnight at RT. The next day wells were washed three times with PBST (400μl/well). Then blocking solution (1% BSA/PBS, 300μl/well) was applied for 1h at RT. In this time Activin A (R&D Systems) standard curve was prepared, included 7 dilutions (concentration less than 30ng/ml) and blank sample. The linear working range of the Activin A assay was established in a pilot experiment and was between 0.25 and 32ng/ml. Standards and samples (in duplicates) were added to wells (100μl/well) and incubated for 2h at RT. Afterwards wells were washed three times (300μl of PBST). Then secondary (biotinylated) antibody (Human/Mouse/Rat Biotinylated Activin A MAb, R&D Systems) was applied (diluted 400-fold to 0.25μg/ml in 1%BSA/PBS) and incubated for 2h at RT. Wells were washed three times (300μl of PBST) and Streptavidin-HRP (diluted in 1%BSA/PBS, R&D Systems) was added and incubated for 20min. at RT. Then wells were washed

three times (300μl of PBST) and 100μl of substrate solution (Quantikine®, R&D Systems) was added and incubated for 30min. at RT in the dark. Afterwards 100μl of stop solution (Quantikine®, R&D Systems) was added to each well and the plate was tapped gently to mix. Microplate reader (Molecular Devices Spectra Max 250, Global Medical Instrumentation, Inc., Minnesota) set to 450nm, with wavelength correction at 540 or 570nm was used to determine the optical density of each well (Greber et al., 2007).

2.2.4 Preparation of Matrigel®-coated plates

Growth Factor-Reduced Matrigel® (Becton Dickinson) was used for coating the plates. First Matrigel® was slowly thawed overnight at 4°C (on ice) to avoid gel formation. On the next day an adequate volume of cold Knockout^{TM} DMEM was added to the 10ml of Matrigel® and mixed well. Bottle containing Matrigel® and falcons were kept on ice, 1ml aliquots were pipetted into each pre-chilled tube and stored at -20°C. Just before coating, plated Matrigel® aliquot was diluted in 14ml of cold Knockout^{TM} DMEM. To coat plates 1.5ml of Matrigel® solution was added to single well of a 6-well plate and 1ml was added to single well of a 12 well-plate. Afterwards plates were wrapped with parafilm and placed at least overnight at 4°C. Matrigel® solution was removed immediately before plating MEFs or hESCs.

2.2.5 Passage of human ES cells on Matrigel®

First Matrigel®-coated plates were prepared to plate cells after splitting. Matrigel® solution was removed, 2ml of CM (supplemented with FGF2) was added and plates were put inside the incubator to warm. Meanwhile differentiated cells were removed from plates using Gilson 10μl tips and 80-100% confluent hESCs colonies were cut manually into pieces of uniform size with a BD Microlance^{TM}3 injection needle (Becton Dickinson, Madrid, Spain). Then cells were placed into the incubator for 10-15 minutes. Afterwards media was aspirated and cells were washed with PBS without $Ca^{2+}Mg^{2+}$ (Gibco, Invitrogen). Into each well of 6-well plate 1ml of dispase (2mg/ml, Gibco, Invitrogen)(Appendix A.5) was added and cells were incubated at 37°C in incubator. Dispase was removed when the edges of the cut colonies start to pull away from the plate and then cells were gently wash thrice with 2ml of UM (Appendix A.3). Then 2ml of UM was placed on cells, they were incubated in 37°C for 10-15 minutes and gently remove with a cell scraper (Biochrom AG, Berlin). Cells clumps were collected and transferred into a 15ml falcon tube for brief centrifugation. Afterwards CM (supplemented with FGF2) was added to cells pellet and gently pipett to avoid a single cell suspension. Human ES cells were seeded into wells of Matrigel®-coated plates, in an optimal split ratio of 1:3. The plates were returned into the incubator and gently agitated left to right and back to front to obtain an even distribution of cells. The day after seeding, undifferentiated cells were visible as small colonies, with subsequent culture these colonies became large and

compact. In this culture system the human ES cells were maintained at high density and split usually once peer week.

2.2.6 Daily maintenance of human ES cells in feeder-free culture

Every day human ES cells were fed with 3-4ml of CM (supplemented freshly with 4ng/ml of FGF2 to a final concentration of 8ng/ml) for each well of a 6-well plate. Only required amount of CM was placed in a 37°C water bath until warm and used immediately because the Knockout^{TM}DMEM and Knockout^{TM}Serum Replacement do not tolerate repeated warming and cooling.

2.2.7 Freezing human ES cells

The procedure for freezing human ES cells is identical to that for splitting these cells. Shortly, colonies were manually cut, washed with PBS, incubated with dispase, washed thrice with UM and gently scrape with a cell scraper. Then clumps were collected, centrifuged and resuspended gently in cold human ES cells freezing media. Human ES cells freezing media (Appendix A.4) were prepared before and placed at 4°C. Usually cells clumps collected from one well of a 6-well plate were suspended in 1ml of freezing media and placed into one cryovial. Then cells were put into a freezing container (Nalgene) and placed immediately at -80°C overnight. The next day vials were transfer to liquid nitrogen for long-term storage. To obtain higher efficiency during thawing, cells clumps for freezing should be slightly larger than those used for splitting.

2.2.8 Thawing human ES cells

The cryovial containing human ES cells was removed from the liquid nitrogen storage tank and thawed by gently swirling in a 37°C water bath until only a small ice pellet remained. Afterwards 70% (v/v) ethanol was used to sterilize the cryovial and the vial was allowed to air dry before opening. Cells were added dropwise, to reduce osmotic shock, into a 15ml falcon containing 10ml of warm UM. Cells were briefly centrifuged, resuspend in 2ml of warm UM and added gently to prepared plate (MEFs plated on Matrigel® and UM). Around 3-4 days before thawing the cells, 6-well plate was coated with Matrigel®. Two days later 250.000 inactivated MEFs per well of 6-well plate were seeded and grown till attached (next day morning). Then MEF media was replaced with UM supplemented with 4ng/ml of FGF2. Usually cells from one cryovial are plated into one well of 6-well plate. Nevertheless it may take even 2 weeks before cells are ready to be expanded.

Human ES cells culture methods and conditions were adapted from Xu et al. (2001).

2.3 Defined Media to growth human ES cells

To perform initial DE differentiation experiments, hESCs after splitting were cultured for 2-3 days in CM and then switched to defined media (N2B27)(Appendix A.8). Cells were adapted to defined media and kept under these conditions till splitting and further culture. Upon reaching 70-80% of confluency, Activin A (100ng/ml, Peprotech) treatment was applied.

2.4 Human ES cells differentiation

Human ES cells can be differentiated through Embryoid Bodies (EBs) formation or directly on culture plate without additional manipulation.

2.4.1 Formation of Embryoid Bodies (EBs)

Human ES cells can be differentiated *in vitro* into three germ layers (ectoderm, endoderm, and mesoderm) by culturing them in suspension. Under this culture condition cells form embryoid bodies (EBs).

Confluent hESCs colonies were cut manually into pieces of uniform size with a BD Microlance^{TM} 3 injection needle (Becton Dickinson, Madrid, Spain), washed with PBS, incubated with dispase, washed thrice with UM, scraped with a cell scraper, collected and centrifuged. Cells clumps were transferred to a 60mm ultra low attachment culture dish (Corning) containing differentiation medium (UM without FGF2). After overnight culture in suspension, human ES cells formed floating spheres called embryoid bodies (EBs). After 2-3 days EBs were transferred into a 15ml tube and aggregates allowed to settle for 5min. in a water bath. The supernatant was aspirated and media was replaced with fresh differentiation medium. Afterwards cells were transferred to low attachment plates for further culture. Media was changed every 3 to 4 days and the EBs were maintained in suspension for 7 and 14 days. Then they were transferred to 12-well culture plates (coated with 0.2% gelatin) to further induce differentiation. To demonstrate their ability to differentiate into all representatives of the three germ layers, they were then fixed with 4% PFA after 7 and 14 days. The immunocytochemistry was performed using antibodies characteristic for cells from the three germ layers. List of used primary and secondary antibodies is in Table 2.1.

2.4.2 Human ES cells differentiation into hepatocyte-like cells

Hay *et al.* differentiation protocol:

Human ES cells H1 and H9 were cultured and propagated on Matrigel®-coated plates in CM supplemented with FGF2. The differentiation was initiated when cells reached 60-70% of confluence. First differentiation into definitive endoderm (DE) was induced by replacing CM with medium “A” (RPMI1640 with 1xB27, Invitrogen), 1mM sodium butyrate (NaB)(Sigma) and 100ng/ml Activin A (PeproTech). After 48 hours,

Table 2.1: Primary and secondary antibodies used

WESTERN BLOT				IMMUNOFLUORESCENCE			
	type	source	dilution		type	source	dilution
primary antibodies							
GAPDH	mouse mono	Ambion	1:5000	OCT4	goat poly	Santa Cruz	1:100
OCT4	goat poly	Santa Cruz	1:5000	SOX2	goat poly	Santa Cruz	1:100
NANOG	goat poly	R&D	1:250	NANOG	goat poly	R&D	1:100
SOX2	goat poly	Santa Cruz	1:5000	TRA-1-81	mouse mono	Chemicon	1:50
ALB	mouse mono	SIGMA	1:2000	TRA-1-60	mouse mono	Chemicon	1:50
AFP	mouse mono	SIGMA	1:200	SSEA-4	mouse mono	Chemicon	1:50
FOXA2 (HNF3β)	goat poly	R&D	1:200	AFP	mouse mono	SIGMA	1:250
HNF4α	rabbit poly	Santa Cruz	1:200	FOXA2 (HNF3β)	goat poly	R&D	1:20
SOX17	goat poly	R&D	1:1000	SOX17	goat poly	R&D	1:50
				T	goat poly	R&D	1:50
				β-Tubulin III (TuJ1)	mouse mono	SIGMA	1:1000
				Nestin	mouse mono	Santa Cruz	1:200
				Smooth Muscle Actin	mouse mono	Dako Cytomation	1:50
				ALB	mouse mono	SIGMA	1:500
				CK18	mouse mono	Invitrogen	1:50
				HNF4α	rabbit poly	Santa Cruz	1:100
				PAX6	rabbit poly	Covance	1:300
				CDX2	rabbit poly	Chemicon	1:50
				A1AT	mouse mono	GeneTex	1:50
secondary antibodies							
anti-mouse HRP	anti-mouse	GE Healthcare	1:5000	Alexa Fluor 488	goat anti-mouse	Invitrogen	1:800
anti-goat HRP	rabbit anti-goat	Calbiochem	1:10000	Alexa Fluor 594	chicken anti-goat	Invitrogen	1:150
anti-rabbit HRP	goat anti-rabbit	Calbiochem	1:1000	Alexa Fluor 546	goat anti-rabbit	Invitrogen	1:500

mono - monoclonal antibody, poly - polyclonal antibody

that medium was changed to medium "B", which was the medium "A" with reduced concentration of sodium butyrate to 0.5mM. Cells were cultured for a further 72 hours under these conditions. Afterwards the cells were split 1:2 onto new Matrigel®-coated plates and cultured for 7 days in UM with 1% of DMSO (Sigma). Finally, the cells were cultured in a maturation medium (CL15 medium: L15 medium supplemented with 8.3% fetal bovine serum, 8.3% tryptose phosphate broth, 10μM hydrocortisone 21-hemisuccinate, 1μM insulin; all from Sigma, and 2mM glutamine) containing 10ng/ml hepatocyte growth factor (HGF, Peprotech) and 20ng/ml oncostatin M (OSM) (R&D Systems Inc.). The medium was changed each day during the differentiation process. Protein and RNA samples were extracted after each step of the differentiation protocol in order to examine the activation of hepatic-associated genes (Hay et al., 2008b).

Agarwal *et al.* differentiation protocol:

The differentiation protocol is basically divided into two parts: differentiation of hESCs into definitive endoderm (DE) and further differentiation of definitive edoderm to hepatocyte-like cells. When hESCs reached 70-80% of confluence they were washed with PBS and CM was replaced with RPMI medium (Mediatech supplemented with glutamine, penicillin/streptomycin and 0.5% of fetal bovine serum (FBS, Biochrom) and 100ng/ml Activin A (Peprotech). Three days later the medium was refreshed using the same RPMI-based medium with 100ng/ml Activin A but FBS was replaced with 2% of Knockout^{TM} Serum Replacement. Cells were maintained under these conditions for another 2 days. Cells differentiated into definitive endoderm were passaged with 0.05% trypsin-EDTA (Gibco, Invitrogen) and plated at a ratio of 1:1 on collagen I (Inamed, Fremont, CA)-coated plates (5μg/cm^2). At this stage of the differentiation protocol, the cells were cultured in RPMI medium supplemented with glutamine, penicillin/streptomycin. Additionally, media was supplemented with 2% of Knockout^{TM} Serum Replacement, 10ng/ml FGF-4 (R&D Systems Inc.) and 10ng/ml HGF (Peprotech). Three days later the cells were switched to minimal MDBK-MM medium (Sigma) supplemented with glutamine, penicillin/streptomycin and containing 0.5mg/ml bovine serum albumin (BSA, Sigma), 10ng/ml FGF-4, and 10ng/ml HGF. After another 3 days, the cells were switched to hepatocyte culture medium (HCM, Lonza) supplemented with SingleQuots (Lonza) and containing 10ng/ml FGF-4, 10ng/ml HGF, 10ng/ml oncostatin M (R&D Systems) and 10^{-7}M dexamethasone (Sigma). In this media differentiation was continued for another 9 days. During the entire protocol the medium was replaced every 2 days. Protein and RNA samples were extracted after each step of the differentiation protocol to examined activation of hepatic-associated genes (Agarwal et al., 2008).

Cai *et al.* differentiation protocol:

In this three-stage differentiation protocol human ES cells were cultured in 1640 medium (Hyclone, Logan, UT) supplemented with 0.5mg/ml albumin fraction V (Sigma) and 100ng/ml Activin A for 1 day. On the following 2 days, 0.1% insulin-transferrin-selenium (ITS, Sigma) was added to the medium. After 3 days of Activin A treatment,

the differentiated cells were cultured in hepatocyte culture medium (HCM, Lonza) containing 30ng/ml FGF4 and 20ng/ml BMP2 for 5 days. Then the differentiated cells were further matured in HCM containing 20ng/ml HGF for 5 days, and 10ng/ml OSM plus 10^{-7}M dexamethasone (Sigma) (Cai et al., 2007).

2.5 Culture of hepatocellular carcinoma (HepG2) and human foreskin fibroblasts (HFF) cells

Cells were thawed by gently agitating the vial in a 37°C water bath. Then cryovials were sprayed with 70% ethanol to sterilized and under sterile conditions cells were transferred into a 15ml falcon containing 10ml of warm complete growth medium (Appendix A.7). Afterwards cells were pelleted by brief centrifugation and resuspended with 10ml fresh growth medium (warm) and plated on T75 flask with 5ml of medium. Both cell lines were incubated at 37°C in a 5% CO_2 in air atmosphere. Media was changed twice a week and cells were split 1:4 to 1:6 ratio using 0.05% trypsin-EDTA solution (Gibco, Invitrogen). Cells were frozen in complete growth medium supplemented with 5% DMSO.

These cell lines were used as a positive (HepG2) and negative (HFF) controls for hepatocytes functional assays and immunocytochemistry.

2.6 Protein isolation

Cells grown in a monolayer were kept on ice and rinsed with PBS (Gibco/Invitrogen). Lysis buffer (25% glycerol, 0.42M NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 20mM HEPES) supplemented with protease inhibitor (Protease Inhibitor Cocktail Tablets, Roche) was added. Cells were detached from the culture plate by using a cell scraper. Usually 200μl (6-well format) or 100μl (12-well format) of lysis buffer with addition of 2 or 1μl protease inhibitor respectively was used. Cells were then disrupted using 1ml syringe and a BD Microlance^{TM} 3 injection needle (Becton Dickinson, Madrid, Spain). Afterwards samples were centrifuged with 10.000g at 4°C for 10min. The supernatant was collected and stored at -80°C.

2.6.1 Protein quantification (Bradford Assay)

Protein samples were quantified using the Bradford assay. Standard protein solutions (Bovine gamma globulin in concentration: 0.125mg/ml, 0.25mg/ml, 0.5mg/ml, 0.75mg/ml, 1.5mg/ml, 2mg/ml; BioRad, Hercules, CA, USA) were used to prepared standard curve. Bradford solution (Bio-Rad Protein Assay; Bio-Rad, Hercules, CA, USA) was diluted 1:5 with 1x PBS and 980μl of the diluted Bradford solution were added to 20μl of each standard and sample. Then samples were mixed by inversion of the 1ml cuvettes (Sarstedt, Nümbrecht, Germany) and incubated for 5min. at RT.

Afterwards they were measured (595nm) using the Ultrospec 3100 pro (GE Healthcare, Munich, Germany) and the provided Bradford Assay programme.

2.6.2 SDS-PAGE gel electrophoresis

Protein gels were prepared in Mini PROTEAN® 3 System protein chambers (Bio-Rad, Hercules, CA, USA). The resolving gel (10%) was prepared by mixing 8.2ml of dH_2O, 5.0ml of 1.5M Tris-Cl pH 8.8 (resolving buffer) (Appendix A.9), 200μl of 10% SDS, 4.6ml of 40% acrylamid (Rotiphorese® Gel 40; Carl Roth, Karlsruhe, Germany), 100μl APS (Ammoniumperoxodisulfate; Carl Roth) and 10μl TEMED (Carl Roth). The edge of the gel was covered with isopropanol to get an even surface. After polymerization the isopropanol was discarded and a 5% stacking gel was poured. Stacking gel consists of: 5.7ml of dH_2O, 2.5 ml of 0.5M Tris-Cl pH 6.8 (stacking buffer) (Appendix A.9), 100μl of 10% SDS, 1.7ml of 40% acrylamid, 100μl APS and 20μl TEMED.

20-30μg of protein was mixed with 3x loading buffer and water (to adjust equal volume) (Appendix A.9). The samples and 7μl of prestained protein marker (New England Biolabs, Beverly, MA, USA) were heated to 95°C for 5min. and afterwards cooled on ice for 1min. before loading. The gel was run in 1x running buffer (appendix A.9) at 110V till the loading buffer reached the end of the stacking gel and then with 130V. Gels were then used to perform Western blotting.

2.6.3 Western blotting

Proteins were transferred from the gel to an Amersham Hybond^{TM} ECL^{TM} nitrocellulose membrane (GE Healthcare). Afterwards "sandwich" consisted of two filter papers, membrane and gel was assembled inside a transfer chamber (Bio-Rad). The blot was run in cold 1x transfer buffer (Appendix A.10) with a 130V for 90min. Cooling boxes were applied additionally. After blotting, the transfer quality was checked by Ponceau S Solution (Sigma) staining. The membrane was rinsed with dH_2O and then blocked with 3% low-fat milk powder in TBST (blocking solution) (Appendix A.10) by shaking for 5min. at RT and then overnight at 4°C.

Membrane was incubated by shaking 1hr at RT, with primary antibody dissolved in TBST with 1% BSA. Afterwards the membrane was extensively washed by shaking 3 times for 10min. in TBST. Then the secondary antibody dissolved in 3% low-fat milk powder in TBST were applied by shaking for 1hr at RT. Afterwards the membrane was again washed by shaking 3 times for 10min. in TBST.

One volume of detection reagent 1 and one volume of detection reagent 2 (GE Healthcare, Amersham ECL Western Blotting Systems) were mixed and kept in the dark for 5 minutes. The membrane was then covered with detection solution and incubated for 1min. Then it was placed in a Hypercassette^{TM} (Amersham). Chemiluminescence was detected using BioMAX XAR film (Kodak, Stuttgart, Germany) and the Curix 60 film developing machine (AGFA, Cologne, Germany). Primary and secondary antibodies

used are listed in Table 2.1.

2.7 RNA isolation and reverse transcription-polymerase chain reaction

Total RNA was isolated using the Rneasy® Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. DNase I (RNase-free DNase set, Qiagen, Hilden, Germany) treatment was performed on column. Reverse transcription was carried out as follows: 500ng of RNA and oligo(dT) (1μg/μl; Invitek, Berlin, Germany) were incubated for 3min in 70°C and cooled on ice. Then supplemented with a master mix consisting of following components: 5.0μl of 5x reaction buffer (Promega), 0.5μl of (25mM) dNTP, 0.1μl of MMLV (Moloney Murine Leukemia Virus) reverse transcriptase (200U/μl; USB) and 9.4μl of dH_2O. After 1 hour incubation at 42°C the reaction was stopped at 65°C for 10min.

2.7.1 Real-time polymerase chain reaction (Real-Time PCR)

Real-time PCR was carried out on the Applied Biosystems 7900 instrument, in 96-Well Optical Reaction Plates (Applied Biosystems, Foster City, CA, United States). The following program was applied: stage 1: 50°C for 2min., stage 2: 95°C for 10min., stage 3: 95°C for 15s and 60°C for 1min., for 40 cycles and, stage 4: 95°C for 15s, 60°C for 15s and 95°C for 15s. Additional dissociation curves of the products were created. The final reaction volume of 20μl consisted of 10μl of SYBR® Green PCR Master Mix (Applied Biosystems), 2.5μM of each primer (3μl), and 7μl of cDNA (1:8 dilution). Each gene was analysed in triplicate. Two biological replicates were used for both H1 and H9-derived hepatocytes. Relative mRNA levels were calculated using the comparative Ct method (ABI instruction manual) and presented as a percentage of the biological controls (undifferentiated hESCs). mRNA levels of β-*ACTIN* and *GAPDH* genes were used as controls for normalisation. Primers are listed in Table 2.2.

2.8 Immunocytochemistry

Cells were washed with PBST (PBS with 0.05% Tween 20) and fixed with 4% paraformaldehyde (PFA) in PBS for 10min. at RT and washed twice with PBST. After washing, cells were permeabilized with 1% Triton X-100 in PBS for 10min. at RT. Then washed twice with PBST and blocked in PBST containing 1% BSA (Bovine Serum Albumin, Fraction V, 99% purity, Sigma) and 5% normal chicken serum (Vector Laboratories Inc. Burlingame) for 45min. at RT with gentle rocking. Afterwards cells were incubated with primary antibodies for 1hr (diluted to working concentration in PBST containing 1% BSA and 1% normal chicken serum). Then cells were washed three times (5min. each) in PBST with 0.1% BSA and incubated with secondary antibodies (diluted in PBST with 1% BSA) for 1h in the dark. Afterwards two washes (PBST with 0.1%

Table 2.2: RT-PCR primer sequences

gene	primer sequence (5'-3')	size (bp)
OCT4	FOR:GTGGAGGAAGCTGACAACAA	119
	REV:ATTCTCCAGGTTGCCTCTCA	
GAPDH	FOR:CTGGTAAAGTGGATATTGTTGCCAT	81
	REV:TGGAATCATATTGGAACATGTAAACC	
ACTB	FOR:TCAAGATCATTGCTCCTCCTGAG	87
	REV:ACATCTGCTGGAAGGTGGACA	
OTX2	FOR:GCCAATCCTTGGTTGAATCTTAGG	120
	REV:CAATCAGTCACACAATTCACACAGC	
PAX6	FOR:CCAGGGCAATCGGTGGTAGT	84
	REV:ACGGGCACTCCCGCTTATAC	
NANOG	FOR:CCTGTGATTTGTGGGCCTG	78
	REV:GACAGTCTCCGTGTGAGGCAT	
HNF4A	FOR:GTGCGGAAGAACCACATGTACTC	102
	REV:GAAGCATTTCTTGAGCCTGCAGTA	
SOX7	FOR:TGCCCACTTCATGCAACTCC	110
	REV:AGGTACCCTGGGTCTTTGGTCA	
FOXA2	FOR:TTCAGGCCCGGCTAACTCTG	97
	REV:CCTTGCGTCTCTGCAACACC	
FGF4	FOR:CCCTTCTTCACCGATGAGTGC	109
	REV:CATTCTTGCTCAGGGCGATG	
GATA6	FOR:TGTGCGTTCATGGAGAAGATCA	83
	REV:TTTGATAAGAGACCTCATGAACCGACT	
SOX2	FOR:TGGCGAACCATCTCTGTGGT	111
	REV:CCAACGGTGTCAACCTGCAT	
SOX17	FOR:TTCGTGTGCAAGCCTGAGATG	99
	REV:GTCGGACACCACCGAGGAA	
AFP	FOR:AGCAGCTTGGTGGTGGATGA	88
	REV:CCTGAGCTTGGCACAGATCCT	
GATA4	FOR:AATGACTCCAGAACAACAACTGGG	111
	REV:CTCCCTCCAGTCCCATCAGC	
ALB	FOR:AGCTGTTATGGATGATTTCGCAG	77
	REV:CCTCGGCAAAGCAGGTCTC	
CYP3A4	FOR:GTGACTTTGCCCATTGTTTAGAAAG	79
	REV:CAGGCGTGAGCCACTGTG	
CYP3A7	FOR:GATTCTGTACGTGCATTGTGCTC	77
	REV:ATTTGGTCATCTCCTCTATATTACCAAGT	
TAT	FOR:CCACACCCACACTCAGATCCT	76
	REV:ATTAGTGAGTCACTCTAGCAGCGC	
TDO2	FOR:GGTGAAAGACGGCTGTCATACAG	77
	REV:TGGAACCTAGGCTCTTCCCTG	
GSC	FOR:ACCTCCGCGAGGAGAAAGTG	101
	REV:CTTCTCCGCGTTCTCCGACT	
A1AT	FOR:GGTCACAGAGGAGGCACCC	76
	REV:AGTCCCTTTCTCGTCGATGGT	

BSA) were applied. Third wash contains DAPI solution (Molecular Probes, Invitrogen). Fluorescence was examined under the confocal microscope (LSM510 Meta, Zeiss, Germany).

The primary antibodies used were: anti-Sox17 (R&D Systems), anti-FoxA2 (R&D Systems), anti-Oct4 (SantaCruz Biotechnology), anti-A1AT (GeneTex), anti-AFP (Sigma), anti-ALB (Sigma), anti-Brachyury (R&D System), anti-CK18 (Invitrogen), anti-HNF4α (SantaCruz Biotechnology), anti-human smooth muscle actin (Dako Cytomation), anti-PAX6 (Covance), anti-β-Tubulin isotype III (TuJ1, Sigma), anti-CDX2 (Chemicon), anti-Nestin (Chemicon). ES Cell Characterization Kit (Chemicon International): anti-SSEA-4, anti-TRA-1-60, anti-TRA-1-81. Secondary antibodies: Alexa Fluor 594 chicken anti-goat IgG (H+L), Alexa Fluor 546 goat anti-rabbit IgG (H+L), Alexa Fluor 448 goat anti-mouse IgG (H+L)(Molecular Probes, Invitrogen)(Table 2.1).

2.9 Genomic DNA isolation

Genomic DNA was isolated from human ES cells using FlexiGene DNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

2.9.1 RNA and genomic DNA quantification

The quantity and quality of RNA and genomic DNA was examined using the NanoDrop (NanoDrop Technologies, Wilmington, DE, USA). 1.2μl of sample was applied to the NanoDrop and measured.

2.9.2 Agarose gel electrophoresis

RNA and DNA quality control was determined using agarose gel electrophoresis and ethidium bromide staining. 0.8-1.5% gels were obtained by mixing agarose (Life Technologies, Paisley, Scotland) and 1x TAE buffer. Ethidium bromide (10mg/ml; Invitrogen) was added directly to the gel before solidifying and mixed. The length of the amplicons was specified according to the GeneRuler^{TM} 1kb DNA ladder (Fermentas, St. LeonRot, Germany). Samples were loaded together with 6x loading buffer (Fermentas) and gels were run in an electrophoresis chamber at 60V. Nucleic acids were visualized with UV light using the AlphaImager^{TM} (Alpha Innotech, San Leandro, CA, USA).

2.10 Illumina 8-Sample BeadChip hybridisation (Gene expression profiling)

Biotin-labelled cRNA was produced by means of a linear amplification kit (Ambion, Austin, TX, USA) using 500ng of quality-checked total RNA as input. Chip hybridisations, washing, Cy3-streptavidin staining, and scanning were performed on an Illumina BeadStation 500 platform (Illumina, San Diego, CA, USA) using reagents and following

protocols supplied by the manufacturer. cRNA samples were hybridised on Illumina human-8 BeadChips. We hybridised the following samples in biological triplicates:

- Hay *et al.* protocol: H1 cell line passage 53 (control), ActA-treated cells, NaB-treated cells, ActA and NaB-treated cells, further differentiated cells (after ActA+NaB and DMSO treatment), hepatocyte-like cells (after 19 days of differentiation),
- Agarwal *et al.* protocol: H1 cell line passage 60 (control), ActA-treated cells, further differentiated cells (after 11 days of differentiation (T11)), hepatocyte-like cells (after 18 days of differentiation (T18)).

In addition, two technical replicates of RNA from fetal human liver (Stratagene) were hybridized.

All basic expression data analysis was carried out using the manufacturer's software BeadStudio 3.4 (Illumina). Raw data were background-subtracted and normalised using the "rank invariant" algorithm, by which negative intensity values may arise. Normalized data were then filtered for significant expression on the basis of negative control beads. Selection for differentially expressed genes was performed on the basis of arbitrary thresholds for fold changes plus statistical significance according to an Illumina custom model (Kuhn et al., 2004).

2.11 Data analysis

For further analysis, the raw data obtained using the manufacturer's software BeadStudio Gene Expression Module v3.4 was imported into the Bioconductor environment (Gentleman et al., 2004) and quantile normalized using the bioconductor package beadarray (Dunning et al., 2007).

In order to test for global gene expression similarities within biological replicates and between different treatments, pair wise Pearson correlation coefficients were calculated for all samples. Variance and cluster analyses were performed using the R environment. In order to identify clusters of genes where all gene members show similar gene expression changes during the several steps of the differentiation protocol, we performed a K-means clustering based on groups of normalized gene level data (Team, 2009). The number of clusters was set to 100.

Filtering and compilations of data were carried out using MS Excel. Differential gene expression and ANOVA analyses were performed using the TIGR-MEV ((Saeed et al., 2003) multiple experiment viewer from the Institute for Genomic Research, versatile microarray data analysis tool, incorporating sophisticated algorithms for clustering, visualization, classification, statistical analysis, and biological theme discovery). Differential gene expression was calculated between all groups by the ANOVA analysis, p-values were calculated based on F-distribution, with a critical p-value of 0.05. For the ANOVA analysis, we created two separated contrast matrices for the Hay *et al.* samples and Agarwal *et al.* samples, respectively. The fetal liver samples were added to both

groups. Based on these results, for each gene we obtained p-values that indicate the strength of gene expression variation throughout the samples of the tested group.

Differentially expressed genes were further filtered according to Gene Ontology terms or mapped to KEGG pathways using DAVID 2008 (http://david.abcc.ncifcrf.gov). For analysis, we used Illumina Gene IDs represented by the corresponding chip oligonucleotides as input.

2.12 Functional Assays for hepatocyte-like cells derived from human ES cells

Periodic Acid-Schiff (PAS) Staining System (Sigma) was applied to identify glycogen storage. The purpose of this method is to identify carbohydrate macromolecules through chemical reaction. In brief, periodic acid oxidizes the glucose residues and creates aldehydes, which react with Schiff reagent producing purple colour. Cells were fixed with 4% PFA for 15min. at RT, washed with distilled water and incubated with Periodic Acid solution for 5min. at RT. Then washed 3 times with water and incubated for 15min. at RT with Schiff's reagent. Afterwards the cells were washed with distilled water (5min.), stained with Hematoxylin (2min.) and again washed with distilled water.

Indocyanine Green (ICG) is an indicator dye used for assessing liver and cardiac function, which after intravenous injection is bound to plasma protein (primarily albumin). This substance is rapidly taken up by the liver and then secreted unchanged into the bile. Cellular uptake and release of ICG (Cardiogreen, ICG, Sigma) was performed to prove the presence of albumin in hepatocyte-like cells derived from human ES cells. Stock solution (5mg/ml) of reagent was prepared in DMSO (Sigma) and freshly diluted in culture media to 1mg/ml just before application. Cells were then incubated in culture media supplemented with ICG for 30min. at 37°C. Afterwards, the cells were washed with PBS (Gibco, Invitrogen) and uptake of dye was documented. To examine the release of ICG the cells were cultured under normal conditions for 6 hours. Afterwards cellular release was analysed.

Both assays were performed using as positive control (Hepatocellular carcinoma, HepG2, ATCC, HB-8065, LGC Promochem), negative control (human foreskin fibroblasts, ATCC, CRL-2429, LGC Promochem) and hepatocyte-like cells derived from human ES cells lines H1 and H9. The results of both assays were examined under an Olympus CK2 phase contrast microscope and representative morphology was recorded at a magnification of X50 using a Canon 300D digital camera.

3

Results

3.1 Optimization of human ES cells culture conditions

FGF signalling has a central role in sustaining human ES cells pluripotent state (Amit et al., 2000). At lower concentrations of FGFs, both Activin A and TGFβ have clear strong effects on human ES cells (Levenstein et al., 2006; Xu et al., 2005), and based on inhibitor studies, it has been suggested that TGFβ/Activin A signalling is essential for human ES cells self-renewal (Amit et al., 2004). These two pathways (FGF2 and TGFβ/Activin/Nodal branch of TGFβ pathway) are crucial for maintaining human ES cell self-renewal. FGF2 stimulates the secretion of beneficial factors in MEFs and suppresses the release of differentiation-inducing activity. FGF2 stimulation of MEFs leads to a morphological change and changes in genes expression in response to FGF2 (Greber et al., 2007). Among genes up-regulated by FGF2 are *Tgfβ1*, *Inhba*, *Grem1*, and *Bmp4* is down-regulated. TGFβ1 and Activin A prevent differentiation via activation of SMAD 2/3 (Valdimarsdottir and Mummery, 2005; Amit et al., 2004), whereas BMP4 initiates differentiation to trophoblast or primitive endoderm via SMAD 1/5/8 signaling (Xu et al., 2002). Exogenous and autocrine FGF2 signalling is upstream of key TGF/beta ligands that, in a concerted manner, maintain *OCT4*, *NANOG* and *SOX2* expression, which in turn, activate endogenous expression of *FGF2* (Greber et al., 2007).

Applying this knowledge initially human ES cells were routinely cultured on mouse fibroblast feeder layers. Subsequently both cell lines, H1 and H9 have been adapted to feeder-free conditions.

3.1.1 Feeder-dependent conditions

Human ES cells were historically derived in serum containing media and on feeder layers of inactivated mouse embryonic fibroblasts that supported them, allowing to maintain their undifferentiated state (Thomson et al., 1998). By substituting fetal calf serum with a combination of the proprietary Knockout^{TM} Serum Replacement (SR) and fibroblast growth factor 2, Amit *et al.* demonstrated enhanced cloning efficiencies and reduced degree of spontaneous differentiation (Amit et al., 2004).

Followed this protocol after thawing the vial of human ES cells, they were plated on inactivated mouse embryonic fibroblast feeder layers and undifferentiated H1 and H9

cell lines have been successfully expanded in this system (Figure 3.1).

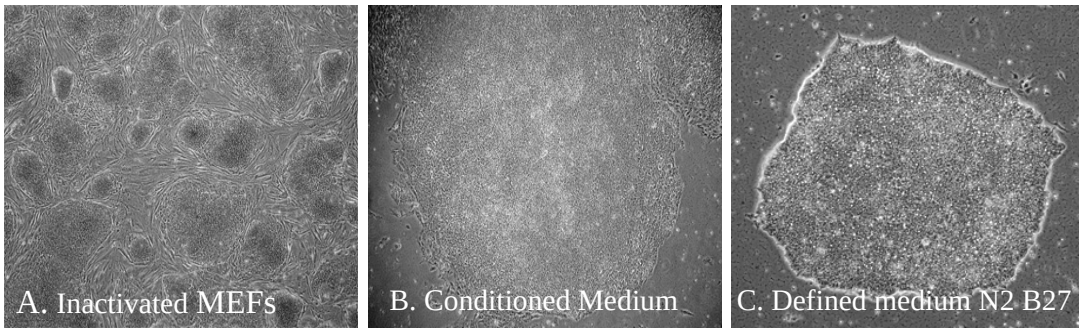


Figure 3.1: The typical morphology of undifferentiated human ES cells cultured in the presence of (A) feeder cells (MEFs), (B) conditioned medium and (C) defined medium.

3.1.2 Feeder-free culture

After expansion and successful maintaining both cell lines in the undifferentiated state (Figure 3.1A) and subsequent frozen stocks preparation cells were adapted to feeder-free culture (Figure 3.1B). Xu et al. (2001) developed a feeder-free human ES cell culture system that involves the conditioning of Amit's medium by MEFs and growing cells on Matrigel®.

To maintain H1 and H9 cell lines in the undifferentiated state for routine cell culture and to perform differentiation experiments, cell were cultured in feeder-free conditions (see Materials and Methods). Cells grown in CM displayed the standard morphology of pluripotent human ES cells (small cell sizes, low nuclear/cytoplasmic ratio, and defined colony borders) (Figure 3.1B).

3.1.2.1 Activin A levels in Conditioned Media

To measure Activin A concentrations in CM, enzyme-linked immunosorbent assay (ELISA) was performed. Since one function of the conditioning process is ActA secretion into the medium by MEFs, ActA is almost not detected in UM (Figure 3.2).

3.1.3 Defined Media based culture conditions

Since Knockout^{TM} Serum Replacement (SR) is complex, poorly defined mixture of proteins and other molecules that influence self-renewal and differentiation. Successful attempts has been made to culture cells in defined media (Lu et al., 2006; Yao et al., 2006). FGF2 is less stable in serum-free medium compared to medium conditioned by MEFs, which in parts explains the use of the high concentrations of FGF2 in N2B27 media (20ng/ml). It is known that heparan sulphate proteoglycans alter the stability and activity of FGFs by forming high affinity binding complexes, but at high concentrations,

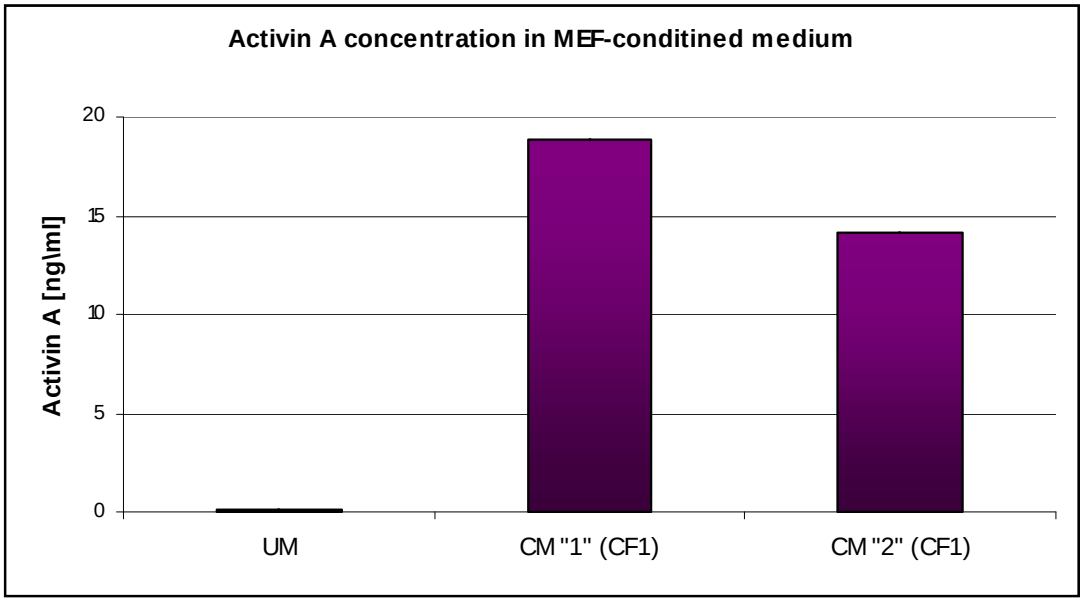


Figure 3.2: Enzyme-linked immunosorbent assay (ELISA)-based measurements of Activin A concentration in conditioned media (CM) prepared with mouse embryonic fibroblasts from CF1 strain. CM was collected for 6 days and then pooled. CM“1" and CM“2" refer to different batch of media.

FGF proteins can signal through direct contact with FGF receptors, and the requirement for these complexes can be avoided (Ludwig et al., 2006). Based on the protocol of Yao *et al.* both cell lines have been cultured in N2B27 media (Yao et al., 2006). Cells were cultured in CM and 3 days before splitting they were switched to N2B27 media. After splitting they were still adapted to defined media and after 3-4 days differentiation experiments were started. Cells cultured in N2B27 are more condensed with a high nucleus/cytoplasm ratio (Figure 3.1C).

3.2 Characterization of human ES cell lines (stem cell markers, *in vitro* differentiation)

The undifferentiated state of human ES cells was demonstrated by the expression of the stem cell markers. To examine the pluripotency of both cell lines (H1 and H9) they were analyzed for expression of transcription factors (OCT4, SOX2, NANOG), and cell surface markers (SSEA4, TRA-1-81, TRA-1-60) by immunocytochemistry. In both H1 and H9 cell lines, more than 95% of cells grown in CM stained positive for each of these stem cell markers (Figure 3.3). In some cases, immunocytochemistry staining with stem cell marker revealed that the hESCs colonies were surrounded by some differentiated cells.

To confirm that hESCs routinely cultured in CM maintain their differentiation potential *in vitro*, embryoid bodies (EBs) formation and differentiation assays were per-

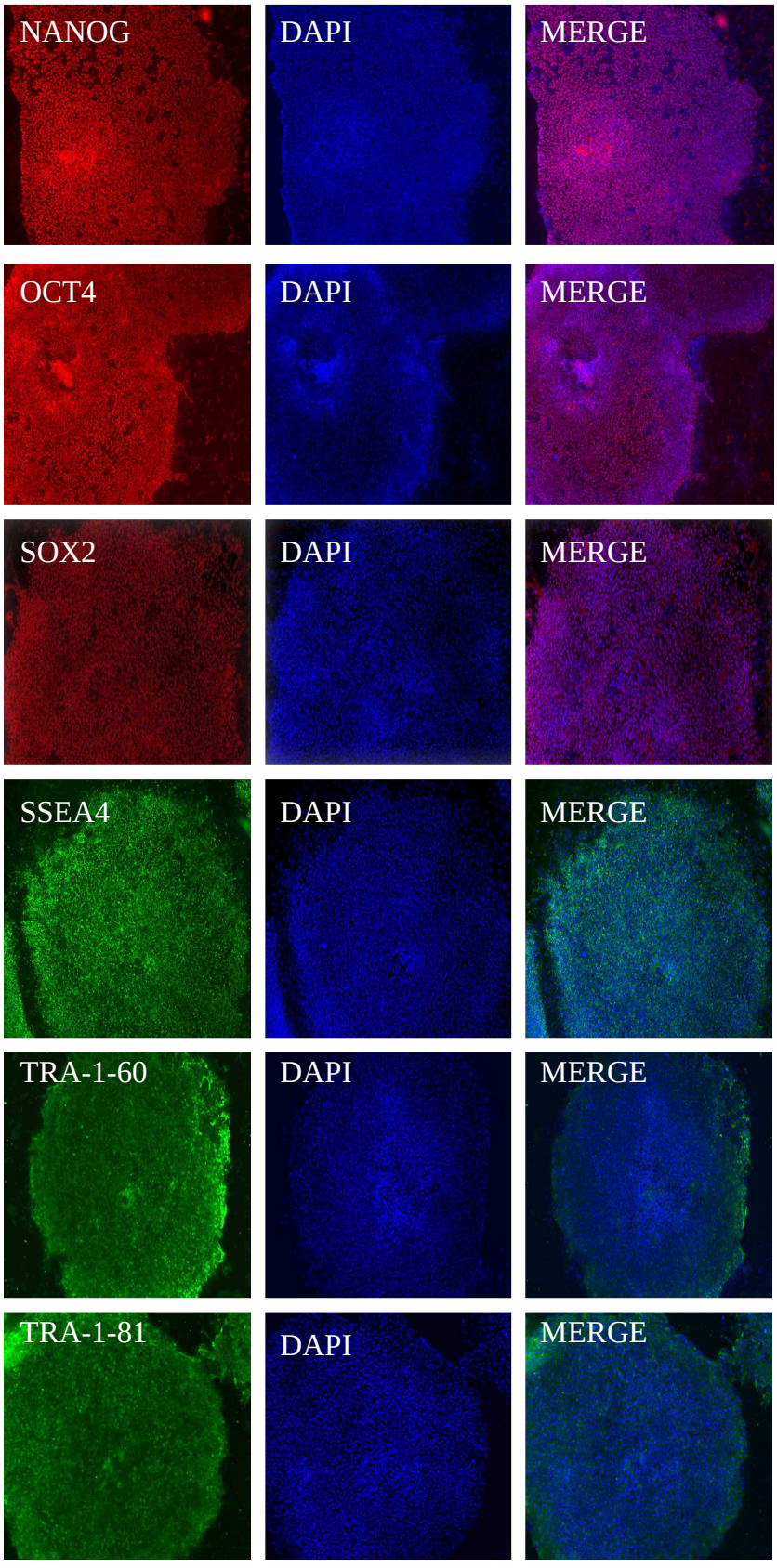


Figure 3.3: Expression of stem cell markers in human ES cells cultured in conditioned medium (CM). Immunofluorescence staining with antibodies to stem cell markers: OCT4, SOX2, NANOG, SSEA-4, TRA-1-60 and TRA-1-81. The nuclei were stained with DAPI.

formed with H1 and H9 cells. Both cell lines formed embryoid bodies in suspension with high efficiency (Figure 3.4A). Subsequently, the embryoid bodies continue to differentiate on gelatine-coated plates for at least 7 days. Expression of endoderm-, mesoderm-, and ectoderm-specific markers in embryoid bodies-derived cells were evaluated by immunocytochemistry. To detect endoderm differentiation AFP, SOX17, FOXA2 antibodies were used, to detect ectoderm differentiation nestin and β-tubulin III (TuJ1) antibodies were used, to detect mesoderm differentiation smooth muscle actin (SMA) antibodies were used (antibodies description in Table 2.1). For both cell lines the embryoid body-derived cells contained cells from the three different lineages (Figure 3.4B). Additionally expression of primitive streak marker brachyury (T) was detected (Figure 3.4).

3.3 Derivation of endoderm lineage from human ES cells

Based on the requirement for high levels of Nodal in the induction of DE during gastrulation, DE was originally derived by D'Amour *et al.* by culturing human ES cells in low serum (0.5% FBS) and high ActA (100ng/ml) in RPMI medium for 5 days. Activin A is used since it is more readily available TGFβ family member that binds Nodal receptor and stimulates similar signalling pathways (D'Amour et al., 2005).

Figure 3.5B depicts the morphology of DE cells. Morphologically, the cells regularly changed from typical, defined, tight human ES colonies (Figure 3.1B) into less dense, flatter cells. The majority of the cells seemed to differentiate cooperatively and resulting in a homogeneous culture. This went together with characteristic appearance of SOX17 protein expression analyzed by immunofluorescence (Figure 3.5C,D). No exclusive expression marker specific for DE has been described yet, since genes expressed in DE are expressed in primitive endoderm or mesoderm (Lavon and Benvenisty, 2005; D'Amour et al., 2005). Cells were hence examined for the distinctive pattern of expression of set of markers, including those that are expected to be expressed in DE and those that are not (Figure 3.5A).

After 5 days of ActA treatment most of the cells lost their expression of the pluripotent marker *OCT4* (Pesce and Scholer, 2001) whereas cells gained strong expression of DE transcription factor markers *SOX17* and *FOXA2*. Importantly, the cells did gain just slight expression of the transcription factors *SOX7* (expressed in primitive endoderm but not DE) on mRNA level. This shows that the expression of *SOX17* and *FOXA2* was not a result of differentiation to primitive endoderm. *PAX6* expression is detectable, demonstrating presence of slight ectoderm differentiation. Moreover brachyury (*T*) expression is present, showing that cells went through primitive streak stage of development. *HNF4A* expressed in part of endoderm intended to develop early hepatic-like fate is up-regulated (Figure 3.5A).

RT-PCR results were supported by Western blot analysis for SOX17 and OCT4 (Figure 3.5E). Comparing protein expression levels in human ES cells to those in Act A induced cells demonstrated a clear gain of SOX17 expression. After 5 days of differenti-

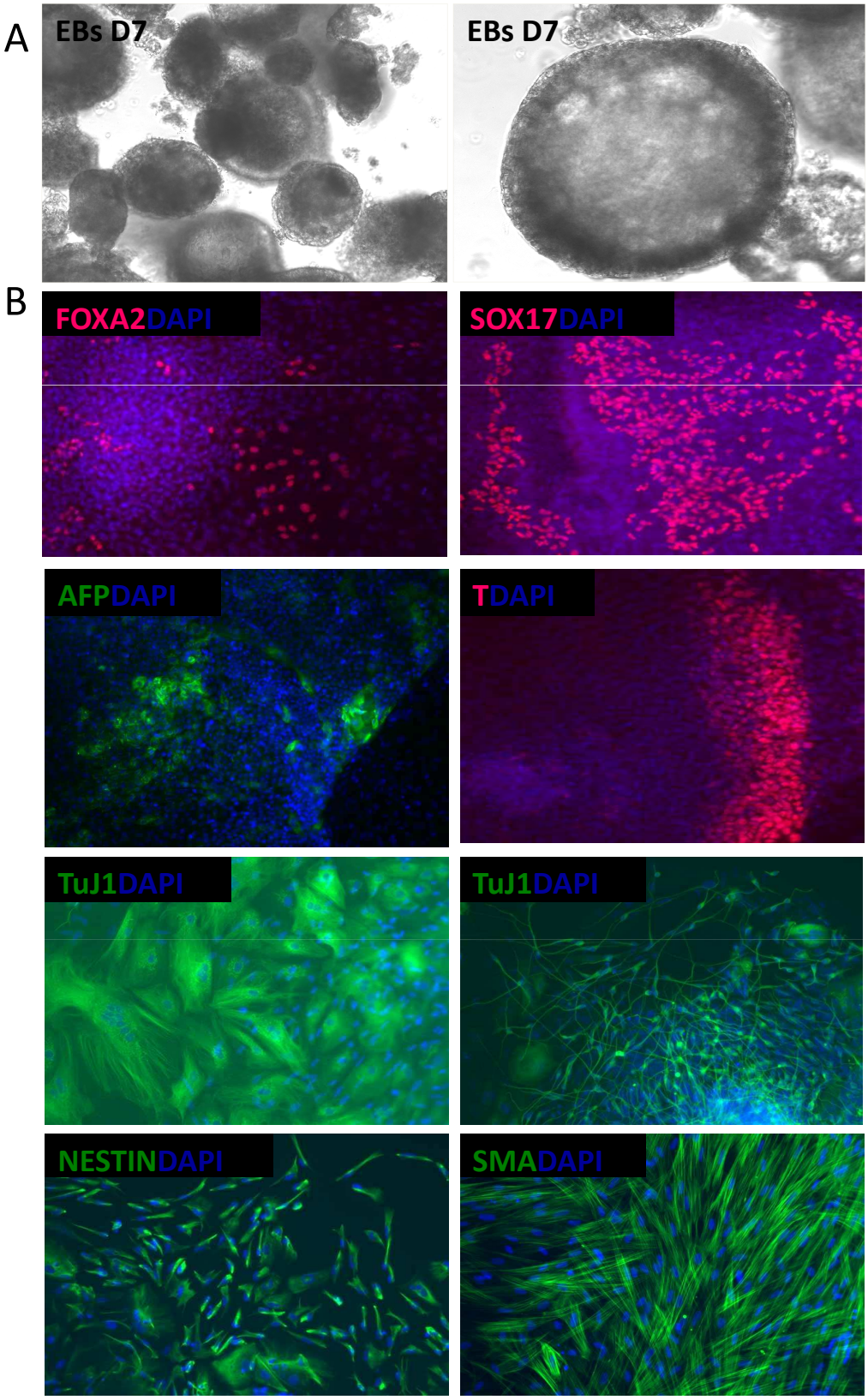


Figure 3.4: Examination of the *in vitro* differentiation potential of human ES cells. Cells were induced to form embryoid bodies (A) and the derived cells were stained with different differentiation markers (B): endoderm (SOX17, AFP, FOXA2), primitive streak (brachyury (T)), mesoderm (SMA) and ectoderm (nestin, β-tubulin (TUJ1)). The nuclei were stained with DAPI.

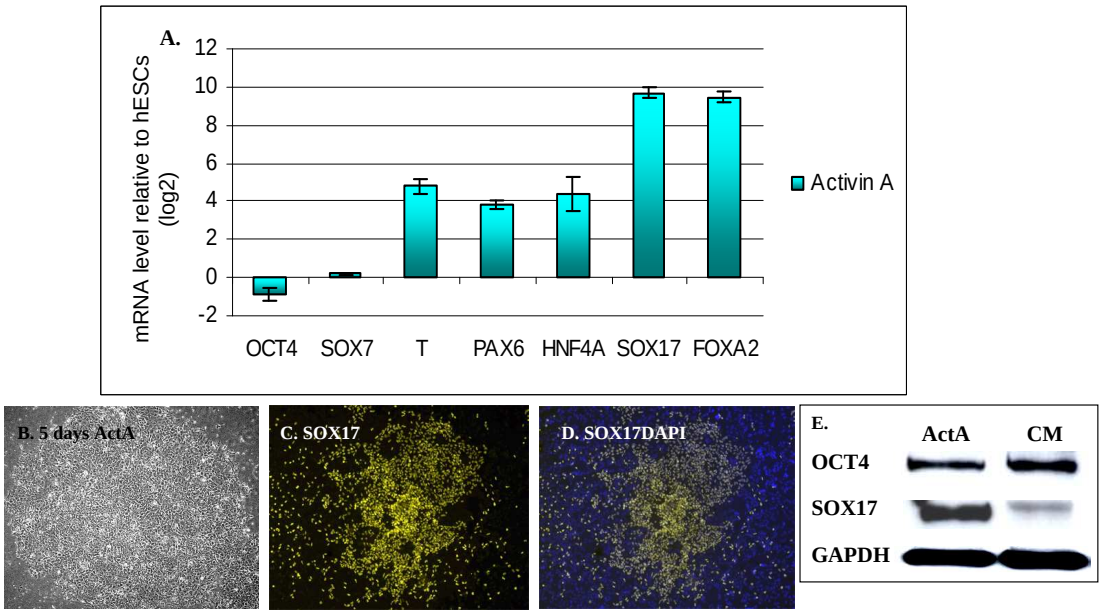


Figure 3.5: Derivation of definitive endoderm from human ES cells. (A) Effect of Activin A on gene expression in human ES cells (H9). (B) Phase contrast image of human ES cells 5 days after Activin A treatment. (C,D) Immunofluorescence labeling of differentiating cultures demonstrating SOX17 expression. The nuclei were stained with DAPI. (E) The detection of protein levels by Western blot.

ation, OCT4 positive cells can be found amongst SOX17 positive cells and slight down regulation of OCT4 in ActA treated cells was evident. These results clearly confirmed induction of DE from human ES cells under these conditions.

3.4 Multi-stage human ES cells hepatic differentiation

I have adopted three available differentiation protocols which involved definitive endoderm (DE) induction step. Cai *et al.* as a first used Activin A treatment in human ES cells hepatic differentiation protocol for both H1 and H9 cell lines (Cai et al., 2007). Modification of Activin A treatment by addition of sodium butyrate has been done by Hay *et al.* for H1 and H7 cell lines (Hay et al., 2008b). Subsequently, Agarwal *et al.* for H1 and H9 cell lines applied conventional Activin A treatment (Agarwal et al., 2008). All further steps in these protocols involve sequential treatments of DE with cytokines important in *in vivo* hepatogenesis.

Strategies for in vitro differentiation of human ES cell into hepatic-like cells are listed in Table 1.1.

3.5 Directed differentiation of definitive endoderm to hepatocytes

In Cai et al. (2007) three-stage differentiation protocol (Figure 3.6) first stage involved differentiation into DE with 3 days of ActA treatment. Afterwards hepatic initiation was induced with FGF4 and BMP2 for 5 days in the culture media. *OCT4*, *SOX17*, *FOXA2* and *AFP* gene expression determined by Real-time RT-PCR was used to confirm DE differentiation and hepatic initiation (Figure 3.7). The combination of FGF4 and BMP2 led to slight increase in *AFP* gene expression, indicating that both FGF and BMP are needed for effective hepatic differentiation (Figure 3.7A). After 5 days of FGF4 and BMP2 treatment, the morphology of the cells was cuboidal which is typical for hepatocytes (Figure 3.6).

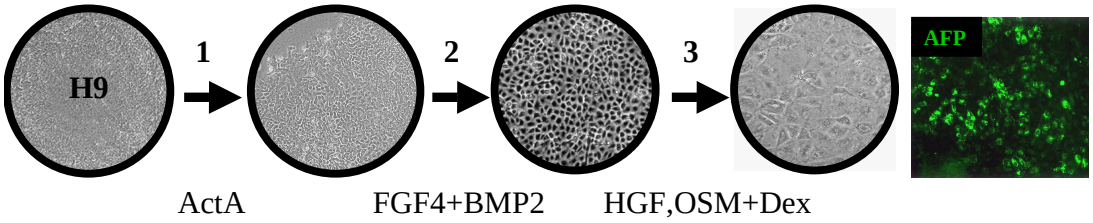


Figure 3.6: Differentiation of human ES cells into hepatocyte-like cells *in vitro*. Schematic display of the differentiation procedure (detailed explanations in text). Phase contrast images of differentiating cells. AFP immunostaining of hepatocyte-like cells after 18 days of differentiation protocol.

Maturation of the cells was induced in the third stage, after 5 days of HGF treatment followed by a further 5 days of OSM/Dex treatment resulting in the cells becoming more flattened (Figure 3.6). In this stage of the differentiation protocol, expression of adult liver cell markers, *ALB*, *A1AT* and fetal liver marker *AFP* was determined by RT-PCR (Figure 3.7B). Expression of hepatic marker genes was compared with fetal liver and showed around half of its expression. The expression of fetal hepatic marker *AFP*, which was earlier demonstrated at the end of the second stage of differentiation, was monitored by immunofluorescence staining. On the basis of the immunofluorescence staining (Figure 3.6) about 10% of the differentiated cells expressed AFP.

SOX17 and OCT4 were determined (after each step of protocol) by Western blot on protein level (Figure 3.8). SOX17 level increased after ActA treatment and maintained during FGF4, BMP2 treatment. SOX17 is not present in human ES cells-derived hepatocyte-like cells (hESCs-DH) and in HepG2 cell line. OCT4 is still present after ActA treatment became undetectable after BMP2 and FGF4 treatment. SOX17 protein was detected in human ES cells. It was demonstrated (Hay et al., 2008a, 2007) that endoderm-associated genes (e.g. *SOX17* and *FOXA2*) are found to be present on protein level to varying levels in undifferentiated human ES cells cultured in CM (Figure 3.8, Figure 3.16).

According to the Hay *et al.* protocol, human ES cells were treated with 100ng/ml

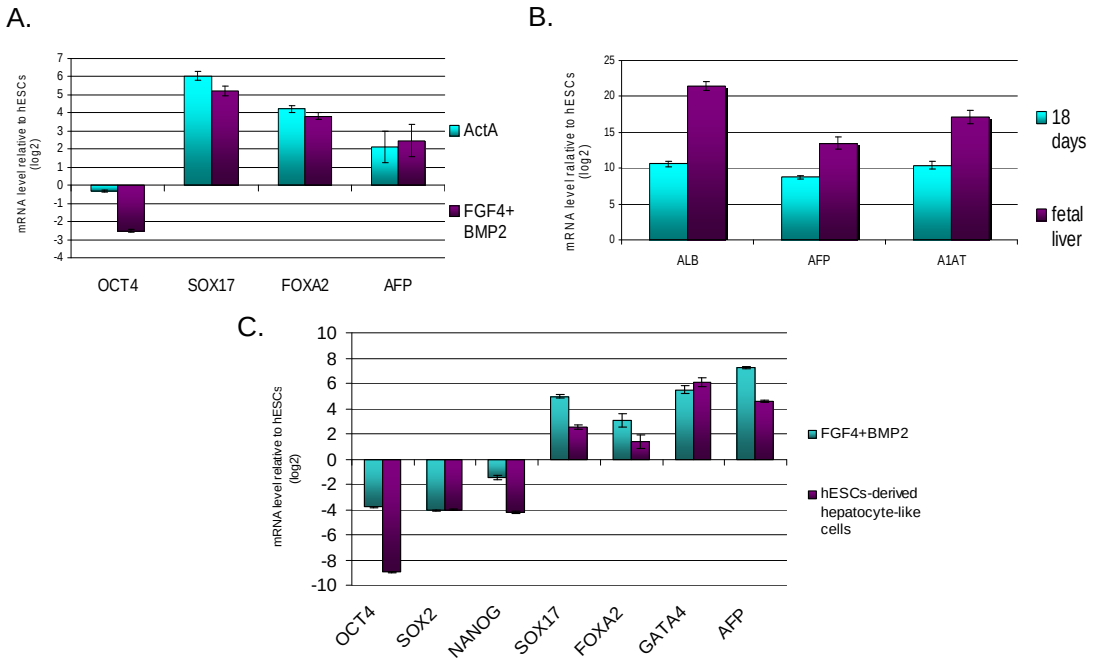


Figure 3.7: Progressive alteration of the gene expression pattern during the differentiation from human ES cells (H9) to hepatocyte-like cells. (A) RT-PCR analysis showing upregulation of definitive endoderm marker genes *SOX17* and *FOXA2*, and the fetal liver marker *AFP*. Downregulation of *OCT4* - marker defining the undifferentiated state, after treatment of human ES cells with ActA and initiation of hepatic differentiation from definitive endoderm by FGF4 and BMP2. (B) RT-PCR analysis of liver marker genes (*ALB*, *AFP*, *A1AT*) in hepatocyte-like cells and fetal liver. (C) Confirmation of gene expression pattern with biological replicates.

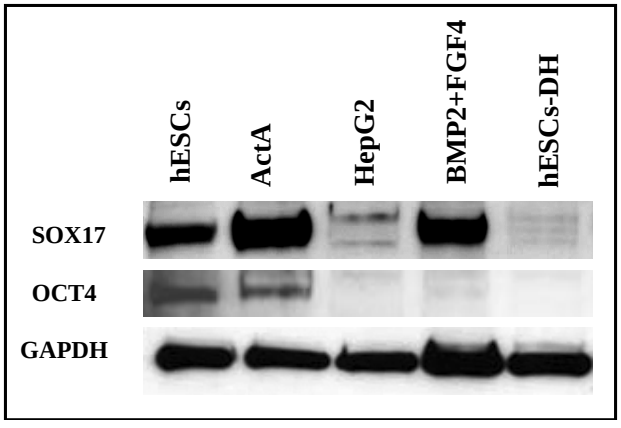


Figure 3.8: Kinetics of protein expression during the differentiation of hESCs (H9) into hepatocyte-like cells. The detection of protein levels by Western blot (detailed description in text)(hESCs-DH - human ES cells-derived hepatocyte-like cells).

ActA and 1mM NaB for 48 hours, followed by 3 days of 100ng/ml ActA and 0.5mM NaB to further improve the differentiation efficiency. During this time remarkable cell death was observed. The cells were then split at a 1:2 ratio and cultured in UM with 1% DMSO for 7 days. The cells displayed morphological changes from a spiny to a polygonal shape (Figure 3.9). At last, the medium was changed to modified L-15 medium supplemented with 10ng/ml HGF and 20ng/ml OSM for further 7 days. Throughout the differentiation process the cells changed their morphology and finally displayed characteristic hepatocyte-like morphology: polygonal in shape and distinct round nuclei (Figure 3.10). In comparison with previous protocols, there is a significant increase in the production of the hepatocyte-like cells. The residual cells were mostly fibroblast-like cells. The human ES cells-derived hepatocyte-like cells could be sustained in the culture for 5-7 days, and then they started to change their morphology into fibroblast-like cells. This is similar to the phenomenon observed for primary human hepatocytes.

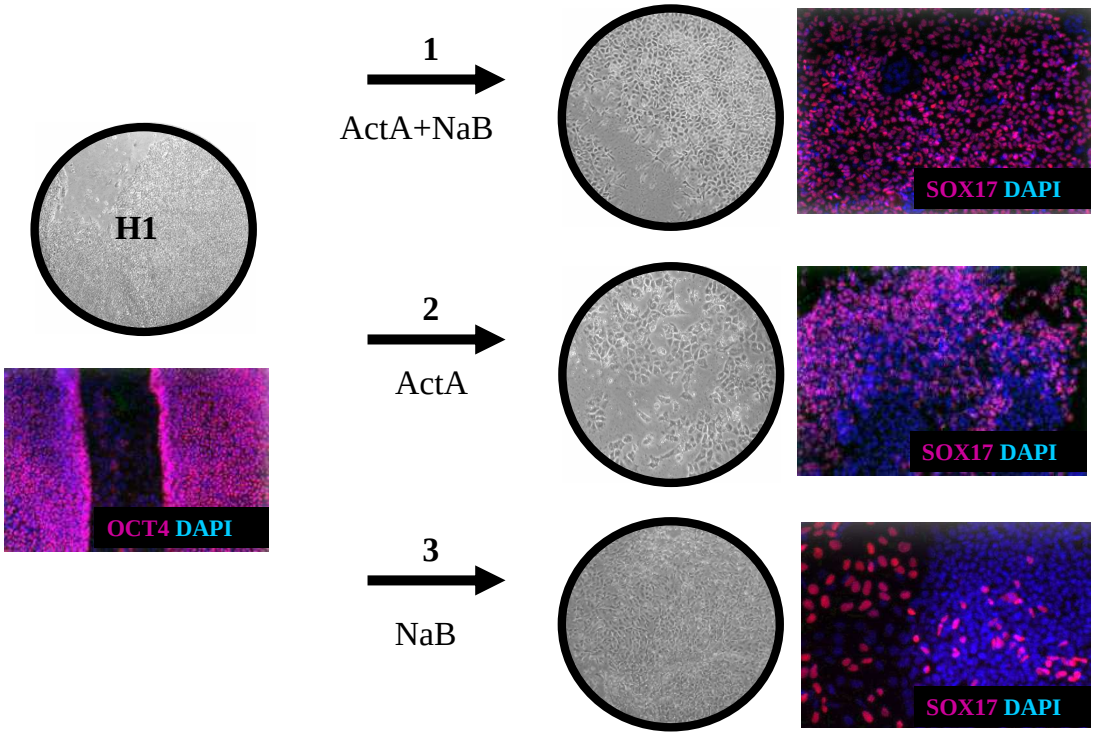


Figure 3.9: Comparison of the effect of Activin A and sodium butyrate in the differentiation of human ES cells to definitive endoderm. Phase contrast images of human ES cells (H1) treated with ActA or NaB alone or with combined ActA and NaB. Immunostaining showing expression of the SOX17 transcription factor.

Sodium butyrate (NaB) has been reported to give more homogenous hepatocyte differentiation and together with ActA induce definitive endoderm differentiation from human ES cells. To further investigate the role of ActA and NaB in the definitive endoderm differentiation, cells were treated with ActA or NaB or with both. After the first 24 hours, the cells treated with NaB displayed significant cell death. After this

step of the differentiation protocol, the cells from all these treatments displayed similar morphologies but the number of SOX17 positive cells in ActA and NaB treated sample was significantly higher (Figure 3.9).

A set of genes was analysed to further investigate differences between ActA, NaB and ActA+NaB. For the H9 cell line, the following genes were analysed: *OCT4*, *SOX2* (pluripotency), *GSC*, *T* (primitive streak), *SOX17*, *FOXA2*, *GATA4*, *GATA6*, *HNF4A* (definitive endoderm), *SOX7* (primitive endoderm), *OTX2*, *PAX6* (neuroectoderm) (Figure 3.11). Pluripotency factors *OCT4* and *SOX2* are dramatically down-regulated upon treatment. *GSC* (goosecoid) which is not only expressed by the primitive streak but continuously expressed by the definitive endoderm progenitors (Tada et al., 2005) is highly up-regulated. Brachyury (*T*) is expressed by the primitive streak mesendoderm and is transiently down-regulated during the transition from mesendoderm to definitive endoderm. Five days of treatment with ActA or both ActA and NaB is definitely too long to maintain Brachyury expression. In NaB treated cells the expression of *T* is significantly up-regulted. Increased expression of definitive endoderm markers such as *SOX17* and *FOXA2* was observed in ActA and both Acta and NaB treated cells at similar levels. In only NaB treated cells, the expression was every low or absent. *GATA4* and *GATA6* were expressed at higher levels in ActA and NaB treated cells. *HNF4A* was up-regulated under these conditions and surprisingly on similar level in NaB treated cells. The primitive endoderm marker SOX7 was significantly down-regulated in ActA and both ActA and NaB treated samples, but up-regulated after NaB treatment. Neuroectoderm markers such as *OTX2* and *PAX6* detected as up-regulated in treatments containing ActA contrary to NaB alone treatment (Figure 3.11).

The profile of gene expression analysed during the endodermal differentiation step revealed that the population treated just with ActA contained more undifferentiated cells which may result in more heterogenous population after further differentiation with DMSO.

After the first stage of differentiation the expression of SOX17 was evident as demonstrated on protein level by immunostaining. A subsequent shift to UM/DMSO induced significant down-regulation of SOX17 and FOXA2 confirmed by Western blot (Figure 3.12A). The gene expression pattern of SOX17 was further analysed by western blotting during the entire differentiation protocol. SOX17 was not detected in human ES cells and further differentiation by ActA treatment evidently induced expression especially strong after both ActA and NaB treatment. Some expression was observed even after NaB treatment what correlates with immunostaining results. After DMSO treatment and in human ES cells-derived hepatocyte-like cells no significant amount of SOX17 was detected (Figure 3.12B).

High expression of AFP was apparent towards the end of stage 2 as well as after stage 3. By stage 3, expression of ALB, the most abundant protein in the liver, was significantly increased and maintained. Other proteins associated with liver functions were also expressed. After DMSO treatment immunostaining confirmed that the cells

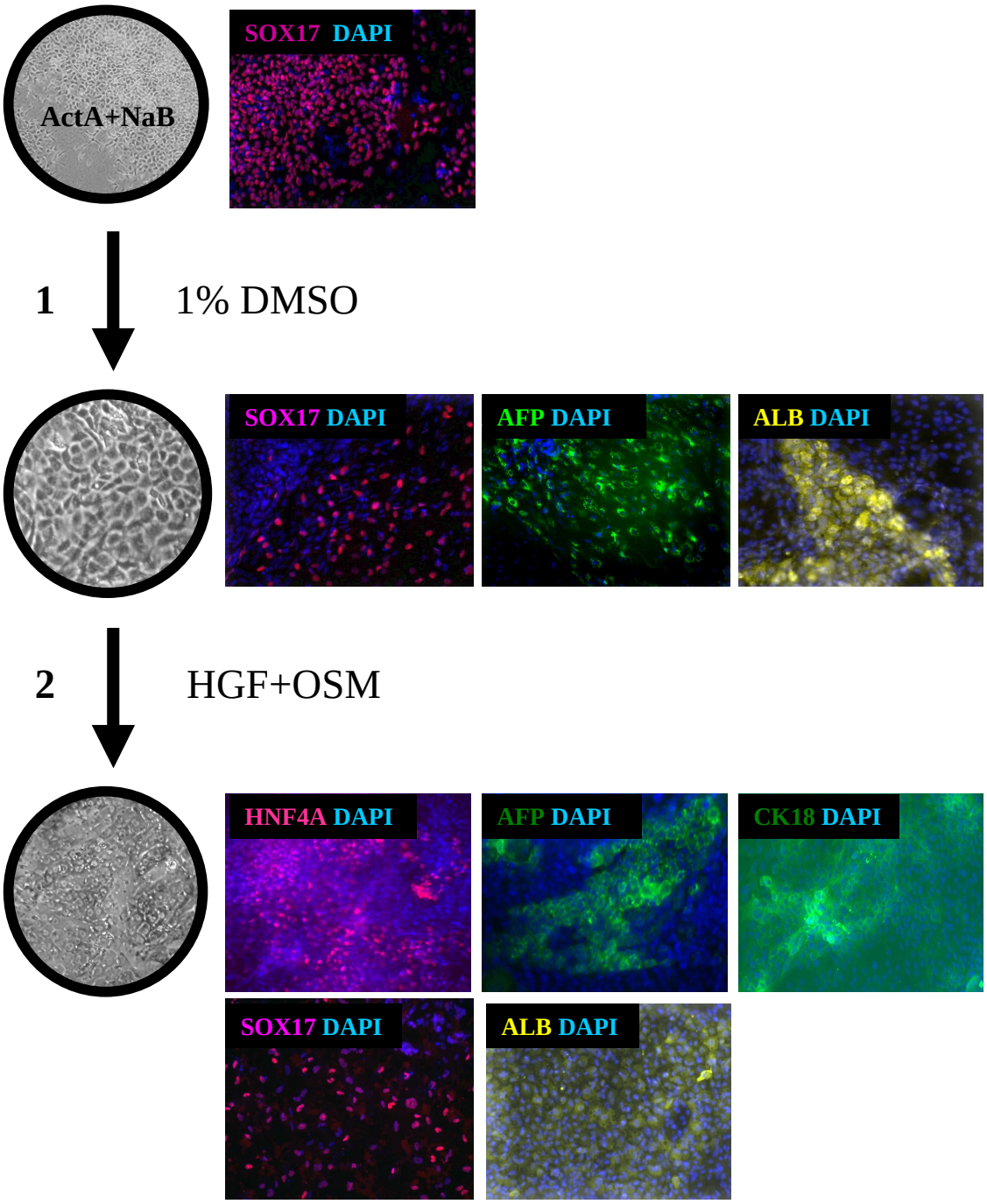


Figure 3.10: Schematic display of the hepatic initiation and differentiation steps (detailed explanations in text). Phase contrast images showing morphological changes during the progression of the protocol. Immunocytochemistry showing expression of various markers during the differentiation process.

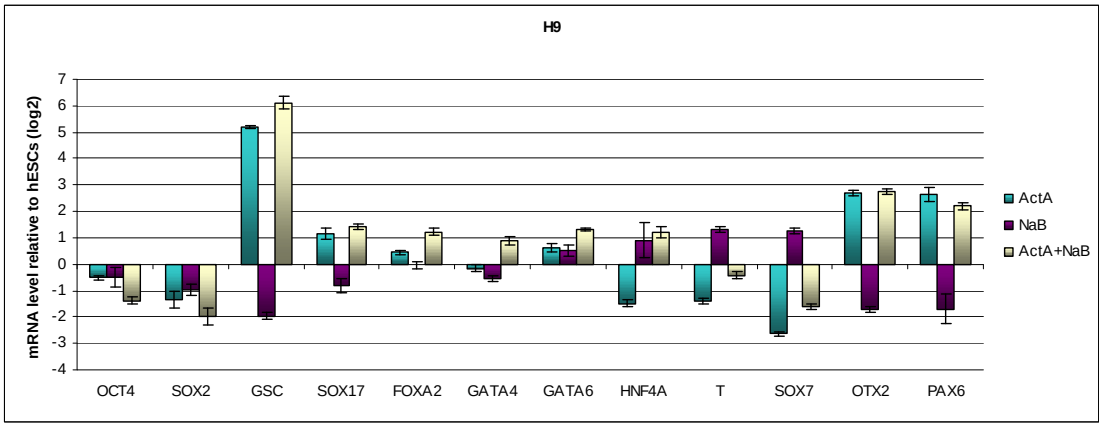


Figure 3.11: Comparison of the effect of Activin A and sodium butyrate on the differentiation of human ES cells (H9) to definitive endoderm.

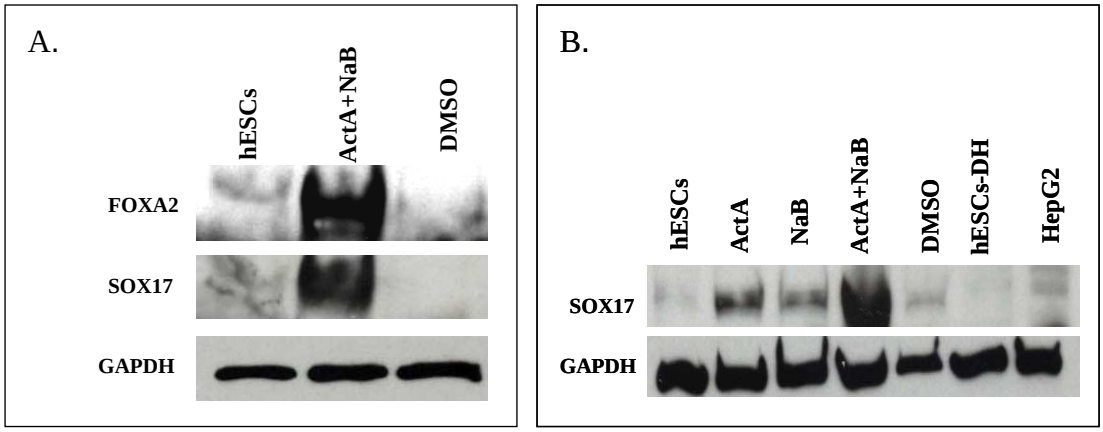


Figure 3.12: Progressive changes in the expression pattern of SOX17 and FOXA2 during the differentiation process.

expressed SOX17, AFP and ALB protein. After further maturation for 7 days, the cells were positive for HNF4A, AFP, CK18, ALB and still some SOX17 positive cells were detected. The results of RT-PCR for *AFP* further confirmed the immunostaining (Figure 3.10). In addition *A1AT* and *TDO* expression was detected in human ES cells-derived hepatocyte-like cells from both H1 and H9 cell lines (Figure 3.13). Gene expression investigation confirmed that the expression of genes throughout the differentiation process of human ES cells simulated that of the hepatogenesis (Figure 3.11 and Figure 3.11).

I followed the Agarwal *et al.* protocol, which mimics hepatogenesis *in vivo* by the sequential addition of inducing factors to human ES cells-derived DE (Figure 3.14). The first step of the protocol generates the DE. Morphologically, the cells changed from the typical, defined tight human ES cells colonies into less dense, flatter cells containing

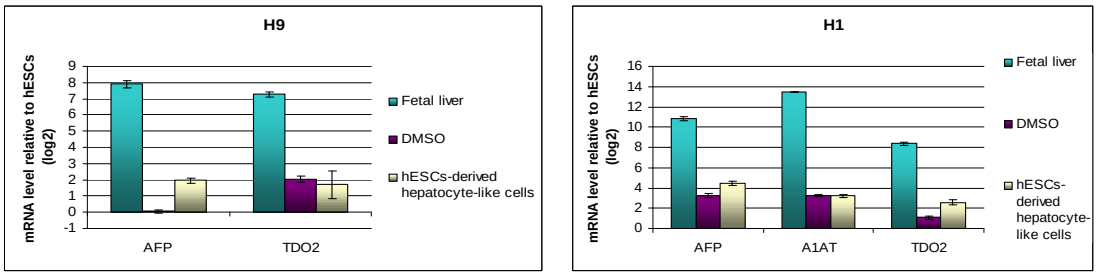


Figure 3.13: Up-regulation of hepatocyte gene expression in human ES cells lines H1 and H9 after UM/DMSO treatment and after 19 days of differentiation into human ES cells-derived hepatocyte-like cells.

prominent nuclei. Comparing human ES cells at the beginning of the differentiation process and after ActA treatment, most of the cells were devoid of the pluripotency marker *OCT4* while gaining strong expression of *GSC*, *SOX17*, *FOXA2*, *HNF4A*, *GATA4*, *GATA6* (Figure 3.15). These RT-PCR results were confirmed by western blot analysis and immunostaining for SOX17 (Figure 3.14 and Figure 3.16).

The differentiation of the cells was examined during the entire protocol. After the induction of definitive endoderm, the cells were replated on Collagen I, reattached and grown further. When the cells matured, they developed morphology resembling primary human hepatocytes (Figure 3.14). Differentiation of DE to hepatocyte-like cells was initially evaluated by immunofluorescence analysis. After each step of the differentiation, the cells were examined by immunostaining to monitor expression in time dependent manner. The well-organized pattern of hepatic and stage-specific proteins, like SOX17, AFP, HNF4A, ALB and CK18 has been observed (Figure 3.14). Additionally, AFP expression was confirmed by Western blot analysis and appears significantly up-regulated in comparison with ActA treated cells (Figure 3.16). The expression of AFP, a marker of the earliest hepatic specification was evident already after the 2nd step of the protocol. The amount of AFP-positive cells increased with time. Albumin (ALB), which is activated further in hepatic differentiation, was detected in human ES cells-derived hepatocyte-like cells. Immunofluorescence analysis revealed that HNF4A, a key transcription factor that regulates a cascade of liver-specific transcription (Zaret, 2002; Duncan, 2003) displayed nuclear localization after 3rd stage of protocol. Comparing protein expression levels in human ES cells to those in induced cells showed a clear loss of expression of OCT4, clear gain of SOX17, FOXA2 and AFP in the induced cells (Figure 3.14).

The expression of hepatic lineage genes during the time course of differentiation was also analyzed at the RNA level by RT-PCR. RNA from fetal liver was examined as a positive control. The late stage markers, *TDO2* and *A1AT* as expected were upregulated in human ES cells-derived hepatic-like cells (hESCs-DH) (Figure 3.17).

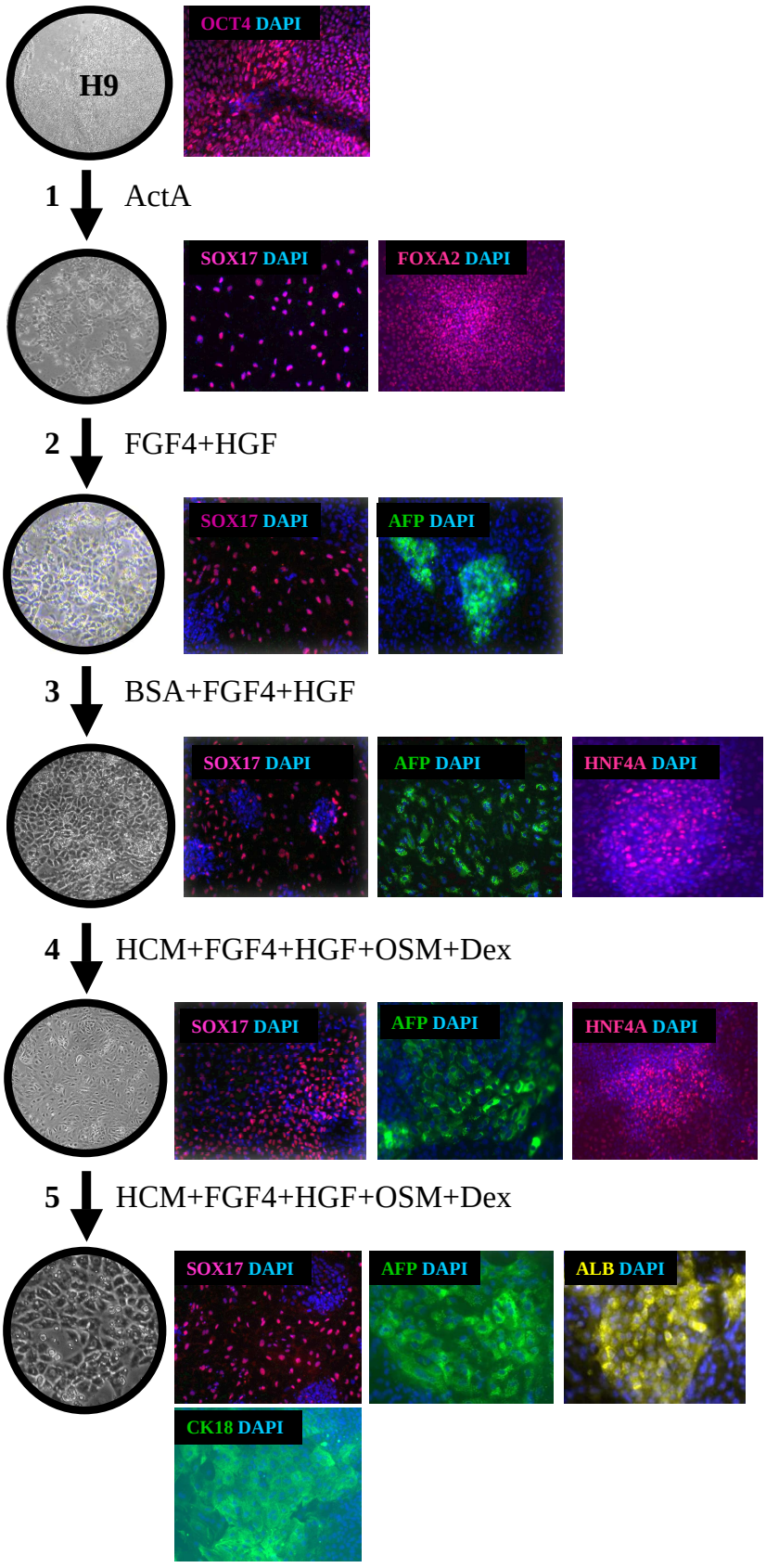


Figure 3.14: Differentiation of human ES cells into hepatocytes *in vitro*. Schematic display of the first three steps of the differentiation protocol (detailed descriptions in text). Phase contrast images of differentiating cells and immunofluorescence analysis of the expression of specific marker genes.

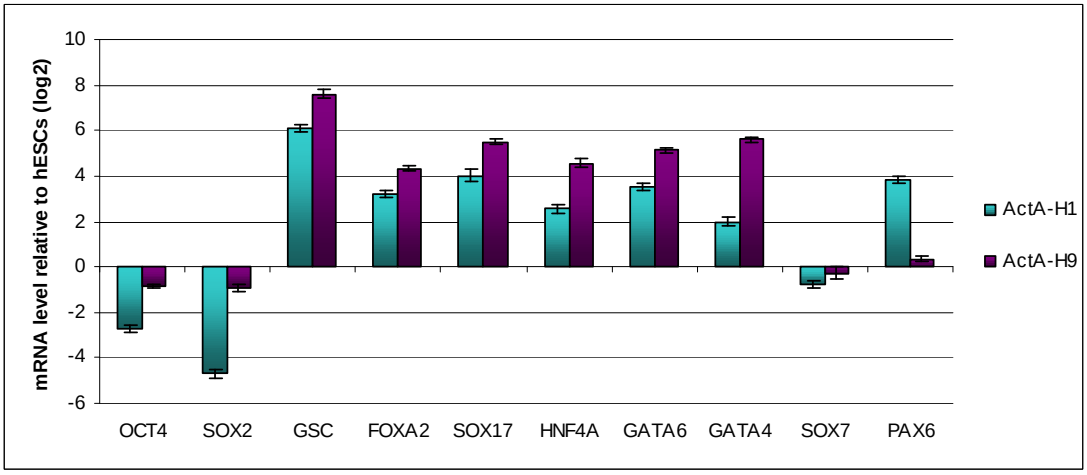


Figure 3.15: Treatment of human ES cells (both H1 and H9) with ActA rapidly up-regulates expression of genes associated with definitive endoderm.

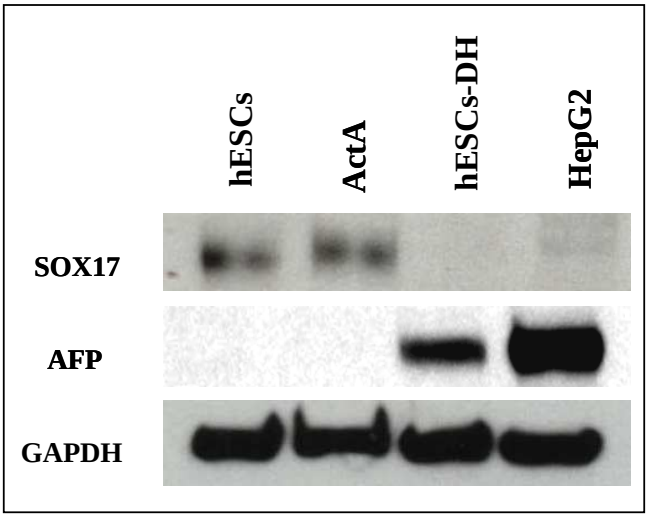


Figure 3.16: The detection of SOX17 and AFP protein by Western blot analysis in ActA treated cells and in human ES cells-derived hepatocyte-like cells (hESCs-DH).

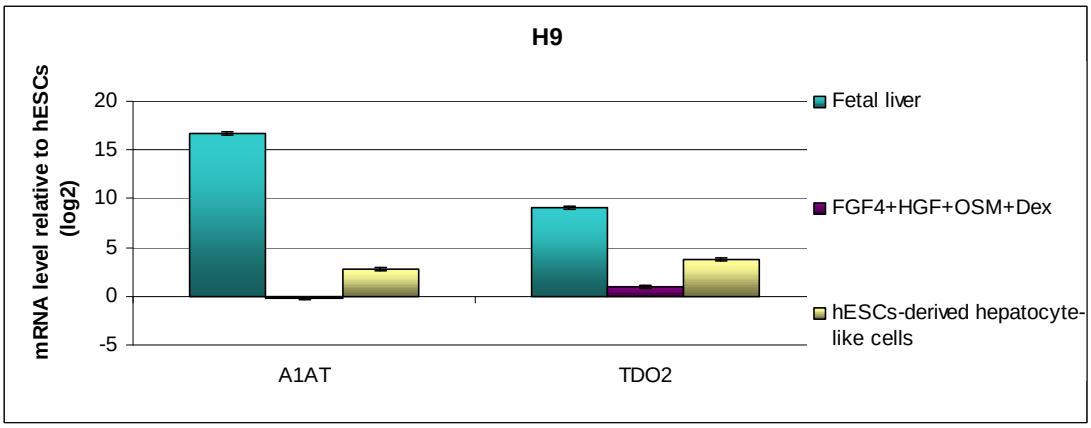


Figure 3.17: RT-PCR analysis of marker genes (*A1AT* and *TDO2*) in the day-11 (FGF4+HGF+OSM+Dex treated cells) and in hESCs-derived hepatocyte-like cells.

3.6 Human ES cell-derived hepatocyte-like cells exhibit hepatocyte-like functions

To study the functionality of human ES cells-derived hepatocyte-like cells, glycogen storage was examined using Periodic Acid-Schiff (PAS) staining. In comparison to fibroblasts, the human ES cells-derived hepatocyte-like cells exhibited evident glycogen storage (Figure 3.18A). The nuclei were counter-stained with Hematoxylin (blue-violet). The hepatocellular carcinoma cell line (HepG2) served as a positive control.

The vital liver cells are able to excrete diverse compounds from the circulation through hepatocellular uptake, conjugate and successive release the compounds. Therefore the ICG uptake was examined (Figure 3.18B), which is one of the liver-specific functions used for the identification of differentiated hepatocytes *in vitro*. Fibroblast did not take up any ICG. In contrast, hepatocyte-like cells could uptake ICG from the medium and exclude the absorbed ICG 6 hours later. The function of HepG2 cells was considered as a positive control. Hepatocyte-like cells generated from human ES cells demonstrate the competence of uptake and excretion of ICG compound.

3.7 Global data analysis

3.7.1 Global gene expression and hierarchical cluster analysis

I provide experimental data at the molecular level based on a genomics approach to investigate changes in gene expression during human ES cells differentiation into hepatocyte-like cells. To identify and further analyse genes, pathways and biological processes altered during two independent differentiation protocols, RNA was isolated from (i) undifferentiated human ES cell line H1, (ii) DE-induced cells, (iii) intermediate differentiation stage and (iv) human ES cells-derived hepatocyte-like cells. For each protocol three biological replicates of four following stages of differentiation were anal-

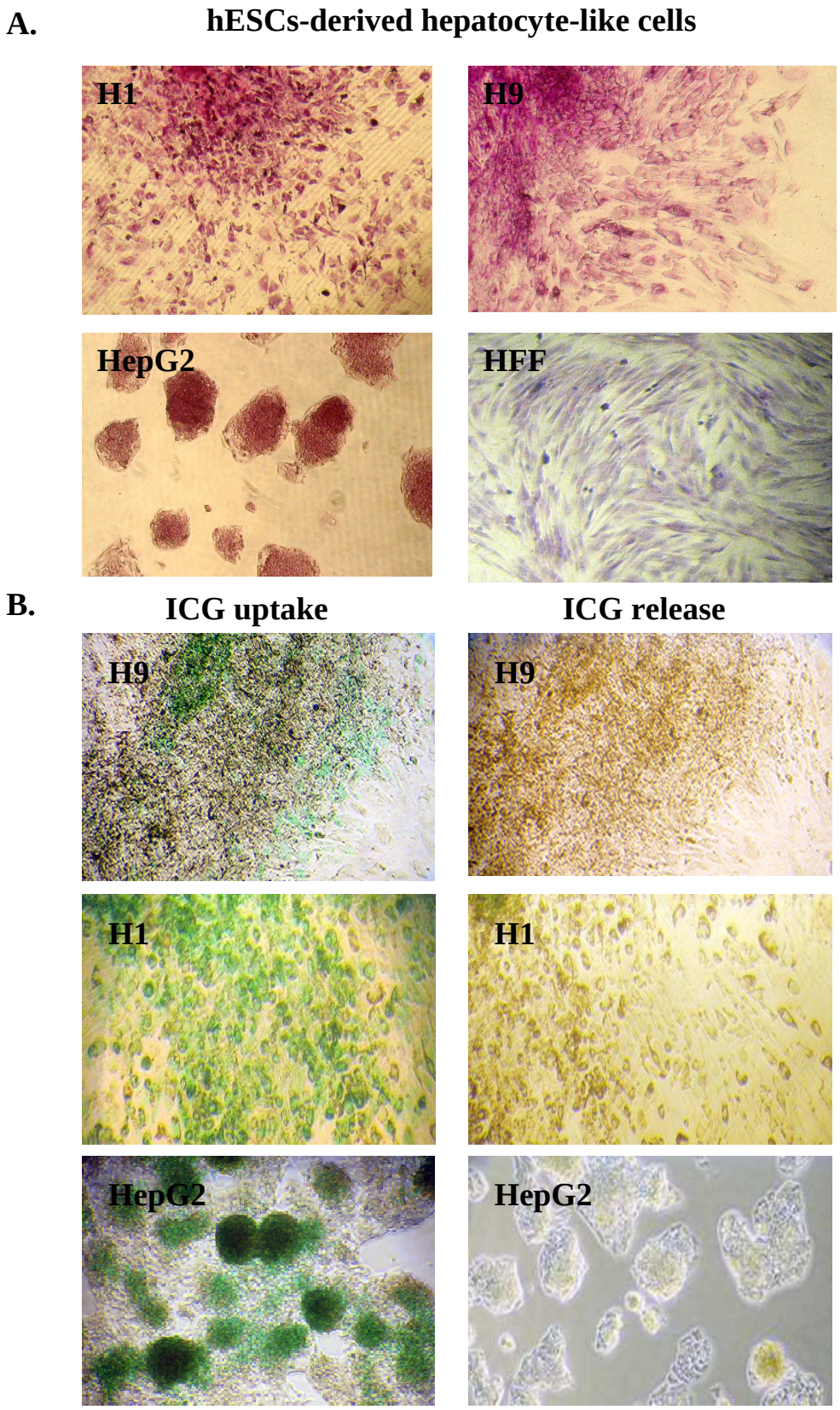


Figure 3.18: Human ES cells-derived hepatocyte-like cells exhibit hepatocyte-like functions. (A) Glycogen storage. Periodic acid Schiff (PAS) assay was performed on human ES cells-derived hepatocyte-like cells (H1 and H9), HepG2 and HFF. Glycogen storage is demonstrated by pink or dark red-purple cytoplasms. (B) Indocyanine Green (ICG) uptake and release. Human ES cells at the end of differentiation protocols and HepG2 (positive control) were examined for their ability to take up ICG and release it 6 hours later.

ysed. Gene expression analysis employing the Illumina Bead Chip platform was used to profile the transcriptomes of these cells.

Data reproducibility is demonstrated by clustering of all hybridized samples and correlations of three biological replicates for one selected group (Figure 3.19). All biological replicates in each group cluster together and the correlations values (0.9835-0.9981) indicate a high degree of correlation between them. A hierarchical cluster analysis of the samples was carried out by employing as input the 100 genes having the highest variance over all samples. Another hierarchical cluster is shown in Figure 3.20 that focuses on the comparison between groups of samples. As expected the clustering shows high diversity between fetal liver and human ES cells-derived hepatocyte-like cells. The transcriptomes of human ES cells-derived hepatocyte-like cells (Hay 19 days, Agarwal T18) are still far from fetal liver (the most distinct sample) but are closer to each other than to undifferentiated hESCs, which cluster together with ActA treated samples. Correlations between hESCs-derived hepatocyte-like cells and fetal liver are in the range 0.6955-0.6795.

3.7.2 Detailed analysis of common expressed genes in human ES cells-derived hepatocyte-like cells and fetal liver

Normalized data were analysed for significant (Illumina detection p-value<0.01 and p-value<0.05) changes in gene expression between the undifferentiated cells and differentiated cells and between fetal liver and human ES cell-derived hepatocyte like cells, with fold-ratios of 1.5 and above. Detailed expression profiling analysis and comparison between hepatocyte-like cells derived with two independent differentiation protocols and undifferentiated cells, elucidate that there are 2459 genes significantly up-regulated (p-val<0.05, detection p-val<0.01, ratio>1.5) in hepatocyte-like cells derived with the Hay *et al.* protocol, 3155 genes up-regulated in hepatocyte-like cells derived with the Agarwal *et al.* protocol and 3596 genes up-regulated in fetal liver. In order to find potential common genes, processes and pathways data sets from two separately generated hepatocyte-like cells and fetal liver were compared using Venn analysis. Venn Diagram in Figure 3.21 illustrates that among significantly up-regulated genes (p-val <0.05, detection p-val <0.01, ratio >1.5) there are 569 genes commonly expressed in all three considered samples. There are 711 common genes between fetal liver and hepatocyte-like cells derived with the Agarwal *et al.* protocol and 553 genes common between fetal liver and hepatocyte-like cells derived from the Hay *et al.* differentiation protocol.

Amongst the genes commonly expressed in both protocols and in fetal liver are already known genes important for hepatic functions (e.g. *SERPINA3*, *SERPINC1*, *FOXA1*, *HOXA5*, *LEAP2*, *MUC1*, *SERPINF1*, *SERPING1*, *SULT1C2*, *ALDH1A2*, *ALDH5A1*, and *CYP51A1*). But more genes crucial for liver functions could be found comparing genes commonly expressed in fetal liver separately with hepatocyte-like cells generated with Hay *et al.* or Agarwal *et al.* protocol (e.g. *ALDH1L1*, *ALDH3A2*,

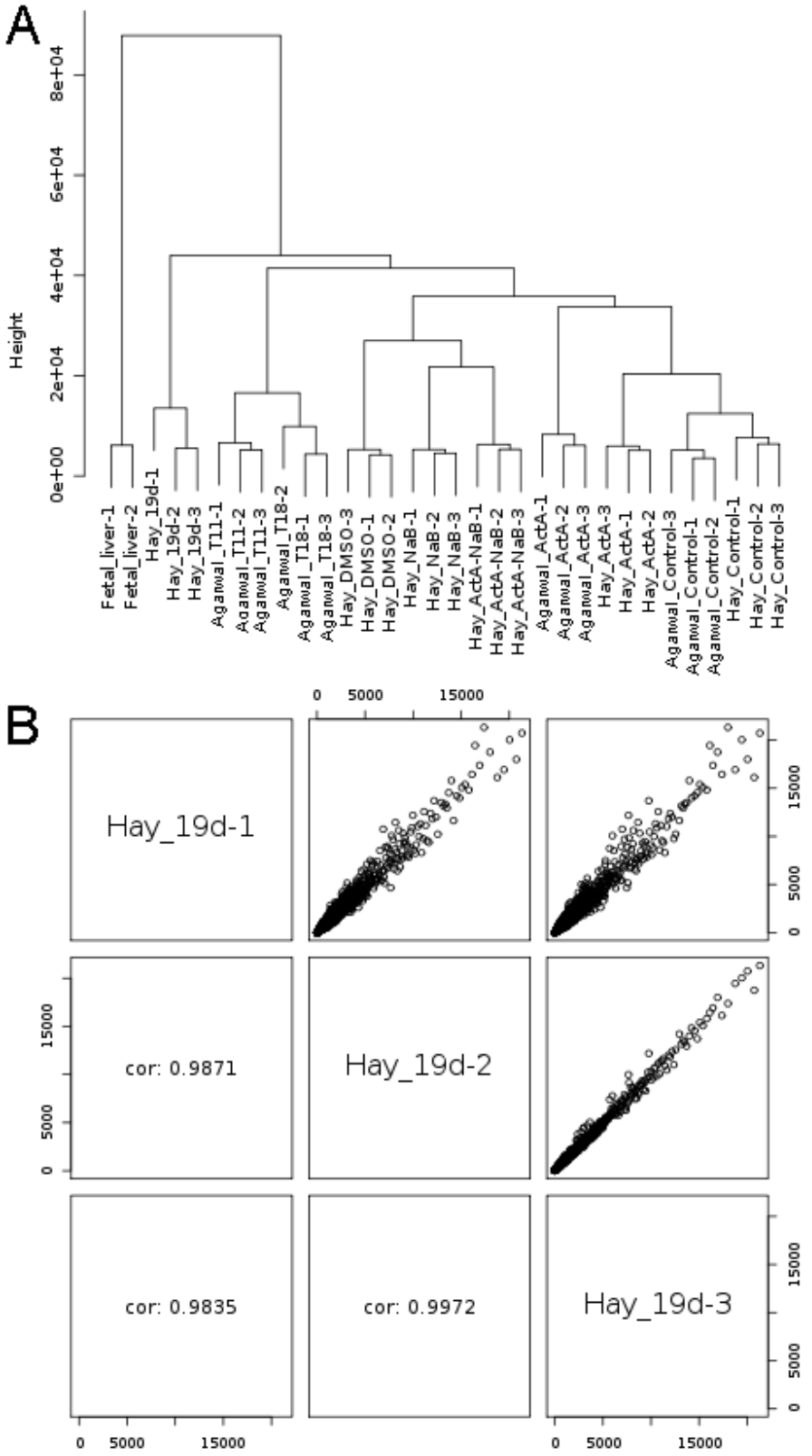
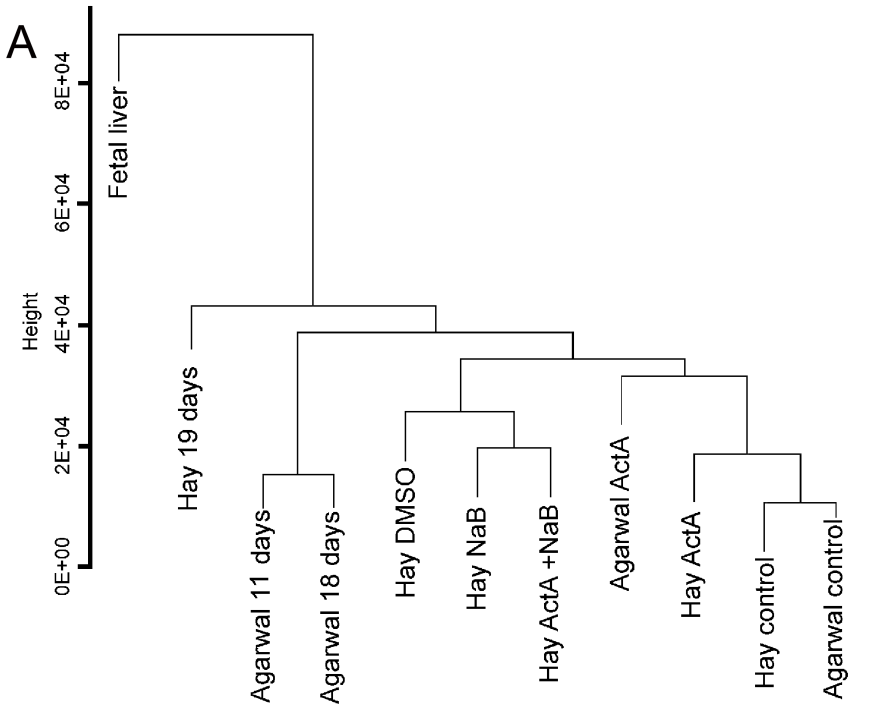


Figure 3.19: Clustering and correlation factors for sample replicates. The figure shows (A) the clustering of all replicates and (B) the one example of corresponding correlation coefficients derived from gene expression analyses.



B

	Hay contr.	Hay ActA	Hay NaB	Hay ActA+NaB	Hay DMSO	Hay 19 days	Agarwal contr.	Agarwal ActA	Agarwal T11	Agarwal T18	Fetal Liver
Hay contr.	1										
Hay ActA	0.9871	1									
Hay NaB	0.9523	0.9632	1								
Hay ActA+NaB	0.9497	0.9703	0.9793	1							
Hay DMSO	0.9575	0.9681	0.9632	0.9628	1						
Hay 19 days	0.9166	0.9212	0.9355	0.9285	0.9469	1					
Agarwal contr.	0.9915	0.9754	0.9313	0.9273	0.9370	0.8962	1				
Agarwal ActA	0.9503	0.9666	0.9326	0.9554	0.9390	0.9053	0.9398	1			
Agarwal T11	0.9168	0.9312	0.9091	0.9086	0.9405	0.8924	0.9105	0.9147	1		
Agarwal T18	0.9171	0.9303	0.9083	0.9051	0.9288	0.8997	0.9102	0.9178	0.9876	1	
Fetal Liver	0.6923	0.6912	0.7021	0.6718	0.6854	0.6955	0.6861	0.6571	0.6752	0.6795	1

Figure 3.20: Clustering and correlation factors for group of samples. The figure shows (A) the clustering and (B) the corresponding correlation coefficients derived from gene expression analyses for groups of samples derived through following steps of both differentiation protocols. Correlation factors are coloured as follows: red = 0.90 – 1.00, orange = 0.80 – 0.90, white = 0.70 – 0.80 and grey <0.70.

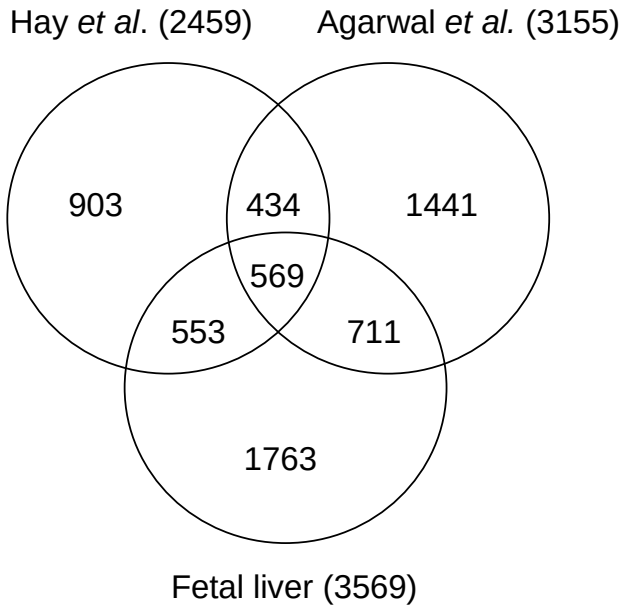


Figure 3.21: Venn diagram illustrating the overlap of common expressed genes between hepatocytes-like cells and fetal liver. The Venn diagram shows the overlap of target gene lists of hepatocyte-like cells and fetal liver.

GSTA4, *GSTM4*, *LHX2*, *RXRA*). It is evident that there are more genes connected to liver functions among common expressed genes between fetal liver and hepatocyte-like cells derived with the Agarwal *et al.* differentiation protocol (e.g. *ALB*, *AFP*, *TTR*, *CEBPA*, *GATA5*, *SERPINA1*, *SERPINF2*, *GSTA1*, *GSTA2*, *GSTK1*, *FGA*, *FGB*, *FGG*, *APOA2*, *APOB*, *APOC3*, *ABCC3*, *ABCC5*, *ABCD1*, and *ABCF3*). The hepatocyte-like cells have a specific gene expression signature related to but distinct from fetal liver.

3.7.2.1 Gene Ontology, pathways and tissue expression signature analysis

To analyse further common characteristics of fetal liver and human ES cells-derived hepatocyte-like cells, the hepatocyte-related gene lists (corresponding Illumina IDs as input) were analysed using the Gene Annotation Tools – DAVID (`http://niaid.abcc.ncifcrf.gov/`) (Huang da et al., 2009; Dennis et al., 2003) to identify common pathways and Gene Ontologies (GOs). Detailed lists of significant GOs (Biological Process, Cellular Component, Molecular Function) and Kegg pathways derived from the DAVID analyses (p-value<0.01) are presented for genes common in both hepatocyte-like cells derived with Hay *et al.* protocol and fetal liver in Table 3.1 and Table B.1; for genes common in both hepatocyte-like cells derived with Agarwal *et al.* protocol and fetal liver in Table 3.2.

In addition, biological processes, cellular components and molecular functions that

Table 3.1: List of overlapping GOs and pathways in hepatocyte-like cells derived with Hay *et al.* protocol and fetal liver

Term	Count	p-value
GO - BP		
response to external stimulus	47	1.14E-07
response to stress	61	2.90E-06
response to stimulus	122	3.14E-06
response to wounding	33	3.84E-06
defense response	39	1.00E-05
inflammatory response	25	2.60E-05
response to chemical stimulus	38	4.08E-05
immune response	39	5.58E-05
lipid metabolic process	43	1.02E-04
aromatic compound metabolic process	13	3.95E-04
steroid metabolic process	15	9.06E-04
lipid transport	11	1.10E-03
blood vessel morphogenesis	14	1.83E-03
protein amino acid glycosylation	12	1.86E-03
amine metabolic process	26	1.92E-03
blood vessel development	15	2.03E-03
glycoprotein biosynthetic process	12	3.53E-03
glycoprotein metabolic process	13	3.83E-03
apoptosis	38	4.13E-03
angiogenesis	12	4.74E-03
GO - CC		
cytoplasm	230	1.65E-07
endoplasmic reticulum	45	1.42E-04
cell fraction	50	3.25E-04
membrane fraction	40	7.20E-04
nuclear envelope-endoplasmic reticulum network	27	1.09E-03
endoplasmic reticulum membrane	27	1.16E-03
organelle membrane	62	1.22E-03
mitochondrion	44	1.45E-03
extracellular space	28	4.38E-03
microsome	13	7.15E-03
vesicular fraction	13	8.54E-03
fibrinogen complex	3	9.28E-03
GO - MF		
catalytic activity	201	9.91E-06
transferase activity, transferring glycosyl groups	20	6.77E-04
vitamin binding	13	1.15E-03
transferase activity	75	3.76E-03
aminopeptidase activity	6	7.19E-03
cytokine binding	9	8.13E-03
steroid binding	7	8.71E-03
interleukin binding	6	9.10E-03
pyridoxal phosphate binding	7	9.74E-03
transferase activity, transferring hexosyl groups	13	1.31E-02
PATHWAYS		
Complement and coagulation cascades	10	3.09E-03

GO - gene ontology, BP - biological process, CC - cellular component, MF - molecular function

Table 3.2: List of overlapping GOs and pathways in hepatocyte-like cells derived with Agarwal *et al.* protocol and fetal liver

Term	Count	p-value
GO - BP		
DNA metabolic process	51	1.49E-05
chromosome organization and biogenesis	32	1.85E-05
establishment and/or maintenance of chromatin architecture	26	8.59E-05
DNA packaging	26	1.21E-04
monocarboxylic acid metabolic process	20	1.13E-03
cell cycle	50	1.64E-03
metabolic process	328	1.78E-03
fatty acid metabolic process	16	1.83E-03
cellular metabolic process	296	2.34E-03
M phase	21	3.70E-03
cellular lipid metabolic process	36	4.16E-03
regulation of transport	12	4.20E-03
cytokinesis	6	5.53E-03
alcohol metabolic process	20	1.33E-02
chromatin assembly	9	1.37E-02
GO - CC		
intracellular part	406	9.15E-08
chromosome	33	1.18E-05
organelle	337	1.49E-05
chromatin	20	4.76E-05
endomembrane system	54	3.69E-03
spindle	9	6.74E-03
cytoplasm	260	8.24E-03
GO - MF		
oxidoreductase activity, acting on the CH-CH group of donors	8	1.36E-03
catalytic activity	216	3.25E-03
chromatin binding	11	8.80E-03
PATHWAYS		
Biosynthesis of steroids	6	7.08E-04
Polyunsaturated fatty acid biosynthesis	5	1.71E-03

GO - gene ontology, BP - biological process, CC - cellular component, MF - molecular function

were identified with high significance (p-value<0.01) by focusing on genes up-regulated in hepatocyte-like cells derived with Hay *et al.* differentiation protocol are shown in Table 3.2 and Table B.2. Similar analysis for genes up-regulated in hepatocyte-like cells derived with Agarwal *et al.* differentiation protocol are shown in Table 3.4 and Table B.3. These subsets of genes were further explored to identify signaling pathways (Table 3.5) tissue expression signature (Table 3.6 and Table 3.7). For each category only the top 20 results are presented if total list exceed this number results are presented in Appendix B.

3.7.2.2 Potential hepatocyte cell surface markers

Comparison of the transcriptional profiles of hepatocyte-like cells and fetal liver revealed several cell surface markers such as *TACSTD2*, *OSMR*, *ANXA1* that can be used for

Table 3.3: List of GOs enriched in hepatocyte-like cells derived with Hay *et al.* protocol

Term	Count	p-value
GO - BP		
organ development	268	1.18E-12
system development	337	6.04E-11
developmental process	561	2.51E-10
cell proliferation	172	1.50E-09
blood vessel development	59	2.88E-09
anatomical structure development	390	3.53E-09
regulation of cell proliferation	115	3.05E-08
positive regulation of biological process	218	5.13E-08
multicellular organismal development	408	1.28E-07
regulation of biological quality	173	1.57E-07
biological adhesion	154	1.63E-07
vesicle-mediated transport	112	2.04E-07
extracellular matrix organization and biogenesis	24	3.38E-07
response to external stimulus	135	4.44E-07
skeletal development	60	1.78E-06
negative regulation of cellular process	212	2.01E-06
blood coagulation	31	2.66E-06
GO - CC		
endoplasmic reticulum	224	2.20E-26
cytoplasm	996	4.60E-21
extracellular matrix	106	2.16E-17
nuclear envelope-endoplasmic reticulum network	117	1.92E-12
endomembrane system	212	3.23E-12
organelle membrane	266	1.86E-09
collagen	21	6.20E-09
cytoplasmic vesicle	95	3.17E-07
contractile fiber	21	7.39E-04
actin cytoskeleton	53	1.40E-03
intrinsic to organelle membrane	31	1.56E-03
pigment granule	23	1.96E-03
membrane part	826	2.45E-03
basolateral plasma membrane	24	2.51E-03
vesicle coat	16	3.11E-03
GO - MF		
protein binding	1090	2.43E-12
extracellular matrix structural constituent	40	6.59E-12
calcium ion binding	194	2.33E-10
cytoskeletal protein binding	92	2.03E-06
collagen binding	13	5.66E-06
enzyme regulator activity	146	2.50E-05
structural molecule activity	128	1.78E-04
actin binding	62	3.50E-04
enzyme activator activity	62	9.72E-04
growth factor binding	21	1.13E-03
protein kinase activity	106	1.63E-03
endopeptidase inhibitor activity	34	1.89E-03
protease inhibitor activity	34	2.32E-03
sulfuric ester hydrolase activity	9	2.97E-03

GO - gene ontology, BP - biological process, CC - cellular component, MF - molecular function

Table 3.4: List of GOs enriched in hepatocyte-like cells derived with Agarwal *et al.* protocol

Term	Count	p-value
GO - BP		
nervous system development	250	1.82E-27
system development	427	2.78E-18
anatomical structure development	502	4.05E-18
multicellular organismal development	532	1.48E-16
developmental process	688	6.79E-15
neurogenesis	99	5.46E-11
generation of neurons	94	8.63E-11
central nervous system development	83	3.32E-09
neurite morphogenesis	54	5.69E-09
neuron morphogenesis during differentiation	54	5.99E-09
cellular morphogenesis during differentiation	55	3.20E-08
cellular component organization and biogenesis	532	1.50E-07
regulation of biological process	851	3.13E-07
axon guidance	29	6.07E-07
anatomical structure morphogenesis	250	7.61E-07
negative regulation of cellular process	245	6.89E-06
negative regulation of transcription	80	2.35E-05
regulation of axonogenesis	16	2.85E-05
cell development	263	3.15E-05
GO - CC		
microtubule cytoskeleton	115	3.35E-10
cytoplasm	1121	2.64E-06
collagen	19	6.09E-06
intracellular part	1677	7.34E-06
extracellular matrix part	36	3.81E-05
organelle	1398	8.31E-05
Golgi apparatus part	92	1.04E-04
endomembrane system	209	1.06E-04
intracellular organelle	1398	2.80E-04
microtubule organizing center	40	5.37E-04
centrosome	37	7.38E-04
synapse	56	1.91E-03
endoplasmic reticulum	174	2.03E-03
neuron projection	31	2.10E-03
axon	18	2.12E-03
cell soma	16	2.45E-03
vesicle	96	2.71E-03
chromatin	46	2.73E-03
GO - MF		
protein binding	1303	6.21E-13
tubulin binding	28	1.40E-06
cytoskeletal protein binding	102	1.01E-05
microtubule binding	20	3.85E-05
transcription repressor activity	56	1.60E-03
transcription factor binding	91	3.03E-03
calcium ion binding	180	3.47E-03
enzyme regulator activity	154	7.30E-03
GPI anchor binding	31	7.52E-03
phospholipid binding	57	8.12E-03
DNA binding	397	8.45E-03
oxidoreductase activity	5	8.93E-03
chromatin binding	30	1.06E-02

GO - gene ontology, BP - biological process, CC - cellular component, MF - molecular function

Table 3.5: List of pathways enriched in hepatocyte-like cells derived with Hay *et al.* and Agarwal *et al.* protocol

PATHWAYS		
hESCs- DH_Hay et al.		
Term	**Count**	**p-value**
Focal adhesion	61	8.87E-07
ECM-receptor interaction	33	2.46E-06
Complement and coagulation cascades	23	1.27E-03
Cell Communication	37	2.20E-03
HIV-I Nef: negative effector of Fas and TNF	18	4.58E-03
3.1.1.1 enzymes	5	6.48E-03
Small cell lung cancer	25	8.46E-03
Pancreatic cancer	22	9.28E-03
hESCs- DH_Agarwal et al.		
Biosynthesis of steroids	14	7.19E-06
Axon guidance	41	9.09E-06
ECM-receptor interaction	29	8.44E-05
Focal adhesion	52	2.42E-04
GAMMA-CAROTENE ⇔ ALPHA-CAROTENE	10	3.53E-04
Colorectal cancer	26	1.64E-03
Fatty acid	15	5.39E-03

hESCs-DH - human ES cells derived hepatocyte-like cells

Table 3.6: List of tissues which signature is enriched in hepatocyte-like cells derived with Hay *et al.* protocol

Tissue expression signature		
Term	**Count**	**p-value**
Pancreas	888	2.28E-116
Globuspallidus	658	1.85E-85
Mammary gland Grade I, ER+, PR+, Her2- invasive ductal carcinoma	328	4.16E-69
Adrenal Cortex	631	7.62E-68
Placenta normal	864	1.70E-67
Uterus Corpus	702	1.06E-61
Trigeminal Ganglion	422	2.77E-53
Mammary gland Grade II, ER+, PR+, Her2- tumor	279	3.46E-52
Medulla Oblongata	825	1.44E-48
Connective tissue normal	663	4.66E-46
Cartilage Dedifferentiated chondrosarcoma lung metastasis	331	2.26E-41
Adipose tissue normal	676	1.46E-35
Parietal Lobe	674	1.59E-35
Chondrosarcoma disease	856	2.34E-34
Ovary	547	2.37E-29
Skeletal Muscle	1169	5.41E-22
Vascular normal	590	6.39E-22

Table 3.7: List of tissues which signature is enriched in hepatocyte-like cells derived with Agarwal *et al.* protocol

Tissue expression signature		
Term	**Count**	**p-value**
Fetal liver	1121	7.58E-101
Appendix	1055	3.34E-81
Olfactory Bulb	886	1.33E-76
Adrenal gland	928	6.06E-72
Subthalamic nucleus	820	5.04E-64
Dorsal root ganglia	545	1.87E-60
Glioma disease	1140	6.69E-58
Colorectal Adenocarcinoma	968	4.17E-57
Kidney	1379	2.75E-50
Brain normal pediatric cortex	612	1.76E-48
Occipital Lobe	867	4.63E-48
Pituitary	858	6.81E-47
Thymus	739	2.25E-46
Brain Anaplastic Grade II Astrocytoma	504	1.06E-45
Brain astrocytoma grade II	452	6.76E-44
Testis	777	1.58E-42
Brain ependymoma	589	1.58E-41
Brain normal, cerebral cortex	518	8.67E-38
Ear normal	670	5.00E-36
Ciliary Ganglion	994	1.16E-35

lineage analysis (Table 3.8).

3.8 Time-resolved analysis of the hepatogenesis using reproducible *in vitro* system

One of the advantages of whole genome transcriptional profiling is the capability to associate genes with new biological processes based on the correlation of their expression with the expression of genes previously shown to play a role in specific biological processes.

Using the Illumina Bead Chip Array we have generated a detailed transcriptional profile of cells after subsequent stages of differentiation for two independent protocols. Although gene signature of human ES cells-derived hepatocyte-like cells is still far from fetal liver to illustrate dynamic changes of gene expression we applied K-means clustering including four stages of hepatic fate induction. The identified clusters were analysed according to the presence of marker genes (i.e. transcription factors) previously described for particular stage. Interestingly, many downstream targets of those primary regulators show similar expression pattern and therefore are often found in the same cluster. Except known genes (e.g. *SOX17*, *FOXA2*, *AFP*, *CEBPA*) also many new potential target genes were identified and therefore qualify for further investigation.

Table 3.8: List of potential hepatocyte cell surface markers

Cell surface component	
hESCs-DH_Hay et al.	
ANXA1	Homo sapiens annexin A1
CBLB	Homo sapiens Cas-Br-M (murine) ecotropic retroviral transforming sequence b
CXCL10	Homo sapiens chemokine (C-X-C motif) ligand 10
ECGF1	***Homo sapiens endothelial cell growth factor 1 (platelet-derived)***
IL6ST	Homo sapiens interleukin 6 signal transducer (gp130, oncostatin M receptor)
INHBB	Homo sapiens inhibin, beta B (activin AB beta polypeptide)
LY6E	Homo sapiens lymphocyte antigen 6 complex, locus E
LY96	***Homo sapiens lymphocyte antigen 96***
MERTK	Homo sapiens c-mer proto-oncogene tyrosine kinase
MPL	***Homo sapiens myeloproliferative leukemia virus oncogene***
PLAUR	Homo sapiens urokinase plasminogen ectivator surface receptor
TACSTD2	Homo sapiens tumor-associated calcium signal transducer 2
hESCs-DH_Agarwal et al.	
ADRB2	***Homo sapiens adrenergic, beta-2-, receptor, surface***
BRD8	***Homo sapiens bromodomain containing 8***
CD14	***Homo sapiens CD14 molecule***
EVL	Homo sapiens Enah/Vasp-like
GLRA2	Homo sapiens glycine receptor, alpha 2
GLRB	***Homo sapiens glycine receptor, beta***
PRKCA	***Homo sapiens protein kinase C, alpha***
TNFRSF14	***Homo sapiens tumor necrosis factor 14 (Herpesvirus entry mediator A)***
hESCs-DH_Hay et al. and hESCs-DH_Agarwal et al.	
BIRC3	***Homo sapiens baculoviral IAP repeat-containing 3***
CD59	***Homo sapiens CD59 molecule, complement regulatory protein***
GEM	Homo sapiens GTP binding protein overexpressed in skeletal muscle
IFNAR2	***Homo sapiens interferon (alpha, beta and omega) receptor 2***
IL13RA1	Homo sapiens interleukin 13 receptor, alpha 1
ISGF3G	***Homo sapiens interferon-stimulated transcription factor 3, gamma***
LIFR	Homo sapiens leukemia inhibitory factor receptor
OSMR	Homo sapiens oncostatin M receptor
OXTR	Homo sapiens oxytocin receptor
PDGFRA	Homo sapiens platelet-derived growth factor receptor, alpha polypeptide
RRAGA	***Homo sapiens Ras-related GTP-binding protein A***
SDC2	***Homo sapiens syndecan-2 precursor***
SST	Homo sapiens somatostatin receptor

GENE NAME - expressed in hESCs-DH and fetal liver

3.8.1 Tracing genes expression patterns

Using the expression data from undifferentiated cells and cells after subsequent stages of differentiation we could see how gene expression changes during the differentiation process and formulate hypotheses concerning when the novel genes may play a significant role.

3.8.1.1 Genes highly expressed in undifferentiated human ES cells

For both protocols, clusters of genes highly expressed in undifferentiated human ES cells and gradually down-regulated upon differentiation have been generated. In the Hay *et al.* protocol we identified three interesting clusters at this step of the time-course of differentiation (Figure 3.22A). Each of mentioned clusters contain well-known pluripotency transcription factor. These clusters are focus around *NANOG*, *SOX2* and *POU5F1*, respectively. In the Agarwal *et al.* protocol two clusters were selected to illustrate mentioned time point (LIN28 and POU5F1) (Figure 3.22B). It is worth to point out that in both POU5F1 clusters are present metallothionein genes (*MT1H*, *MT1E* and *MT1F*). In *SOX2* cluster other genes required in self-renewal network like *ZIC3* and *HEY2* are present. In *POU5F1* cluster of Hay *et al.* protocol there are present *RCOR2* and *ZSCAN10* that plays a role in regulating pluripotency of ES cells.

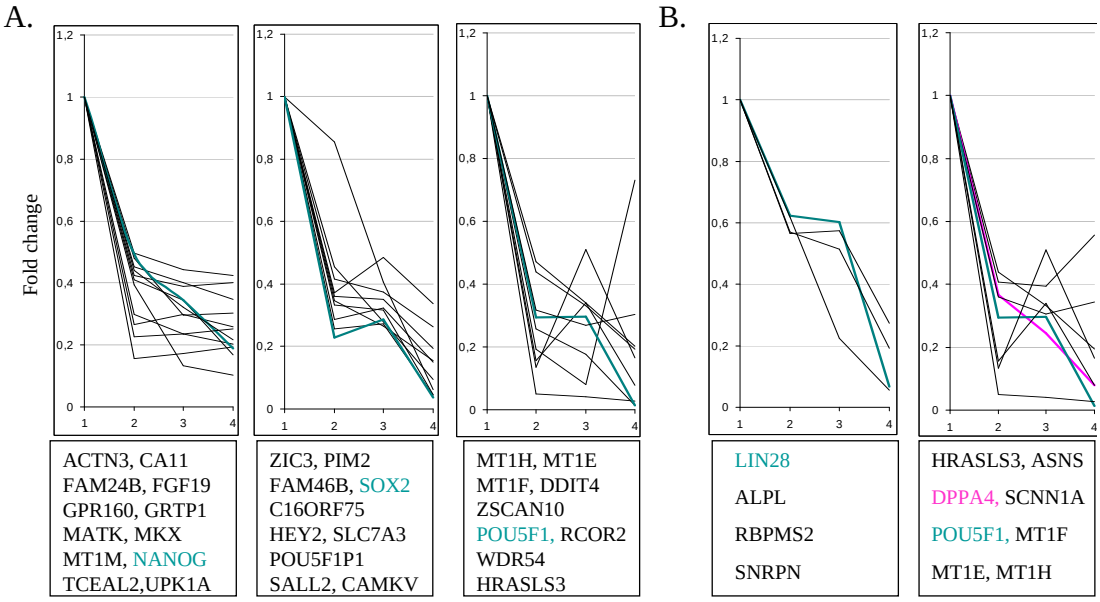


Figure 3.22: Tracing gene expression patterns (undifferentiated human ES cells). K-means clusters of genes highly expressed in undifferentiated human ES cells. (A) Hay *et al.* differentiation protocol, (B) Agarwal *et al.* differentiation protocol. (1) undifferentiated human ES cell line H1, (2) DE induced cells, (3) intermediate differentiation stage and (4) human ES cells-derived hepatocyte-like cells.

Looking in details into genes cluster together with *POU5F1* over time-course of Hay *et al.* differentiation I found *RCOR2* (REST (RE1-silencing transcription factor)

co-repressor 2) which is a component of transcriptional repression pathway that repress neuronal gene transcription in non-neuronal cells (McGraw et al., 2007; Ching and Liem, 2009). It is known that *REST* levels are high in ES cells, where its presence was shown to be essential for keeping neuronal gene expression low. *REST* is a direct part of the *OCT4/SOX2/NANOG* transcriptional network in ES cells and many *REST* targets are other transcription factors that are also regulated by the *OCT4/SOX2/NANOG* factors in ES cells. Deletion of *REST* in ES cells was reported to markedly decreased expression of members of the transcriptional network required for the self-renewal of ES cells, i.e., *OCT4*, *SOX2*, and *NANOG*. Interestingly, a major group of genes targeted by *REST* preferentially in ES cells are signaling molecules associated with the Wnt pathways. Since Wnts constitute a major class of extracellular signal substances, and different Wnts affect specific intracellular signaling pathways with profound effects on cell proliferation, death, migration, polarity, progenitor expansion, differentiation, and maturation thus changes in Wnt signaling induce effects on several key aspects of the ES cells phenotype (Johnson et al., 2008).

As well three genes encoding metallothioneins (*MT1H*, *MT1E*, and *MT1F*) are present in this cluster. Main function of these proteins is protection against oxidative stress. In the human body large quantities are synthesized in liver and kidneys. Physiological MT synthesis and MT concentrations are increased transiently several folds during cell proliferation. MT interchanges its zinc with zinc-finger proteins *in vitro* and plays a contributory role in zinc-dependent processes involved in gene expression.

In this cluster is also present *ZSCAN10* (zinc finger and SCAN (alpha-synuclein) domain containing 10) also known as *ZNF206*. This transcription factor plays a role in regulating pluripotency of ES cells. Upon differentiation of human and mouse ES cells, *ZNF206* (*Zfp206* in mouse) expression is quickly repressed. *ZNF206* is a direct downstream target of *POU5F1* and *SOX2* which are able to regulate its transcription (Wang et al., 2007).

Second cluster of genes highly expressed in undifferentiated human ES cells consist of genes cluster together with *SOX2*. This cluster contains *SALL2* (Sal-like 2). Although mouse *Sall2* was reported to be expressed during development and abundantly in the adult brain, precise expression patterns and the physiological function of *SALL2* have remained unknown (Sato et al., 2003).

ZIC3 (Zinc finger protein of the cerebellum 3) a member of the Gli family of zinc finger transcription factors share as well the same cluster with *SOX2*. *ZIC3* is expressed in ES cells and its expression is repressed upon differentiation. The expression of *ZIC3* in pluripotent ES cells is also directly regulated by *OCT4*, *SOX2*, and *NANOG*. In context of this work it is especially important that repression of *ZIC3* in human and mouse ES cells, induced expression of several markers of the endodermal lineage. Since expression of *Nanog* (repressor of extraembryonic endoderm specification in ES cells), is significantly reduced in *Zic3* knockdown cells this suggests that *Zic3* plays an important role in the maintenance of pluripotency by preventing endodermal lineage specification

in ES cells (Ware et al., 2006).

HEY2 (HES-related repressor protein 2) is present in this same cluster with *ZIC3*. It is known that Notch signaling pathway maintains stem cells through transcriptional activation of *HES*/*HEY* family members to repress tissue-specific transcription factors. *HES1*, *HEY1* and *HEY2* are expressed in endothelial cells and are target genes of Notch signaling pathway. Whereas *HES*1 and *HES3* are expressed in undifferentiated ES cells and are target genes of the ES cell-specific network of transcription factors (Katoh and Katoh, 2007).

3.8.1.2 Genes up-regulated upon Activin A treatment

For both protocols, clusters of genes highly expressed upon the second step of differentiation (definitive endoderm cells induction) have been generated. For each differentiation protocol three clusters have been chosen for further analysis. For the Hay *et al.* protocol it was *FOXA2*, *HHEX* (*GSC*, *GATA4*) and *NODAL* (*MIXL1*, *EOMES*) clusters (Figure 3.23A). For the Agarwal *et al.* protocol *MIXL1* (*NODAL*, *SOX17*), *GSC* (*LHX1*, *GDF3*) and *CER1* (*KRT8*, *CYP26A1*) clusters have been investigated (Figure 3.23B).

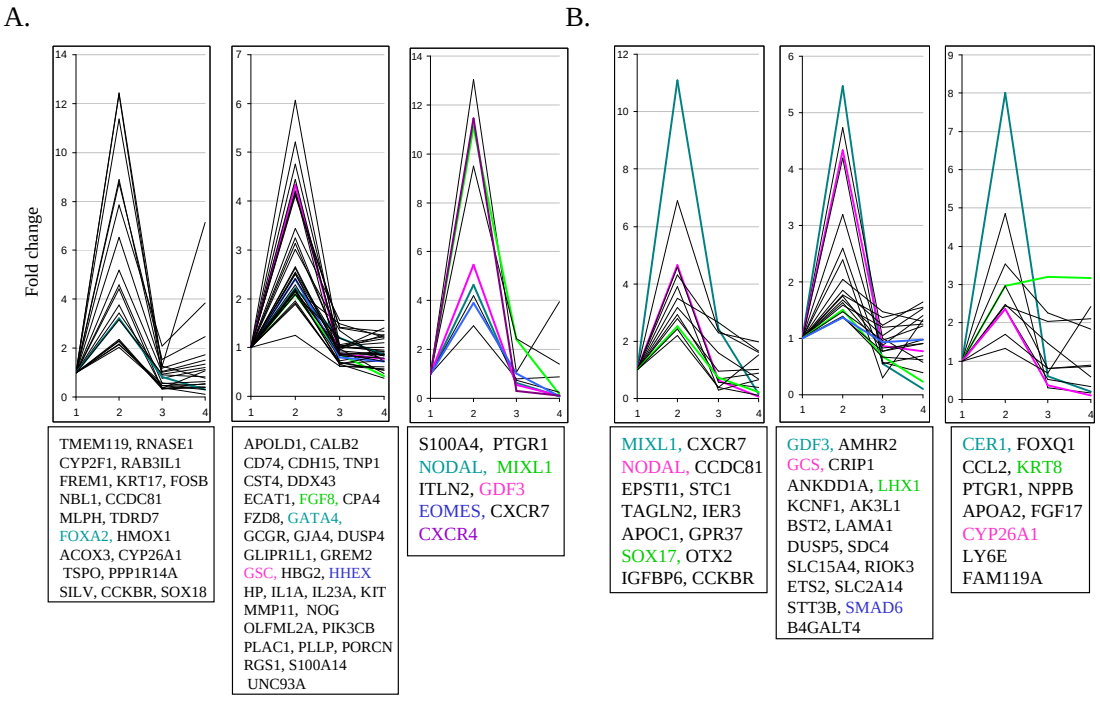


Figure 3.23: Tracing genes expression patterns (DE differentiated cells). K-means clusters of genes highly expressed in DE differentiated human ES cells. (A) Hay *et al.* differentiation protocol, (B) Agarwal *et al.* differentiation protocol. (1) undifferentiated human ES cell line H1, (2) DE induced cells, (3) intermediate differentiation stage and (4) human ES cells-derived hepatocyte-like cells.

Among genes highly up-regulated in the second step of differentiation (Activin A and NaB treatment) which cluster together with *FOXA2* are i.a., *CYP26A1*, *SOX18*

and *NBL1*.

CYP26A1 (cytochrome P450 26A1) is the retinoic acid (RA)-degrading enzyme which play a critical role in defining the normal anterior limit of the pancreatic field. Pancreatic cell fates are specified by retinoic acid (RA), and proper size and localization of the pancreatic field are dependent on tight control of RA signaling. Endodermal expression of cyp26a1 genes is subject to positive regulation by RA, additionally feedback loop can maintain consistent levels of RA signaling, despite environmental fluctuations. Disruption of cyp26a1 function causes a dramatic expansion of pancreatic cell types toward the anterior of the embryo (Kinkel et al., 2009).

SOX18 (SRY-box 18) is a member of the F-subgroup of Sox transcription factors family mostly expressed in endothelial compartments and known to be important for the development of blood vessels and hair follicles in mice. Together with SOX7 is expressed in the lateral plate mesoderm during somitogenesis (Olsson et al., 2001; Pendeville et al., 2008).

The *NBL1* (neuroblastoma 1) gene product is the founding member of the evolutionarily conserved CAN (Cerberus and DAN) family of proteins, which contain a domain resembling the CTCK (C-terminal cystine knot-like) motif found in a number of signaling molecules. These proteins are secreted, and act as BMP (bone morphogenetic protein) antagonists by binding to BMPs and preventing them from interacting with their receptors. They may thus play an important role during growth and development but till now a role of *NBL1* in endoderm development has not been described (Gazzerro and Canalis, 2006; Katoh and Katoh, 2006).

In another cluster possessing this pattern are present genes well known to be expressed in definitive endoderm (DE) like: *CXCR4*, *CXCR7*, *EOMES*, *GDF3*, *MIXL1*, *NODAL* (Zaret, 2002). In addition, there are as well genes early not connected to this process like *ITLN2*, *PTGR1* and *S100A4* (Figure 3.23A).

The *CXCR4* (C-X-C chemokine receptor 4) plays a decisive role in physiological cell migration both in developmental processes and adult tissues. During gastrulation, its expression is observed in the epiblast at the level of the primitive streak and in the endoderm. Later, expression is noticeable in the ventral foregut portal, developing somites, tail bud, neural tube, the intermediate mesoderm, the lateral plate mesoderm and the developing blood vessels of the chicken embryo. However, little is known about how the movement of endodermal cells is regulated during gastrulation it has been shown that *sdf1a* (stromal cell-derived factor, cxcl12) expressing mesodermal cells control the movements of the cxcr4a-expressing endodermal cells and guide them to the dorsal side of the embryo during gastrulation. The stromal cell-derived factor-1 (CXCL12) chemokine engages the CXCR4 and CXCR7 receptors and regulates homeostatic and pathologic processes, including organogenesis, leukocyte homeostasis, and tumorigenesis. Both receptors are widely expressed in mammalian cells, but how they cooperate to respond to CXCL12 is not well understood. CXCR7 plays as well an essential role in the CXCL12/CXCR4-mediated transendothelial migration (TEM)

of CXCR4(+)CXCR7(+) human tumor cells (Zabel et al., 2009).

Fibroblast-specific protein 1 (FSP1; encoded by the S100A4 gene) is a member of the S100 family of calcium-binding proteins and is constitutively expressed in the cytoplasm of fibroblasts or epithelial cells converted into fibroblasts by means of epithelial-mesenchymal transition (EMT). Its possible role in definitive endoderm differentiation can be suggested since EMT transition is crucial for this process (Yamaguchi et al., 2009).

3.8.1.3 Genes up-regulated in intermediate differentiation stage

For Hay *et al.* protocol one cluster has been chosen to illustrate genes up-regulated in intermediate differentiation stage. Cells on this stage went through 3 days of Activin A and NaB treatment and 7 days of 1% DMSO treatment. This cluster contains *APOA4* (apolipoprotein A-IV) and *FGA* (fibrinogen alpha chain) (Figure 3.24A). For Agarwal *et al.* protocol as well one cluster containing *WNT3A* has been selected for further investigation (Figure 3.24C).

Fibrinogen (FBG) is one of the soluble precursors of fibrin, which is the main protein constituent of the blood clot, it is synthesized in hepatocytes in the form of a hexamer composed of two sets of three polypeptides (A-alpha, B-beta, and G-gamma). Although fibrinogen genes are expressed constitutively in hepatocytes, their transcription can be greatly increased during inflammatory stress. During an acute phase response, interleukin-6 (IL-6) and glucocorticoids up-regulate expression of the three fibrinogen genes (*FGA*, *FGB*, and *FGG*) in liver and lung epithelium (Vu and Neerman-Arbez, 2007).

CCN1/CYR61 (cysteine-rich angiogenic inducer 61) is a member of the protein family that can be promptly induced by growth factors and is present in the *APOA4* cluser. *CCN1/CYR61* promotes cell proliferation, adhesion, and differentiation. It plays important roles in angiogenesis and extracellular matrix production. In addition, *CCN1/CYR61* has many potential functions in tumorigenesis, development, embryo implantation, as well as formation of endometriotic lesions. Expression of *CCN1/CYR61* is regulated by a variety of agents including cytokines, growth factors, steroid hormones, and some drugs. It has been reported that *CCN1/CYR61* exerts its functions via interacting with at least five integrins as well as heparan sulfate proteoglycan. By activating Wnt, NF-kappaB, or tyrosine kinase signaling pathways, *CCN1/CYR61* is not only able to control the growth of epithelial cells and fibroblasts, but also induce or suppress apoptosis in a cell type-specific manner. Chromatin immunoprecipitation analysis indicates that *CCN1/CYR61* is a direct target of canonical Wnt/β-catenin signalling (Chen and Du, 2007).

FBN2 (Fibrillin 2) is as well present in the *APOA4* cluster. Fibrillin-rich microfibrils are extracellular assemblies that impart structural properties of the connective tissue and contribute to organogenesis. Knockout mice models demonstrated that fibrillins 1 (FBN1) and 2 (FBN2) perform partially overlapping functions during aortic devel-

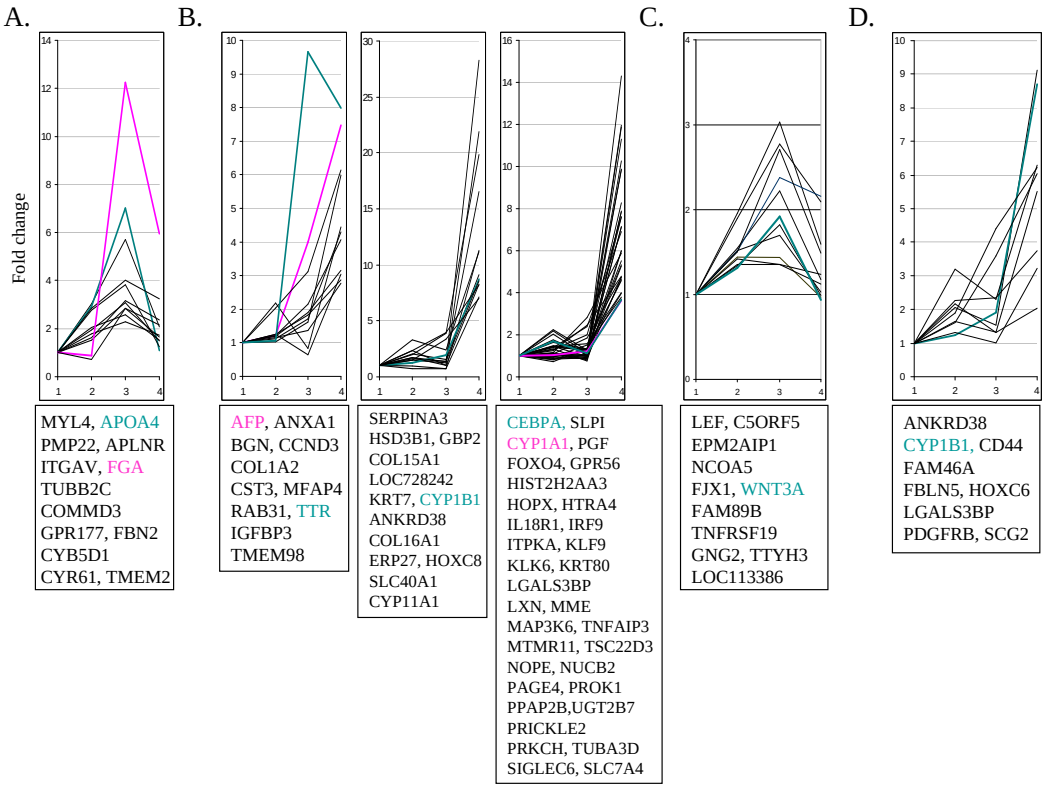


Figure 3.24: Tracing genes expression patterns. (A) K-means clusters of genes up-regulated in intermediate differentiation stage of Hay *et al.* differentiation protocol, (B) genes up-regulated in hepatocyte-like cells derived with Hay *et al.* differentiation protocol, (C) K-means clusters of genes up-regulated in intermediate differentiation stage of Agarwal *et al.* differentiation protocol, (D) genes up-regulated in hepatocyte-like cells derived with Agarwal *et al.* differentiation protocol. (1) undifferentiated human ES cell line H1, (2) DE induced cells, (3) intermediate differentiation stage and (4) human ES cells-derived hepatocyte-like cells.

opment. Transcriptional profiling revealed that heart failure progression in Fbn1 null mice is accompanied by unproductive up-regulation of gene products normally involved in tissue repair and vascular integrity, such as plasminogen activator inhibitor-1, activin A, and cysteine-rich angiogenic protein 61. *FBN2* and *CYR61* appears as a promising targets since they are present in one cluster and their common regulation has been already described during aortic development (Carta et al., 2006).

3.8.1.4 Genes up-regulated in human ES cells-derived hepatocyte-like cells

Amongst the genes which cluster together with *AFP* in hepatocyte-like cells derived employing the Hay *et al.* protocol are *ANXA1* (annexin A1), *TTR* (transthyrethin), and *IGFBP3* (insulin-like growth factor binding protein 3) (Figure 3.24B).

Annexins are a family of structurally related, Ca2+-sensitive proteins that bind to

negatively charged phospholipids and establish specific interactions with other lipids and lipid microdomains. They are present in all eukaryotic cells and share a common folding motif, the "annexin core", which incorporates Ca2+- and membrane-binding sites. Annexins participate in a variety of intracellular processes, ranging from the regulation of membrane dynamics to cell migration, proliferation, and apoptosis (Della Gaspera et al., 2001). Annexin A1 (*ANXA1*) is up-regulated during liver regeneration and hepatocyte proliferation (Harashima et al., 2006). It is endowed with anti-inflammatory properties and IL-6 and TNF-α regulates *ANXA1* expression at the transcriptional level.

Transthyretin (TTR) is an evolutionarily conserved serum and cerebrospinal fluid protein that transports holo-retinol-binding protein and thyroxine. Its serum concentration has been widely used to assess clinical nutritional status. Most TTR in the systemic circulation is produced and secreted by the liver. TTR is found in other organs as a result of local synthesis or transport, suggesting that it may have other, as yet undiscovered, functions. It is possible that its capacity to bind many classes of compounds allows it to serve as an endogenous detoxifier of molecules with potential pathologic effects (Buxbaum and Reixach, 2009).

Present in the same cluster, *IGFBP3* (insulin-like growth factor binding protein 3) is expressed in adult liver (Casillas-Ramirez et al., 2009).

In the *CYP1B1* (cytochrome P450 1B1) cluster (Figure 3.24B) there are present genes like *CYP11A1*, *SERPINA3* and *COL15A*.

CYP1B1 encodes a member of the cytochrome P450 superfamily of enzymes. The cytochrome P450 proteins are monooxygenases that catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids. The enzyme encoded by this gene localizes to the endoplasmic reticulum and metabolizes procarcinogens such as polycyclic aromatic hydrocarbons and 17β-estradiol. Human cytochrome P450 1B1 (CYP1B1) is found mainly in extrahepatic tissues and is overexpressed in a variety of human tumors. In this same cluster is present *CYP11A1*, which is considered as not liver specific but more expressed in the gonads and the placenta. This protein localizes to the mitochondrial inner membrane and catalyzes the conversion of cholesterol to pregnenolone, the first and rate-limiting step in the synthesis of the steroid hormones (Lavoie and King, 2009).

Present in the same cluster antichymotrypsin (*SERPINA3*) is a widely expressed member of the serpin superfamily, required for the regulation of leukocyte proteases released during an inflammatory response and is highly expressed in the liver.

COL15A1 (collagen Type XV alpha1) appears in the same cluster. It was reported to be localized mainly in the basement membrane zone, but their distributions in blood vessels and nonvascular tissues have yet to be thoroughly clarified. Type XV collagen is a large macromolecule distinguished by a highly interrupted collagenous domain and many utilized sites of attachment for CS (chondroitin sulfate) and HS (heparan sulfate) glycosaminoglycan chains. It is present in most basement membrane zones of human tissues, where it is found closely associated with large collagen fibrils.

There is a third cluster of genes up-regulated in hepatic-like cells derived with the Hay *et al.* protocol. The genes, which cluster together with *CEBPA* and *CYP1A1*, are *FOXO4*, *KLF9* and *IL18R1* (Figure 3.24B). Amongst the genes highly expressed in hepatocyte-like cells derived with Agarwal emphet al. protocol and cluster together with *CYP1B1* are *HOXC6* and *CD44* (Figure 3.24D).

3.8.1.5 Genomic data validation by RT-PCR

To validate the accuracy of the gene expression calculated from Illumina Bead Chip the expression levels of 13 genes were verified by real time RT-PCR (Table 3.9). Slight differences in the predicted fold change can be explained by the fact that Real-time RT-PCR is a more sensitive method for mRNA detection. The high degree of consistency with real time RT-PCR validations suggests that the data set is of high quality. Positive ratios indicate increased expression and negative ratios decreased expression. The super- and subscript numbers next to the ratios show the positive and negative standard deviations, respectively.

3.9 Sodium Butyrate (NaB) and its role in the differentiation protocol

Sodium Butyrate (NaB) under endoderm-stimuling conditions, selectively induced apoptosis of non-(endodermal) differentiating cells, and simultaneously promoted the survival of endodermal differentiating cells (Hay et al., 2008b). From a mechanistic standpoint, NaB has been shown to inhibit HDAC (histone deacethylase) activity. This leads to hyperacetylation of histones that results in chromatin remodeling and destabilization probably leading to transcription and subsequent alterations in gene expression. Detailed expression profiling analysis and comparison between NaB-treated samples, ActA-treated samples and both ActA and NaB treated samples revealed that there are 2488 genes significantly up-regulated (p-val<0.05, detection p-val<0.01, ratio>1.5) upon NaB treatment, 1033 genes up-regulated upon ActA treatment and 2480 genes up-regulated upon both ActA and NaB treatment (Figure 3.25).

Gene expression signature of ActA treated samples, NaB treated samples and the combined ActA and NaB treated samples were compared using Venn diagram analysis. This analysis revealed 481 genes common between all treatments and 74 genes common between ActA treated samples and NaB treated samples. There are 1224 common genes between NaB treated samples and the combined ActA and NaB treated samples. There are 177 genes common between ActA-treated cells and cells treated with both ActA and NaB. Further gene lists (2488 and 709) were employed to find potential GOs and pathways regulated in cells treated with NaB. Table 3.10 presents biological processes and pathways significantly regulated upon treatment. In addition similar analysis has been performed for subset of genes (709) exclusively expressed in NaB treated cells. Results of this analysis are presented in Table 3.11.

Table 3.9: Array data confirmation by Real time RT-PCR

		Ratio Hay et al.							
		ActA		NaB		ActA+NaB			
Name	**Definition**	**Array**	**PCR**	**Array**	**PCR**	**Array**	**PCR**
POU5F1	Homo sapiens POU domain, class 5, transcription factor 1	$-0.09^{+0.10}_{-0.10}$	$-0.51^{+0.07}_{-0.07}$	$-2.05^{+0.03}_{-0.03}$	$-0.48^{+0.36}_{-0.36}$	$-1.74^{+0.04}_{-0.04}$	$-1.37^{+0.14}_{-0.14}$
SOX2	Homo sapiens SRY (sex determining region Y)-box 2	$-0.39^{+0.08}_{-0.08}$	$-1.34^{+0.31}_{-0.31}$	$-2.65^{+0.02}_{-0.02}$	$-0.96^{+0.22}_{-0.22}$	$-2.13^{+0.02}_{-0.02}$	$-1.97^{+0.32}_{-0.32}$
GSC	Homo sapiens goosecoid homeobox	$1.24^{+0.23}_{-0.23}$	$5.18^{+0.06}_{-0.06}$	$-0.33^{+0.09}_{-0.09}$	$-1.97^{+0.12}_{-0.12}$	$2.12^{+0.45}_{-0.45}$	$6.11^{+0.24}_{-0.24}$
SOX17	Homo sapiens SRY (sex determining region Y)-box 17	$0.73^{+0.51}_{-0.51}$	$1.16^{+0.20}_{-0.20}$	$-0.09^{+0.26}_{-0.26}$	$-0.82^{+0.25}_{-0.25}$	$1.33^{+0.74}_{-0.74}$	$1.41^{+0.10}_{-0.10}$
FOXA2	Homo sapiens forkhead box A2	$0.36^{+0.06}_{-0.06}$	$0.46^{+0.08}_{-0.08}$	$0.26^{+0.05}_{-0.05}$	$-0.03^{+0.11}_{-0.11}$	$1.69^{+0.46}_{-0.46}$	$1.23^{+0.11}_{-0.11}$
GATA4	Homo sapiens GATA binding protein 4	$0.59^{+0.22}_{-0.22}$	$-0.15^{+0.14}_{-0.14}$	$0.62^{+0.21}_{-0.21}$	$-0.55^{+0.11}_{-0.11}$	$1.14^{+0.33}_{-0.33}$	$0.88^{+0.16}_{-0.16}$
GATA6	Homo sapiens GATA binding protein 6	$0.21^{+0.22}_{-0.22}$	$0.62^{+0.14}_{-0.14}$	$0.35^{+0.15}_{-0.15}$	$0.53^{+0.20}_{-0.20}$	$0.49^{+0.22}_{-0.22}$	$1.34^{+0.05}_{-0.05}$
HNF4A	Homo sapiens hepatocyte nuclear factor 4, alpha	$0.07^{+0.16}_{-0.16}$	$-1.50^{+0.12}_{-0.12}$	$0.26^{+0.04}_{-0.04}$	$0.90^{+0.66}_{-0.66}$	$0.14^{+0.10}_{-0.10}$	$1.20^{+0.21}_{-0.21}$
SOX7	Homo sapiens SRY (sex determining region Y)-box 7	$-0.02^{+0.04}_{-0.04}$	$-2.64^{+0.07}_{-0.07}$	$0.15^{+0.06}_{-0.06}$	$1.28^{+0.11}_{-0.11}$	$0.11^{+0.08}_{-0.08}$	$-1.61^{+0.10}_{-0.10}$
PAX6	Homo sapiens paired box 6	$0.86^{+0.21}_{-0.21}$	$2.66^{+0.27}_{-0.27}$	$-0.18^{+0.05}_{-0.05}$	$-1.69^{+0.57}_{-0.57}$	$0.58^{+0.09}_{-0.09}$	$2.19^{+0.12}_{-0.12}$

		DMSO		hESC-DH	
		Array	**PCR**	**Array**	**PCR**
SERPINA1	Homo sapiens serpin peptidase inhibitor, clade A member 1	$1.64^{+0.89}_{-0.89}$	$3.22^{+0.11}_{-0.11}$	$1.97^{+1.18}_{-1.18}$	$3.21^{+0.14}_{-0.14}$
AFP	Homo sapiens alpha-fetoprotein	$1.98^{+1.83}_{-1.83}$	$3.27^{+0.03}_{-0.03}$	$2.85^{+3.38}_{-3.38}$	$4.47^{+0.16}_{-0.16}$
TDO2	Homo sapiens tryptophan 2,3-dioxygenase	$0.02^{+0.09}_{-0.09}$	$1.10^{+0.13}_{-0.13}$	$0.01^{+0.08}_{-0.08}$	$2.56^{+0.25}_{-0.25}$

hESC-DH - human ES cells-derived hepatocyte-like cells

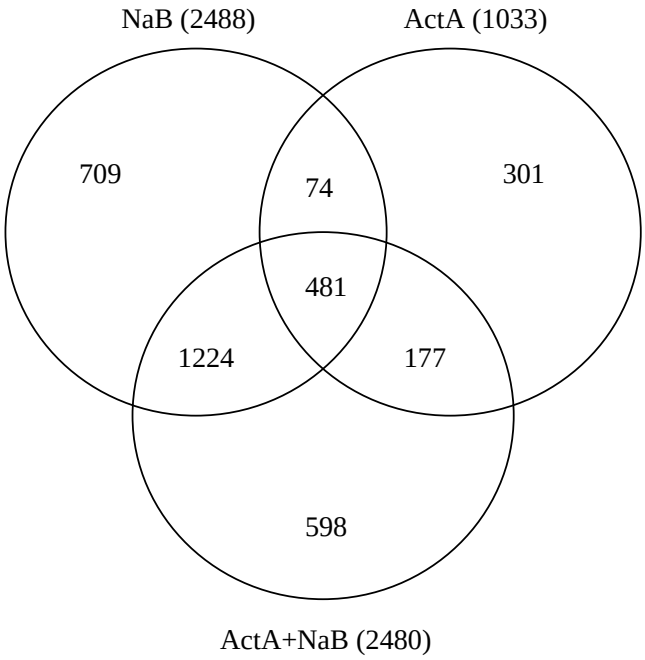


Figure 3.25: Venn diagram illustrating the overlap of common expressed genes between Activin A, sodium butyrate and both Activin A and sodium butyrate treated cells. The Venn diagram shows the overlap of target gene lists for Activin A and sodium butyrate treated cells.

3.10 Comparison with available data sets

There is one available expression profiling data set published in 2008 by Chiao *et al.* which describes expression profiling of hepatocyte-like cells derived from human ES cells. The cells were derived using EBs technique and AFP:eGFP reporter was applied to enrich for hepatic cell lineage. They found 609 genes as enriched in AFP:eGFP+ fractions (Chiao et al., 2008). Amongst these there were transcription factors and signalling molecules known to play significant role in liver development. Among the transcription factors *FOXA1*, *FOXA2*, *FOXA3*, *HNF4A*, *TCF2*, *PROX1*, and *CEBPA* were all enriched in the AFP:eGFP+ cells. Several genes involved in WNT/β-catenin signalling were found to be enriched in AFP:eGFP+ cells including *FZD5*, *DACT2* and *DKK3* as well as genes involved in signalling that have not previously been associated with liver development like *COBL1* and *GMCL1*. Among these genes was also present cell surface marker *CDH17* (cadherin 17) which is found in my data set in hepatocyte-like cells derived with Hay *et al.* protocol (p-val = 1.5E-11, detection p-val = 0, ratio = 12.71 fold). Other genes proposed by Chiao *et al.* that have not previously been identified as playing a role in hepatic development are *TDGF1*, *GRHL2*, *DACT2*, *NRG1*, *RA12*, *NTN4*, *KLF4* and *GMCL1*. These genes were found among genes expressed in human ES cells-derived hepatocyte-like cells.

Since advances have been made in deriving endoderm from human ES cells, yet a

Table 3.10: List of GOs and pathways enriched in cells treated with sodium butyrate (NaB) generated with 2488 significantly regulated genes

Term	Count	p-value
GO - BP		
organ development	263	2.77E-11
lipid metabolic process	160	2.94E-09
sterol metabolic process	32	7.31E-08
blood vessel development	55	1.55E-07
alcohol metabolic process	73	1.65E-06
cell adhesion	149	2.05E-06
amine metabolic process	91	2.82E-05
enzyme linked receptor protein signaling pathway	65	3.34E-05
lipid transport	30	6.91E-05
nitrogen compound metabolic process	94	1.09E-04
cell motility	86	1.69E-04
positive regulation of I-kappaB kinase/NF-kappaB cascade	27	4.31E-04
phosphate transport	25	4.89E-04
carboxylic acid metabolic process	101	1.03E-03
negative regulation of transcription, DNA-dependent	48	1.10E-03
negative regulation of transcription from RNA polymerase II promoter	36	1.63E-03
transmembrane receptor protein serine/threonine kinase signaling pathway	22	2.01E-03
PATHWAYS		
Biosynthesis of steroids	15	1.90E-06
ECM-receptor interaction	27	1.46E-03
Cell Communication	38	1.67E-03
Focal adhesion	51	1.91E-03
Acute Myocardial Infarction	8	2.12E-03
Cholera - Infection	16	2.80E-03
Extrinsic Prothrombin Activation Pathway	7	3.77E-03
Fatty acid	17	4.18E-03
Sphingolipid metabolism	15	4.69E-03
Cyanoamino acid metabolism	6	5.81E-03
PAR signaling pathway	22	5.91E-03

better understanding of definitive and extraembryonic endoderm is necessary. In 2007 Sherwood *et al.* performed microarray analysis of E8.25 mouse definitive and visceral endoderm. They developed an early endoderm gene expression signature and clarified the transcriptional similarities and differences between definitive and visceral endoderm. In this data set genes used as markers for endoderm in differentiated ES cells such as *MIXL1*, *GSC*, *T*, *CER1* and *HHEX* were not found to be expressed at higher levels in E8.25 definitive endoderm than in non-endodermal tissues (Sherwood et al., 2007).

The profiling of definitive and visceral endoderm gave me the opportunity to analyse the transcriptional regulatory similarities and differences between these tissues and endoderm derived from human ES cells. Efficient discrimination between definitive and visceral endoderm is a crucial and limiting step for further hepatic differentiation of human ES cells. In Sherwood *et al.* data set from a GO (gene ontology) search, only 4 transcription factors were expressed >2-fold in definitive endoderm and > 3-fold

Table 3.11: List of GOs and pathways enriched in cells treated with sodium butyrate (NaB) generated with 709 exclusively regulated genes

Term	Count	p-value
GO - BP		
lipid metabolic process	62	6.12E-08
organic acid metabolic process	43	3.42E-05
anatomical structure development	123	4.44E-05
amine metabolic process	32	1.27E-03
membrane lipid metabolic process	19	1.91E-03
nitrogen compound metabolic process	33	2.13E-03
developmental process	161	2.16E-03
glycosylceramide metabolic process	4	2.27E-03
steroid biosynthetic process	10	4.81E-03
fatty acid metabolic process	16	5.02E-03
glycolipid metabolic process	6	6.58E-03
embryonic limb morphogenesis	7	9.67E-03
embryonic appendage morphogenesis	7	9.67E-03
glucosylceramide metabolic process	3	9.97E-03
amino acid metabolic process	21	1.08E-02
embryonic development	22	1.09E-02
PATHWAYS		
Arachidonic acid metabolism	11	6.16E-04
Tight junction	15	8.52E-03
Taurine and hypotaurine metabolism	4	1.10E-02
Retinol metabolism	3	1.70E-02
Hypoxia-Inducible Factor in the Cardiovascular System	4	2.36E-02
Ether lipid metabolism	6	2.60E-02
Intrinsic Prothrombin Activation Pathway	4	2.85E-02
Sphingolipid metabolism	6	6.18E-02
Cyanoamino acid metabolism	3	6.85E-02
Extrinsic Prothrombin Activation Pathway	3	7.41E-02
Linoleic acid metabolism	5	8.93E-02

higher in visceral endoderm than any other tissue. These four transcription factors are *Sox17*, *FoxA1*, *Ripk4* (not essential for endoderm formation but is necessary for proper morphogenesis of the oral, nasal and esophageal endoderm) and *5730467H21Rik* (role unknown). The 10 transcription factors which were expressed > 2-fold higher in definitive endoderm than in any other tissue are involved in endoderm organogenesis. Four of the eight transcription factors that were expressed > 3-fold higher in visceral endoderm than in all other tissues (*Irf5*, *Nfact2*, *Npas2*, *Vdr*) were previously uncharacterized. Of the characterized genes, *Hnf4a* and *Tcf2* are necessary for visceral endoderm formation, *Cited1* is necessary for proper extraembryonic development and *FoxA3* is necessary for visceral endoderm formation.

In this publication comparison on transcription factors not exclusive to endoderm was performed to detect transcriptional differences between definitive and visceral endoderm. The results showed that transcription factors expressed > 3-fold higher in definitive than visceral endoderm but also expressed in non-endodermal tissues included 12 homeobox family transcription factors (*HoxA1*, *HoxA3*, *HoxA9*, *HoxB1*, *HoxB2*, *HoxB3*

HoxB9, *HoxC4*, *HoxC8*, *HoxD1*, *HoxD8*, *HoxD9*) and members of others homeobox families involved in embryonic patterning such as paired (*Pax1*, *Pax3*, *Pax6*, *Pax8*, *Pax9*), distal-less (*Dlx2*), Iroquois related (*Irx2*, *Irx3*, *Irx5*), sin oculis-related (*Six1*, *Six3*) and SRY-box con-taining (*Sox9*, *Sox11*, *Sox21*). Transcription factors expressed > 3-fold higher in visceral than definitive endoderm but also expressed in non-endodermal tissues included one homeobox transcription factor (*HoxB8*) but contained transcription factors such as *Gata4*, *Gata6*, *Lhx1*, and *Sox7* that are implicated in endoderm formation in lower vertebrates.

Table 3.12 presents expression ratios of genes described by Sherwood *et al.* as endoderm-enriched transcription factors in human ES cells-derived endoderm. Three of four pan-endodermal transcription factors are significantly up-regulated in Activin A treatments for both protocols as well as in Acitvin A and NaB treatment.

Table 3.12: List of endoderm-enriched transcription factors described by Sherwood *et al.* in E8.25 mouse definitive and visceral endoderm

Name	p-value (Hay)	p-value (Agarwal)	Ratio (Hay ActA/Contr.)	Ratio (Hay ActA+NaB/Contr.)	Ratio (Agarwal ActA/Contr.)
All endoderm enriched					
FOXA1	7.13E-09	7.52E-07	1.63	1.89	7.52
RIPK4	2.23E-05	1.06E-07	1.21	1.71	2.13
SOX17	2.90E-09	6.03E-07	1.82	3.09	19.53
Definitive endoderm enriched					
DLX3	3.12E-08	2.90E-02	1.09	1.32	0.83
DLX5	6.44E-09	1.91E-08	0.93	2.01	4.78
FOXG1B	4.14E-01	5.46E-01	1.06	0.21	1.26
GATA3	2.34E-11	2.80E-05	0.67	0.87	3.01
ID4	9.64E-02	1.73E-01	0.31	0.20	-7.97
PAX9	4.27E-02	3.24E-01	-2.45	-1.03	1.63
SP6	2.87E-04	2.36E-02	1.34	1.84	1.44
TP73L	6.43E-08	7.20E-04	9.37	10.70	2.46
Visceral endoderm enriched					
CITED1	4.65E-02	6.26E-05	3.65	2.67	0.43
FOXA3	2.56E-09	1.39E-11	0.80	0.61	1.41
HNF4A	2.69E-01	5.32E-02	2.41	1.90	6.46
IRF6	3.25E-03	3.19E-05	1.63	2.15	2.26
NFATC2	5.36E-02	3.07E-02	-2.87	-2.74	5.71
NPAS2	5.30E-02	2.27E-01	2.44	1.70	1.22
TCF2	1.46E-02	5.17E-02	-1.42	-0.78	-0.05
VDR	2.62E-02	2.42E-02	1.95	1.40	-0.28

In accordance with Sherwood *et al.* data set the six (*CITED1*, *FOXA3*, *IFR6*, *NFATC2*, *TCF2*, *VDR*) of eight genes described as visceral endoderm enriched are significantly regulated in my data set. Unfortunately too high detection p-values do not allow to consider them as truly significantly regulated (Table 3.12). Genes enriched in definitive endoderm as well are difficult to consider because of high detection p-values. The four of eight genes are significantly regulated upon Activin A treatment in both protocols. In Hay *et al.* protocol two of them are up-regulated (1.9-3.6 fold)

and in Agarwal *et al.* protocol three of them are up-regulated (1.4-2.6 fold). For both protocols there are two genes down-regulated (Table 3.12).

Among genes described by Sherwood *et al.* as non-endoderm enriched transcription factors that distinguish definitive and visceral endoderm and are enriched in definitive endoderm, in my data set generated with differentiated human ES cells there are 10 genes up-regulated (> 1.5 fold) in Hay *et al.* protocol and 14 in Agarwal *et al.* protocol (Table 3.13).

Table 3.13: List of non-endoderm enriched transcription factors described by Sherwood *et al.* that distinguish definitive and visceral endoderm and are enriched in definitive endoderm

Name	p-value (Hay)	p-value (Agarwal)	Ratio (Hay ActA/Contr.)	Ratio (Hay ActA+NaB/Contr.)	Ratio (Agarwal ActA/Contr.)
ARNT2	7.31E-08	2.22E-09	0.95	0.40	0.87
CDX4	5.06E-05	2.04E-03	1.04	0.72	0.29
CUTL2	4.80E-06	7.76E-07	0.76	0.62	0.69
DLX2	2.63E-03	9.59E-02	-10.09	-4.33	1.65
DMRTA1	3.76E-05	3.28E-06	1.23	1.38	0.71
EVX1	5.31E-01	3.28E-01	1.74	1.83	0.74
FOXC1	2.50E-07	2.34E-05	0.96	1.19	5.32
FOXC2	1.69E-02	9.73E-02	1.56	1.96	1.35
GLI3	3.50E-04	4.15E-07	1.01	1.03	0.86
HEY2	9.30E-10	3.14E-09	0.75	0.36	0.16
HOXA1	3.56E-02	4.76E-02	3.05	4.13	1.02
HOXA3	7.97E-02	3.46E-04	5.04	6.52	4.81
HOXA9	3.22E-10	1.52E-10	0.75	3.07	1.31
HOXB1	9.84E-02	7.17E-03	1.27	1.43	1.33
HOXB2	4.03E-11	1.21E-10	0.57	0.67	0.41
HOXB3	5.24E-04	2.25E-09	2.99	3.35	0.41
HOXB9	1.36E-02	7.37E-06	-15.07	-41.60	1.44
HOXC4	3.81E-08	1.20E-08	1.21	3.24	1.48
HOXC8	2.56E-12	5.36E-09	0.99	1.75	0.56
HOXD1	9.39E-05	7.43E-10	-4.35	-5.37	10.00
HOXD8	4.90E-04	1.75E-01	1.57	2.55	2.33
HOXD9	5.38E-02	7.91E-02	-2.76	-1.52	9.58
IRX2	3.06E-09	2.16E-09	0.62	0.19	0.16
IRX3	2.09E-09	3.01E-07	0.52	0.41	1.68
IRX5	1.69E-02	7.20E-09	-11.48	-9.41	3.47
MEOX1	1.62E-04	7.02E-03	1.25	3.16	6.55
MORF4L1	3.08E-06	3.23E-04	7.45	16.47	1.17
CITED2	1.69E-06	1.36E-05	0.58	1.54	3.17
PAX1	2.72E-02	1.39E-01	1.40	0.33	1.29
PAX3	6.32E-01	1.57E-03	3.11	-1.80	0.17
PAX6	1.05E-08	8.62E-06	13.34	9.67	-81.55
PAX8	5.83E-03	3.02E-03	2.95	4.30	2.27
PBX1	4.99E-09	3.75E-09	0.57	0.33	0.39
PKNOX1	3.60E-05	2.18E-03	3.32	2.66	1.94
RFX3	9.51E-03	3.04E-03	0.90	0.75	0.78
SIX1	5.22E-01	5.38E-05	-0.92	0.01	3.14
SIX3	5.75E-01	2.92E-03	1.29	1.56	1.89
SOX11	3.19E-10	2.88E-10	1.17	1.11	1.31
SOX21	7.14E-14	5.18E-07	1.57	5.57	1.03
SOX9	2.19E-08	1.05E-09	1.20	3.68	0.78
SSBP2	9.51E-07	4.15E-09	0.71	0.54	0.53
T	1.13E-05	3.25E-09	1.05	3.24	0.43
ZHX2	6.09E-06	1.30E-09	0.94	0.77	1.73

In this same category but genes enriched in visceral endoderm there are 24 genes. In human ES cells derived endoderm, I could find 7 genes (> 1.5 fold) for Hay *et al.* protocol and 10 for Agarwal *et al.* protocol (Table 3.14).

Based on these results one obvious conclusion is that human ES cells-derived endoderm is a mixed population of both definitive and visceral endoderm cells if we consider as a reference mouse E8.25 tissue.

Table 3.14: List of non-endoderm enriched transcription factors described by Sherwood *et al.* that distinguish definitive and visceral endoderm and are enriched in visceral endoderm

Name	p-value (Hay)	p-value (Agarwal)	Ratio (Hay ActA/Contr.)	Ratio (Hay ActA+NaB/Contr.)	Ratio (Agarwal ActA/Contr.)
ASB8	6.76E-07	1.05E-04	0.97	0.97	1.22
ATP6V0A1	2.53E-08	6.75E-10	0.90	1.36	1.23
EHF	4.63E-02	2.38E-01	3.84	3.43	1.12
ELF1	8.77E-13	2.15E-09	1.18	1.73	1.84
FOXF1	7.91E-08	6.26E-01	0.69	1.24	0.99
FOXQ1	5.81E-13	4.32E-13	2.60	5.44	7.42
GATA4	3.11E-09	1.87E-08	1.92	3.40	6.45
GATA6	3.81E-03	9.56E-09	3.07	5.68	16.53
HOXB8	2.60E-11	1.64E-08	0.67	1.25	0.89
IPF1	1.17E-02	4.06E-04	-2.04	-5.24	2.03
IRF2BP2	2.34E-05	3.09E-06	0.62	0.81	0.78
LASS2	3.83E-12	1.65E-12	1.18	0.96	0.91
LHX1	1.40E-07	2.40E-10	1.28	1.78	9.27
NFATC1	1.90E-07	2.12E-08	0.82	1.90	2.53
NFIX	7.63E-13	1.84E-08	-0.86	-2.74	6.03
PCBD1	4.36E-09	7.54E-09	0.93	0.88	1.69
PPARGC1A	1.95E-07	3.17E-05	1.36	2.16	1.30
RUNX1	1.92E-08	4.45E-05	2.95	2.06	2.54
SOX7	5.03E-05	8.57E-03	3.15	5.68	1.39
STAT5A	9.84E-12	1.40E-08	0.46	0.27	0.38
STAT5B	2.89E-08	4.18E-06	0.79	0.47	0.83
TCF19	1.11E-01	5.62E-02	5.70	3.57	0.85
TFEC	4.11E-01	3.62E-01	-1.30	-1.10	-12.82
TWIST1	1.37E-08	1.52E-05	1.31	2.10	1.01

4

Discussion

4.1 Differentiation protocols imitate hepatogenesis *in vivo*

The three described multistep protocols for the efficient derivation of hepatocyte-like cells from human ES cells resemble the progressive specification of definitive endoderm (DE) and hepatocytes during mammalian development and leads to a synchronous population of hepatocyte-like cells. Initially, pluripotent human ES cells were induced to generate DE (D'Amour et al., 2005). The cells I have obtained were positive for *SOX17*, *FOXA2*, *GATA4* and negative for *SOX7*. The induction of brachyury (*T*) (expressed in definitive but not primitive endoderm), excluded the possibility of generation of primitive endoderm in these conditions. The sequential application of a suitable mixture of hepatic development-based inducers, in suitable medium formulations and stages, resulted in the coordinated differentiation of the cells which exhibited a temporal regulation of hepatic stage specific gene expression. The majority of the cells at the end of differentiation protocol stayed largely homogeneous, resembled hepatocytes morphologically, expressed a set of genes present in mature (*GATA4*, *HNF4A*, *A1AT*, *TDO2*) and fetal (*AFP*) hepatocytes and displayed hepatocyte-like functions (Figure 3.7, 3.10, 3.11, 3.14, 3.18). The expression of other mature hepatocyte markers, like ALB and TAT has been investigated at the mRNA level and appeared not to be significantly elevated in the Hay *et al.* and Agarwal *et al.* protocols. Moreover the expression of the cytochrome P450 enzymes was of particular interest. CYP3A4 is the most abundant CYP protein in the human liver and involved in the metabolism of a large percent of current pharmaceutical drugs (Ek et al., 2007) and CYP3A7 is the major cytochrome in fetal liver. Both of these genes could not be detected at the mRNA level in human ES cells-derived hepatocyte-like cells (hESC-DH). The presence of drug-metabolizing enzymes in human ES cells-derived hepatocyte-like cells is critical to their application in drug metabolism and hepatotoxicity screens *in vitro* (Soderdahl et al., 2007).

The remarkable persistence of homogeneity in the culture implies the coordinated stimulation of signaling pathways in the cells since the cells grown in monolayer are uniformly exposed to inducing factors. The selection of inducing factors is mainly directed by their known roles in hepatic development. The sequential treatment of cells is crucial for highly efficient hepatic differentiation. As has been reported, FGFs could replace cardiac mesoderm (Jung et al., 1999) for liver initiation in from foregut endo-

derm. BMPs, working simultaneously with FGF, were also found to be critical for the morphogenetic growth of the hepatic endoderm (Rossi et al., 2001). Since there is an obvious difference between *in vivo* and *in vitro* conditions, it is sometimes more effective to change the expected order. BMP4 is the best example, since is not required for human ES cells differentiation into hepatocytes (Agarwal et al., 2008) and result in increasing the heterogeneity in the cultures. As BMP4 was reported to be essential for the generation of hepatocytes from mouse ES cells it could reflect species difference and substantial technical differences in the EBs-based approach, or the compensation for added BMP4 by other factors such as HGF. It was shown in mutant mouse liver explants (impaired in the BMP4 superfamily, TGFβ signaling) that HGF could supply equivalent signals to TGFβ pathway (Weinstein et al., 2001).

These systems of hepatic differentiation, with its progression through different stages of lineage commitment, could a potent tool to analyze molecular pathways involved at each stage.

Employing the Activin A priming protocol a significant increase in the number of hepatocyte-like cells was observed. The results indicate that gene expression in the *in vitro* differentiation process from human ES cells to hepatocytes recapitulates liver *in vivo*. In stage 1, human ES cells were altered to the definitive endoderm cells through the cells resembling primitive streak mesendoderm. This was characterized by the gradual down-regulation of ES markers, such as *OCT4* and *NANOG* and up-regulation of differentiation markers *GSC*, *SOX17* and *FOXA2* expression. During stage 2 of differentiation, the DE cells further differentiate to hepatocyte progenitor cells as shown by the persistent up-regulation of *HNF4A*, *AFP* and *A1AT*. Finally at stage 3 of differentiation, the hepatocyte progenitor cells matured further as shown by the increased expression of *TDO2* and *A1AT*.

It is important of this point to underline that endoderm-associated genes (e.g. *SOX17* and *FOXA2*) (Hay et al., 2007, 2008b) are often found to be present on protein level to varying levels in undifferentiated human ES cells cultured in CM (Figure 4.8 and Figure 4.16).

4.2 Epigenetic modifications during human ES cells differentiation into hepatocyte-like cells

Positive and negative regulation of eukaryotic transcription has been shown to be mediated in part by two opposing types of enzymatic activities, histone acetylases (HATs) and histone deacetylases (HDACs). While HATs and histone deacethylases inhibitors (HDACi) are associated with activation of gene transcription, HDACs mediate transcriptional repression. The genuine proposal of changing cell fate through direct interference with the local chromatin structure of plastic cells only became introduced a few years ago. In 2003 Rambhatla *et al.* published that exposure of human ES cells to 5mM sodium butyrate (NaB) allow to 10-15% enrichment with pure hepatic cells. In 2007

Zhou *et al.* published differentiation of hepatic progenitor cells from mouse ES cells induced by sodium butyrate (Zhou et al., 2007).

The role of epigenetic modifiers, particularly HDACi (histone deacetylase inhibitor), in mediating differentiation of human ES cells is still elusive. Although it is the fact that the covalent histone modification is central in processes determining lineage-specific gene expression and cell fate determination. In general, epigenetic modifiers affect a broad variety of cellular processes, including cell cycle, differentiation and apoptosis.

The combination of ActA and NaB treatment directs human ES cells more efficiently after further differentiation to hepatocytes than ActA alone. NaB is a short-chain fatty acid, which is histone deacetylase inhibitor (HDACi) and has been reported to induce growth arrest, differentiation and apoptosis in a number of cancer cells (Shahbazian and Grunstein, 2007). HDACs catalyze the removal of acetyl groups from the histones, thereby mediate condensation of nucleosomes. HDAC inhibitors (e.g. NaB) promote histone acetylation, relax the DNA wrapped around the core histones, and permit transcription of genes. NaB treatment results in significant cell death so possibly may function as a selecting reagent to reduce number of cells that have not differentiated. Essentially, mixed application of epigenetic modification and stepwise introduction of cytokine stimuli significantly contribute to homogeneity of the endpopulation and acquirement of hepatic functionality (Hay et al., 2008b). Analysis of GOs and pathways in NaB treated cells revealed that genes significantly regulated are enriched is such biological processes as organ development (263), lipid metabolic process (160), cell adhesion (149) and negative regulation of transcription (48). Some of the transcriptional targets regulated by NaB are known. NaB activates the transcription of p21(CIP/WAF1) by increasing the acetylation of histone H3 and H4 and eventually blocked the cell cycle at G2/M phase. Activation of the NHE3 gene promoter by NaB depends on the activity of Ser/Thr kinases, in particular protein kinase A (PKA) (Kiela et al., 2007) and transmembrane receptor protein Ser/Thr kinase signaling pathway components are enriched upon NaB treatment in my data set.

Despite the extensive knowledge on histone acetylation, the relationship between butyrate and transcriptional activation remains relatively unclear. In microarray analyses of colon epithelium, butyrate modulated expression of a significantly larger fraction of cellular genes than a specific HDAC inhibitor, Trichostatin A (TSA). These observations suggest that butyrate has multiple mechanisms of action involving more than just the inhibition of histone deacetylation traditionally ascribed to it (Shahbazian and Grunstein, 2007). Among biological processes exclusively enriched in NaB treated cells are lipid metabolic process (62), organic acid metabolic process (43), anatomical structure development (123) and embryonic development (22). Among pathways significantly regulated are arachidonic acid metabolism (11), tight junction (15) and taurine metabolism (4).

Arachidonic acid is a polyunsaturated fatty acid that is present in the phospholipids (especially phosphatidylethanolamine, phosphatidylcholine and phosphatidylinositides)

of membranes of the body's cells, and is abundant in the brain and muscles. It is known that phospholipid metabolism is altered in NaB treated C6 glioma cells. Upon treatment (i) increased arachidonic acid incorporation into phosphatidylcholine, and (ii) decreased arachidonic acid incorporation into phosphatidylinositol and (iii) phosphatidylethanolamine have been observed. As well inositol triphosphate (IP3) was decreased in these cells. The decreased IP3 formation and its subsequent action, i.e., Ca^2+ mobilization, may play an early but pivotal role by which sodium butyrate induces C6 glioma cell differentiation (Sun et al., 1997). Based on these evidences and results obtained in my data set the most probable mechanism responsible for cell death and enhanced differentiation of human ES cells is connected to lipid metabolic processes and signal transduction by secondary messengers like diacylglycerol (DAG), phosphatidylinositol (PI) and inositol triphosphate (IP3).

4.3 Maturation state of human ES cells-derived hepatocyte-like cells

Even though the hESC-DH described here exhibit some characteristics of mature hepatocytes (gene expression/protein profile and hepatic functions), it should be noted that they also appear to maintain some immature characteristics such as relatively low levels of expression of the cytochrome P450 transcripts and continued expression of AFP, a marker of fetal rather than adult hepatocytes. Further investigation is required to examine whether hepatocytes derived *in vitro* from human ES cells can be matured further in culture (with additional key inducing factors and/or more time) and whether the final maturing steps require an in vivo environment.

Human ES cells-derived hepatic-like cells express various adult liver markers, like *A1AT* and *TDO2*. Furthermore, these cells possess liver-specific functions, such as glycogen storage and ICG uptake (Figure 4.18). Due to the fact that the single functional test may not be uniquely specific to liver cells (for example, ICG could be taken up by liver cells, trophoblast cells; glycogen could be detected in liver and muscle) we combined these complementary functional tests to characterize the differentiated cells. Certain markers such as albumin are relatively easy to induce. In contrast, the induction of other factors, such as CYP3A is more difficult.

To be able to use human ES cells-derived hepatocytes for drug screening, further improvements are required, particularly on maturation. It remains a challenge how to further mature human ES cells-derived hepatocytes. Liver development *in vivo* take place in a 3-dimensional (3D) environment but *in vitro* culture is 2-dimensional (2D). Even though 2D system may give support to efficient differentiation as cells receive more synchronous induction, the 3D culture environment promotes cell-cell interactions, which may further enhance maturation. In addition, the current culture conditions may not be optimal for further hepatocytes maturation. Further improvement of the culture conditions may help further maturation and enhance functionality. In conclusion, with these multiple standards, the induced differentiated cells were similar to the adult only

in certain extent.

4.4 Global gene expression profiling approach as a tool to trace dynamic changes during the process of hepatic differentiation

Transcriptome analysis is a powerful tool to evaluate the molecular phenotype and developmental status of human ES cells-derived cell types. Comparing the gene expression pattern of somatic cells generated in tissue culture with their counterparts in developing organs also interlinks *in vitro* and *in vivo* differentiation. Published in 2008 by Ciao *et al.* expression profiling of AFP:eGFP positive cells generated with EBs technology demonstrated that the population is highly enriched for genes characteristic of hepatic cells. The authors claimed that following this differentiation protocol purified AFP:eGFP positive cells express genes found in all three early hepatic cell types (hepatoblast, hepatocytes and cholangiocytes). Moreover they conclude that this system may be used to analyse genetic programs regulating the differentiation of the bipotential hepatoblast into either hepatocytes or cholangiocytes. The EB/acidic FGF differentiation protocol results in multilayered cells with a wide spectrum of cellular morphologies.

There remains the question if the hepatocytes generated in the complex cellular environment are different from the monolayer differentiation protocol. To try to answer this question on each step of differentiation for both protocols I provide lists of genes that are significantly up-regulated and cluster together with genes known to play significant role in liver development (Figure 3.22, Figure 3.23, Figure 3.24). Many genes that have not been previously identified as playing a role in hepatic development cluster together with genes known to be involved in this process and therefore deserve further investigation. In addition genes described by Chiao *et al.* that have not previously been identified as playing role in hepatic development (*TDGF1*, *GRHL2*, *DACT2*, *NRG1*, *RA12*, *NTN4*, *KLF4*, *GMCL1*) were found among genes expressed in human ES cells-derived hepatocyte-like cells.

To investigate in details endoderm differentiation step the comparison with data set generated with E8.25 mouse definitive and visceral endoderm has been done. Reported differentiation of human ES cells into definitive endoderm rely on the expression of a relatively small number of markers genes. That is why comparison with Sherwood et al. data set has been crucial for investigation a status of differentiated cells. Comparison of genes expressed in mouse definitive and visceral endoderm and in definitive endoderm derived from human ES cells gives insight into similarities and differences between in vivo and in vitro systems (Table 3.12, Table 3.13 and Table 3.14). Detailed comparison with mouse definitive and visceral endoderm reveal that both cell types are generated and it is difficult to describe the final population. Surely there is some species difference in generation of endoderm even though the Nodal signalling pathway is highly conserved.

In this work I have investigated in details the expression of cell surface proteins. The presence of *OSMR* and *IL6ST* (both Oncostatin M receptors) proof that cells indeed

responded to OSM stimualtion present in differentiation media. The occurance of genes expressed in fetal and adult liver such as *ANXA1*, *ECGF1*, *TNFRSF14* (Herpesvirus entry mediator A) additionally confirm the hepatocyte-like fate of cells. As well *CDH17*, that has been proposed together with *HAVCR1* as potential candidates to help further purify and characterize the hepatic cell type by Ciao *et al.*, is highly expressed in my data set in human ES cells-derived hepatocyte-like cells. Since to date only one publication (Chiao et al., 2008) have employed gene expression analysis of hepatocyte-like cells derived from human ES cells. In that case my results presenting the GOs, pathways and tissue signature analysis can be directly compared only with publications considering fetal and adult liver. Among biological processess regulated in hepatocyte-like cells derived with Hay *et al.* protocol are organ development (268), cell proliferation (172) and response to external stimulus (135). Among highly regulated pathways are focal adhesion (61), ECM-receptor interaction (33) and complement and coagulation cascades (23). In coagulation cascade serpins (*SERPINA1*, *SERPIND1*, *SERPINA5*, *SERPINC1*, *SERPIND1*) and macroglobulin (*A2M*) produced by liver are the most important components. The tissue expression signature revealed that signature of the pancreas is the highest enriched.

In hepatocyte-like cells derived with the Agarwal *et al.* protocol highly enriched biological processess are nervous system development (250), developmental process (688) and anatomical structure morphogenesis (250). Since developmental processes leading to differentiation into different germ layers and tissues possess common subset of regulators this can be the reason of enrichment in nervous system development. The most significantly regulated pathway is biosynthesis of steroids (14) which take place in liver. The fetal liver and appendix expression signature is highly elevated in hepatocyte-like cell derived wit Agarwal *et al.* protocol. The only common pathway between fetal liver and hepatocyte-like cells derived with Hay *et al.* protocol is complement and coagulation cascades. Biosynthesis of steroids and polyunsaturated fatty acid biosynthesis are common pathways between fetal liver and hepatocyte-like cells derived with Agarwal *et al.* protocol.

In summary, the Agarwal *et al.* protocol, which employed fully defined conditions (including extracellular matrix, collagen I) seem to induce cells that are closer to fetal hepatocytes on transcription level. Even though Hay *et al.* protocol engaged sodium butyrate and DMSO (chemicals which role and mechanism of action are not fully understand), the efficiency is comparable with Agarwal *et al.* protocol and resulted hepatocyte-like cells seem to be at similar stage of maturation.

5

Conclusion

The studies conducted to investigate the differentiation of human ES cells to hepatocyte lineage confirmed that at the transcriptional level human ES cells-derived hepatocyte-like cells have some similarities to fetal liver. However in some aspects a distinct pattern was observed. The hepatocyte-like cells obtained with different protocols exhibit some characteristic of mature hepatocytes but they also appear to maintain some immature characteristic such as relatively low levels of expression of the cytochrome P450 and continued expression of AFP. In addition tracing of changes in gene expression during the entire process of *in vitro* differentiation identified many new target genes (cell surface receptors, transcriptional regulators) and associated signaling pathways that might be involved in hepatogenesis *in vivo*.

Furthermore, the analysis of the differentiation time-course on the transcriptional level and comparison with available data set (mouse E8.25 definitive and visceral endoderm) revealed that endoderm obtained during Activin A treatment is a mixture of definitive and visceral endoderm even though the expression of *SOX7* is low.

As well the investigation of sodium butyrate (NaB) function in the differentiation protocol at the stage of endoderm induction confirmed its role in inducing apoptosis and differentiation through changes in phospholipids metabolism as the arachidonic acid metabolism pathway is significantly regulated in human ES cells upon NaB treatment.

In addition, the results demonstrate that:

- Sequential addition of growth factors during the differentiation process enable tracking the changes in gene expression.
- Gradual morphological and phenotypic alterations correspond to the sequential regulation of genes important for the subsequent stages of differentiation.
- Clear downregulation of pluripotency factors (*NANOG*, *SOX2*, *POU5F1*) was observed in both protocols upon Activin A treatment.
- Activin A induced expression of genes crucial for definitive endoderm (*FOXA2*, *GSC*, *HHEX*, *GATA4*, *NODAL*, *CXCR4*, *SOX17*, *CER1*).
- Succesive acquisition of hepatic fate was evident by the upregulation of genes important for hepatic functions (*CYP1B1*, *AFP*, *TTR*, *APOA4*, *FGA*, *CYP1A1*, *CEBPA*).

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- **Jozefczuk J**, Stachelscheid H, Chavez L, Herwig R, Lehrach H, Zeilinger K, Gerlach JC, Adjaye J. Molecular characterization of cultured adult human liver progenitor cells. *Tissue Engeenering Part C Methods*, Epub 2009 Oct 29.
- **Jozefczuk J**, Chavez L, Herwig R, Lehrach H, Adjaye J. Analysis of dynamic regulatory events during human Embryonic Stem Cells differentiation into hepatocytes (in preparation).

Curriculum Vitae

For reasons of data protection, the curriculum vitae is not included in the online version

A

Supplementary protocols

A.1 MEF culture medium (components to make 500ml of media)

450ml of DMEM (High Glucose, Gibco, Invitrogen)
50ml of FBS (10% (v/v), Biochrom AG, Berlin)
5ml of 200mM L-glutamine (1/100 (v/v), Gibco, Invitrogen)
5ml of Penicillin-Streptomycin (1/100 (v/v), Gibco, Invitrogen)
Mix all components and filter (Corning, 0.22μM, PAS)

A.2 MEFs freezing media

10% of DMSO (Sigma)
40% of DMEM (High Glucose, Gibco, Invitrogen)
50% of FBS (Biochrom AG, Berlin)

A.3 Human Embryonic Stem Cells Media (UM, unconditioned medium)

400ml of Knockout^{TM} DMEM (Gibco, Invitrogen)
100ml of Knockout^{TM} Serum Replacement (20% (v/v), Gibco, Invitrogen)
5ml of 200mM L-glutamine (1/100 (v/v), Gibco, Invitrogen)
5ml of Penicillin-Streptomycin (1/100 (v/v), Gibco, Invitrogen)
5ml of Non-Essential Amino Acids (1/100 (v/v), Gibco, Invitrogen)
35μl of 0.14 M β-Mercaptoethanol (Sigma), diluted before 1:10 in PBS (filter)
Mix all components and filter (Corning, 0.22μM, PAS)

A.4 Human ES cells freezing media

10% of DMSO (Sigma)
40% of Knockout^{TM} DMEM (Gibco, Invitrogen)
50% of Knockout^{TM}Serum Replacement (Gibco, Invitrogen)

A.5 Dispase solution

Dispase (5g, Gibco, Invitrogen) was dissolved in 10ml of Knockout^{TM} DMEM (Gibco, Invitrogen) and placed in water bath for 15 minutes to dissolve completely before filtration. Then solution was filtered (0.2μM Acrodisc® Syringe Filters, Pall Corporation) and 500μl (100x) aliquots were frozen in $-20°$C. Working solution was prepared by dissolving 500μl in 50 ml of Knockout^{TM} DMEM and aliquots were placed into $-20°$C.

A.6 β-Mercaptoethanol solution for hESCs media

14.3M β-Mercaptoethanol (Sigma) was diluted 1:10 in PBS, filtered and stored at $-20°$C in 40μl aliquots. Aliquots were thawed and used immediately.

A.7 HepG2 and HFF culture medium

450ml of DMEM (high glucose, Gibco, Invitrogen)
50ml of FBS (10% (v/v), Biochrom AG, Berlin)
5ml of 200mM L-glutamine (1/100 (v/v), Gibco, Invitrogen)
5ml of Penicillin-streptomycin (1/100 (v/v), Gibco, Invitrogen)

A.8 Defined (N2B27) culture media to growth hESCs (components to make 50ml)

47ml of DMEM/F12 (Hyclone)
0.5ml of N2 Supplement (100x, Invitrogen)
1ml of B27 Supplement minus Vitamin A (50x, Invitrogen)
340μl of BSA (Bovine Albumin Fraction V, 7.5%, Gibco, Invitrogen)
0.5ml of 200mM L-glutamine (1/100 (v/v), Gibco, Invitrogen)
0.5ml of Penicillin-Streptomycin (1/100 (v/v), Gibco, Invitrogen)
0.5ml of Non-Essential Amino Acids (1/100 (v/v), Gibco, Invitrogen)
3.5μl of 0.14 M β-Mercaptoethanol (Sigma), diluted before1:10 in PBS (filter)
Mix all components and filter (Corning, 0.22μM, PAS)

A.9 Buffers for SDS-PAGE gel electrophoresis

Resolving Buffer	
1.5M Tris-HCl pH 8.8:	180g Tris base (121g/mol)
	ad 900ml dH_2O
	adjust pH8.8 with 37% HCl (approx. 26ml)
	ad 1000ml dH_2O

Stacking Buffer

0.5M Tris-HCl pH 6.8:	60g Tris base (121g/mol)
	ad 900ml dH_2O
	adjust pH6.8 with 37% HCl (approx. 47ml)
	ad 1000ml dH_2O

3x Loading Buffer (Sample Buffer)

3x SDS-PAGE SB:	9.375ml Stacking Buffer
	17.2ml 87% glycerol
	15ml 10% SDS
	some grains bromophenol blue
	ad 47.5ml dH_2O
	store at RT
working solution:	950μl (47.5ml) 3x Loading Buffer
	50μl (2.5ml) β-Mercaptoethanol
	store at $-20°C$

10x Running Buffer

10x SDS-PAGE RB:	250mM Tris base (121g/mol) $\rightarrow$ 30.3g
	1.92M Glycine (75g/mol) $\rightarrow$ 144.1g
	100ml 10% SDS
	ad 1000ml dH_2O

A.10 Buffers for Western blotting

10x Transferbuffer:	250mM Tris base (121g/mol) $\rightarrow$ 30.3g
	1.92M Glycine (75g/mol) $\rightarrow$ 144.1g
	ad 1000ml dH_2O
1x Transferbuffer:	100ml 10x Transferbuffer
	200ml Methanol
	ad 1000ml dH_2O
1M Tris-HCl pH7.6:	120g Tris base (121g/mol)
	ad 900ml dH_2O
	adjust pH7.6 with 37% HCl
	ad 1000ml dH_2O

1x TBS:	8g Sodium Chloride 20ml 1M Tris-HCl pH7.6 ad 1000ml dH_2O
1x TBST:	1000 ml 1x TBS 1 ml Tween 20
Blocking solution:	3% milk powder → 1.5g ad 50ml 1x TBST

B

Supplementary tables

Table B.1: List of overlapping GOs and pathways in hepatocyte-like cells derived with Hay *et al.* protocol and fetal liver

Term	Count	p-value
GO - BP		
aromatic amino acid family catabolic process	4	5.43E-03
acute inflammatory response	8	5.67E-03
nitrogen compound metabolic process	26	5.70E-03
amino acid and derivative metabolic process	21	6.49E-03
positive regulation of cell proliferation	16	6.55E-03
catecholamine metabolic process	5	7.10E-03
programmed cell death	38	7.54E-03
phenol metabolic process	5	7.81E-03
positive regulation of cell proliferation	16	8.40E-03
response to unfolded protein	8	8.89E-03
response to protein stimulus	8	9.11E-03

Table B.2: List of GOs enriched in hepatocyte-like cells derived with Hay *et al.* protocol

Term	Count	p-value
GO - BP		
protein kinase cascade	89	2.68E-06
anatomical structure morphogenesis	210	3.58E-06
response to wounding	93	6.62E-06
organ morphogenesis	91	8.25E-06
inorganic anion transport	43	9.23E-06
hemostasis	31	9.47E-06
cell motility	91	1.02E-05
localization of cell	91	1.27E-05
cell-substrate adhesion	29	1.53E-05
negative regulation of cell proliferation	59	1.93E-05
cell-matrix adhesion	28	1.93E-05
intracellular signaling cascade	255	2.45E-05
anatomical structure formation	47	2.83E-05
cell development	223	2.84E-05
angiogenesis	40	2.88E-05
cell development	223	2.98E-05
angiogenesis	40	3.05E-05
apoptosis	149	3.19E-05
anatomical structure formation	47	3.32E-05
GO - CC		
vesicle membrane	30	3.28E-03
myofibril	17	4.78E-03
vacuole	45	5.16E-03
cytoskeleton	174	5.74E-03
adherens junction	16	6.88E-03
coated vesicle membrane	16	6.88E-03
COPI coated vesicle membrane	6	7.82E-03
cell-matrix junction	12	9.21E-03
sarcomere	15	9.90E-03
GO - MF		
enzyme inhibitor activity	52	5.15E-03
oxidoreductase activity	28	5.27E-03
serine-type endopeptidase inhibitor activity	23	5.80E-03
phospholipid binding	50	6.68E-03
transferase activity, transferring hexosyl groups	38	6.88E-03
phosphotransferase activity, alcohol group as acceptor	122	7.03E-03

Table B.3: List of GOs enriched in hepatocyte-like cells derived with Agarwal *et al.* protocol

Term	Count	p-value
GO - BP		
negative regulation of metabolic process	105	1.04E-04
cell migration	70	1.07E-04
cell differentiation	364	1.18E-04
cell cycle	189	1.36E-04
sterol biosynthetic process	16	1.86E-04
negative regulation of nucleobase	82	1.98E-04
cell motility	99	2.22E-04
localization of cell	99	2.34E-04
transmission of nerve impulse	84	4.99E-04
heart development	37	7.43E-04
regulation of cell differentiation	43	1.19E-03
synaptic transmission	73	1.41E-03
forebrain development	20	1.61E-03
muscle development	47	2.01E-03
vasculature development	50	2.03E-03
vesicle-mediated transport	108	3.66E-03
nucleosome assembly	23	3.90E-03
stem cell division	5	3.91E-03
chromatin assembly	26	4.04E-03
somatic stem cell division	5	4.07E-03
GO - CC		
intracellular membrane-bound organelle	1230	3.57E-03
chromosome	83	4.32E-03
cytoplasmic membrane-bound vesicle	78	6.38E-03
dendrite	16	7.14E-03
endoplasmic reticulum part	107	7.91E-03
lytic vacuole	48	8.10E-03
nucleosome	19	8.30E-03

Table B.4: List of tissues which signature is enriched in hepatocyte-like cells derived with Hay *et al.* protocol

Tissue expression signature		
Term	**Count**	**p-value**
Atrioventricular node	312	9.32E-22
Pancreas primary adenocarcinoma	490	2.84E-21
Brain normal fetal astrocyte, GFAP positive, cerebral cortex	342	2.88E-20
Pancreatic tumor disease	601	3.13E-20
PB-BDCA4+Dentritic Cells	157	3.89E-20
Bone normal	747	1.56E-19
Peritoneum normal	326	1.70E-19
Fetal brain	553	2.09E-19
Brain Glioblastoma	372	8.31E-18
Subthalamic nucleus	561	1.07E-17
Ear normal	486	1.96E-17
Mammary glandnormal breast stroma	222	1.93E-16
Peritoneum malignant peritoneal mesothelioma	452	1.97E-16
Pancreatic Islets	248	4.02E-16
Heart normal	683	1.31E-15
White blood cells plaque macrophage	223	1.75E-15
Neonate (< 4 weeks old) development	467	2.89E-15
Uterus normal	728	3.41E-15
Lung focal fibrosis and chronic inflammation	274	3.94E-15
White blood cells plaque macrophage	219	2.83E-14
Vascular hemangioma	304	3.07E-14
Thyroid	282	5.04E-12
Kidney normal	67	9.57E-12

Table B.5: List of tissues which signature is enriched in hepatocyte-like cells derived with Agarwal *et al.* protocol

Tissue expression signature		
Term	**Count**	**p-value**
Cerebellum medulloblastoma	572	2.18E-35
Brain normal thalamus	585	6.54E-34
Brain anaplastic astrocytoma	424	4.18E-32
Ovary	640	1.55E-30
Prefrontal Cortex	766	5.70E-30
Spinal cord normal spinal cord	557	2.95E-25
Uterus Corpus	686	9.56E-25
Prostate	1816	7.26E-24
Retina normal macula	429	2.63E-22
Mammary gland Grade I, ER+, PR+, Her2- invasive ductal	354	3.54E-21
Cerebellum	498	4.76E-21
Kidney tumor disease	810	3.51E-18
Adrenal gland normal	777	1.13E-17
Heart normal	836	8.26E-15
Adipose tissue normal	721	1.87E-14
Bone normal	868	3.08E-11
Prostate normal epithelium	418	1.84E-10
Testis Interstitial	872	6.90E-10
Parathyroid normal	677	2.43E-09
Vascular normal	639	3.76E-08