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ROLE OF THE CONSTITUTIVE ANDROSTANE RECEPTOR IN THE DERIVATION OF HEPATIC-LIKE CELLS FROM HUMAN EMBRYONIC STEM CELLS

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by

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ABSTRACT

The liver performs an array of functions vital to life including detoxification, production of serum proteins, maintenance of cholesterol homeostasis, production and clearance of bile components, assembly and inter-conversion of amino acids, synthesis and breakdown of glucose, and processing of fatty acids. Current treatments for liver failure are inadequate, relying on liver or hepatocyte transplantation, both of which are significantly limited by insufficient donor tissue, donor-to-donor variability in tissue quality, and the risk of rejection, infection, or adverse immune response in the recipient. Human embryonic stem cells (hESCs) – derived from the inner cell mass of developing blastocysts and capable of giving rise to any cell type in the body upon exposure to the appropriate conditions – offer promise as an alternative source of cells from which a supply of hepatocytes may be derived for therapeutic transplantations. Hepatocytes derived from hESCs would also potentially provide a repository of cells for pharmacological and toxicological studies which rely on hepatocytes obtained from human donors as models for drug metabolism research and predictors of toxicological responses that may be associated with exposure to xenobiotic compounds. While a number of studies have demonstrated that hESCs are capable of differentiating into hepatic precursors, the precise means by which these cells may be derived, and the genes governing this multifaceted process, have yet to be fully elucidated. In this investigation we employed a unique hepatic differentiation protocol in which hESCs are cultured for only 10 days on collagen matrix in our hepatocyte media (William's E Media supplemented with HEPES, glutamine, antibiotics, dexamethasone, insulin, transferrin, selenium, and linoleic acid/albumin). The resulting cell population exhibits hepatic-like cell morphology and decreased expression of 'stemness' markers including certain transcription factors, surface antigens, and enzymes. The hESC-derived hepatic-like cells express enhanced levels of hepatic markers including transcription factors, nuclear receptors, liver-generated plasma proteins, protease inhibitors, metabolic enzymes, and biotransformation enzymes. Acquisition of hepatic function is confirmed by the cells' ability to transport anionic compounds and store glycogen. Notably, expression of the constitutive androstane receptor (CAR) – a nuclear receptor which, in the adult liver, is involved in the regulation of diverse physiological processes including all three phases of hepatic biotransformation and elimination as well as energy metabolism and lipid homeostasis – is highly increased in the hepatic-like cells, to levels approaching those of cultures of primary human hepatocytes. CAR is also expressed robustly and consistently in human fetal liver tissue obtained from subjects of a range of gestational ages. Modulation of CAR levels in differentiating hESCs using a lentivirus system – which we demonstrate to stably and robustly transduce both hESCs and cultures of primary human hepatocytes without affecting markers of pluripotency or the hepatic phenotype, respectively – reveals that CAR over-expression and siRNA-mediated attenuation of CAR mRNA levels result in corresponding changes in expression of hepatic markers previously assessed. In contrast, expression of pregnane X receptor (PXR) – the nuclear receptor exhibiting the most sequence homology to CAR – is not increased during hepatic differentiation and is negligible in human fetal liver tissue. Further, exogenous expression of PXR in hESCs induced to differentiate along a hepatic lineage does not result in enhanced expression of markers of the hepatic phenotype. Taken together, these results illuminate a unique, expeditious means by which functional hepatic-like cells may be derived from hESCs and further define a novel role for CAR in human hepatic specification.

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LIST OF ABBREVIATIONS

AF……..activation function
AHR……aryl hydrocarbon receptor
AKR1B7……aldo-keto reductase family 1, member 7
AR……..androgen receptor
ASSL……argininosuccinate synthetase
A1AT……alpha-1-antitrypsin
BCR……breast cancer resistance
BMP……bone morphogenetic protein
BSEP……bile salt export pump
CAR……..constitutive androstane receptor
CCRP……CAR cytoplasmic retention protein
C/EBPα……CCAAT/enhancer binding protein alpha
CITCO……….6-(4-chlorophenyl)imidazo[2,1–b] [1,3] thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime
C-MYC……cellular myelocytomatosis
CPS……..carbamyl-phosphate synthetase I
CPT1A……..carnitine palmitoyltransferase 1 gene
CYP……cytochrome P450
DAX-1…………… dosage-sensitive sex reversal-adrenal hypoplasia congenital critical region X chr. gene 1
DBD……DNA-binding domain
DKK-1……dickkopf-1
DR……..direct repeat
ECM……..extracellular matrix
ERR……estrogen-related receptor
FASC……fluorescence-activated cell sorting
FBS……fetal bovine serum
FBX15……..F-box-containing protein 15
FGF……fibroblast growth factor
FL……fetal liver
FOXA……..forkhead box A
FOXO1……..forkhead box O1

FRP2……frizzled-related protein 2

GFP……green fluorescent protein

GR……glucocorticoid receptor

GRIP-1……glutamate receptor-interacting protein 1

G-6-Pase……glucose-6-phosphatase

HEK……human embryonic kidney

hESC……human embryonic stem cell

hFF……human foreskin fibroblast

HGF……hepatocyte growth factor

HH……human hepatocyte

HMGCS2……3-hydroxy-3-methylglutaryl-coenzyme A synthase 2

HNF1……hepatic nuclear factor 1

HNF3……hepatic nuclear factor 3

HNF4α……hepatic nuclear factor 4 alpha

HSP90……heat shock protein 90

ICG……indocyanine green

INSIG-1……insulin-induced gene 1

iPS……induced pluripotent stem

KLF4……krueppel-like factor 4

LBD……ligand-binding domain

LIF……leukemia inhibitory factor

LRH-1……liver receptor homolog 1

MCL-1……myeloid cell leukemia 1

MDR……multi-drug resistance protein

mESC……mouse embryonic stem cell

MOI……multiplicity of infection

MRP……multi-drug resistance-associated protein

NANOG……nanog homeobox

NOD/SCID……immune-deficient non-obese diabetic/severe combined immune deficient

NR……nuclear receptor

NTCP……Na+-taurocholate co-transporting polypeptide

OAT……organic anion transporter

OATP……organic anion-transporting polypeptide

OCT……organic cation transporter

OCT4……octamer-binding transcription factor 4

OSM……oncostatin M

OTC……ornithine transcarbamylase

PAS……periodic acid-Schiff

PB……phenobarbital

PEG……polyethylene glycol

PEPCK……phosphoenolpyruvate carboxykinase

PGC-1……peroxisome proliferator-activated receptor gamma coactivator 1

PMO……phosphorodiamidate morpholino oligomer

PNR……photoreceptor cell-specific nuclear receptor

PPAR……peroxisome proliferator-activated receptor

PP2A……protein phosphatase 2A

PR……progesterone receptor

PXR……pregnane X receptor

RAR……retinoic acid receptor

ROR……retinoic acid receptor-related orphan receptor

RXR……retinoid X receptor

SHP……short heterodimer partner

shRNA……small hairpin RNA

siRNA……small interfering RNA

SMC-1……structural maintenance of chromosomes protein 1

SOX2……SRY-box containing gene 2

SRC-1……steroid receptor coactivator 1

SREBP-1……sterol regulatory element binding protein 1

SSEA-3……stage-specific embryonic antigen 3

TGF-β……transforming growth factor beta

TR……thyroid hormone receptor

TRA-1-60……tumor rejection antigen 1-60

TRA-1-81……tumor rejection antigen 1-81

TU……transducing unit

UGT1A1……………………………………………………………………………………………………….UDP-glucuronosyltransferase

VDR……..vitamin D receptor

WNT………........…………wingless-int

XRE………xenobiotic responsive element

Chapter 1

Literature Review

1.1 Introduction and Significance

As the largest internal organ in mammals, the liver performs many functions that are essential to the body including detoxification, production and secretion of serum proteins, maintenance of cholesterol homeostasis, production and elimination of bile constituents, synthesis and inter-conversion of amino acids, fatty acid processing, and the synthesis and breakdown of glucose (Fig. 1-1). Unfortunately, the liver is the target of a number of congenital and acquired diseases. In the United States alone, more than 40,000 deaths annually may be attributed to liver failure resulting from disease [1]. Both orthotropic liver transplantation, currently the most successful treatment option [2], and hepatocyte transplantation are limited by numerous caveats including insufficient donor tissue, significant donor-to-donor variability in tissue quality, and the risk of rejection, infection, or adverse immune response in the recipient [3]. Primary human hepatocytes maintained in culture also serve as models for drug metabolism research and predictors of toxicological responses that may be associated with exposures to xenobiotic compounds. However, the scarcity of hepatic tissue, as well as the challenges inherent in maintaining differentiated hepatocytes in culture, limit this vital research. Thus, the development of functional hepatocytes from alternative sources, such as human embryonic stem cells (hESCs), is an attractive proposition for the generation of an inexhaustible supply of hepatocytes for both therapeutic transplantation and pharmacological and toxicological research purposes, as well as to serve as models to study the regulatory networks governing hepatic differentiation.

This review provides a synopsis of the process of hepatic differentiation beginning with the pluripotent embryonic stem cell. Particular emphasis is placed on the signals that induce hepatic lineage commitment and the transcription factors that mediate the gene expression changes through which the specialized hepatic phenotype is manifested. A summary of progress in the generation of hepatic-like cells from hESCs using defined culture conditions is provided, together with a brief description of our optimized conditions for the maintenance of the differentiated hepatic phenotype in culture. As this work defines a novel role for the constitutive androstane receptor (CAR) in hepatic specification, a summary of the roles of nuclear receptors in the maintenance of pluripotency and induction of differentiation as well as the biology of CAR – with emphasis on physiological roles, regulation, and species differences and expression during hepatogenesis – is included as well.

1.2 Stem Cells

Embryonic stem cells, derived from the inner cell mass of the blastocysts of developing embryos, are capable of indefinite self-renewal, yet retain a plasticity which permits their differentiation into derivatives of all three germ layers under the appropriate conditions [4]. ‘Stemness’ is broadly defined by four properties: i) self-renewal capacity, ii) pluripotency, iii) stable tissue reconstitution, and iv) successive transplantability [5]. Commitment of stem cells to a particular lineage leads to reduction in expression of pluripotency factors, increases in expression of specific differentiation regulators, and epigenetic modifications including changes in DNA methylation status and chromatin structure [6].

The molecular basis of ‘stemness’ is attributable to the expression of three key transcription factors that together serve to preserve embryonic stem cell plasticity: octamer-binding transcription factor 4 (OCT4), SRY-box containing gene 2 (SOX2), and nanog homeobox (NANOG) [6]. Combining chromatin

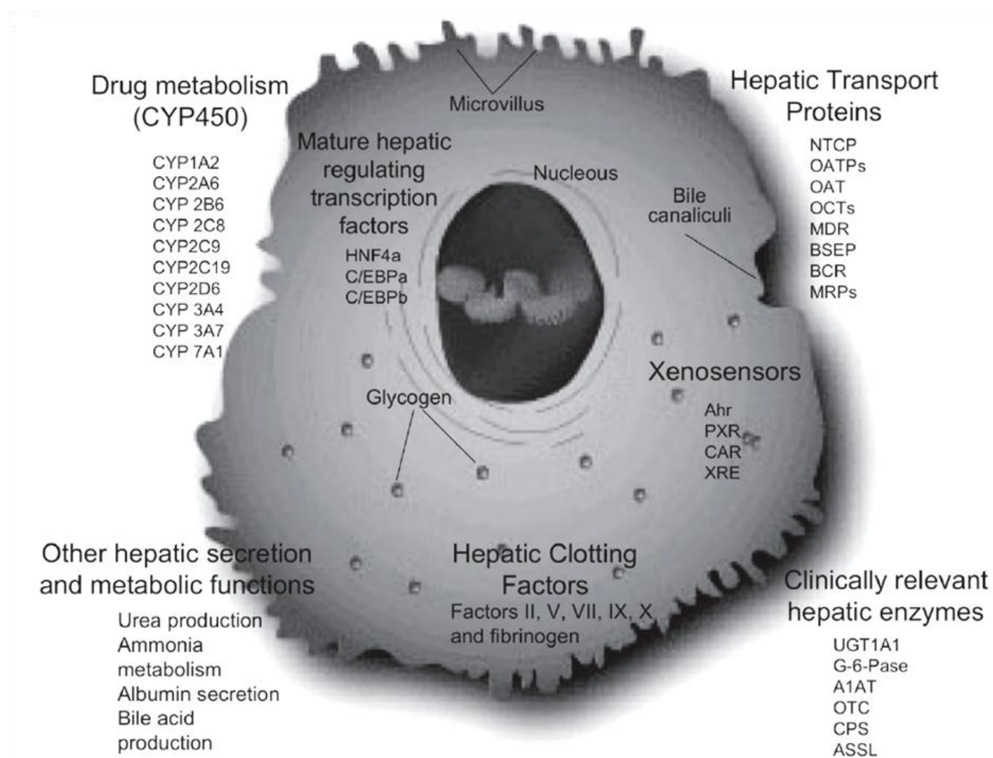

Figure 1-1. Specialized functions of the hepatocyte. Hepatocytes, the functional cells of the liver, perform a variety of functions vital to life including metabolism and transport of drugs and xeno and endobiotics, production and secretion of plasma proteins such as albumin and clotting factors, glycogen storage, and a variety of other metabolic functions including urea production and ammonia processing, generation and clearance of bile acid components, as well as maintenance of cholesterol homeostasis, assembly and inter-conversion of amino acids, synthesis and breakdown of glucose, and processing of fatty acids. AHR, aryl hydrocarbon receptor; ASSL, argininosuccinate synthetase; A1AT, alpha-1-antitrypsin; BCR, breast cancer resistance; BSEP, bile salt export pump; CAR, constitutive androstane receptor; C/EBPα or β, CCAAT-enhancer binding protein alpha or beta; CPS, carbamyl-phosphate synthetase I; G-6-Pase, glucose-6-phosphatase; HNF4α, hepatocyte nuclear factor 4 alpha; MDR, multi-drug resistance protein; MRP, multi-drug resistance associated protein; NTCP, Na+-taurocholate co-transporting polypeptide; OAT, organic anion transporter; OATP, organic anion-transporting polypeptide; OCT, organic cation transporter; OTC, ornithine transcarbamylase; PXR, pregnane X receptor; UGT1A1, UDP-glucuronosyltransferase; XRE, xenobiotic responsive element. *Reproduced from Soto-Gutierrez et al. Differentiating stem cells into liver. Biotechnol Genet Eng Rev 2008; 25:149-164 with permission of corresponding author, with whom copyright privileges lie. Copyright 2008, Ira J. Fox.*

immunoprecipitation with microarray analysis to map the genome-wide promoter occupancy of these core ES cell transcription factors, Boyer et al. found that OCT4, SOX2, and NANOG occupy the promoters of – and positively regulate – each other as well as an overlapping set of target genes also implicated in the maintenance of pluripotency including transcription factors, components of the wingless-int (WNT) signaling pathway, and members of the transforming growth factor beta (TGF-β) signaling pathway [7].

The vital role played by OCT4 and SOX2 in conferring pluripotency is further underscored by studies performed by Takahashi and Yamanaka in which a retroviral system was utilized to stably express these pluripotency factors – along with the two oncogenes cellular myelocytomatosis (C-MYC) and krueppel-like factor 4 (KLF4) – in mouse embryonic and adult human fibroblasts [8;9]. Upon selection for expression of F-box-containing protein 15 (FBX15) – an OCT4 target gene – the resulting population, designated 'induced pluripotent stem' (iPS) cells, exhibited morphology, growth patterns, and gene expression profiles similar to that of ES cells and were capable of contributing to embryonic

development when injected into the blastocyst and of forming teratomas when transplanted into mice [8;9]. Interestingly, expression of the pluripotency factor NANOG was superfluous for this reprogramming process [8].

1.3 Liver Development

During mouse development, the liver bud forms from the ventral foregut endoderm on embryonic day (E)9 [10]. As the liver bud develops the hepatic precursors, or hepatoblasts, delaminate and migrate as cords through the basement membrane which separates the foregut from the adjacent septum transversum [10]. Hepatoblasts in the newly emerged liver bud are bipotential, capable of giving rise to definitive hepatocytes or cholangiocytes [11]. At E10, the migrating hepatoblast cords associate intimately with rudimentary sinusoidal endothelial cells which form basic capillary-like sinusoids that run between the hepatocyte cords [12]. Prior to E12 in mouse development, hepatoblasts are morphologically undifferentiated; compared to the mature hepatocyte, hepatoblasts exhibit irregular shape, have few cellular organelles, and have a large nuclear to cytoplasmic ratio [13]. Although still lacking the spaces and fenestrations that characterize sinusoids in the mature liver, by E14 sinusoidal endothelium has been definitively established [12]. Continuing hepatic differentiation is marked by changes in hepatocyte ultrastructure including increases in the number of Golgi apparatus and endoplasmic reticulum, which facilitate the necessary enhancement in protein synthesis, as well as the emergence of peroxisomes and glycogen rosettes [13]. Hepatocyte polarity is established with the development of bile canaliculi at the apical hepatocyte surface [13].

1.3.1 Inductive Signaling

Liver development is governed by a complex series of inductive events mediated by extracellular signals such as fibroblast growth factor (FGF) [14], bone morphogenetic protein (BMP) [15], oncostatin M (OSM) [16], hepatocyte growth factor (HGF) [17], glucocorticoids [16;18], and extracellular matrix (ECM) components [19]. Hepatic induction by these factors initiates intracellular signaling cascades that activate hepatic-enriched transcription factors, including members of the hepatic nuclear factor 1 (HNF1) and forkhead box A (FOXA) – also known as hepatic nuclear factor 3 (HNF3) – families, as well as hepatic nuclear factor 4 (HNF4α) and CCAAT/enhancer binding protein alpha (C/EBPα) [20], resulting in global changes in patterns of gene expression and manifestation of the hepatic phenotype.

Initiation of hepatogenesis requires inductive signals from the adjacent cardiac mesoderm [20]. To elucidate the molecular mechanisms of hepatic induction, Jung et al. employed mouse embryonic tissue explants to demonstrate that specific FGFs can induce hepatic specification *in lieu* of cardiac mesoderm, and that hepatic induction can be ablated by blocking FGF signaling [14]. Although necessary, this cardiac signaling is not sufficient for hepatic specification [21].

Acting cooperatively with FGF signaling from the cardiac mesoderm, inductive signals from the septum transversum mesenchyme are critical for hepatic morphogenesis [20]. BMP is a member of the TGF-β family of secreted signaling molecules, proteins that play important roles in a number of developmental processes [22]. Using BMP-null mice and specific inhibitors, Rossi et al. demonstrated that BMP signaling from the septum transversum mesenchyme is necessary for hepatic specification and morphogenetic growth of the liver bud from the ventral foregut endoderm [15].

Secreted by hematopoietic cells in the fetal liver, OSM, a member of the interleukin-6 family of cytokines that is structurally similar to leukemia inhibitory factor (LIF), acts to induce hepatic

differentiation of the surrounding cells [23]. The importance of this cytokine in hepatic differentiation is underscored by the fact that livers from mice deficient in a subunit of the OSM receptor display functional abnormalities [16].

Inductive signaling by HGF plays an important role in liver development as well. HGF-null mice die *in utero* prior to completion of development*;* HGF-null embryos further display reduced liver size and significant parenchymal cell loss [17]. Additionally, treatment of murine fetal liver cells with HGF in the presence of dexamethasone results in increased expression of hepatic marker genes as well as intracellular glycogen accumulation [18].

The synthetic glucocorticoid dexamethasone is required for hepatic induction by both OSM and HGF [16;18]. Using cultures of murine fetal hepatocytes, Kamiya et al. demonstrated that treatment with a combination of dexamethasone and OSM results in morphological changes consistent with fully differentiated hepatocytes, augmented expression of hepatic marker genes, and intracellular glycogen accumulation [16]. Further, hematopoiesis, a function of the liver transiently manifested exclusively during the early stages of development, is terminated by dexamethasone/OSM treatment, underscoring the importance of glucocorticoid signaling in hepatic maturation [24]. In contrast to OSM stimulation alone, treatment with dexamethasone alone promotes a number of hepatic specification markers, albeit to a much lesser extent than the dexamethasone/OSM combination [25]. Studies assessing the role of HGF in hepatic differentiation yield similar results; in the absence of dexamethasone, HGF is unable to induce differentiation, as measured by the increased expression of hepatic marker genes and intracellular glycogen accumulation [18].

In addition to regulation by cell-cell interactions and soluble signaling factors, hepatocyte function in the liver microenvironment is influenced by the ECM, a dynamic network of insoluble proteins and proteoglycans existing within the liver architecture in the space of Disse, the region between the basal surface of the hepatocyte cords and the extensively fenestrated endothelial cells lining the sinusoids [19]. Liver ECM is comprised primarily of collagen type I and fibronectin, as well as collagen types III, IV, and VI, tenascin, and laminin [19]. While primary hepatocytes maintained on plastic dishes rapidly de-differentiate in culture, a number of studies have shown that hepatocytes cultured on collagen type I gel (fibers comprised of multiple collagen molecules held together by hydrogen bonds under physiological conditions) or Matrigel™ (a mouse sarcoma cell-derived basement membrane matrix) retain their differentiated phenotype [19;26-29]. ECM regulation of hepatocyte differentiation is mediated in part through the hepatic-enriched transcription factor C/EBPα (see below). In contrast to hepatocytes cultured on plastic dishes, which rapidly lose expression of C/EBPα, hepatocytes cultured on Matrigel™ retain robust expression of this integral hepatic regulator [30].

1.3.2 Liver-Enriched Transcription Factors

In response to inductive signaling, the hepatic-specific gene set is induced by liver-enriched transcription factors, notably members of the HNF1 and FOXA/HNF3 families, as well as HNF4α and C/EBPα [20]. While expression of these factors is not restricted to liver, and none are independently capable of specifying hepatic differentiation, together they comprise a combinatorial regulatory network that controls transcription of most genes in the hepatic program [31].

The HNF1 family of transcription factors – comprised of HNF1α and HNF1β (also known as HNF1 and vHNF1, respectively) – is characterized by a POU-homeodomain DNA-binding sequence [20]. Mice

deficient in total HNF1 exhibit decreased expression of albumin and α-1-antitrypsin, liver enlargement, and wasting syndrome that results in death within 1-3 months of birth [32].

The FOXA (also known as HNF3) family of transcription factors – including FOXA1, FOXA2, and FOXA3 (or HNF3α, HNF3β, and HNF3γ, respectively) – contains a highly conserved 'winged helix,' or forkhead box, DNA binding domain [20]. Mouse embryos lacking FOXA1 in the foregut endoderm fail to develop a liver bud and cultured FOXA1-deficient mouse endoderm lacks the ability to induce albumin and transthyretin expression in response to FGF signaling [33].

HNF4α belongs to the nuclear receptor (NR) superfamily of transcription factors characterized by a highly conserved zinc finger DNA-binding domain [20]. HNF4α-null mice exhibit impaired gastrulation due to a visceral endoderm abnormality and do not survive beyond E6.5 [34]. Using tetraploid complementation in which HNF4α-null embryos are coupled with HNF4α-wt visceral endoderm, however, investigators have been able to facilitate the survival of the embryos until E12, into the early stages of hepatocyte differentiation [35]. At E12, despite the surprising absence of morphological abnormalities, HNF4α-null embryos exhibited attenuated expression of many genes of vital importance for hepatic function including genes encoding metabolic proteins, serum proteins, and apolipoproteins [36]. Using a conditional knockout technique, the survival of HNF4α-deficient mice can be increased to E18.5 [37]. When analyzed at this developmental stage, HNF4α-null mice livers exhibit morphological disruptions in both cell shape and liver architecture as well as functional abnormalities [37]; microarray analyses reveal attenuated expression of numerous adhesion and cell junction assembly proteins which orchestrate epithelial morphogenesis of the developing liver [38]. Investigations probing the transcriptional regulatory circuitry of human hepatocytes have revealed a vital role for HNF4α in the maintenance of the fully differentiated hepatic phenotype. Combining chromatin immunoprecipitation with microarray analysis, Odom et al. found that HNF4α bound the promoter regions of 1575 genes (12% of the array) in human hepatocytes [39]. Of the genes simultaneously bound by RNA polymerase II (indicative of active transcription), 42% were also bound by HNF4α [39].

C/EBPα is a member of C/EBP family of basic leucine zipper DNA-binding domain transcription factors [20]. C/EBPα is first expressed in the developing liver of mouse embryos on E13 [40]. C/EBPα-null mice exhibit reduced post-natal expression of glycogen synthase and the gluconeogenic enzymes PEPCK and glucose-6 phosphatase; the inability to build sufficient hepatic glycogen reserves results in hypoglycemia and death within 8 hours post-partum [41]. Studies by Suzuki et al. have shown that HGF treatment induces expression of C/EBPα and ablation of C/EBPα prevents differentiation along a hepatic lineage, suggesting that the hepatogenic effects of HGF are mediated, at least in part, through C/EBPα [42].

1.4 Directed Hepatic Differentiation of Human Embryonic Stem Cells

To date, a number of investigations have sought to direct differentiation of hESCs along a hepatic lineage. For a comprehensive overview regarding progress in derivation of hepatic-like cells from hESCs, the reader is referred to a recent review by Snykers et al. [43]. The majority of the studies have employed defined culture conditions to reconstitute the *in vivo* liver microenvironment, supplementing culture media with FGF [3;44-51], BMP [46], HGF [3;44;45;48-50;52-55], dexamethasone [44;45;48-50;55;56], insulin [49;56], OSM [44;48;49;53;55], activin A [44-46;48;52-55;57;58], WNT3A [55;57], and sodium butyrate [53;59]. Research has also shown the importance of a collagen type I ECM in hepatic induction [3;44;49;49;56]. While promising, current differentiation methodologies have thus far been insufficient to derive fully functional hepatocytes from embryonic stem cells.

Acquisition of hepatic character of the hESC-derived hepatic-like cells is generally assessed *in vitro* at the morphological, mRNA, protein, and functional/activity level [43]. Molecular markers evaluated include expression of hepatic-enriched transcription factors (HNF1, FOXA/HNF3, HNF4α, and C/EBPα), liver-generated plasma proteins (α-fetoprotein, albumin, and transthyretin), cytoskeleton proteins (cytokeratins 8 and 18), metabolic enzymes (tryptophan 2,3-dioxygenase and tyrosine amino transferase), and biotransformation enzymes (cytochrome P450s). Functional endpoints assessed include glycogen storage, anion transport, urea metabolism, albumin secretion, and inducible biotransformation capacity. The ultimate test of the hESC-derived hepatic-like cells is whether or not they can restore function to animal models of liver damage .

1.5 Optimized Culture Conditions for Maintenance of the Hepatic Phenotype

Cultures of primary human hepatocytes serve as valuable *in vitro* models to study liver biology. However, these cells rapidly dedifferentiate under most standard culture conditions, limiting their utility as predictors of xenobiotic responses in humans. Thus, significant effort has been put forth to explore culture methodologies that best mimic the *in vivo* liver microenvironment and serve to maintain the differentiated hepatic phenotype.

Work in our laboratory has shown that the two-dimensional sandwich culture system, in which primary hepatocytes are plated on a collagen type I substrate, overlaid with dilute concentrations of ECM (Matrigel™), and maintained in a highly defined, serum-free media supplemented with insulin and nanomolar concentrations of dexamethasone, is highly successful for maintaining hepatocyte differentiation character [26-29;60;61]. Primary hepatocytes maintained in this culture system exhibit hepatocyte cell morphology, expression of hepatic-specific genes, and phenobarbital gene induction capacity, a uniquely sensitive measure of hepatocyte differentiation status [29]. Further, global gene expression analyses comparing human liver tissue to primary human hepatocytes cultured using this system and established human hepatoma cell lines reveal that, of biological processes comparatively over-represented among probe sets expressed in the *in vitro* systems, greater than 77% of the probe sets remain unchanged between liver tissue and cultures of primary hepatocytes, while less than 48% and 55% remain unchanged between liver tissue and HepG2 and Huh7 hepatoma cell lines, respectively [26]. The importance of extracellular matrix in our culture system is underscored by the finding that Matrigel™ enhances hepatocyte morphology, expression of hepatic-specific genes, and the phenobarbital induction response of primary hepatocytes in culture [27]. Taken together, these findings suggest that our culture system is highly reflective of the *in vivo* hepatocyte.

1.6 Roles for Nuclear Receptors in Pluripotency and Differentiation

As the molecular mechanisms governing the balance between the maintenance of 'stemness' and differentiation begin to emerge, increasing evidence points to roles for members of the NR superfamily in these processes. A comprehensive overview of the functions of specific nuclear receptors in stem cell biology can be found in a recent review by Jeong and Mangelsdorf, highlights of which include analysis of dosage-sensitive sex reversal-adrenal hypoplasia congenital critical region on the X chromosome gene 1 (DAX-1), estrogen-related receptor beta (ERRβ), and liver receptor homolog 1 (LRH-1) in the maintenance of pluripotency; vitamin D receptor (VDR), glucocorticoid receptor (GR), retinoic acid receptor beta (RARβ), androgen receptor (AR), and peroxisome proliferator-activated receptor gamma (PPARγ) in adipogenesis; the AR in myogenesis; the VDR, GR, and RAR (α & β) in chondrogenesis; the VDR and PPARγ in osteoclastogenesis; and the PPARγ in osteoblastogenesis [62]. As noted previously, a key role for HNF4α in hepatogenesis is well-established [36]. A synopsis of the roles of the nuclear

receptors DAX-1, ERRβ, and LRH-1 in regulation of 'stemness' and the role of the VDR – which shares the NR1I subfamily designation with CAR and PXR – in a variety of developmental processes is furnished below.

The nuclear receptors DAX-1, ERRβ, and LRH-1 play integral roles in conferring stem cell pluripotency, primarily through their interactions with the core 'stemness' transcriptional regulatory factors OCT4 and NANOG. While DAX-1 is regulated in part by NANOG [63] and OCT4 [64], ERRβ acts in concert with NANOG to induce transcription of OCT4 [65] and in concert with OCT4 to enhance expression of NANOG [66]. LHR-1's role in 'stemness' is manifested through its co-localization with OCT4 in the inner cell mass and its role in governing expression of this central regulator in embryonic stem cells [67]. Through integrated and cross-regulatory networks that are only beginning to be characterized, these nuclear receptors play key roles in stem cell biology.

As noted above, the VDR has been implicated in a number of developmental processes. The VDR acts to inhibit adipogenesis in the mouse through a variety of mechanisms including suppression of pro-adipogenic factors C/EBPα, PPARγ, and sterol regulatory element binding protein 1 (SREBP-1) in preadipocytes [68] and suppression of dickkopf-1 (DKK-1) and frizzled-related protein 2 (FRP2) in bone marrow stromal cells [69]. The VDR also modulates chondrogenesis, albeit in a tissue- or species-specific manner, as VDR activation induces chondrogenesis in chick embryo limb bud mesenchymal cells [70] but inhibits chrondrogenesis in the murine chondrogenic ATDC5 cell line [71]. Activation of the VDR in murine chondrocytes also stimulates osteoclastogenesis [72].

1.7 Constitutive Androstane Receptor

The constitutive androstane receptor (CAR; NR1I3) is a member of the nuclear receptor (NR) superfamily that is expressed primarily in the liver. CAR exhibits a modular structure (Fig. 1-2A) comprised of [73]:

- A/B domain: a shortened N-terminal domain which lacks the ligand-independent activation function 1 (AF-1) characteristic of most NR family members.
- C domain: a highly conserved zinc-finger DNA-binding domain (DBD).
- D domain: a flexible hinge region
- E domain: a ligand-binding domain (LBD) which contains the dimerization region and the ligand-dependent activation function 2 (AF-2).
- F domain: a variable C-terminal domain.

1.7.1 Physiological Roles

CAR was originally characterized as a 'xenosensor' because of its role in regulating expression of genes whose products act at all three phases of hepatic biotransformation and disposition, including cytochrome P450s (phase I), sulfotransferases, glutathione S-transferases, glucoronosyltransferases (phase II), multidrug resistance-associated proteins, and organic anion transporting polypeptides (phase III), in response to potentially toxic stimuli [74]. Through the regulation of these genes, CAR plays a key role in the metabolism and transport of exogenous compounds including drugs [75] and carcinogens [76]. As these systems are also integral to the metabolism and disposition of numerous endogenous substances such as steroids [76], heme/bilirubin [76], thyroid hormone [77], and cholesterol/bile acids [78;79], CAR also plays a role in endobiotic homeostasis and is important for a variety of physiological processes.

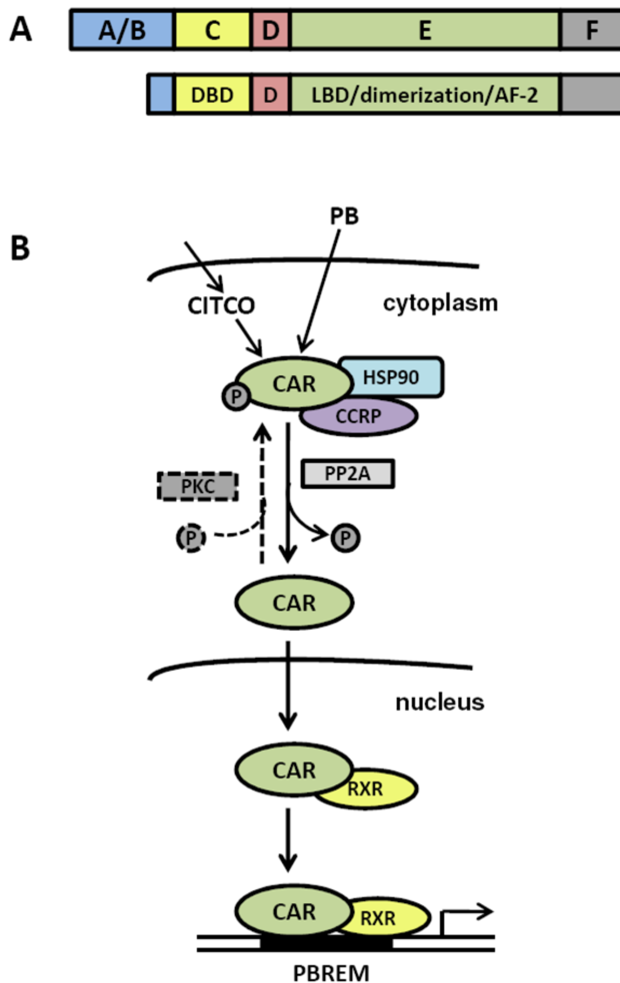

Figure 1-2. Constitutive androstane receptor (CAR) biology. (A) CAR exhibits a modular structure comprised of the following domains: A/B - a shortened N-terminal domain which lacks the ligand-independent activation function 1 (AF-1) characteristic of most nuclear receptor family members, C - a highly conserved zinc-finger DNA binding domain (DBD), D - a flexible hinge region (H), E - a ligand binding domain (LBD) which also contains the dimerization region and the ligand-dependent activation function (AF-2), and F - a variable C-terminal domain. (B) In the canonical model of CAR activation in the absence of inducing agent the threonine 38 residue in CAR's DBD is phosphorylated by protein kinase C (PKC) and CAR is sequestered in the cytoplasm in association with CAR cytoplasmic retention protein (CCRP) and heat shock protein 90 (HSP90). Upon exposure to inducer such as the human CAR agonist CITCO or the activator phenobarbital (PB), protein phosphatase 2A (PP2A) is recruited to the complex facilitating the dephosphorylation of threonine 38 and the subsequent release and nuclear translocation of CAR. Once in the nucleus, CAR heterodimerizes with retinoid X receptor (RXR), binds to DNA response elements (such as the prototypical phenobarbital-responsive enhancer module, or PBREM) in the promoter regions of target genes, and recruits coactivators to activate target gene transcription.

Of particular interest, CAR is emerging as an important regulator of lipid and energy metabolism. As mentioned above, through induction of hepatic metabolism and transport systems CAR mediates cholesterol and bile acid metabolism, helping to prevent the accumulation of lipids and bile acids which can result in cholestasis and liver injury [79]. Additional work has defined a role for CAR in the inhibition of fatty acid β-oxidation mediated through its repression of expression of the mitochondrial carnitine palmitoyltransferase 1 gene (CPT1A), the rate-limiting enzyme of mitochondrial fatty acid β-oxidation [80], and inhibition of lipogenesis mediated through induction of insulin-induced gene 1 (INSIG-1), a protein with anti-lipogenic properties [81]. CAR has also been implicated in the inhibition of gluconeogenesis through its role in i) functioning as a co-repressor of forkhead box O1 (FOXO1) target genes containing insulin response sequences such as the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK) [82], and ii) competing with the positive regulator HNF4α for binding to the co-activators glutamate receptor-interacting protein 1 (GRIP-1) and peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α) and the direct repeat (DR)-1 response element upstream of key genes in glucose metabolism [83]. Through inhibition of these processes, CAR decreases levels of lipids and glucose and thus may represent an important therapeutic target for the treatment hyperlipidemia and hyperglycemia, both of which are often present in patients who are obese and/or diabetic [84].

CAR positively regulates expression of genes involved in other diverse biological processes including the human cathepsin E gene (an aspartic protease involved in the innate immunity response) [85], mouse aldo–keto reductase family 1, member 7 (AKR1B7) (an enzyme involved in modulating lipid peroxidation) [86], mouse myeloid cell leukemia-1 (MCL-1) (an anti-apoptotic protein) [87], and mouse C-MYC (a proto-oncogene) [88].

1.7.2 Regulation

A number of investigations have elucidated the means by which CAR mRNA expression is regulated. Employing deletion analysis, mutagenesis, electromobility shifts, and chromatin immunoprecipitation assays, Pascussi et al. identified a GR response element at -4432 bp [89] and an HNF4α response element at -144 bp [90] in the CAR upstream regulatory region. Further observations revealed that in the developing liver there is a positive correlation between expression levels of CAR mRNA and those of HNF4α [90], a aforementioned essential regulator of hepatic development [36]. PPARα also regulates CAR gene expression through binding of a DR-1 response element at -980 bp [91] and studies by Patel et al. have demonstrated that CAR mRNA levels are subject to further regulation by the aryl hydrocarbon receptor (AHR) [92].

The regulation of CAR protein activity is complex and not yet fully elucidated. In the absence of inducing agent, CAR is retained in the cytoplasm where it exists in a complex with two accessory proteins, CAR cytoplasmic retention protein (CCRP) and heat shock protein 90 (HSP90) [93]. Upon exposure to inducer, protein phosphatase 2A (PP2A) is recruited to the complex mediating the release and subsequent nuclear translocation of CAR [73]. Interesting, CAR translocation occurs without a strong nuclear localization signal and independently of the AF-2 domain [94]. Recent studies by Mutoh et al. identified threonine 38 (located on an α-helix in the zinc finger DBD) as the residue which, through its phosphorylation status, governs CAR translocation [95]. Further, protein kinase C acts to phosphorylate threonine 38, presumably destabilizing the α-helix, and resulting in CAR's sequestration in the cytoplasm [95]. Activator exposure mediates dephosphorylation of this residue and subsequent nuclear translocation [95]. Once in the nucleus, CAR heterodimerizes with retinoid X receptor (RXR), binds to DNA response elements in the promoter/enhancer regions of target genes, and recruits coactivators including GRIP-1, PGC-1, steroid receptor coactivator 1 (SRC-1), and structural maintenance of chromosomes protein 1 (SMC-1) to activate gene transcription [73]. Human CAR activity may be modulated by indirect activators, such as the prototypical inducer phenobarbital (PB), which induces nuclear translocation and subsequent target gene induction without directly binding to the receptor [96]. Alternatively, CAR activity in humans may be modulated directly by agonists such as 6-(4-chlorophenyl)imidazo[2,1–b] [1,3] thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime (CITCO) [97], as well as inverse agonists including clotrimazole [98], meclizine [99], androstanol, and androstenol (Fig. 1-2B) [100].

1.7.3 Expression during Hepatogenesis

So-called 'xenosensors,' including CAR, pregnane X receptor (PXR), and AHR, are best known for their roles in mediating expression of hepatic biotransformation and transport genes in response to potentially toxic stimuli. Studies of the AHR-null mouse, however, reveal an integral role for this protein in a number of other biological processes including hepatic development and growth [101]. As noted previously, CAR impinges on a variety of biological processes beyond that of hepatic biotransformation and transport. To date, however, only limited data exist regarding CAR's role in hepatic differentiation and liver function.

Results from a recent study by Xie et al. comparing NR expression levels in human and mouse undifferentiated ESCs and during embryoid body differentiation show increased expression of CAR during hESC early embryoid body differentiation (Fig. 1-3A). These findings are confirmed by Ek et al. who show expression of CAR in hepatic-like cells generated from hESCs using a 30-day differentiation protocol [102]. Further, during hepatogenesis human CAR mRNA expression correlates with that of its transcriptional regulator HNF4α [90] which, as mentioned previously, is a key mediator of hepatic development [36]. However, a study of CAR mRNA expression in ~60 human fetal liver samples ranging in age from 11 to 32 weeks of gestation found that average fetal liver CAR expression is ~4-fold greater than that of HNF4α [103]. Findings from the same study showed that, despite extensive inter-individual and developmental stage variability, CAR expression in human fetal liver is on average ~40% of that of postnatal liver [103]. Microarray expression data from panels of human tissue, publicly available through the UCSC Genome Browser website (http://genome.ucsc.edu/) [104;105], confirm that CAR is robustly expressed in human fetal liver. While these studies show that CAR is expressed relatively highly during human liver development, how this expression correlates with function has yet to be evaluated.

1.7.4 Species Differences

To further elucidate CAR's role in hepatic gene induction, Wei et al. generated a CAR-null mouse model by disrupting the receptor's DBD through the substitution of exons 1 and 2 with *neo* and *B-gal* resistance genes [75]. CAR-deficient mice, fertile and born at expected mendelian frequencies, exhibit no overt morphological abnormalities [75]. The lack of an obvious phenotype in CAR-null mice, however, does not preclude the possibility that CAR plays a role in liver specification, as functional redundancy built into the hepatic specification process may compensate for the lack of CAR function or CAR's role in hepatogenesis may be species-dependent.

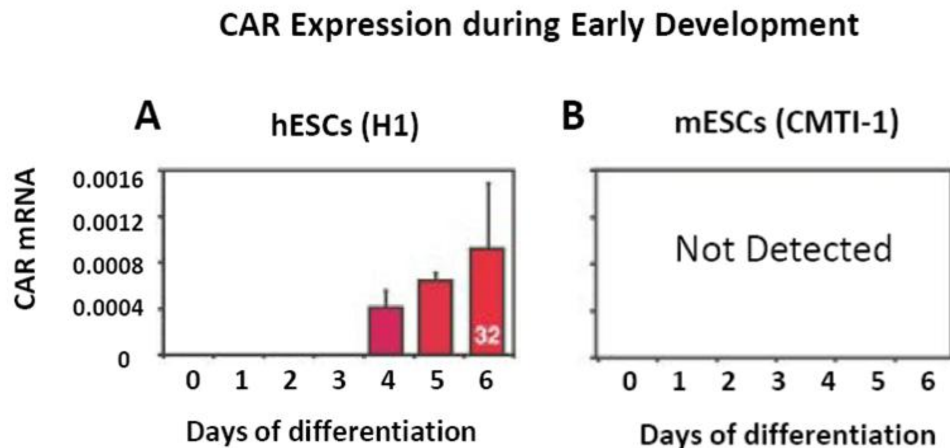

Figure 1-3. CAR expression in human and mouse embryonic stem cells and during early embryoid body differentiation. Graphs depict mRNA levels of CAR in (A) the H1 human ESC line and (B) the CMTI-1 mouse ESC line in the undifferentiated state (Day 0) and during embryoid body differentiation (Days 1-6). Embryoid body differentiation was induced by culturing the cells on ultra-low attachment plates in ESC media without human basic fibroblast growth factor and with 20% fetal bovine serum in place of knockout serum replacement. Values are expressed as mean +/- SD from triplicates of each sample. The Ct of the highest expressing value is indicated inside its corresponding bar. *Adapted from Xie et al. Expression profiling of nuclear receptors in human and mouse embryonic stem cells. Mol Endocrinol 2009; 23:724-733 with permission of publisher. Copyright 2009, The Endocrine Society.*

In support of the latter hypothesis, there are significant interspecies differences in both the expression profiles and roles of members of the NR superfamily in the maintenance of pluripotency and differentiation. Indeed, in the aforementioned review by Jeong and Mangelsdorf summarizing advances in the study of the roles of NRs in 'stemness' and early cell lineage commitment in a variety of species, the authors conclude that NRs function in an integral but highly species-specific manner to regulate pluripotency and differentiation [62]. Lending further support to this hypothesis, the aforementioned study by Xie et al. comparing NR expression levels in human and mouse undifferentiated ESCs and during embryoid body differentiation showed that only 29 NRs were expressed in both human and mouse undifferentiated ESCs [106]. Five NRs were expressed exclusively in hESCs (retinoic acid receptor-related orphan receptor beta (RORβ), thyroid hormone receptor beta (TRβ), HNF4γ, PPARγ, and PXR), while eight were expressed exclusively in mESCs (photoreceptor cell-specific nuclear receptor (PNR), progesterone receptor (PR), short heterodimer partner (SHP), RORγ, RXRγ, ERRβ, ERRγ, and PPARα) [106]. Notably, CAR is one of only seven NRs not expressed in undifferentiated ESCs of either species [106]. During the early stages of embryoid body differentiation 21 NRs exhibit expression patterns that differ between the two species [106]. Interestingly, NRs expressed exclusively in either human or mouse undifferentiated ESCs generally maintained this exclusive expression profile during embryoid body differentiation, with the single notable exception of CAR [106]. While CAR was not expressed in undifferentiated hESCs, it was expressed increasingly in hESCs undergoing embryoid body differentiation, as mentioned previously [106]. In contrast, CAR expression was not detected in either undifferentiated mESCs or mESCs undergoing embryoid body differentiation (Fig. 1-3A and B) [106]. Microarray expression data from panels of human and mouse tissue, publicly available through the UCSC Genome Browser website (http://genome.ucsc.edu/) [104;105], further support this hypothesis. While CAR is expressed robustly in human fetal liver tissue, expression in the mouse embryo is negligible. Based on the remarkable differences observed between nuclear receptor expression profiles in human and mouse ESCs in the undifferentiated state and during differentiation, Xie et al. concur that the transcriptional programming of undifferentiated and early-differentiated embryonic stem cells is highly species-specific [106].

That species-specific differences may exist with respect to CAR's role in liver differentiation and development is further supported by the fact that the LBDs of CAR and PXR exhibit the highest degree of inter-species variation of all NR family members [107]. Indeed, X-ray crystallographic analyses of human and mouse CAR bound to various ligands reveal considerable sequence divergence at amino acid positions that interact directly with the ligand, explaining the differences in CAR activators that exist between human and mouse [107]. These findings indicate that of all the NRs, CAR may be among the most likely to exhibit species-specific differences. Taken together, these data suggest that CAR may play a role in hepatic lineage commitment in humans.

1.8 Hypothesis and Specific Aims

Hypothesis:

The constitutive androstane receptor plays a unique role in the hepatic differentiation of hESCs mediated by regulating expression of hepatic-enriched transcription factors, nuclear receptors, and liver-generated plasma proteins.

Aim #1:

To derive hepatic-like cells from hESCs for use in therapeutic cell transplantation, pharmacological and toxicological research, and as models to study the regulatory networks governing hepatic differentiation. In this aim, we will employ defined culture conditions to promote hepatic differentiation of hESCs. The extent of differentiation will be assessed by morphological evaluation, expression of integral 'stemness' and hepatic marker genes, and functional analyses.

Rationale:

Although promising, to date studies undertaking to induce differentiation of hESCs along a hepatic lineage have been insufficient to derive functional, transplantable hepatocytes. In this investigation, we hypothesize that culture media optimized for the maintenance of primary human hepatocytes, coupled with collagen substrate, will provide an enhanced means of generating functional hepatic-like cells from hESCs.

Aim #2:

To assess the effects of lentiviral transduction on the differentiation status of the mature hepatocyte. In this aim, we will transduce cultures of primary human hepatocytes with lentiviral vectors and assess expression levels of key hepatic marker genes and retention of the capacity for CYP3A4 induction. Additionally, an siRNA targeted to CAR will be developed in order that CAR levels may be modulated in subsequent studies.

Rationale:

The ability to modulate gene expression without compromising cellular differentiation status is paramount for studies of cell lineage determination. The versatility of lentiviral vectors – retroviral vectors that transduce both dividing and non-dividing cells and integrate stably into the host genome [108-110] – for genetic manipulation of cultures of primary human hepatocytes [111-114] is well-documented. However, whether or not lentiviral transduction affects hepatocyte differentiation status – i.e., expression of specific nuclear receptors, hepatic secretory proteins, and metabolic enzyme induction capacity – has not been evaluated.

Aim #3:

To elucidate the role of CAR in the promotion of hepatic differentiation. In this aim we will evaluate CAR expression levels in the developing liver and utilize CAR over-expression and siRNA-mediated mRNA reduction techniques to manipulate CAR levels in hESCs undergoing hepatogenesis. The effects of CAR modulation on hepatic differentiation will be assessed by measuring changes in patterns of gene expression. Corresponding studies using PXR – the NR most similar to CAR in primary sequence, sharing 40% sequence homology in the DBD and 45% in the LBD [73] – will be performed to determine whether any observed effects are CAR-specific.

Rationale:

Given the emerging role of members of the NR superfamily in pluripotency and differentiation, and considering that CAR is expressed both during embryoid body [106] and hepatic-directed differentiation

of hESCs [102] and is expressed relatively highly in human fetal liver – at levels on average nearly 4-fold greater than HNF4α, a key regulator of hepatic differentiation [103] – we hypothesize that CAR plays a unique role in human hepatic differentiation and liver development.

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Chapter 2

An Efficient Method for the Differentiation of Human Embryonic Stem Cells along a Hepatic Lineage

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2.1 Abstract

The limited availability of hepatic tissue suitable for the treatment of liver disease and drug discovery research advances the generation of hepatic-like cells from alternative sources as a valuable approach. In this investigation, we exploited a unique hepatic differentiation approach to generate hepatocyte-like cells from human embryonic stem cells (hESCs). hESCs were cultured for only 10 days on collagen substrate in highly defined and serum free hepatocyte media. The resulting cell populations exhibited hepatic cell-like morphology and were characterized with a variety of biological endpoint analyses. mRNA expression of the 'stemness' markers NANOG and alkaline phosphatase in the differentiated cells was significantly reduced, findings that were functionally validated using alkaline phosphatase activity detection measures. Immunofluorescence studies revealed attenuated levels of the 'stemness' markers OCT4, SOX2, SSEA-3, TRA-1-60, and TRA-1-81 in the hepatic-like cell population. The hepatic character of the cells was confirmed by the demonstration of increased mRNA expression of the hepatic transcription factors FOXA1, C/EBPα, and HNF1α, the nuclear receptors CAR, RXRα, PPARα, and HNF4α, the liver-generated plasma proteins α-fetoprotein, transthyretin, transferrin, and albumin, the protease inhibitor α-1-antitrypsin, metabolic enzymes HMGCS2, PEPCK, and biotransformation enzymes CYP3A7, CYP3A4, CYP3A5, and CYP2E1. Indocyanine green uptake and glycogen storage capacity further confirmed acquisition of hepatic function. These studies define an approach that facilitates the differentiation of hESCs along a hepatic lineage and provide a framework for subsequent use in pharmacological and toxicological research applications requiring a renewable supply of human hepatocytes.

2.2 Introduction

As the largest internal organ in mammals, the liver performs myriad functions that are essential to the body including detoxification, production and secretion of plasma proteins, maintenance of cholesterol homeostasis, production and elimination of bile constituents, synthesis and inter-conversion of amino acids, fatty acid processing, and the synthesis and breakdown of glucose [1]. Unfortunately, the liver is the target of a number of diseases processes; in the United States alone more than 40,000 deaths annually may be attributed to liver failure resulting from disease [2]. Both whole liver transplantation, currently the most successful treatment option [3], and hepatocyte transplantation are limited by the paucity of donor tissue, marked donor-to-donor variability in tissue quality, and the risk of infection, tissue rejection, or adverse immune response in the recipient [4]. Primary human hepatocyte cultures also serve as models for drug metabolism research and as predictors of toxicological processes that may be associated with exposures to xenobiotic compounds. However, the scarcity of hepatic tissue, as well as difficulties inherent in maintaining differentiated hepatocytes in culture, currently limits this vital research. Thus, the development of an unlimited supply of functional hepatocytes from alternative sources such as human embryonic stem cells (hESCs) is an attractive proposition.

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[1] Designed and conducted experiments, analyzed data, wrote manuscript.
[2] Assisted with cell maintenance and experiments.
[3] Principal investigator.

HESCs, derived from the inner cell mass of the blastocysts of developing embryos, have the potential for indefinite self-renewal yet retain a plasticity which, under appropriate conditions, permits their differentiation into derivatives of all three germ layers [5]. To date a number of studies have undertaken to induce differentiation of hESCs along a hepatic lineage. The majority of these studies have employed defined culture conditions to promote differentiation, supplementing culture media with FGFs [4;6-13], BMPs [8], hepatocyte growth factor (HGF) [4;6;7;10-12;14-17], dexamethasone [6;7;10-12;17;18], insulin [11;18], oncostatin M (OSM) [6;10;11;15;17], activin A [6-8;10;14-17;19;20], wnt3a [17;19], and sodium butyrate [15;21]. Collagen type I extracellular matrix (ECM) is also important in hepatic specification [4;6;18]. Although promising, these approaches have thus far been insufficient to direct differentiation of hESCs into fully functional, transplantable hepatocytes and subsequently require additional model development.

Primary human hepatocytes rapidly dedifferentiate in culture, losing *in vivo* hepatic hallmarks such as biotransformation enzyme induction capacity, and studies by our laboratory have been instrumental in defining culture conditions that help to preserve the differentiated hepatic phenotype in culture. By maintaining hepatocytes in the presence of extracellular matrix components [22] and in serum-free media supplemented with physiological concentrations of insulin [23] and the synthetic glucocorticoid dexamethasone [24], we have been able to facilitate retention of markers of the differentiated hepatic phenotype. Building upon a report by Shirahashi et al., where mouse and human ESCs were induced to differentiate along a hepatic lineage in the presence of collagen type I, dexamethasone, and insulin, although cultured in Iscove's Modified Dulbecco's Media supplemented with 20% fetal bovine serum (FBS) and differentiated through an embryoid body intermediate [18], we hypothesized that our previously developed and highly defined culture media – optimized for the maintenance of mature primary human hepatocytes, when coupled with a collagen substrata, would provide an enhanced means of generating hepatic-like cells from hESCs.

Indeed, the hepatic-like cell population derived by culturing hESCs for 10 days under these conditions displayed hepatic cell-like morphology, attenuated expression of pluripotency markers and 'stemness' function, increased expression of genes representing a wide range of hepatic functions including transcription factors, nuclear receptors, plasma proteins, and metabolic and biotransformation enzymes, as well as augmented hepatic function. Thus, our results define a unique, expeditious model approach to the differentiation of hESCs along a hepatic lineage.

2.3 Materials and Methods

Transformed cell line culture

All cell culture reagents were obtained from Gibco (Grand Island, NY) unless otherwise indicated. Human foreskin fibroblasts (hFFs), acquired from ATCC (Manassas, VA), were cultured in Dulbecco's Modified Eagle Media + GlutaMAX supplemented with 0.75 g/l sodium bicarbonate and 15% fetal bovine serum (FBS). HEK 293T/17 transformed human embryonic kidney cells (ATCC) were maintained in Dulbecco's Modified Eagle Media + GlutaMAX supplemented with 0.1 mM non-essential amino acids, 0.75 g/l sodium bicarbonate, 1 mM sodium pyruvate, 10 mM HEPES, 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% FBS. HepG2 human hepatoma-derived cells (ATCC) were cultured in Minimum Essential Media + Earle's Salts + ʟ-glutamine supplemented with 0.1 mM non-essential amino acids, 0.75 g/l sodium bicarbonate, 1 mM sodium pyruvate, 10 mM HEPES, 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% FBS.

Primary human hepatocyte culture

Primary human hepatocyte cultures, secured through the Liver Tissue Procurement and Distribution System (NIH Contract #N01-DK-9-2310), were isolated by collagenase perfusion and plated on rat-tail collagen as described previously [25]. Hepatocytes were cultured in our hepatocyte media: William's E Media supplemented with 10 mM HEPES, 2 mM GlutaMAX, 100 units/ml penicillin, 100 μg/ml streptomycin, 25 nM dexamethasone (Sigma; St. Louis, MO), 10 nM insulin (Sigma), 5 ng/ml selenium (Sigma), 5 μg/ml transferrin (Sigma), and 1% linoleic acid/albumin (Sigma). Hepatocyte media was replenished every other day.

Human embryonic stem cell culture

WA09 (H9) human embryonic stem cells, acquired through the National Stem Cell Bank at the WiCell Research Institute (Madison, WI), were maintained on irradiated hFF feeder layer cells in hESC media: Dulbecco's Modified Eagle Media F-12 supplemented with 20% knock-out serum replacement, 0.1 mM non-essential amino acids, 1 mM GlutaMAX, 100 ng/ml basic fibroblast growth factor (National Cancer Institute; Bethesda, MD), and 0.1 mM β-mercaptoethanol (Sigma). Media was replenished daily, differentiated colonies were removed 2-3 times per week by manual dissociation, and cells were passaged weekly and plated on fresh hFF feeder layers.

Hepatic differentiation of human embryonic stem cells and treatments

To induce hepatic differentiation, human embryonic stem cells were plated in wells coated with ~3 μg/cm^2 of rat tail type I collagen (Sigma) in hESC media. After 2 days, media was switched to hepatocyte media and cells were maintained for 8 additional days with daily replenishment of hepatocyte media for a total of 10 days in culture. Treatments were carried out with phenobarbital (Sigma) and 6-(4-chlorophenyl)imidazo[2,1–b] [1,3] thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime (CITCO) (BioMol; Plymouth Meeting, PA).

RNA isolation, cDNA archiving, and real-time PCR

RNA was isolated using TRIzol Reagent (Invitrogen; Carlsbad, CA) and converted to cDNA using High Capacity cDNA Archive Kit (Applied Biosystems; Foster City, CA), both according to manufacturers' instructions. Real-time PCR was performed using Assays-on-Demand Gene Expression Products (Applied Biosystems), according to manufacturer's protocol. Briefly, 100 ng of cDNA template, 25 μl 2X Taqman Universal Master Mix, and 2.5 μl 20X Target Assay Mix were combined into 50 μl reactions. Reactions were divided in half and run as technical replicates on an ABI 7300 Real-time PCR System. Data were analyzed using the $\Delta\Delta C_T$ method as described by Livak and Schmittgen [26]. Due to variability in the gene expression between hESCs of different passages, all data were normalized to a low-passage number (passage 33) hESC control population.

Immunofluorescence

Immunofluorescence was carried out using Human Embryonic Stem/Induced Pluripotent Stem Cell Characterization Kit (Applied StemCell Inc; Sunnyvale, CA) and all reagents were obtained from Applied StemCell Inc. unless otherwise indicated. A modified manufacturer's protocol was followed for immunofluorescence analysis. Briefly, cells were grown in 12-well plates and immersed in cell fixation solution for 1 h, washed 3 times with PBS for 5 min each, incubated in permeabilization solution for 30

minutes, and immersed in blocking solution for 1 h. Pre-diluted primary antibody solution (OCT4 anti-rabbit, SOX2 anti-rabbit, SSEA-3 anti-rat, TRA-1-60 anti-mouse, or TRA-1-81 anti-mouse) was added and cells were incubated overnight in a humidified 4° chamber. Cells were washed twice with PBS for 10 min each, incubated with either Alexa Fluor 546 goat anti-rabbit, Alexa Fluor 546 goat anti-rat, or Alexa Fluor 488 goat anti-mouse secondary antibody (Invitrogen) diluted 1:200 in PBS for 1 h in the dark, and washed in PBS for 10 min in the dark. Cells were examined microscopically on a Nikon inverted fluorescent microscope (Melville, NY) using a Nikon B-2E/C or G-2E/C filter and images were taken using a digital camera and SpotRT software (Diagnostic Instruments; Sterling Heights, MI).

Alkaline phosphatase staining

Alkaline phosphatase staining was performed using Alkaline Phosphatase Detection Kit (Millipore; Billerica, MA) according to manufacturer's protocol. Briefly, cells were fixed with 4% formaldehyde in PBS for 1-2 min, washed with TBST (20mM Tris-HCl, 0.15M NaCl, 0.05% Tween-20), stained in the dark for 15 min with a 2:1:1 ratio of Fast Red Violet:Naphthol:water, and washed with TBST prior to microscopic examination and imaging.

Indocyanine green uptake

Indocyanine green (ICG) uptake analysis was carried out essentially as described by Yamada et al. except that a final ICG concentration of 100 µg/ml was used due to toxicity observed in hESC cultures at the recommended 1 mg/ml concentration [27]. Briefly, Cardiogreen (Sigma) was dissolved in water to make a fresh 5 mg/ml stock solution and then diluted in culture medium to a final concentration of 100 µg/ml. Cells were immersed in the diluted ICG solution, incubated for 15 min at 37°C, washed with PBS, and observed and imaged microscopically.

Periodic acid-Schiff staining

Periodic acid-Schiff (PAS) staining was performed using Periodic Acid-Schiff Kit (Sigma) according to manufacturer's instructions with minor adaptations. Briefly, cells were fixed in 4% formaldehyde in PBS for 1-2 min, washed with TBST, stained for 5 min with periodic acid, and washed 3x with PBS. Cells were then stained with Schiff's reagent for 15 min, washed 3 times with PBS, counterstained for 90 sec with hematoxylin solution, and washed 3 times with PBS prior to microscopic examination and imaging.

Statistical analyses

A Student's t-test (one-tailed; two-sample, unequal variance) was used for all statistical analyses.

2.4 Results

HESCs subjected to hepatic differentiation exhibit hepatic cell-like morphology

We subjected the H9 hESC line – selected because it has been extensively characterized and employed for numerous studies of hepatic differentiation – to a unique hepatic differentiation approach in which the cells were maintained on rat-tail collagen type I substrate in highly defined hepatocyte media (William's E Media supplemented with HEPES, glutamine, antibiotics, dexamethasone, insulin, transferrin, selenium, and linoleic acid/albumin) for 10 days. A large subset (~40%) of the resulting cell population displayed a uniform morphology similar to that of hepatic-like cells including the hepatoma-

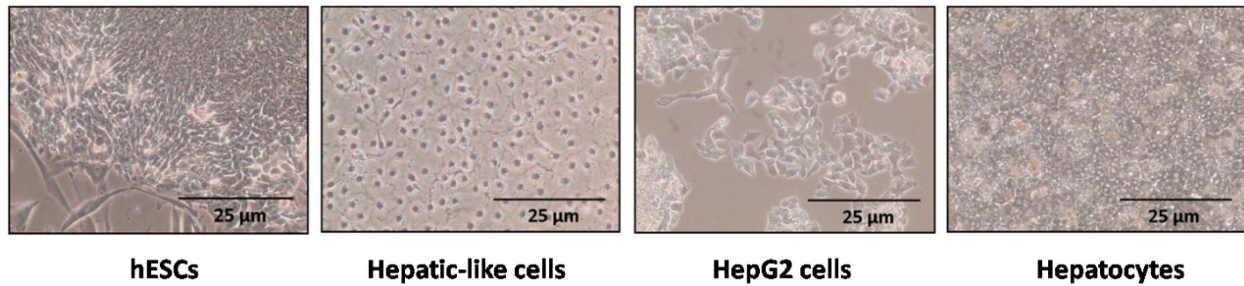

Figure 2-1. HESCs subjected to hepatic differentiation exhibit hepatic cell-like morphology. Images depict phase-contrast micrographs of a hESC colony (hESCs), hESC-derived hepatic-like cells generated by culturing hESCs for 10 days on type I rat-tail collagen in hepatocyte media (Hepatic-like cells), HepG2 hepatoma cells (HepG2 cells), and cultures of primary human hepatocytes (Hepatocytes).

derived HepG2 cell line, a model widely employed in hepatic studies, and cultures of primary human hepatocytes. In contrast to hESCs which exhibit a small, flat morphology and grow in culture in distinct colonies, the hepatic-like cells exhibit a larger, more cuboidal shape and a homogenous distribution more similar to that of cultures of primary human hepatocytes (Fig. 2-1).

'Stemness' marker mRNA expression is significantly decreased in hepatic-like cells

Differentiation of hESCs is accompanied by the loss of expression of markers of pluripotency or 'stemness,' such as the transcription factor nanog homeobox (NANOG) and alkaline phosphatase. The hepatic-like cell population resulting from our differentiation protocol exhibited a significant reduction in mRNA expression of both NANOG – which together with octamer-binding transcription factor 4 (OCT4) is widely considered to be the key factor in the conferral of stem cell pluripotency [28] – and alkaline phosphatase, to levels approximately one-third of those of hESC controls (Fig. 2-2).

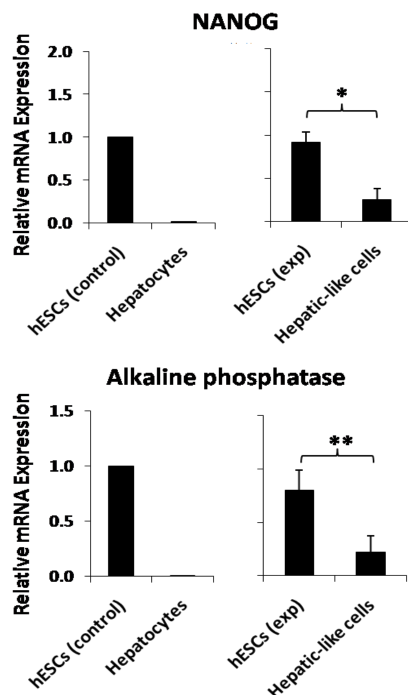

Figure 2-2. 'Stemness' marker mRNA expression is significantly decreased in hepatic-like cells. HESCs were cultured on either hFF feeder layers in hESC media (hESCs exp) or on collagen in hepatocyte media (Hepatic-like cells). After 10 days in culture cells were harvested and RNA was isolated, converted to cDNA, subjected to real-time PCR, and the data analyzed using the $\Delta\Delta C_T$ method to determine expression levels of NANOG and alkaline phosphatase relative to low-passage hESCs. Control data (left graph) depict expression levels of low-passage hESCs (hESCs control) which serve as a negative control and pooled samples from six primary human hepatocyte donors (Hepatocytes) which serve as a positive control. Experimental data (right graph) depict expression levels of hESCs (hESCs exp) and passage-matched hESCs subject to our hepatic differentiation protocol (Hepatic-like cells). Experimental data are expressed as mean +/- standard deviations of at least two independent trials using hESCs from different passages. * $p < 0.05$; ** $p < 0.01$.

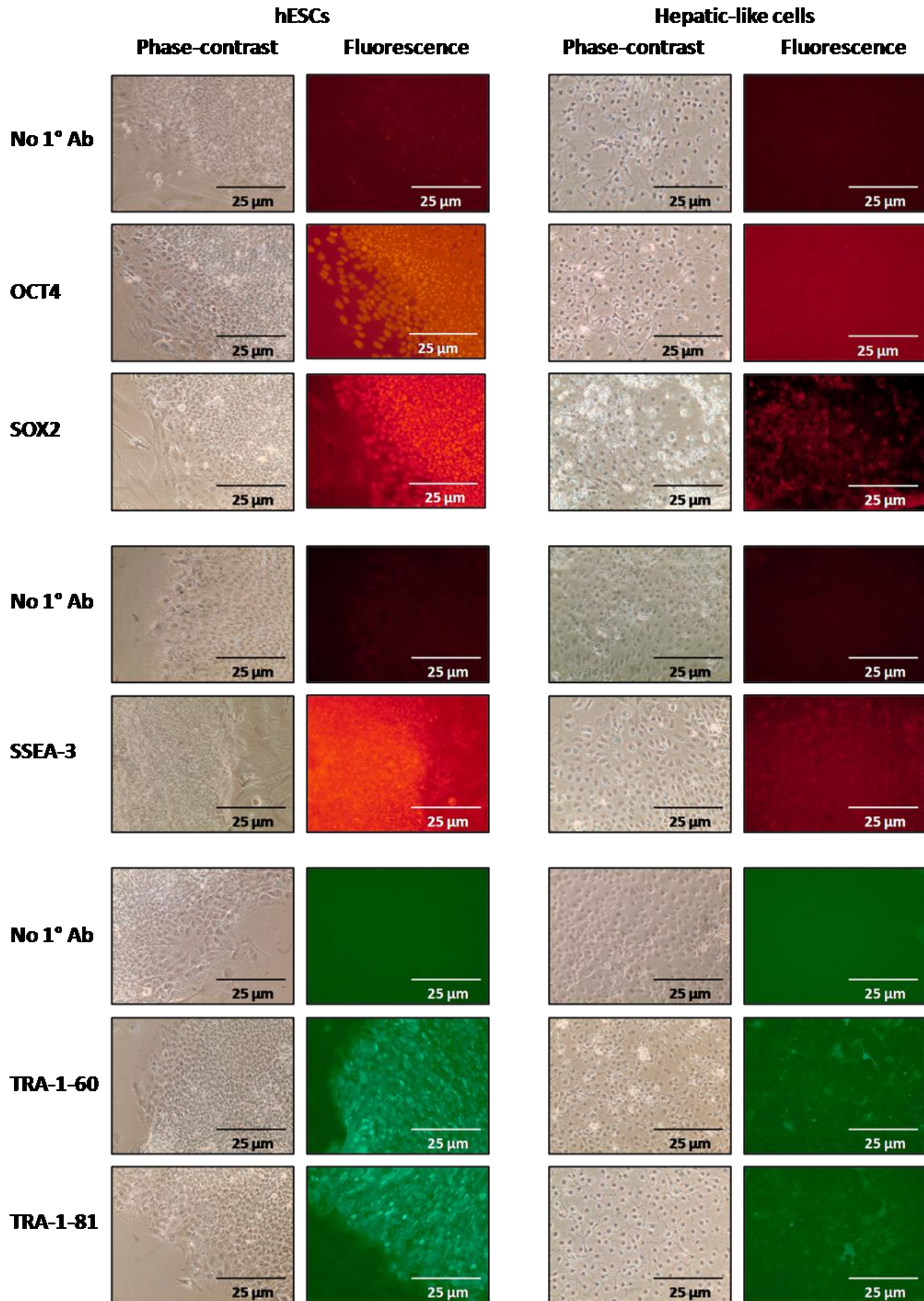

Figure 2-3. 'Stemness' marker protein expression is reduced in hepatic-like cells. HESCs were cultured on either hFF feeder layers in hESC media (hESCs) or on collagen in hepatocyte media (Hepatic-like cells). At 10 days in culture cells were probed for OCT4, SOX2, SSEA-3, TRA-1-60, and TRA-1-81 protein expression by immunofluorescence using antibodies specified in the Materials and Methods. Cells were examined microscopically and phase-contrast and fluorescence images were captured.

'Stemness' marker protein expression is reduced in hepatic-like cells

Changes in gene expression at the protein level were assessed by immunofluorescence analysis which showed that while hESCs exhibited robust, uniform expression of the 'stemness' transcription factors OCT4 and SRY-box containing gene 2 (SOX2) and the surface antigens stage-specific embryonic antigen 3 (SSEA-3), tumor rejection antigen 1-60 (TRA-1-60), and tumor rejection antigen 1-81 (TRA-1-81), expression of these markers in the hepatic-like cells was noticeably attenuated (Fig. 2-3). Of the markers assessed, expression of the key pluripotency factor OCT4 exhibited the most dramatic decrease upon hepatic differentiation (Fig. 2-3, second row).

Hepatic-like cells exhibit decreased 'stemness' function

To assess functional changes in the hESC-derived hepatic-like cells we utilized alkaline phosphatase activity staining. Staining revealed robust enzymatic activity in the hESCs (pink color), activity which was undetectable in both the hepatic-like cell population and primary human hepatocytes (Fig. 2-4). These data confirm the functional relevance of the reduced alkaline phosphatase mRNA expression observed previously in the hepatic-like cells and, together with the data showing the reduction in mRNA and protein expression of an array of select other markers of 'stemness,' suggest that the hepatic-like cell population has indeed transitioned from a pluripotent state.

Hepatic transcription factor mRNA expression is increased in hepatic-like cells

To determine the extent of acquisition of hepatic character in the hepatic-like cell population we assessed mRNA expression of select hepatic-enriched transcription factors. Compared to hESCs, the hepatic-like cells exhibited significantly increased expression of forkhead box A1 (FOXA1), CCAAT/enhancer binding protein alpha (C/EBPα), and hepatic nuclear factor 1 alpha (HNF1α) (Fig. 2-5A), integral members of the combinatorial regulatory network that controls transcription of most genes in the hepatic program [29], as well as augmented expression of the hepatic nuclear receptors constitutive androstane receptor (CAR), retinoid X receptor alpha (RXRα), peroxisome proliferator-activated receptor alpha (PPARα), and hepatic nuclear factor 4 alpha (HNF4α) (Fig. 2-5B). Notably, expression of FOXA1, a transcription factor vital for the onset of hepatogenesis [30], was enhanced an average of 80-fold in hepatic-like cells compared to hESCs, to levels comparable to those of primary human hepatocytes (Fig. 2-5A, left).

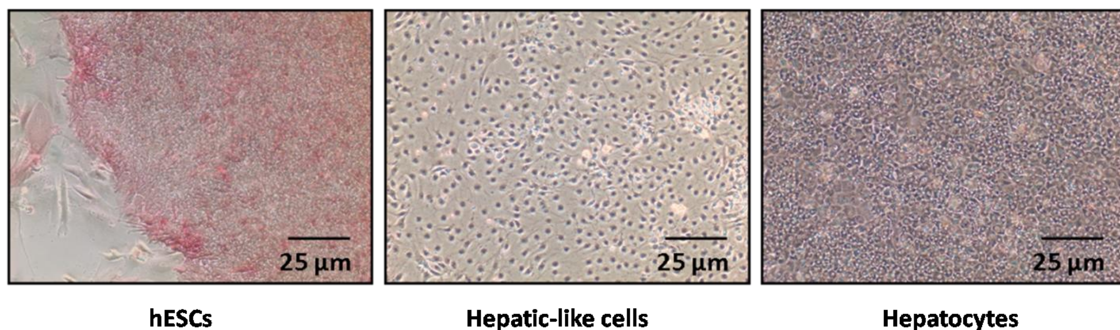

Figure 2-4. Hepatic-like cells exhibit decreased 'stemness' function. HESCs were cultured on either hFF feeder layers in hESC media (hESCs) or on collagen in hepatocyte media (Hepatic-like cells). At 10 days in culture cells were stained for alkaline phosphatase activity (pink color) and phase-contrast images were captured. Primary human hepatocytes (Hepatocytes) stained in parallel serve as a negative control.

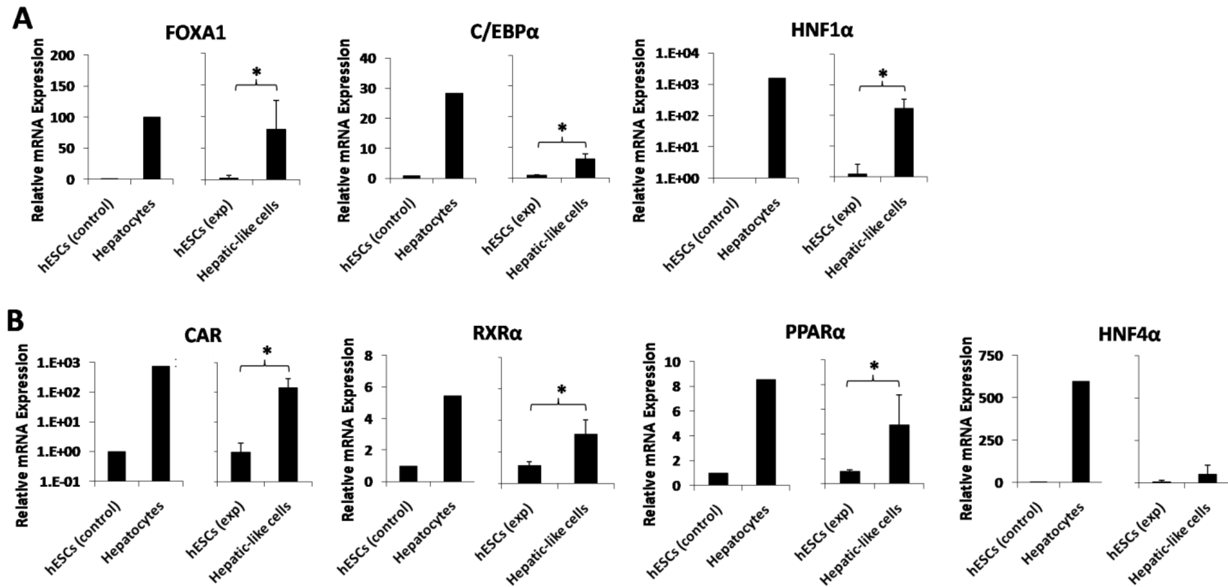

Figure 2-5. Hepatic transcription factor mRNA expression is increased in hepatic-like cells. HESCs were cultured on either hFF feeder layers in hESC media (hESCs exp) or on collagen in hepatocyte media (Hepatic-like cells). At 10 days in culture cells were harvested and RNA was isolated, converted to cDNA, subjected to real-time PCR, and the data analyzed using the $\Delta\Delta C_T$ method to determine expression levels relative to low-passage hESCs (hESCs control) of (A) the hepatic transcription factors FOXA1, C/EBPα, and HNF1α, and (B) the hepatic nuclear receptors CAR, RXRα, PPARα, and HNF4α. Control data (left graph) depict expression levels of low-passage hESCs (hESCs control) which serve as a negative control and pooled samples from six primary human hepatocyte donors (Hepatocytes) which serve as a positive control. Experimental data (right graph) depict expression levels of hESCs (hESCs exp) and passage-matched hESCs subject to our hepatic differentiation protocol (Hepatic-like cells). Experimental data are expressed as mean +/- standard deviations of at least three independent trials using hESCs from different passages. * $p < 0.05$.

Hepatic marker mRNA expression is augmented in hepatic-like cells

While expression of hepatic transcription factors is indicative of differentiation along a hepatic lineage, hepatic character is ultimately defined by acquisition of hepatic function. Thus, we assessed mRNA expression of hepatic functional marker genes in the hepatic-like cell population. Indeed, compared to hESCs, the hepatic-like cells exhibited increased mRNA expression of the liver-generated plasma proteins α-fetoprotein, transthyretin, transferrin, and albumin (Fig. 2-6A) and the protease inhibitor α-1-antitrypsin (Fig. 2-6B). mRNA levels of the metabolic enzymes 3-hydroxy-3-methylglutaryl-coenzyme A synthase 2 (HMGCS2) and phosphoenolpyruvate carboxykinase (PEPCK) (Fig. 2-6C), and members of the cytochrome P450 (CYP) family of biotransformation enzymes including CYP3A7, CYP3A4, CYP3A5, and CYP2E1 (Fig. 2-6D) were also enhanced in the hepatic-like cell population.

Hepatic-like cells exhibit the hepatic-specific functions of ICG uptake and glycogen storage

Cellular uptake of indocyanine green (ICG), an organic anion that is a substrate of hepatic transporter systems, is a commonly used indicator of hepatic function [27] which we employed to further assess the hepatic character of our hESC-derived hepatic-like cell population. Despite the differences in confluence between the cell populations, indocyanine green uptake by the hepatic-like cells (cyan-green color) approached those of primary human hepatocyte cultures, while hESC cultures excluded the anionic solution (Fig. 2-7, top row). We next assessed the ability of the hepatic-like cells to store glycogen, a

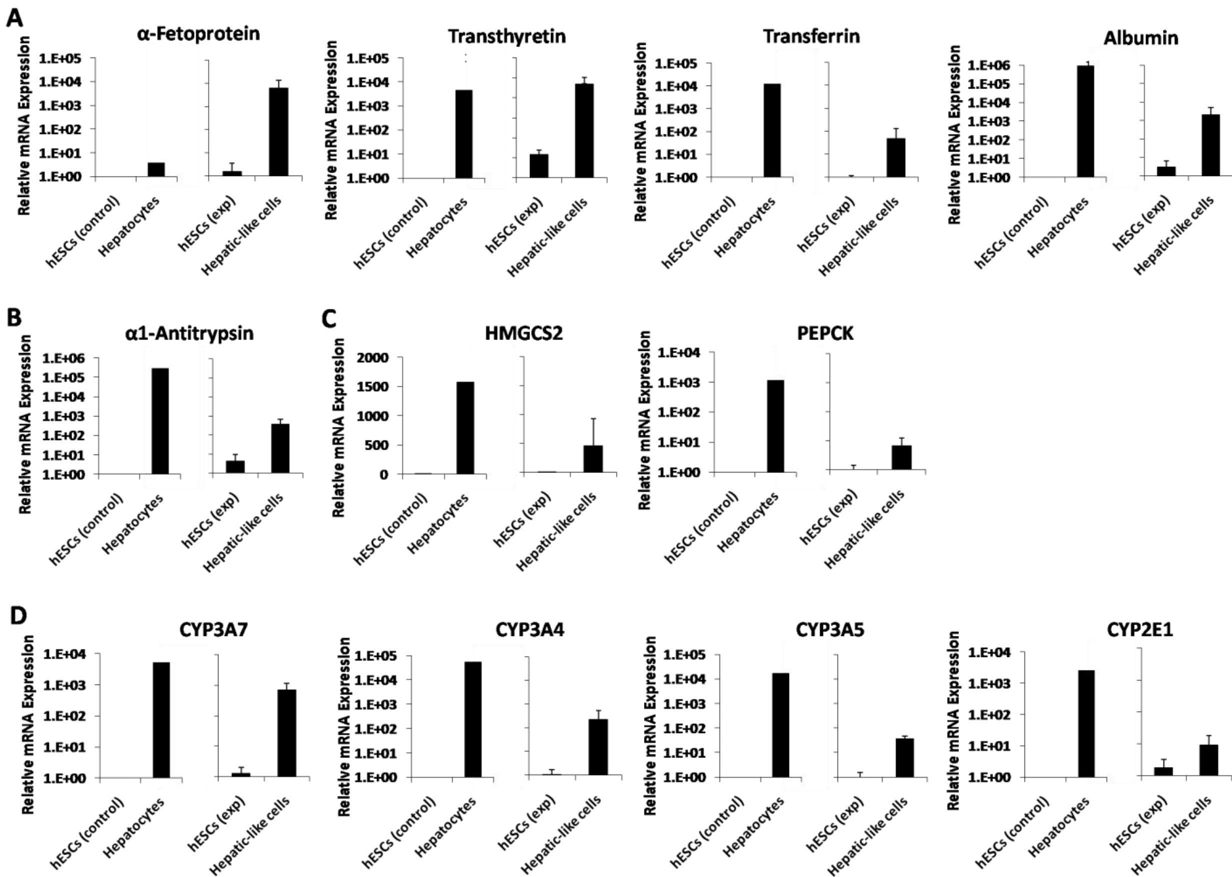

Figure 2-6. Hepatic marker mRNA expression is augmented in hepatic-like cells. HESCs were cultured on either hFF feeder layers in hESC media (hESCs exp) or on collagen in hepatocyte media (Hepatic-like cells). At 10 days in culture cells were harvested and RNA was isolated, converted to cDNA, subjected to real-time PCR, and the data analyzed using the $\Delta\Delta C_T$ method to determine expression levels relative to low-passage hESCs (hESCs control) of (A) the plasma proteins α-fetoprotein, transthyretin, transferrin, and albumin, (B) the protease inhibitor α-1-antitrypsin, (C) the metabolic enzymes HMGCS2 and PEPCK, and (D) the biotransformation enzymes CYP3A7, CYP3A4, CYP3A5, and CYP2E1. Control data (left graph) depict expression levels of low-passage hESCs (hESCs control) which serve as a negative control and pooled samples from six primary human hepatocyte donors (Hepatocytes) which serve as a positive control. Experimental data (right graph) depict expression levels of hESCs (hESCs exp) and passage-matched hESCs subject to our hepatic differentiation protocol (Hepatic-like cells). Experimental data are expressed as mean +/- standard deviations of at least three independent trials using hESCs from different passages.

unique function of the specialized hepatocyte, using periodic acid-Schiff (PAS) staining. Again, although levels of confluence varied considerably between the cell populations, the hepatic-like cell population exhibited levels of glycogen accumulation (pink color) approaching those of primary human hepatocyte cultures (Fig. 7, bottom row). Although hESCs exhibited limited PAS staining as well, the stain was distributed uniformly throughout the cells, unlike the hepatic-like cells and hepatocytes in which glycogen was present only in the cytoplasm, suggesting that the staining observed in the hESCs may be artifactual in nature. Notably, the cytoplasmic glycogen stores in the hepatic-like cells serve to further highlight the cells' enhanced nuclear-to-cytoplasmic ratio, cuboidal morphology, and prominent nucleoli, characteristics of a mature hepatic phenotype (Fig. 2-7, bottom row). Measurement of albumin secretion, another indicator of mature hepatic function, was not undertaken due to the high levels of albumin present in the hepatocyte media.

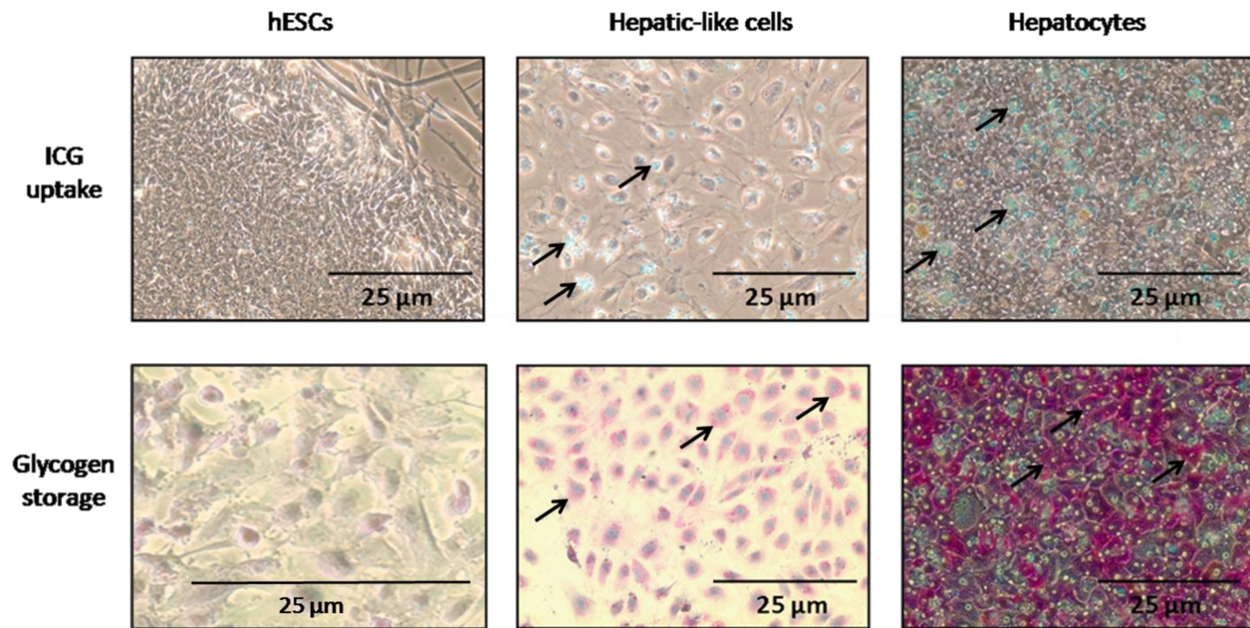

Figure 2-7. Hepatic-like cells exhibit the hepatic-specific functions of indocyanine green uptake and glycogen storage. HESCs were cultured on either hFF feeder layers in hESC media (hESCs) or on collagen in hepatocyte media (Hepatic-like cells) for 10 days. Cells were evaluated for indocyanine green (ICG) uptake (cyan-green color) by incubation with 100 µg/ml ICG and for glycogen storage capacity (pink color) using periodic acid-Schiff (PAS) staining, both as described in Materials and Methods. Cells were examined microscopically and phase-contrast images were captured. Cultures of primary human hepatocytes (Hepatocytes) assessed in parallel were included as positive controls.

2.5 Discussion

The hepatic differentiation approach defined in this study – in which hESCs are cultured in hepatocyte media (William's E media supplemented with HEPES, glutamine, antibiotics, dexamethasone, insulin, transferrin, selenium, and linoleic acid/albumin) on collagen type I substrate for 10 days – provides a novel approach by which hESCs may be directed to differentiate along a hepatic lineage. Extensive characterization of our hESC-derived hepatic-like cell population revealed attenuated mRNA expression of markers of pluripotency including the transcription factor NANOG and the alkaline phosphatase enzyme (validated functionally by activity staining) and reduced protein expression of the 'stemness' transcription factors OCT4 and SOX2 as well as the surface antigens SSEA-3, TRA-1-60, and TRA-1-81. The hepatic-like cells exhibited increased mRNA expression of the hepatic-enriched transcription factors FOXA1, C/EBPα, and HNF1α, nuclear receptors CAR, RXRα, PPARα, and HNF4α, plasma proteins α-fetoprotein, transthyretin, transferrin, and albumin, the protease inhibitor α-1-antitrypsin, metabolic enzymes HMGCS2, PEPCK, and biotransformation enzymes CYP3A7, CYP3A4, CYP3A5, and CYP2E1. Indocyanine green uptake and glycogen storage capacity by the differentiated cells confirmed acquisition of hepatic function.

In our differentiation protocol, hESCs were cultured on collagen type I, a protein component of ECM that along with soluble signaling factors such as those found in our defined hepatocyte media, influences hepatocyte development and function in the liver microenvironment. Liver ECM is comprised primarily of collagen type I and fibronectin, as well as collagen types III, IV, and VI, tenascin, and laminin [31]. While primary human hepatocytes maintained on plastic dishes rapidly de-differentiate in culture, a number of studies have shown that primary hepatocytes cultured on collagen type I gel retain their

differentiated functions, including expression of liver-specific genes, albumin secretion, and urea production [31]. While culturing of hESCs on collagen substrate in the absence of hepatocyte media resulted in limited expression of hepatic differentiation markers (data not shown), the combination of collagen substrate and hepatocyte media was necessary to achieve maximal hepatic induction.

The unique hepatocyte media used in this study is comprised of a number of factors that likely act in concert to facilitate hepatic differentiation of hESCs. In particular, dexamethasone and insulin play integral roles in normal liver development and thus are potentially the primary factors inducing differentiation in this approach. Dexamethasone is required for hepatic induction by both OSM and HGF [32;33]. Using cultures of murine fetal hepatocytes, Kamiya et al. demonstrated that treatment with a combination of dexamethasone and OSM results in morphological changes consistent with fully differentiated hepatocytes, augmented expression of hepatic marker genes, and intracellular glycogen accumulation [32]. In contrast to OSM stimulation alone, treatment with dexamethasone alone promoted a number of hepatic specification markers, albeit to a much lesser extent than the dexamethasone/OSM combination [34]. Studies assessing the role of HGF in hepatic differentiation yielded similar results; in the absence of dexamethasone, HGF is unable to induce differentiation, as measured by the up-regulation of hepatic marker genes and intracellular glycogen accumulation [33]. The liver is also subject to continual exposure to insulin secreted by the pancreatic islets through the portal vein, the importance of which is underscored by the liver atrophy which results from the removal of this signal [35].

While the hepatic-like cells derived from this protocol expressed high mRNA levels of an extensive array of hepatic markers, expression levels of the majority of the genes assessed were lower than those of primary human hepatocytes. Further, the hepatic-like cell population expressed the fetal liver markers α-fetoprotein and CYP3A7 and, while they exhibited increased levels of CYP3A4 compared to hESCs, treatment with 500 μM phenobarbital or 100 nM CITCO did not result in further induction of CYP3A4, a hallmark of the differentiated hepatocyte (data not shown). These findings indicate that our hepatic-like cell population is not yet fully mature and may be better characterized as hepatoblasts or hepatic progenitor cells. Thus, further studies are necessary to elucidate the factors needed to facilitate completion of the hepatic differentiation process as well as to determine the mechanism(s) by which hepatic differentiation is mediated.

In summary, the results presented in this study underscore the importance of collagen type I together with dexamethasone and insulin – factors hitherto thought to play a lesser role in hepatogenesis and to act at a later developmental stage than FGFs, BMP, OSM, HGF, or activin A [36] – in hepatic differentiation. We posit that our differentiation approach, when combined with other hepatic induction methodologies, may provide the supplementary factors needed to generate fully functional hepatocytes from human embryonic stem cells and ultimately provide a valuable source of hepatocytes for therapeutic transplantations and for direct application in pharmacological and toxicological research investigations.

2.6 References

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Chapter 3

Preservation of Hepatic Phenotype in Lentiviral-Transduced Primary Human Hepatocytes

Adapted from:

Zamule SM[1], Strom SC[2], and Omiecinski CJ[3]. Preservation of hepatic phenotype in lentiviral-transduced primary human hepatocytes. Chem Biol Interact 2008; 173:179-186.

3.1 Abstract

Lentiviral vectors effectively transduce both dividing and non-dividing cells and stably integrate into the genome of the host cell. In this study, we evaluated the usefulness of a lentiviral system for genetic modulation of primary human hepatocyte cultures. Infection with GFP expressing lentivectors shows that Huh7 and HepG2 cell lines, as well as primary cultures of human hepatocytes, are efficiently transduced by lentiviral vectors. Real-time RT-PCR analyses demonstrate that infection with lentivectors does not alter hepatic hallmarks such as the expression of the nuclear receptors CAR, PXR, RXRα, or HNF4α, or expression of the secretory protein, albumin. Additionally, infected hepatocytes retain the capacity for CYP3A4 induction in response to treatment with phenobarbital, a uniquely sensitive indicator of hepatic differentiation status. Lentivectors may be used for both over-expression and knockdown analyses in primary hepatocytes, as demonstrated in this study by >200-fold CAR over expression and knockdown of CAR to less than 40% of endogenous levels, with corresponding effects on CYP2B6 expression. In summary, lentiviral vectors provide a novel methodology by which primary human hepatocytes may be stably genetically manipulated, with minimal effects on the differentiated hepatic phenotype. These approaches offer considerable advantage over current methodologies, providing a valuable alternative for use in pharmacological and toxicological investigations involving primary human hepatocyte models and potentially for cell-based therapeutics to treat hepatic dysfunction *in vivo*.

3.2 Introduction

Healthy hepatic function is integral to the disposition and metabolism of a diverse array of endogenous and exogenous substances. However, the liver is the target of many congenital and acquired diseases, some of which are amenable to gene therapy. Additionally, cultures of primary human hepatocytes serve as vital models for drug metabolism research and as predictors of toxicological out-comes that may result following exposure to xenobiotic agents. The ability to modulate gene expression within the fully differentiated hepatocyte is imperative for the realization of the full potential of hepatic gene therapy, and for manipulating the hepatocyte model in research investigations.

Development of an ideal methodology for the genetic modulation of primary human hepatocyte cultures has proven challenging. Primary human hepatocytes are refractory to most common transfection techniques. While adenoviral vectors transduce hepatocytes effectively, the vectors are non-replicating and remain episomal, and thus gene expression is transient [1;2]. Retroviral vectors integrate into the host genome, but most require a round of cell division for the integration event to

[1] Designed and conducted experiments, analyzed data, wrote manuscript.
[2] Provided primary human hepatocyte cultures.
[3] Principal investigator.

occur [3–5]. Such viral vectors are therefore ineffectual for stable genetic modulation in quiescent primary hepatocyte cultures [6]. Baculovirus vectors are reasonably efficient for hepatocyte transduction, however, previous studies in our laboratory have demonstrated that the baculoviral infection event adversely affects the differentiated hepatic phenotype [7;8], with infection reducing hepatic albumin levels and ablating the phenobarbital induction response; both sensitive indicators of hepatocyte differentiation status [9].

Lentiviral vectors are unique within the retroviral vector family because lentivirus is capable of infecting both dividing and non-dividing cells and stably integrates into the host genome, thereby facilitating long-term transgene expression [10–12]. Several studies have shown that lentiviral vectors effectively transduce the widely used hepatoma cell lines, Huh7 and HepG2 [13–18]. More recently, researchers demonstrated that lentivectors afford high efficiency transduction of primary cultures of human hepatocytes [14;19–21]. While these results are promising, it is imperative to evaluate whether the infection event alters the differentiated hepatic phenotype, or otherwise adversely affects hepatocytes in primary culture.

In this investigation, we verify that lentiviral vectors efficiently transduce Huh7 and HepG2 hepatoma-derived cell lines, and demonstrate that transgene expression is preserved through cell division. We further confirm that primary human hepatocyte cultures are effectively transduced by lentivectors and that hepatocytes retain transgene expression for the duration of the culture period. Importantly, the results of our studies demonstrate that lentiviral infection does not alter mRNA expression levels of albumin or of key nuclear receptors in primary human hepatocytes, including constitutive androstane receptor (CAR), retinoid X receptor alpha (RXRα), pregnane X receptor (PXR), or hepatocyte nuclear factor four alpha (HNF4α). Additionally, we show that lentiviral-infected hepatocytes retain the capacity for cytochrome P450 3A4 (CYP3A4) induction in response to treatment with phenobarbital, a response consistent with a highly differentiated hepatic phenotype [22]. Further, we demonstrate that a lentiviral infection strategy in hepatocytes is effective for achieving both over-expression and knockdown of select genes with concomitant modulation of specific target gene responses, and identify a novel siRNA sequence capable of knocking down CAR to ∼40% of endogenous levels in human hepatocytes.

3.3 Materials and Methods

Materials

Cell lines were purchased from ATCC (Manassas, VA). Dulbecco's Modified Eagle's Media (DMEM)+ GlutaMAX, Minimum Essential Media (MEM) + Earle's Salts + ʟ-glutamine, William's E Media, non-essential amino acids, sodium bicarbonate, sodium pyruvate, HEPES, penicillin, streptomycin, GlutaMAX, and fetal bovine serum (FBS) were acquired from Gibco/Invitrogen (Grand Island,NY). Dexamethasone, insulin, selenium, transferrin, and linoleic acid/albumin were obtained from Sigma (St. Louis, MO). 6-(4-Chlorophenyl)imidazo[2,1–b] [1,3] thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime (CITCO) was purchased from BioMol (Plymouth Meeting, PA). Phenobarbital (PB) was purchased from Sigma (St. Louis, MO). All oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA). pTracer-CMV2 was purchased from Invitrogen (Carlsbad, CA). pCDH1-MCS1-EF1-copGFP cDNA lentivector, pSIH1-H1-copGFP shRNA lentivector, and pPACKH1 packaging plasmid mix were purchased from System Biosciences (Mountain View, CA). QIAquick Gel Extraction Kit and QIAfilter Plasmid Maxi Kit were obtained from Qiagen (Valencia, CA). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA). PEG-8000 was acquired from Sigma (St. Louis, MO). Nikon inverted fluorescence microscope was purchased from Nikon USA (Melville, NY), and digital camera and SpotRT software were

Donor	Age	Gender	Ethnicity	Cause of Death
HH-A	54	M	C	N/A (Resection)
HH-B	45	M	C	Head Trauma
HH-C	54	M	C	N/A (Resection)
HH-D	55	M	C	Cerebrovascular Accident
HH-E	74	F	C	N/A (Resection)
HH-F	49	M	C	N/A (Resection)
HH-G	69	F	C	N/A (Resection)
HH-H	44	M	C	N/A (Resection)
HH-I	57	F	C	N/A (Resection)

Table 3-1. Human hepatocyte donor information.

purchased from Diagnostic Instruments (Sterling Heights, MI). TRIzol Reagent was obtained from Invitrogen (Carlsbad, CA). High Capacity cDNA Archive Kit, Assays-on-Demand Gene Expression Products, and ABI 7300 Real-time PCR System were purchased from Applied Biosystems (Foster City, CA).

Cell culture and treatment

293T/17 transformed human embryonic kidney cells were maintained in DMEM + GlutaMAX supplemented with 0.1 mM non-essential amino acids, 0.75 g/l sodium bicarbonate, 1 mM sodium pyruvate, 10 mM HEPES, 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% FBS. HepG2 and Huh7 human hepatoma-derived cell lines were maintained in MEM + Earle's Salts + ʟ-glutamine supplemented with 0.1 mM non-essential amino acids, 0.75 g/l sodium bicarbonate, 1 mM sodium pyruvate, 10 mM HEPES, 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% FBS. Primary human hepatocyte cultures were obtained through the Liver Tissue Procurement and Distribution System under NIH Contract #N01-DK-9-2310 (refer to Table 3-1 for hepatocyte donor information). Hepatocytes were isolated by a three-step collagenase perfusion technique and plated on rat-tail collagen as previously described [23]. Hepatocytes were maintained in William's E Media supplemented with 10 mM HEPES, 2 mM GlutaMAX, 100 units/ml penicillin, 100 µg/ml streptomycin, 25 nM dexamethasone, 10 nM insulin, 5 ng/ml selenium, 5 µg/ml transferrin, and 1% linoleic acid/albumin.

Lentiviral cDNA expression vector construction

Human CAR (NM_005122) was initially PCR amplified from human liver cDNA using the primer sequences: F 5'-GATC<u>GAATTC</u>GTCATGGCCAGTAGGGAAGATGAG-3'
R 5'-GATC<u>GATATC</u>TCAGCTGCAGATCTCCTGGAGCCAG-3'
The amplicon was then digested with EcoRI and EcoRV (restriction sites underlined) and cloned by ligation into pTracer-CMV2. The hCAR fragment was subsequently sub-cloned into pCDH1-MCS1-EF1-copGFP (pCDH1) cDNA lentivector. Briefly, hCAR was digested from the original vector using EcoRI and EcoRV, electrophoresed through a 0.6% agarose gel, purified using QIAquick Gel Extraction Kit, and cloned by ligation into EcoRI and SwaI sites of the pCDH1 vector. Plasmids were purified using QIAfilter Plasmid Maxi Kit.

siRNA design and lentiviral siRNA expression vector construction

Small interfering RNA (siRNA) target sequences within the hCAR mRNA were identified using GenScript Corporation's "siRNA Target Finder" (https://www.genscript.com/ssl-bin/app/rnai) and confirmed by

searching a number of other publically accessible siRNA target sequence identifiers. Template sequences encoding a short hairpin RNA (a stem-loop structure consisting of both the sense and anti-sense strands of the targeted sequence separated by a loop sequence) were generated and cloned into pSIH1-H1-copGFP (pSIH1) shRNA lentivector. Briefly, two complementary oligonucleotides (Table 3-2, restriction sites underlined) were annealled, digested, electrophoresed through a 3% agarose gel, purified using QIAquick Gel Extraction Kit, and ligated into BamH1 and EcoRI sites of the pSIH1 vector. Plasmids were purified using QIAfilter Plasmid Maxi Kit.

Lentiviral production, titering and target cell infection

Lentivirus production and subsequent target cell infections were performed essentially according to manufacturer's instructions, except that Lipofectamine 2000 was utilized for transfection of the 293T/17 packaging cells with pPACKH1 packaging plasmids and either pCDH1 or pSIH1 expression vectors. Pseudoviral supernatant harvested from packaging cells was either used to infect target cells directly, or was concentrated prior to infection by 10% PEG-8000 precipitation. Viral titer was estimated by infecting 100,000 293T/17 cells with 10-fold dilutions of virus immediately after plating. 48 h post-infection the percentage of GFP + cells in three to five random fields was determined by counting cells at 200x magnification and used to calculate the number of transducing units per milliliter of viral supernatant (TU/ml). GFP expression in infected cells was observed using a Nikon inverted fluorescence microscope, and images were captured using SpotRT software with a digital camera.

Real-time RT-PCR

Primary human hepatocyte RNA was isolated using TRIzol Reagent and converted to cDNA using High Capacity cDNA Archive Kit, both according to manufacturers' instructions. Real-time RT-PCR was performed using Assays-on-Demand Gene Expression Products according to manufacturer's protocol. Briefly, 100 ng of cDNA template, 25 µl 2x Taqman Universal Master Mix, and 2.5 µl 20x Target Assay Mix were combined into 50 µl reactions. Each reaction was divided in half and run on an ABI 7300 Real-time PCR System. Real-time RT-PCR data were analyzed using the $\Delta\Delta C_T$ Method as previously described [24]. Briefly, C_T values for each half-reaction were averaged and this value used in subsequent

Template I.D.	Strand 1	Strand 2
CAR 210	5'GGTACCGGATCCGCCACAGGCTACCA CTTTAATCTTCCTGTCAGAATTAAAGTG GTAGCCTGTGGCTTTTTGAATTCGAATT CGGTACC 3'	5'GGTACCGAATTCGAATTCAAAAAGCCAC AGGCTACCACTTTAATTCTGACAGGAAGA TTAAAGTGGTAGCCTGTGGCGGATCCGG TACC3'
CAR 379	5'ACCGGATCCCTGGCATGAGGAAAGAC ATGACTTCCTGTCAGATCATGTCTTTCC TCATGCCAGTTTTTGAATTCGGT3'	5'ACCGAATTCAAAAACTGGCATGAGGAA AGACATGATCTGACAGGAAGTCATGTCTT TCCTCATGCCAGGGATCCGGT3'
CAR 572	5'ACCGGATCCAGCTCATCTGTTCATCCA TCACTTCCTGTCAGATGATGGATGAACA GATGAGCTTTTTTGAATTCGGT3'	5'ACCGAATTCAAAAAAGCTCATCTGTTCA TCCATCATCTGACAGGAAGTGATGGATGA ACAGATGAGCTGGATCCGGT3'
CAR 755	5'ACCGGATCCGGAAATCTGTCACATCG TACTCTTCCTGTCAGAAGTACGATGTGA TGACATATTTCCTTTTTGAATTCGGT3'	5'ACCGAATTCAAAAAGGAAATCTGTCACA TCGTACTTCTGACAGGAAGAGTACGATGT GACAGATTTCCGGATCCGGT3'

Table 3-2. Sequences of oligonucleotides used to generate pSIH1 lentivectors expressing siRNAs targeted to CAR (restriction sites underlined).

calculations. Target gene expression levels for each sample were normalized to those of the corresponding 18S endogenous control by calculating the ΔC_T [$\Delta C_T = C_{Ttarget} - C_{T18S}$]. Target gene expression levels of samples infected with gene- or siRNA-expressing virus were further normalized to those infected with empty virus by computing the $\Delta\Delta C_T$ [$\Delta\Delta C_T = \Delta C_{Tgene/siRNA\text{-}exp.virus} - \Delta C_{Temptyvirus}$]. The relative fold change of target gene expression levels of samples infected with gene- or siRNA-expressing virus relative to those infected with empty virus was calculated by raising 2 to the $-\Delta\Delta C_T$ power [$2^{-\Delta\Delta CT}$].

3.4 Results

Lentiviral vectors effectively transduce Huh7 and HepG2 cells, as well as primary human hepatocyte cultures

In order to evaluate the usefulness of a lentiviral system for genetic modulation of hepatic models, we first tested the ability of the lentiviral vectors to transduce two commonly used hepatic model cell lines, Huh7 and HepG2. Indeed, when infected with lentiviral vectors engineered to express green fluorescent protein (GFP) at a multiplicity of infection (MOI) of ~5 TU/cell, >95% of Huh7 cells, and >75% of HepG2 cells were estimated to be GFP+ at 5 days post-infection (Fig. 3-1A and B, upper panels). These results are consistent with those of previous studies which have reported that Huh7 cells are more efficiently transduced by lentivectors than HepG2 cells [14;16]. To confirm retention of transgene expression throughout cell division, infected Huh7 and HepG2 cells were maintained for over 3 months, throughout 20 passages, in the absence of any selective agent. Results indicated that GFP expression is retained in close to 100% of the cells in the initial GFP+ Huh7 and HepG2 populations, through cell proliferation and without selective pressures, although there is a decrease in mean fluorescent intensity of the GFP+ cells over time, most markedly in the HepG2 cells (Fig. 3-1A and B, middle and lower panels).

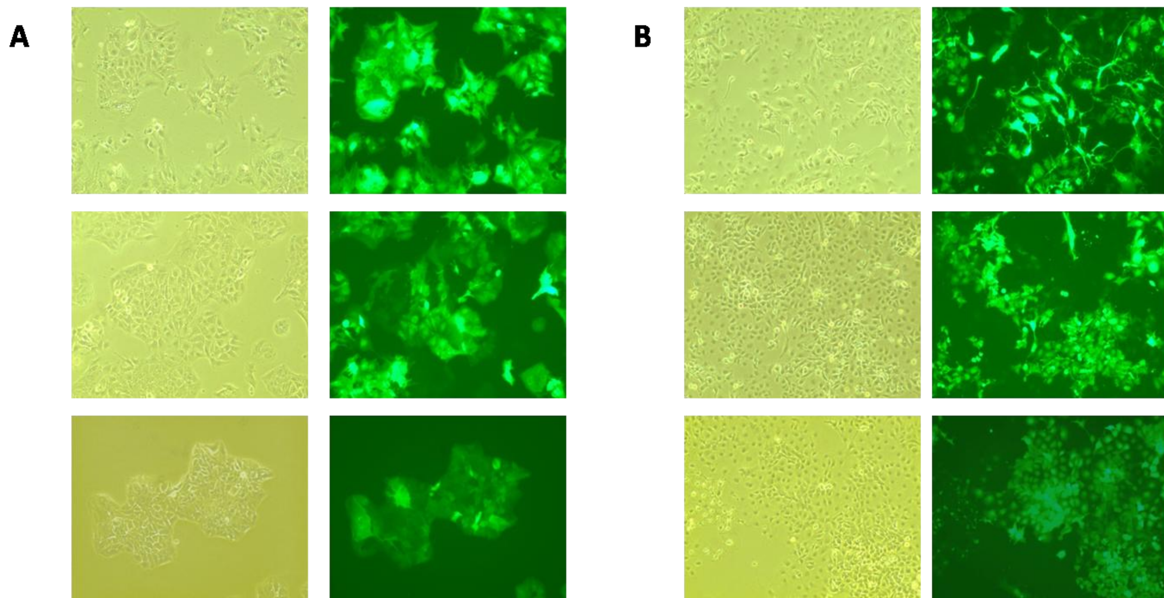

Figure 3-1. GFP expression in Huh7 and HepG2 cells infected with GFP-expressing lentiviral vectors. Huh7 and HepG2 cells were infected while in suspension with pSIH1empty lentivectors at a MOI of ~5 transducing units/cell (TU/cell) and maintained through multiple passages in the absence of selective pressures. Cells were imaged at 100x magnification in both phase-contrast and fluorescence microscopy. (A) Upper panel, Huh7 cells at 5 days post-infection; middle panel, Huh7 cells after 5 passages; lower panel, Huh7 cells after 20 passages. (B) Upper panel, HepG2 cells at 5 days post infection; middle panel, HepG2 cells after 5 passages; lower panel, HepG2 cells after 20 passages.

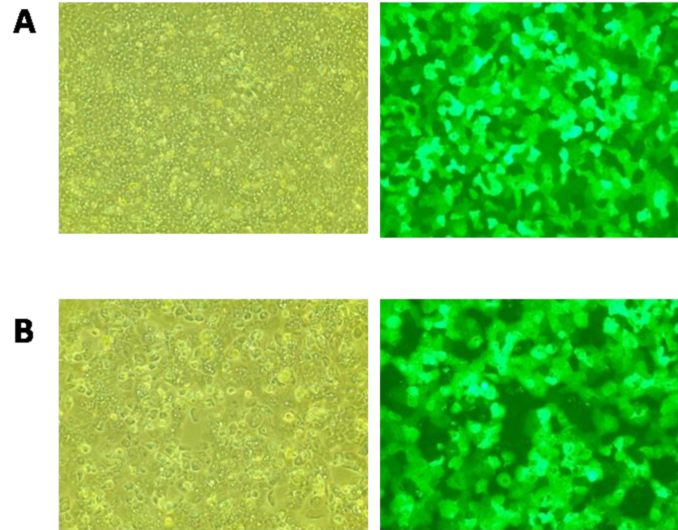

Figure 3-2. GFP expression in primary human hepatocyte cultures infected with GFP-expressing lentiviral vectors. Hepatocyte cultures (HH-C) were infected with pSIH1 empty lentivectors at a MOI of ~5 TU/cell. Cells were imaged at 100x magnification in both phase-contrast and fluorescence microscopy. (A) Hepatocyte cultures at 2 days post-infection. (B) Hepatocyte cultures at ~2 weeks post-infection.

Primary cultures of human hepatocytes are the current gold standard in hepatic models. Thus, subsequent experiments assessed the effectiveness of a lentiviral system for genetic manipulation of difficult-to-transfect primary human hepatocyte cultures (refer to Table 3-1 for hepatocyte donor information). Primary human hepatocytes were infected with GFP-expressing lentiviral vectors at a MOI of ~5 TU/cell. Infected hepatocytes displayed high levels of GFP expression (>75% GFP+ cells) at 2 days post-infection (Fig. 3-2A), and expression of GFP was retained for the duration of the hepatocyte culture time (14 days) (Fig. 3-2B). Microscopic examination revealed that neither the hepatic cell lines nor the primary human hepatocytes exhibited morphological abnormalities upon lentiviral infection.

Lentiviral infection does not affect select markers of the differentiated hepatic phenotype

For primary human hepatocyte culture models to most accurately reflect *in vivo* responses, it is vital that the cultures maintain a highly differentiated status, i.e. that they retain hepatic signature hallmarks such as expression of specific nuclear receptors and hepatic secretory proteins, as well as metabolic enzyme induction capacity. As a genetic manipulation tool, the lentivirus system would be most valuable for use in a primary human hepatocyte model if the infection event does not alter these hepatic characteristics, or otherwise adversely affect the cell. To examine these parameters, we next evaluated whether lentiviral infection would alter an array of hepatic markers, including expression of the nuclear receptors CAR, RXRα, PXR, and HNF4α, albumin expression, or induction of the CYP3A4 gene following phenobarbital treatment. Primary human hepatocyte cultures were transduced with lentivectors at a MOI of ~10 TU/cell, a level sufficient to infect >80% of the cells as demonstrated by GFP marker gene expression (data not shown). Under these conditions, we observed no morphological changes in lenti-infected hepatocytes. Additionally, real-time RT-PCR analyses demonstrated that infection with lentiviral vectors did not alter hepatic mRNA expression of CAR, RXRα, PXR, or HNF4α (Fig. 3-3A). Albumin mRNA expression remained similarly unchanged by viral infection (Fig. 3-3B). Although variable between donors, as documented previously [25], induction of CYP3A4 upon phenobarbital treatment was preserved in lentivirally infected hepatocytes of individual donors (Fig. 3-3C).

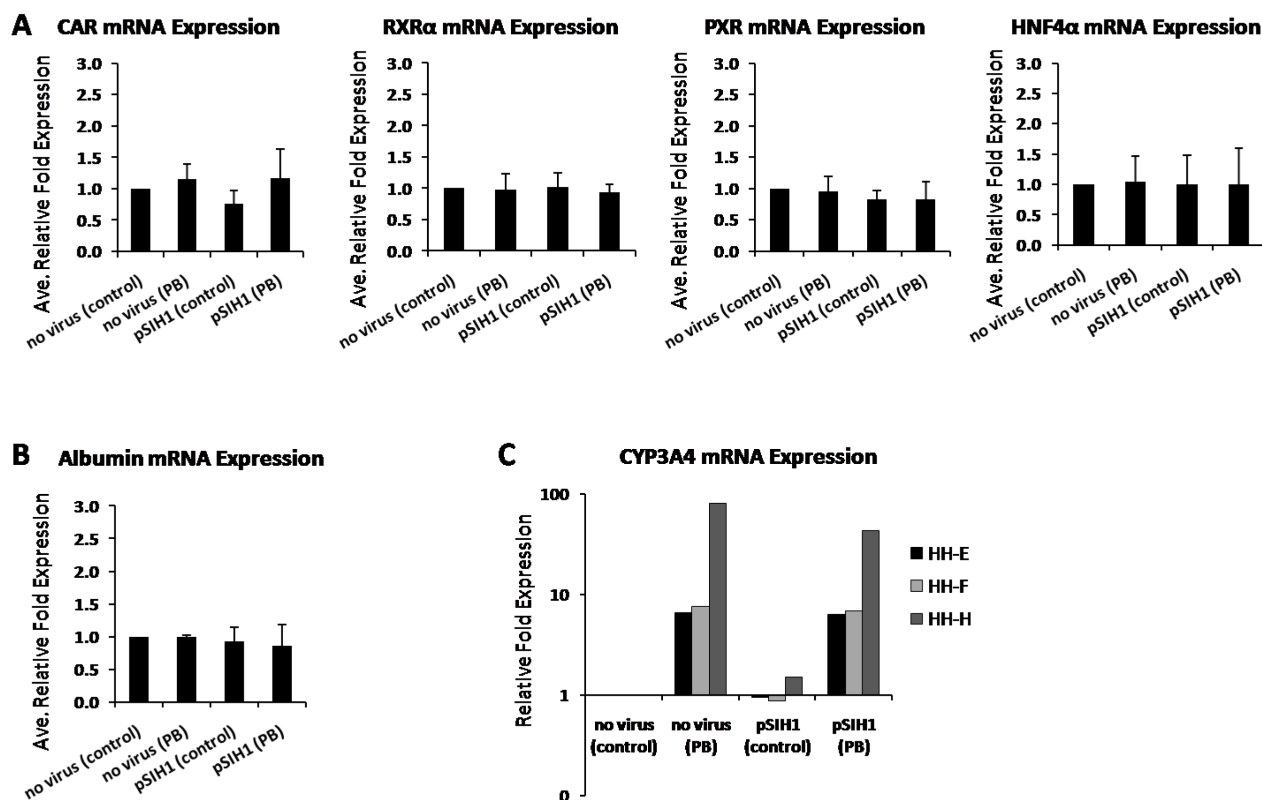

Figure 3-3. mRNA expression levels of hepatic hallmarks in cultures of primary human hepatocytes infected with lentiviral vectors. Hepatocyte cultures from three individual donors (HH-E, HH-F, and HH-H) were infected with pSIH1 empty lentivectors at a MOI of ~10 TU/cell. Hepatocytes were treated with 500 µM PB for 24 h. RNA was extracted 3–4 days post-infection, converted to cDNA, subjected to real-time RT-PCR, and the data analyzed using the $\Delta\Delta C_T$ method to determine relative mRNA expression levels. (A) mRNA expression levels of the hepatic nuclear receptors CAR, RXRα, PXR and HNF4α; values are mean ±S.D. of the three hepatocyte donors. (B) Albumin mRNA expression levels; values are mean ±S.D. of the three hepatocyte donors. (C) mRNA expression levels of CYP3A4 hepatic enzyme; values are individual expression levels for each of the three donors.

Effectiveness of the lentiviral system for both over-expression and knockdown of CAR

In our studies, lentiviral vectors efficiently transduced primary human hepatocyte cultures with little effect on the expression of the assessed hepatic hallmarks and hepatic-specific responses. We next evaluated the usefulness of the lentiviral genetic modulation system for over-expression and knockdown analyses in primary human hepatocyte cultures.

For CAR over-expression assessment, hepatocytes were infected with lentivirus expressing CAR. Real-time RT-PCR results showed that CAR mRNA expression levels were increased >200-fold in cells infected with CAR-expressing lentivirus (Fig. 3-4A). Whether such over-expression was functionally relevant was assessed by measuring mRNA expression levels of the CAR target gene, CYP2B6 (Fig. 3-4B). Consistent with previous findings, human hepatocytes exhibited inter-individual variability in the level of CYP2B6 induction in response to treatment with the human CAR agonist, CITCO (2- to 6-fold) [25,26]. Hepatocytes infected with CAR expressing lentivirus exhibited a highly variable increase in basal CYP2B6

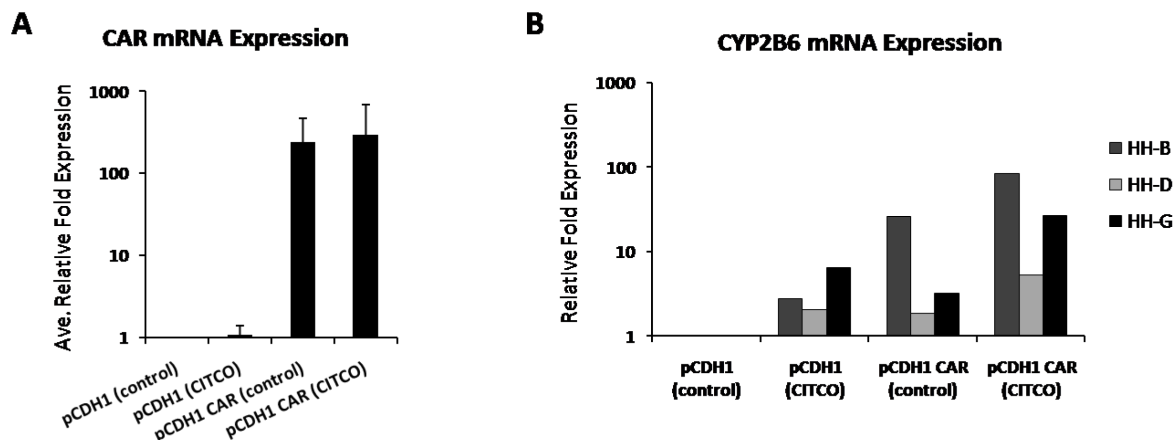

Figure 3-4. CAR and CYP2B6 mRNA expression in primary human hepatocyte cultures infected with CAR-expressing lentiviral vectors. Hepatocyte cultures from three individual donors (HH-B, HH-D, and HH-G) were infected with lentivectors expressing CAR or pCDH1 empty lentivectors. Hepatocytes were treated with 100 nM CITCO for 24–48 h. RNA was extracted 2–5 days post-infection, converted to cDNA, subjected to real-time RT-PCR, and the data analyzed using the $\Delta\Delta C_T$ method to determine relative mRNA expression levels. (A) CAR mRNA expression levels; values are mean ±S.D. of the three hepatocyte donors. (B) CYP2B6 mRNA expression levels; values are individual expression levels for each of the three donors.

expression levels (2-to 26-fold). CYP2B6 induction in hepatocytes infected with CAR-expressing lentivirus was slightly enhanced upon CITCO treatment compared to that of hepatocytes infected with empty lentivirus (empty virus induction: HH-B 3-fold, HH-D 2-fold, HH-G 6-fold, and CAR-expressing virus induction: HH-B 3-fold, HH-D 2.5-fold, and HH-G 9-fold). These results indicate that some portion of the over-expressed CAR pool is engaged in cycling between the cytosolic and nuclear compartments and, due to its constitutive activity, that fraction that equilibrates to the nucleus participates in transactivation of endogenous gene targets similarly as would the resident activated receptor.

For CAR knockdown analysis, primary human hepatocytes were infected with lentivirus expressing four unique siRNAs targeted to CAR at specific locations along the mRNA sequence. Real-time RT-PCR analyses indicated that the siRNA targeted to CAR at position 755 exerted the most effective knockdown (Fig. 3-5A). CAR mRNA expression was reduced to ~40% of endogenous cellular levels in hepatocytes infected with lentivirus expressing the siRNA against CAR at position 755 at a MOI of ~3 TU/cell (virus sufficient to infect >60% of the cells) (Fig. 3-5B). Functional significance of the CAR knockdown was confirmed by assessing mRNA expression levels of CYP2B6 in the presence and absence of phenobarbital. Results demonstrated that CAR knockdown led to a corresponding ~50% decrease in both basal and phenobarbital-induced mRNA expression levels of CYP2B6 (Fig. 3-5C).

3.5 Discussion

Previous investigations demonstrated that lentiviral vectors efficiently transduce the hepatic model cell lines Huh7 and HepG2 [13–18], as well as cultures of primary human hepatocytes [14;19–21], with Nguyen and colleagues reporting a MOI of 5 TU/cell as sufficient for transduction of ~75% of the primary hepatocyte culture [20]; all findings with which our results are consistent. Although these investigations demonstrated that lentivectors transduce primary human hepatocyte cultures with high

efficiency, they did not evaluate whether the infection event alters hepatic signature hallmarks, such as expression of nuclear receptors or secreted proteins, or metabolic enzyme induction.

To determine whether lentiviral infection may adversely affect the differentiated hepatic character, we measured expression levels of a number of hepatic-enriched nuclear receptors subsequent to lentiviral infection. The nuclear receptors CAR, PXR, RXRα, and HNF4α were selected for evaluation as they regulate expression of a myriad of hepatic genes encoding, generally, drug metabolizing enzymes (such as cytochromes P450, sulfotransferases, UDP-glucuronosyltransferases, and glutathione *S*-transferases),

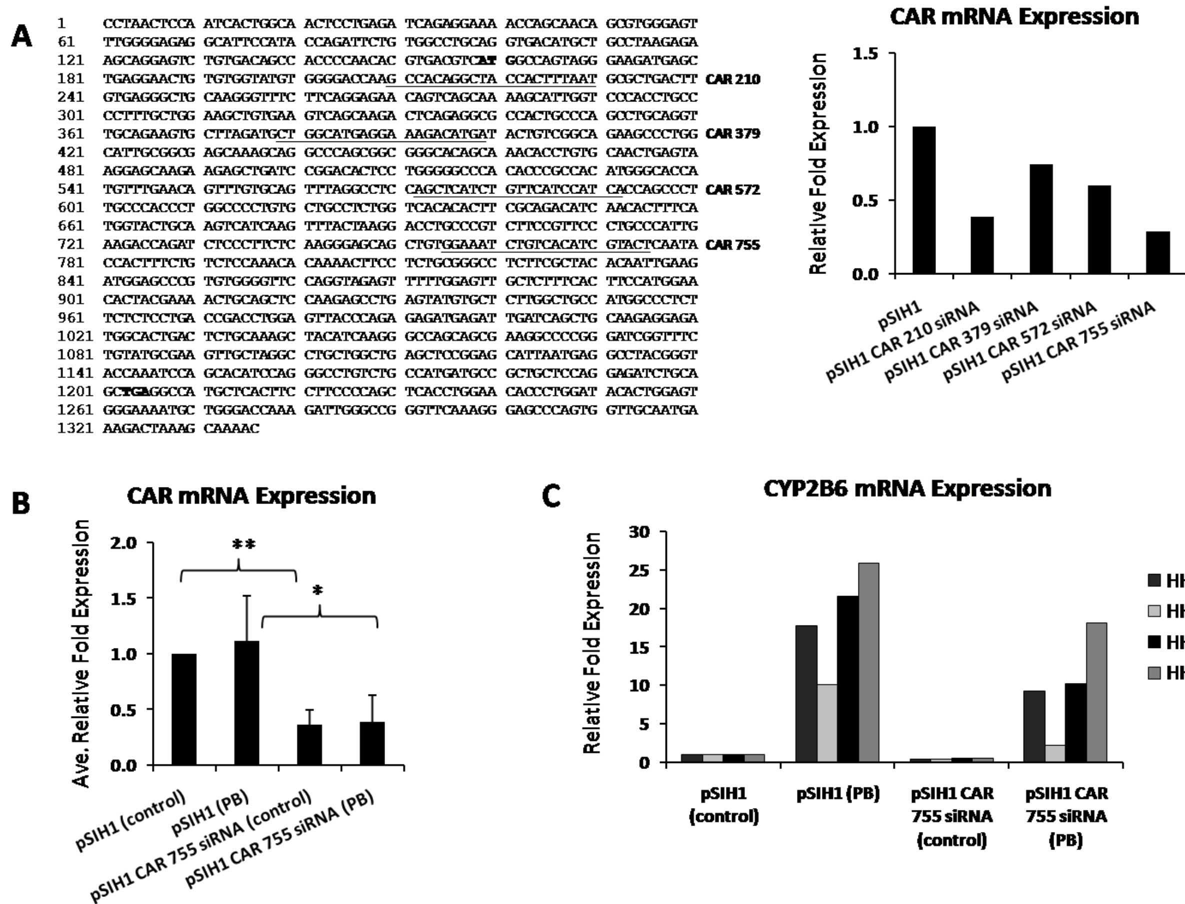

Figure 3-5. CAR and CYP2B6 mRNA expression in hepatocyte cultures infected with lentiviral vectors expressing siRNA against CAR. Hepatocyte cultures from four individual donors (HH-A, HH-E, HH-H, and HH-I) were infected with pSIH1 lentivectors expressing siRNA against CAR or pSIH1 empty lentivectors. RNA was extracted 4–7 days post-infection, converted to cDNA, subjected to real-time RT-PCR, and the data analyzed using the $\Delta\Delta C_T$ method to determine relative mRNA expression levels. (A) Left panel, schematic showing target locations of the CAR siRNAs assessed for CAR knockdown capability. Underlined sequence indicates siRNA target location; bolded sequence denotes translation initiation and termination codons. Right panel, graph illustrating the effect of the siRNA lentivectors on CAR expression. (B) CAR mRNA expression levels from hepatocytes infected with pSIH1 empty lentivectors or pSIH1 CAR 755 siRNA lentivectors (MOI of ~3 TU/cell) and treated with 500 μM PB for 24–48 h; values are mean ±S.D. of the four hepatocyte donors (**p* < .05, ***p* < .01 by Student's *t*-test, one-tailed, two sample unequal variance). (C) CYP2B6 mRNA expression levels from hepatocytes infected with pSIH1 empty lentivectors or pSIH1 CAR 755 siRNA lentivectors (MOI of ~3 TU/cell) and treated with 500 μM PB for 24–48 h; values are individual expression levels from each of the four donors.

drug transporters (such as multidrug-resistance proteins, multidrug resistance-associated proteins, and organic anion-transporting proteins), as well as other nuclear receptors [27;28]. Our data demonstrate that the levels of these hepatic nuclear receptors remain constant despite lentiviral transduction at a MOI of ~10 TU/cell (virus sufficient to infect >80% of hepatocytes), therefore indicating that hepatic gene regulation by these transcription factors is not impaired by the infection event. This conclusion is further confirmed by the retention of albumin expression levels in lentivector-infected hepatocyte cultures. In contrast, albumin expression was ablated by baculoviral infection of primary hepatocytes [9]. Importantly, phenobarbital-mediated induction of CYP3A4, a sensitive indicator of hepatocyte differentiation status [22], was fully preserved in lentivirally transduced cells.

Results from this study show that lentivectors may be employed for both over-expression and knockdown investigations in primary human hepatocyte cultures (as demonstrated here by CAR modulation), with the functional significance of these manipulations apparent by measure of effects on downstream target gene expression. Since the lentivectors integrate into the genome of the host cell, duration of transgene expression is theoretically limited only by the length of time that hepatocytes can be maintained in culture. Our results demonstrate that lentivirus-infected Huh7 and HepG2 cell lines maintain robust GFP transgene expression for over 3 months in culture, spanning 20 passages (duration of experiment). Likewise, transgene expression is retained for at least 14 days post-infection in primary cultures of human hepatocytes. Further, we identified a novel siRNA sequence capable of knocking down CAR to ~40% of endogenous levels, with specific knockdown of CAR mRNA expression remaining evident at 7 days post-infection. More lengthy time-course studies to confirm preservation of transgene and siRNA expression are underway.

Using a lentivector expressing a siRNA targeted to CAR at position 755, we were able to demonstrate knockdown of CAR to ~40% of endogenous levels in human hepatocytes. The data in Fig. 3-5 represent the CAR mRNA expression levels from the entire hepatocyte culture, of which ~60% of the cells were infected. It is possible that the ~40%of uninfected hepatocytes may account for the remaining CAR expression, suggesting that this siRNA may approach close to 100% knockdown efficiency in infected cells. Experiments are being conducted in our laboratory to enrich for the infected population by sorting the cells by FACS. Recently, Chen et al. identified a siRNA capable of knocking down CAR to ~30% of endogenous levels [29]. However, the knockdown experiment was performed in Caco-2 cells transiently transfected with both a CAR expression vector and the siRNA against CAR. Thus, to our knowledge, our report is the first to demonstrate direct knockdown of endogenous CAR.

This study demonstrates that lentiviral vectors may be utilized successfully to infect Huh7 cells, HepG2 cells, and, importantly, cultures of primary human hepatocytes. Data presented herein show that lentiviral infection does not alter hepatic markers including expression of albumin and an array of hepatic nuclear receptors. Further, infected hepatocytes retain the capacity for CYP3A4 induction by phenobarbital treatment, a response that is specific to highly differentiated hepatocytes. Both over-expression and knockdown analyses may be successfully undertaken using a lentiviral system in human hepatocytes. Lentiviral vectors thus offer a powerful methodology for achieving stable genetic modulation of primary human hepatocytes. These strategies offer a promising approach for both pharmacological and toxicological investigations within the primary hepatocyte model, as well as ultimately for use in *ex vivo* gene therapy to ameliorate hepatic disease.

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Chapter 4

A Novel Role for the Constitutive Androstane Receptor in Human Hepatic Specification

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4.1 Abstract

Human embryonic stem cells (hESCs) induced to differentiate along a hepatic lineage offer promise for therapeutic transplantations to ameliorate liver dysfunction and for pharmacological and toxicological research. While a number of studies have shown that hESCs are capable of differentiating into hepatic precursors, the precise mechanisms by which hESCs are induced to differentiate into fully function hepatocytes and the identity of the genes governing this process remain elusive. The constitutive androstane receptor (CAR) is a nuclear receptor that regulates expression of genes involved in all three phases of hepatic biotransformation and transport. Recent studies have also defined roles for CAR in other biological processes and have shown expression of CAR in the early phases of liver development. We have previously shown that CAR expression is specifically and significantly augmented in hepatic-like cells derived from hESCs using a novel differentiation approach. Thus, we hypothesized that CAR plays a role in human hepatogenesis. To expand upon our previous findings we screened a panel of fetal liver tissue and found that CAR mRNA expression in fetal liver is higher than that of adult liver at each gestational stage assessed. We next employed a lentivirus system for genetic modulation of hESCs which resulted in robust, stable transgene expression with no effect on hESC markers of pluripotency. Stable CAR over-expression and siRNA-mediated attenuation in hESCs subject to hepatic differentiation resulted in corresponding changes in mRNA expression of hepatic markers including the transcription factors C/EBPα, HNF1α, HNF4α, and FOXA1, and liver-generated plasma proteins α-fetoprotein, transthyretin, transferrin, and albumin. In contrast, expression of the pregnane X receptor (PXR) – the nuclear receptor most similar to CAR in primary sequence – was not increased upon hepatic differentiation and was negligible in fetal liver tissue. Further, stable over-expression of PXR in hepatic-induced hESCs did not lead to enhanced expression of markers of the hepatic phenotype. Taken together, these results define a novel role for CAR in hepatic lineage commitment.

4.2 Introduction

To date a number of studies have sought to direct differentiation of hESCs along a hepatic lineage in order to derive a repository of cells for therapeutic transplantation and to serve as models for studies of drug and xenobiotic metabolism. The majority of the studies have employed defined culture conditions to reconstitute the *in vivo* liver microenvironment by plating cells on a variety of matrices and supplementing culture media with fibroblast growth factor [1-9], bone morphogenetic protein [3], hepatocyte growth factor [1;2;5-8;10-13], dexamethasone [1;2;6-8;13;14], insulin [7;14], oncostatin M [1;6;7;11;13], activin A [1-3;6;10-13;15;16], wnt3a [13;15], and sodium butyrate [11;17]. The reader is referred to a recent review by Snykers et al. for a comprehensive synopsis on progress in the field of hepatocyte derivation from hESCs [18]. While promising, differentiation methodologies defined thus far have been insufficient to derive fully functional hepatocytes from hESCs. Further, few data exist regarding the mechanism(s) by which these factors induce hepatic differentiation and the specific genes involved in this complex process.

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[1] Designed and conducted experiments, analyzed data, wrote manuscript.
[2] Principal investigator.

We have previously defined a unique hepatic differentiation approach in which hESCs are cultured for 10 days on collagen in specialized, highly-defined hepatocyte media (William's E Media supplemented with HEPES, glutamine, antibiotics, dexamethasone, insulin, transferrin, selenium, and linoleic acid/albumin). The resulting hepatic-like cell population exhibits decreased expression of 'stemness' markers and enhanced expression of a variety of hepatic transcription factors, nuclear receptors, liver-generated plasma proteins, protease inhibitors, metabolic enzymes, and biotransformation enzymes. Further, the hESC-derived hepatic-like cells have the capacity to transport anionic compounds and store glycogen. Notably, expression of the constitutive androstane receptor (CAR) is highly increased in the hepatic-like cells, to levels approaching those of cultures of primary human hepatocytes.

CAR is a member of the nuclear receptor (NR) superfamily expressed primarily in the liver [19]. In the adult liver, CAR acts as a transcriptional regulator of genes whose products act at all three phases of hepatic biotransformation and transport including certain cytochrome P450s (Phase I monooxygenase enzymes), sulfotransferases and glucoronosyltransferases (Phase II conjugation enzymes), and multidrug resistance-associated proteins and organic anion transporting polypeptides (Phase III transporters) [20] in response to potentially toxic stimuli. Through the regulation of these genes, CAR plays a key role in the metabolism and transport of exogenous compounds including drugs [21] and carcinogens [22] as well as endogenous substances such as steroids [22], heme/bilirubin [22], thyroid hormone [23], and cholesterol/bile acids [24;25]. CAR is also emerging as an important regulator of lipid and energy metabolism mediated through a variety of mechanisms [26], and has been shown to regulate genes involved in diverse physiological processes including the human cathepsin E gene (an aspartic protease involved in the innate immunity response) [27], mouse aldo-keto reductase family 1, member 7 (AKR1B7) (an enzyme involved in modulating lipid peroxidation) [28], mouse myeloid cell leukemia-1 (MCL-1) (an antiapoptotic protein) [29], and mouse cellular myelocytomatosis (C-MYC) (a proto-oncogene) [30].

Emerging evidence suggests that NRs play integral roles in both the maintenance of 'stemness' and induction of differentiation. For a comprehensive overview on the functions of specific nuclear receptors in stem cell biology in a variety of species, the reader is referred to a recent review by Jeong and Mangelsdorf [31]. However, limited data exist regarding CAR expression and its potential role in the hepatic specification process. Recent studies by Xie et al. comparing expression levels of members of the NR superfamily during embryoid body differentiation show that hESCs express increasing levels of CAR during the first six days of development [32]. These findings are corroborated by Ek et al. who document increasing expression of CAR in hepatic-like cells derived from hESCs using a 30-day differentiation protocol [33]. In the developing liver CAR mRNA expression correlates with that of its transcriptional regulator HNF4α [34], a NR which plays an integral role in hepatic differentiation [35]. Interestingly, however, a study of CAR mRNA expression in a panel of human fetal liver samples found that average fetal liver CAR expression is ~4-fold greater than that of HNF4α [36]. Findings from the same study showed that, despite wide inter-individual and developmental stage variability, CAR expression in fetal liver is ~40% of that of postnatal liver [36]. Given these findings, and considering our previous results, we hypothesized that CAR plays a role in the differentiation of hESCs along a hepatic lineage.

Results presented herein confirm that CAR is expressed highly during hepatic differentiation and in fetal liver obtained from donors of a variety of gestational ages. Further, lentiviral-mediated CAR over-expression and siRNA-mediated mRNA reduction in hESCs subject to hepatic differentiation results in corresponding changes in expression of a variety of markers of the hepatic phenotype. In contrast, PXR is not expressed during hepatic differentiation and is expressed only marginally in fetal liver.

Additionally, lentiviral-mediated over-expression of PXR in hepatic-induced hESCs does not enhance expression of hepatic-specific markers, suggesting that the effects observed are indeed specific to CAR.

4.3 Materials and Methods

Primary human hepatocyte culture

Cultures of primary human hepatocytes, obtained through the Liver Tissue Procurement and Distribution System (NIH Contract #N01-DK-9-2310), were isolated by a three-step collagenase perfusion technique and plated on rat-tail collagen as previously described by Strom et al. [37]. Hepatocytes were maintained in our hepatocyte media comprised of William's E Media (Gibco; Grand Island, NY) supplemented with 10 mM HEPES (Gibco), 2 mM GlutaMAX (Gibco), 100 units/ml penicillin and 100 µg/ml streptomycin (Gibco), 25 nM dexamethasone (Sigma; St. Louis, MO), 10 nM insulin (Sigma), 5 ng/ml selenium (Sigma), 5 µg/ml transferrin (Sigma), and 1% linoleic acid/albumin (Sigma).

Human embryonic stem cell culture

The WA09 (H9) human embryonic stem cell line was acquired from the National Stem Cell Bank through WiCell Research Institute (Madison, WI). The cells were maintained on irradiated human foreskin fibroblast (hFF) feeder layer cells (ATCC; Manassas, VA) in hESC media consisting of Dulbecco's Modified Eagle Media F-12 (Gibco) supplemented with 20% knock-out serum replacement (Gibco), 1 mM GlutaMAX (Gibco), 0.1 mM non-essential amino acids (Gibco), 100 ng/ml basic fibroblast growth factor (National Cancer Institute; Bethesda, MD), and 0.1 mM β-mercaptoethanol (Sigma). Media was changed daily and differentiated colonies were removed from the culture by manual dissociation 2-3 times per week, depending on the culture density. Cells were passaged weekly by manual dissociation and plated on fresh hFF feeder layers.

Hepatic differentiation of human embryonic stem cells and treatments

To induce hepatic differentiation hESCs were plated in wells coated with ~3 $\mu g/cm^2$ of rat tail type I collagen (Sigma) in hESC media. After 2 days media was switched to hepatocyte media (see above) and cells were maintained for 8 additional days with daily replenishment of hepatocyte media for a total of 10 days in culture.

Alkaline phosphatase staining

Alkaline Phosphatase Detection Kit (Millipore) was used for alkaline phosphatase staining essentially according to manufacturer's instructions. Briefly, cells were fixed with 4% formaldehyde in PBS, rinsed with TBST (20mM Tris-HCl, 0.15M NaCl, 0.05% Tween-20), stained for 15 minutes with a 2:1:1 ratio of Fast Red Violet:Naphthol:water, rinsed with TBST, and covered with PBS. Cells were visualized using a Nikon inverted fluorescent microscope (Nikon USA; Melville, NY) and images were captured using a digital camera and SpotRT software (Diagnostic Instruments; Sterling Heights, MI).

Lentiviral cDNA and siRNA expression vector construction

Lentivectors were obtained from System Biosciences (Mountainview, CA). pCDH1-MCS1-EF1-copGFP cDNA lentivector expressing human CAR (NM_005122) and pSIH1-H1-copGFP shRNA lentivector expressing a small interfering RNA targeted to CAR were generated as described previously [38].

pCDH1-MCS1-EF1-copGFP cDNA lentivector expressing human PXR (NM_003889) was engineered as follows. Briefly, PXR was PCR-amplified from human liver cDNA using the primer sequences: F 5'-GATCGAATTCGACATGGAGGTGAGACCCAAAGAAAG -3', R 5'- GATCGATATCTAGAAGGCACAGTCGAGG-3' (restriction sites underlined). The amplicon and vector were then digested with EcoRI/EcoRV and EcoRI/SwaI, respectively, electrophoresed through a 0.6% agarose gel, purified using QIAquick Gel Extraction Kit (Qiagen; Valencia, CA), and cloned by ligation. Plasmids were purified using QIAfilter Plasmid Maxi Kit (Qiagen).

Lentiviral production and target cell infection

Production of lentiviral particles and target cell transductions were performed essentially according to manufacturer's instructions (System Biosciences) with minor adaptations. Briefly, human embryonic kidney (HEK) 293T/17 transformed virus packaging cells (ATCC), cultured as previously described [38], were transfected with lentiviral expression plasmids and pPACKH1 packaging plasmid mix (System Biosciences) using Lipofectamine 2000 (Invitrogen; Carlsbad, CA). Psuedoviral supernatant was collected from packaging cell cultures at 72 hours post-transfection, filtered, and used for direct target cell infections in the presence of 6 µg/ml polybrene. Target cell media was replaced the following day. Transduction efficiency was assessed using green fluorescent protein marker gene expression monitored with a Nikon inverted fluorescence microscope and images were captured using a digital camera and SpotRT software.

RNA isolation, cDNA archiving, and real-time PCR

RNA was isolated using TRIzol Reagent (Invitrogen)and converted to cDNA using High Capacity cDNA Archive Kit (Applied Biosystems; Foster City, CA), both according to manufacturers' protocols. Real-time RT-PCR was carried out using Assays-on-Demand Gene Expression Products (Applied Biosystems), according to manufacturer's instructions. Briefly, 100 ng of cDNA template, 25 µl 2x Taqman Universal Master Mix, and 2.5 µl 20x Target Assay Mix were combined into 50 µl reactions. The reactions were divided in half to generate technical replicates and run on an ABI 7300 Real-time PCR System. Data were analyzed using the $\Delta\Delta C_T$ Method as previously described [38;39]. Due to variability in the gene expression between hESCs of different passages, all data were normalized to a low-passage number (passage 33) hESC control population.

Statistical analyses

A Student's t-test (one-tailed; two-sample, unequal variance) was used for all statistical assessments.

4.4 Results

Hepatic differentiation of hESCs results in increased CAR expression

Employing a novel hepatic differentiation approach in which hESCs are cultured for 10 days on collagen substrate in specialized, highly-defined hepatocyte media (William's E Media supplemented with HEPES, glutamine, antibiotics, dexamethasone, insulin, transferrin, selenium, and linoleic acid/albumin), we have previously generated hepatic-like cells which show HepG2 hepatoma cell-like morphology, attenuated expression of pluripotency markers and 'stemness' function, enhanced expression of hepatic markers including transcription factors, nuclear receptors, plasma proteins, and metabolic and biotransformation enzymes, as well as augmented hepatic function including transport of anionic

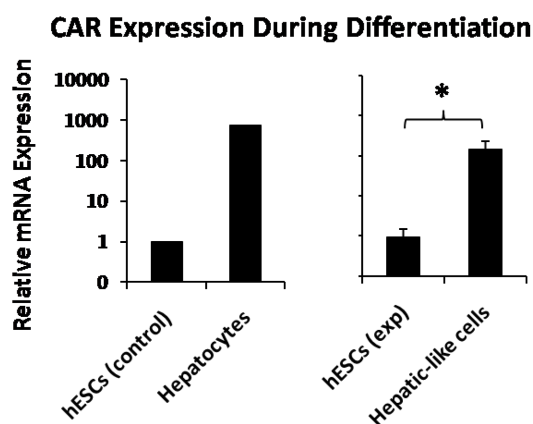

Figure 4-1. Hepatic differentiation of hESCs results in increased CAR mRNA expression. HESCs were cultured on either hFF feeder layers in hESC media (hESCs exp) or on type I rat-tail collagen in hepatocyte media (Hepatic-like cells). At 10 days in culture RNA was isolated, converted to cDNA, subjected to real-time PCR, and the data analyzed using the $\Delta\Delta C_T$ method to determine expression levels of CAR relative to low-passage hESCs (hESCs control). Control data (left graph) depict expression levels of low-passage hESCs (hESCs control) which serve as a negative control and pooled samples from six primary human hepatocyte donors (Hepatocytes) which serve as a positive control. Experimental data (right graph) depict expression levels of hESCs (hESCs exp) and passage-matched hESCs subject to hepatic differentiation (Hepatic-like cells). Experimental data are expressed as mean +/- standard deviations of four independent trials using hESCs from different passages. * $p < 0.05$.

compounds and glycogen storage. Notably, CAR mRNA expression is significantly increased in these hepatic-like cells, to levels only slightly lower than those of primary human hepatocyte positive controls (Fig. 4-1).

CAR is expressed highly in human fetal liver tissue

To determine the extent to which CAR is expressed in human fetal liver tissue, we measured CAR mRNA expression in human fetal liver tissue acquired from ten subjects at a range of gestational stages. While CAR mRNA levels varied extensively between samples, its expression was higher than that of the adult liver pooled control in all fetal liver tissue samples screened (Fig. 4-2). The differences between our findings and those of Vyhildal et al., who found that CAR expression in fetal liver is approximately 40% of that of adult liver [36], are likely attributable to both inter-individual and gestational stage differences in CAR expression. Microarray expression data from a panel of human tissue generated using U133A and GNF1H chips (publicly available through the UCSC Genome Browser website http://genome.ucsc.edu/ [40;41]), further confirms that CAR mRNA is robustly and selectively expressed in fetal liver tissue.

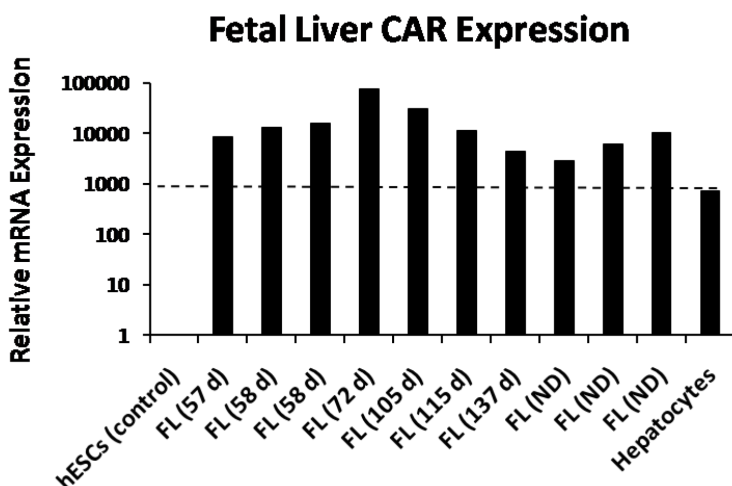

Figure 4-2. CAR mRNA is expressed highly in human fetal liver tissue. Human fetal liver (FL) tissue was obtained from ten subjects at the indicated stages of gestation. RNA was isolated, converted to cDNA, subjected to real-time PCR, and the data analyzed using the $\Delta\Delta C_T$ method to determine expression levels of CAR relative to low passage number hESC controls. Primary human hepatocyte cultures pooled from six individual donors (Hepatocytes) were included as positive controls. ND, no data.

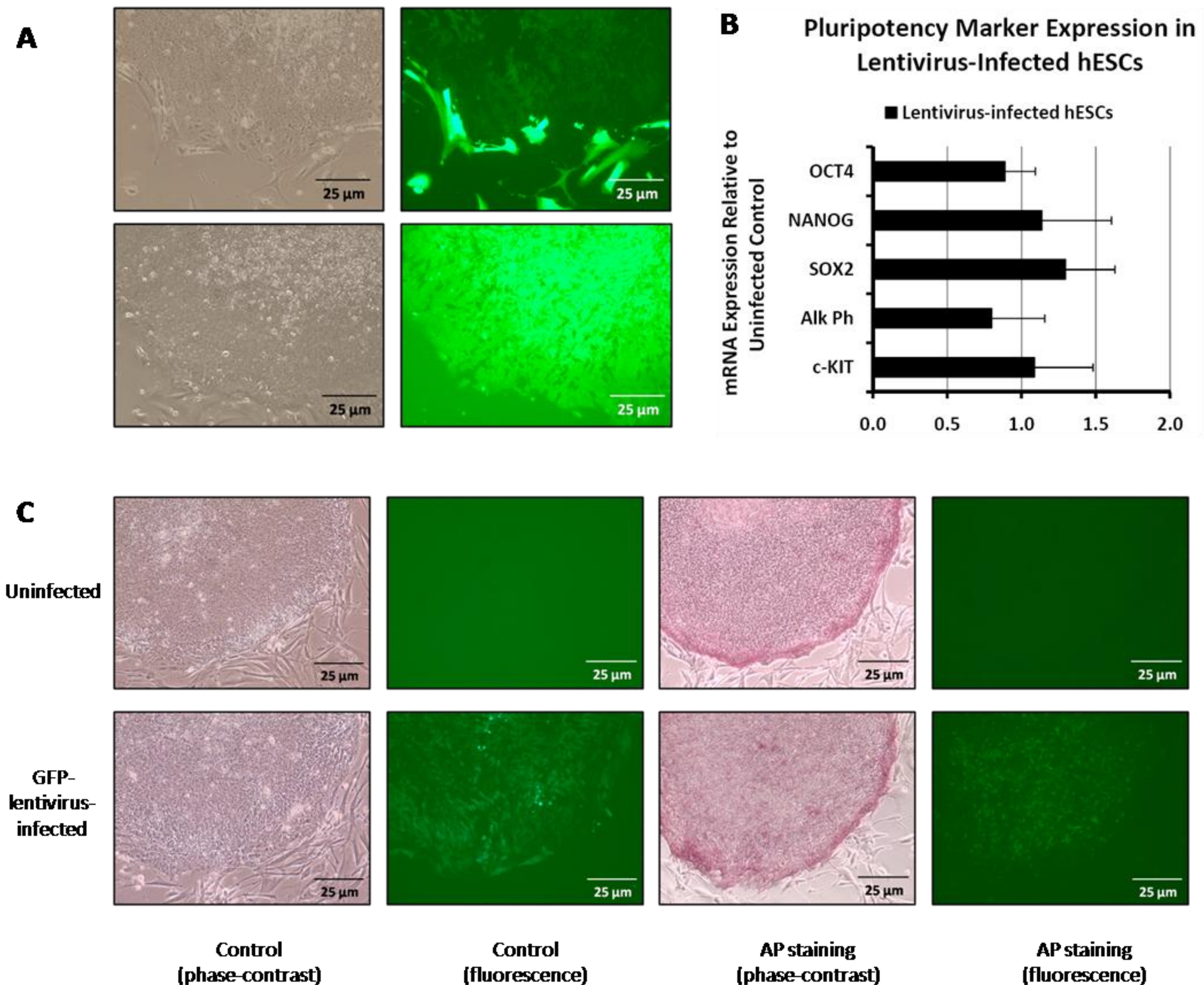

Figure 4-3. Lentivirus efficiently transduces hESCs and does not alter select markers of 'stemness.' HESCs colonies were infected with lentiviral particles expressing GFP and maintained in culture on hFF feeder layers in hESC Media for six weeks. Media was replenished daily and cells were passaged weekly by manually dissociating the most homogenously GFP+ colonies and replating the cells onto fresh feeder layers. (A) HESC colonies were imaged in both phase-contrast and fluorescence microscopy at ~1 week post-infection (upper panels) and ~6 weeks post-infection (lower panels). (B) A sample of hESC colonies was obtained at ~3-4 weeks post-infection (after three passages). RNA was isolated, converted to cDNA, subjected to real-time PCR, and the data analyzed using the $\Delta\Delta C_T$ method to determine relative mRNA expression levels of select 'stemness' markers OCT4, NANOG, SOX2, Alkaline phosphatase, and C-KIT compared to uninfected cells. Values are mean +/- S.D. from three independent trials using hESCs from three different passages. (C) HESCs infected with GFP-lentivirus were stained for alkaline phosphatase (AP) activity (pink) at ~ 1 week post-infection and compared to alkaline phosphatase stained uninfected hESC passage-matched controls.

Lentivirus efficiently transduces hESCs and does not alter select markers of 'stemness'

Before we could ascertain whether exogenous expression of CAR could enhance induction of hepatic differentiation of hESCs, it was necessary to develop a stable genetic modulation system effective in hESCs. To this end, hESC colonies were transduced with lentivirus expressing green fluorescent protein (GFP). Despite inter- and intra- colony variability in expression level, infected hESC colonies generally exhibited high levels of GFP expression (Fig. 4-3A, upper panels). The most homogenously GFP+ colonies were selected for subsequent propagation and GFP expression was retained in infected hESCs

for over two months in culture (throughout six passages), in the absence of selective pressures (Fig. 4-3A, lower panels). Available data suggest that lentiviral infection does not affect hESC pluripotency [42;43], but to more comprehensively evaluate the effects of lentiviral transduction on hESC differentiation status, hESCs were infected with lentivirus expressing GFP and relative mRNA expression levels of select 'stemness' markers implicated in self-renewal and pluripotency were assessed. Infected hESC colonies exhibited no morphological abnormalities or signs of toxicity. Further, lentiviral infection did not alter mRNA expression levels of the 'stemness' markers octamer-binding transcription factor 4 (OCT4), nanog homeobox (NANOG), SRY-box containing gene 2 (SOX2), alkaline phosphatase, or the tyrosine-protein kinase C-KIT compared to the uninfected control (Fig. 4-3B). Confirmation that lentivirus infection does not alter alkaline phosphatase expression was attained at the functional level using an alkaline phosphatase activity staining technique (Fig. 4-3C).

CAR expression enhances hepatic differentiation of hESCs

We next employed the lentivirus system to stably express exogenous CAR in hESCs induced to differentiate along a hepatic lineage in order to determine if CAR expression can enhance hepatic differentiation capacity. HESCs were transduced with lentiviral vectors expressing CAR and subject to our hepatic differentiation protocol. hESCs infected with CAR-expressing virus exhibited significantly increased expression of CAR mRNA compared to cells infected with empty virus (Fig. 4-4A). Among populations subjected to the hepatic differentiation protocol, hESCs infected with CAR-expressing virus showed increased mRNA expression of the hepatic transcription factors CCAAT/enhancer binding protein alpha (C/EBPα), hepatic nuclear factor 1 alpha (HNF1α), and hepatic nuclear factor 4 alpha (HNF4α) (Fig. 4-4B), primary members of the combinatorial regulatory network that governs transcription of most genes in the hepatic program [44], as well as the liver-generated plasma proteins α-fetoprotein, transthyretin, transferrin, and albumin (Fig. 4-4C).

siRNA-mediated CAR reduction attenuates hepatic differentiation of hESCs

To further assess the importance of CAR in hepatic differentiation, we sought to determine whether a reduction in CAR expression in hESCs induced to differentiate along a hepatic lineage would attenuate differentiation capacity. Prior to hepatic induction using our hepatic differentiation protocol, hESCs were transduced with lentiviral particles expressing an siRNA targeted to CAR [38]. CAR mRNA was substantially reduced in hESCs infected with siRNA-expressing virus, compared to the population infected with empty virus (Fig. 4-5A). Further, the siRNA-mediated reduction in CAR mRNA attenuated the differentiation-dependent increase in mRNA expression of the hepatic transcription factors C/EBPα, HNF1α, HNF4α, and forkhead box A1 (FOXA1) (Fig. 4-5B) and the plasma proteins α-fetoprotein and transthyretin (Fig. 4-5C).

PXR is expressed at very low levels during hepatic differentiation

To determine whether these findings are specific to CAR, we examined the ability of the pregnane X receptor (PXR) – the NR most similar to CAR in primary sequence, sharing 40% sequence homology in the DBD and 45% in the LBD [45] – to enhance the differentiation capacity of hESCs induced to differentiate along a hepatic lineage. In contrast to CAR, PXR mRNA expression is not increased in hESC-derived hepatic-like cells (Fig. 4-6A). Further, PXR mRNA expression in human fetal liver tissue samples was negligible (Fig. 4-6B), findings confirmed by microarray expression data from a panel of human tissue generated using U133A and GNF1H chips (publicly available through the UCSC Genome Browser website http://genome.ucsc.edu/ [40;41]).

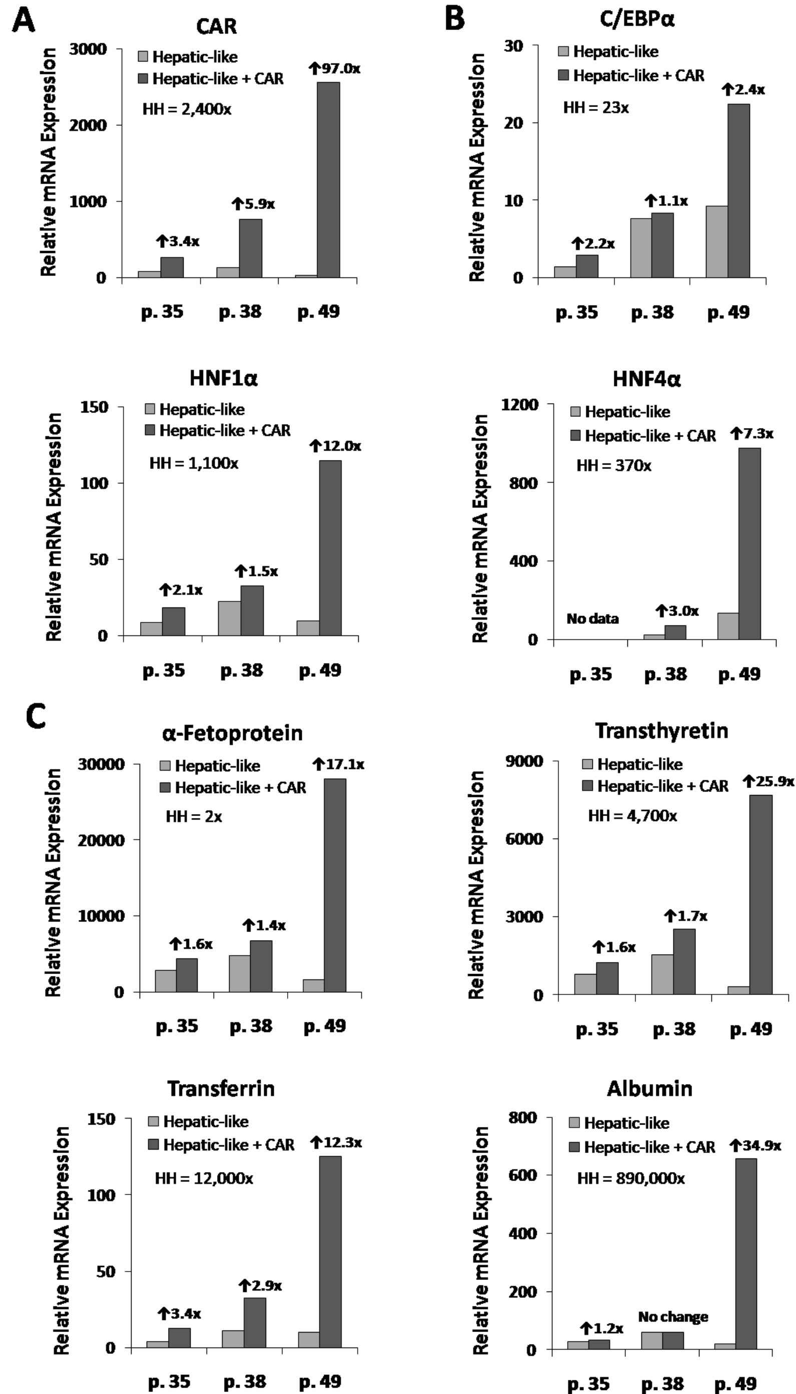

Figure 4-4. CAR expression enhances hepatic differentiation of hESCs. Prior to induction of hepatic differentiation by culturing for 10 days on type I rat-tail collagen in hepatocyte media, hESCs were transduced with either empty lentiviral vectors (Hepatic-like) or lentiviral vectors expressing CAR (Hepatic-like + CAR). RNA was isolated, converted to cDNA, subjected to real-time PCR, and the data analyzed using the $\Delta\Delta C_T$ method to determine expression levels relative to low passage number hESCs cultured on hFF feeder layers in hESC media of the following target genes: (A) CAR, (B) the hepatic transcription factors C/EPBα, HNF1α, and HNF4α and, (C) the plasma proteins α-fetoprotein, transthyretin, transferrin, and albumin. Numerical values denote target gene expression levels of primary human hepatocytes (HH) pooled from six individual donors relative to low passage number hESCs cultured on hFF feeder layers in hESC media. Data are from at least two independent trials using hESCs from different passages.

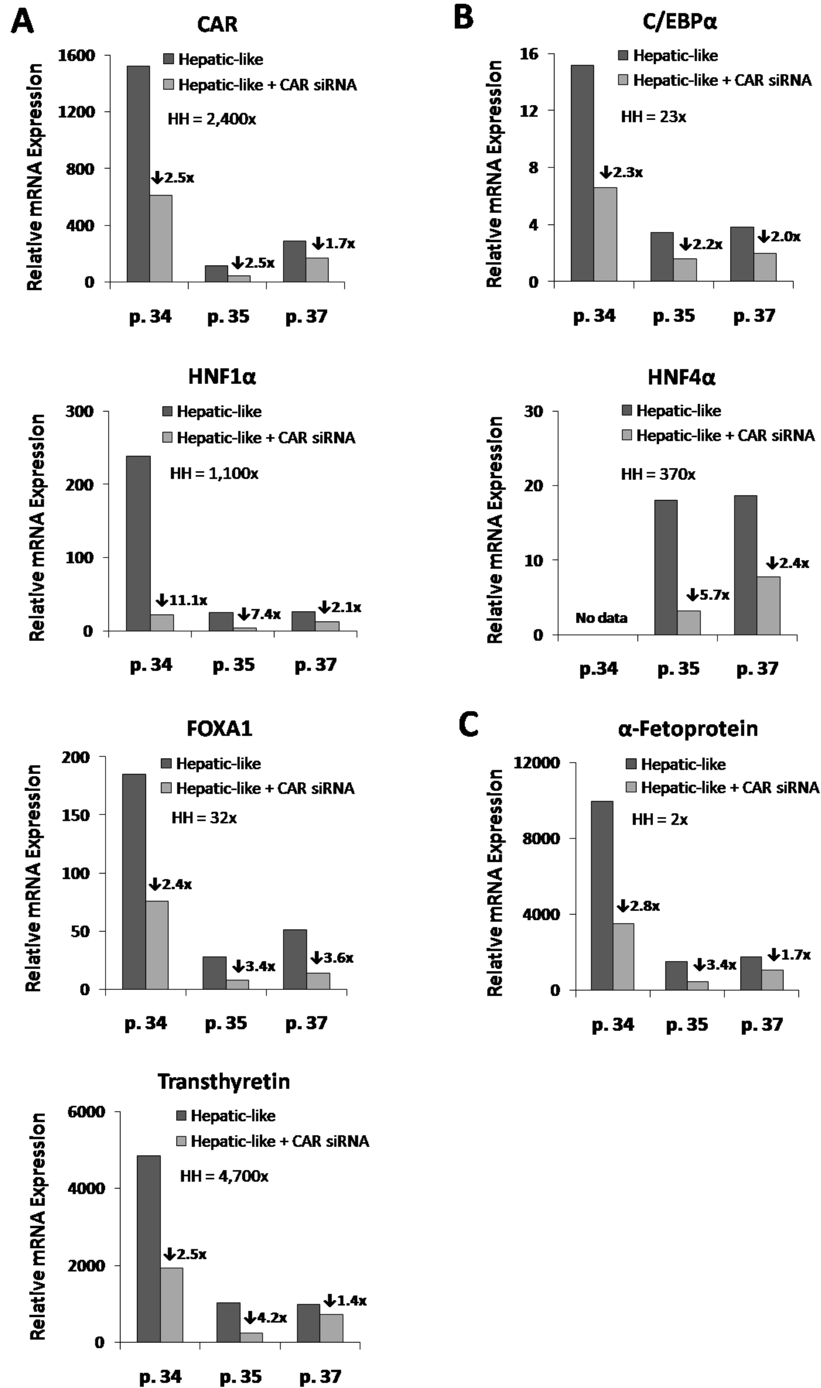

Figure 4-5. siRNA-mediated reduction in CAR attenuates hepatic differentiation of hESCs. Prior to induction of hepatic differentiation by culturing for 10 days on type I rat-tail collagen in hepatocyte media, hESCs were transduced with either empty lentiviral vectors (Hepatic-like) or lentiviral vectors expressing an siRNA targeted to CAR (Hepatic-like + CAR siRNA). RNA was isolated, converted to cDNA, subjected to real-time PCR, and the data analyzed using the $\Delta\Delta C_T$ method to determine expression levels relative to low passage number hESCs cultured on hFF feeder layers in hESC media of the following target genes: (A) CAR, (B) C/EBPα, HNF1α, HNF4α, and FOXA1 and (C) plasma proteins α-fetoprotein and transthyretin. Numerical values denote target gene expression levels of primary human hepatocytes (HH) pooled from six individual donors relative to low passage number hESCs cultured on hFF feeder layers in hESC media. Data are from at least two independent trials using hESCs from different passages.

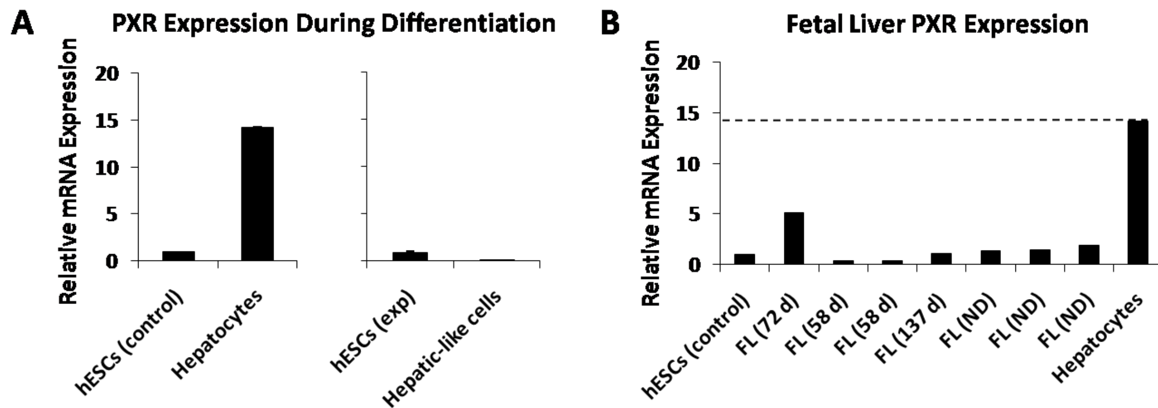

Figure 4-6. PXR mRNA is expressed at very low levels during hepatic differentiation. (A) HESCs were cultured on either hFF feeder layers in hESC media (hESCs exp) or on type I rat-tail collagen in hepatocyte media (Hepatic-like cells). At 10 days in culture RNA was isolated, converted to cDNA, subjected to real-time PCR, and the data analyzed using the $\Delta\Delta C_T$ method to determine mRNA expression levels of PXR relative to low-passage hESCs (hESCs control). Control data (left graph) depict expression levels of low-passage hESCs (hESCs control) which serve as a negative control and pooled samples from six primary human hepatocyte donors (Hepatocytes) which serve as a positive control. Experimental data (right graph) depict expression levels of hESCs (hESCs exp) and passage-matched hESCs subject to hepatic differentiation (Hepatic-like cells). Experimental data are expressed as mean +/- standard deviations of two independent trials using hESCs from different passages. (B) Human fetal liver (FL) tissue was obtained from seven subjects at the indicated stages of gestation. RNA was isolated, converted to cDNA, subjected to real-time PCR, and the data analyzed using the $\Delta\Delta C_T$ method to determine mRNA expression levels of PXR relative to low passage hESCs (hESCs control). Primary human hepatocyte cultures pooled from six individual donors (Hepatocytes) were included as positive controls. ND, no data.

PXR expression dos not enhance hepatic differentiation of hESCs

To expand upon these findings, we utilized a lentivirus system to stably express exogenous PXR in hESCs directed to differentiate along a hepatic lineage in order to determine whether or not PXR, like CAR, can enhance hepatic differentiation capacity. To this end, hESCs were transduced with lentiviral vectors expressing PXR and subsequently stimulated to differentiate along a hepatic lineage. Indeed, PXR mRNA expression was enhanced in hESCs infected with PXR-expressing virus, compared to cells infected with empty virus (Fig. 4-7A). In contrast to CAR, hESCs infected with PXR-expressing virus and subject to hepatic differentiation actually exhibited decreased mRNA expression of the hepatic transcription factors C/EBPα, HNF1α, HNF4α, and FOXA1 (Fig. 4-7B) as well as the liver-generated plasma proteins α-fetoprotein, transthyretin, and transferrin (Fig. 4-7C).

4.5 Discussion

Our results confirm that CAR expression is significantly augmented by this differentiation approach, findings consistent with our previous observations and those of Ek et al., who noted increased CAR expression in hepatic-like cells derived from hESCs [33]. To ascertain whether CAR expression can drive hepatic differentiation, we stably expressed CAR in hESCs. Although our results show that expression of CAR alone is insufficient to induce hepatic specification of hESCs (data not shown), in conjunction with hepatic factor supplementation in culture, CAR expression considerably enhanced the hepatic character of the resulting cell population. Conversely, CAR knockdown attenuated the differentiation-dependent increase in expression of a number of hepatic markers. The observed effects on hepatogenesis are specific to CAR, as our results show that PXR is expressed at very low levels during hepatic differentiation and in the fetal liver. Further, overexpression of PXR in hepatic-induced hESCs does not result in increased levels of markers of the hepatic phenotype.

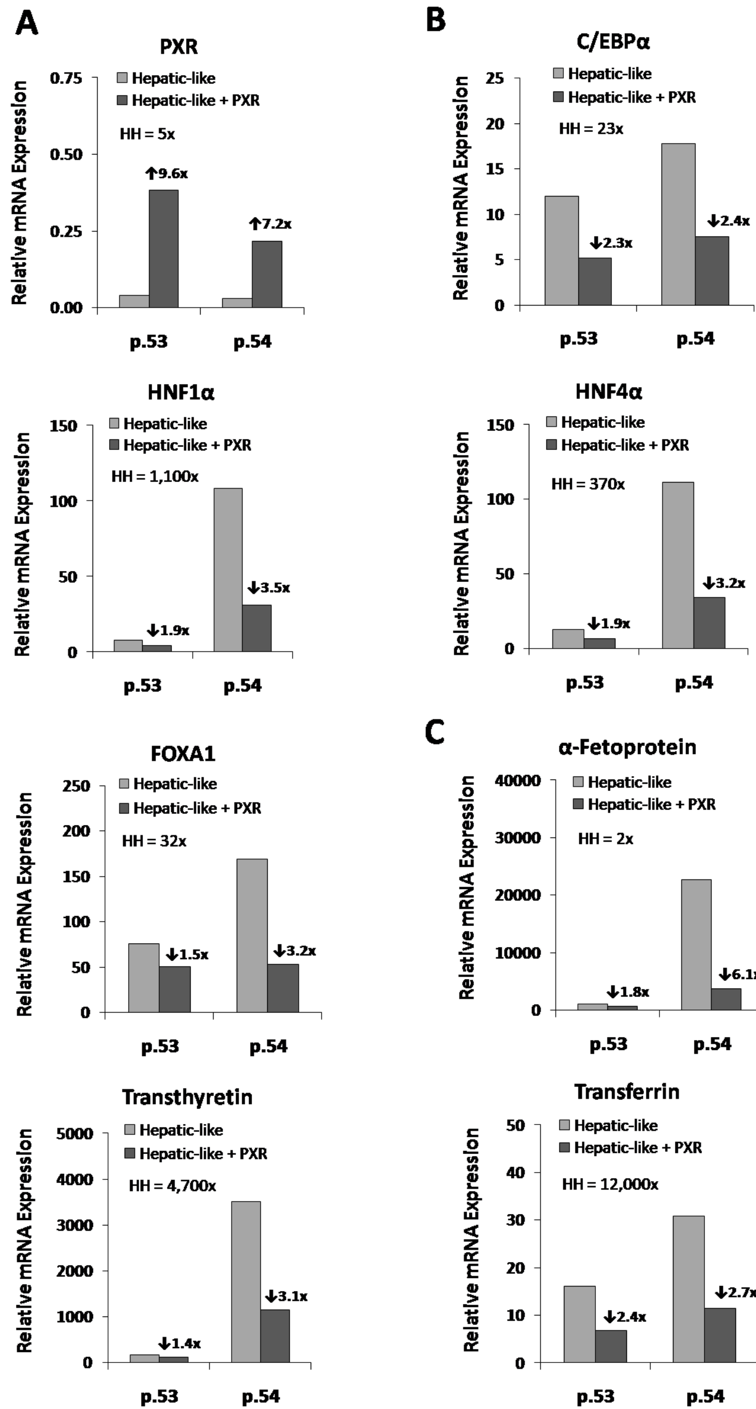

Figure 4-7. PXR expression does not enhance hepatic differentiation of hESCs. Prior to induction of hepatic differentiation by culturing for 10 days on type I rat-tail collagen in hepatocyte media, hESCs were transduced with either empty lentiviral vectors (Hepatic-like) or lentiviral vectors expressing PXR (Hepatic-like + PXR). RNA was isolated, converted to cDNA, subjected to real-time PCR, and the data analyzed using the $\Delta\Delta C_T$ method to determine expression levels relative to low passage number hESCs cultured on hFF feeder layers in hESC media of the following target genes: (A) PXR, (B) C/EBPα, HNF1α, HNF4α, FOXA1 and (C) α-fetoprotein, transthyretin, and transferrin. Numerical values denote target gene expression levels of primary human hepatocytes (HH) pooled from six individual donors relative to low passage number hESCs cultured on hFF feeder layers in hESC media. Data are from two independent trials using hESCs from different passages.

The precise mechanism by which CAR is acting to mediate hepatic differentiation, however, remains unclear. To determine if CAR is directly regulating expression of the hepatic marker genes evaluated in this investigation, a 3 kb section of the promoter regions immediately upstream of the transcription start sites was analyzed using NHR-Scan [46] for CAR-binding direct repeat (DR)-4 elements. No DR-4 elements were identified in the promoter regions of the C/EBPα, HNF1α, HNF4α, FOXA1, α-fetoprotein, transthyretin, or albumin genes. However, four putative DR-4 elements were identified in the transferrin gene promoter at -750 bp, -1.3 kb, -1.6 kb, and -2.5 kb, respectively, upstream of the transcription start site. While further studies are necessary to determine whether CAR regulates expression of transferrin directly by binding to one or more of these DR-4 response elements, it is unlikely that such a direct mechanism accounts for CAR's effects on the remaining hepatic markers.

Because CAR-knockout mice exhibit no overt morphological abnormalities [21], either functional redundancy built into the hepatic differentiation process is such that mechanisms are in place to compensate for the lack of CAR function or CAR's role in hepatic differentiation is species-specific. Indeed, the aforementioned review by Jeong and Mangelsdorf summarizing advances in the study of the roles of NRs in 'stemness' and early cell lineage commitment in a variety of species, supports the latter hypothesis. The authors note significant interspecies differences in the expression profiles and roles of members of the NR superfamily in the maintenance of pluripotency and differentiation and conclude that NRs function in these roles in a highly species-specific manner [32]. The study by Xie et al. referenced above lends further support to this hypothesis. The results from this study show that while CAR is increasingly expressed during embryoid body differentiation in both H1 and H9 human ESC lines, expression of CAR in mouse ESCs is not detectable at any developmental stage [32]. Microarray expression data from panels of human and mouse tissue, publicly available through the UCSC Genome Browser website (http://genome.ucsc.edu/) [40;41], further support this hypothesis. While CAR is expressed robustly in human fetal liver tissue, expression in the mouse embryo is negligible. Together, these data suggest that CAR's function in the developing liver may indeed be species-specific.

In summary, this work lends further insight into the genetic regulatory mechanisms governing hepatic differentiation – a complex and multifaceted process which has yet to be fully elucidated – by defining a novel role for CAR in this process. Exogenous expression of CAR augmented the hepatic character of hESCs directed toward this lineage, suggesting CAR over-expression as a potential strategy to enhance the generation of hESC-derived hepatic-like cells for use in therapeutic cell transplantations, as models for pharmacological and toxicological investigations, and to study cell lineage commitment.

4.6 References

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Chapter 5

Conclusion

5.1 Summary

The results presented herein define a novel hepatic differentiation approach in which human embryonic stem cells (hESCs) are cultured for 10 days on collagen matrix in specialized hepatocyte media (William's E Media supplemented with HEPES, glutamine, antibiotics, dexamethasone, insulin, transferrin, selenium, and linoleic acid/albumin). The resulting cell population displays hepatic-like cell morphology and exhibits a reduction in expression of 'stemness' markers at the mRNA, protein, and functional levels. The hESC-derived hepatic-like cells also show augmented mRNA expression of hepatic markers including transcription factors, nuclear receptors, liver-generated plasma proteins, protease inhibitors, metabolic enzymes, and biotransformation enzymes. Glycogen storage capacity and indocyanine green uptake by the differentiated cells confirm acquisition of hepatic function. Notably, expression of the constitutive androstane receptor (CAR) is highly increased in the hepatic-like cells, to levels approaching those of primary human hepatocytes. Further investigations confirm that CAR is correspondingly expressed in fetal liver tissue obtained from donors of various gestational ages. CAR over-expression and siRNA-mediated mRNA reduction in hESCs induced to differentiate along a hepatic lineage using a lentvirus system – which stably and robustly transduces both hESCs and cultures of primary human hepatocytes without affecting markers of 'stemness' or liver differentiation, respectively – results in corresponding alterations in expression of select hepatic-specific transcription factors, nuclear receptors, and secreted plasma proteins produced by the liver. In contrast, pregnane X receptor (PXR) expression is not enhanced during hepatic differentiation and is correspondingly negligible in human fetal liver tissue. Further, PXR over-expression in hepatic-induced hESCs does not result in increased expression of markers of the hepatic phenotype.

5.2 Discussion

The hepatic differentiation protocol defined in this work represents a relatively simple, expeditious approach by which functional hepatic-like cells may be derived from hESCs. Our unique culture system was developed to mimic the *in vivo* liver microenvironment and serves to maintain cultures of primary hepatocytes in a highly differentiated state [1-6]. By subjecting hESCs to a slightly modified version of this culture system, we were able to derive cells exhibiting gene expression profiles and functional characteristics reflective of the fully differentiated hepatocyte that may be useful for cell-based therapeutics and research purposes.

The human liver specification process is multifaceted and not yet fully elucidated. Thus, in this investigation we utilized our hepatic differentiation protocol as a unique model of human liver development in order to gain further insight into the gene regulatory networks governing this complex process. Interestingly, in addition to the expected increases in expression of liver-enriched transcription factors known to mediate hepatic differentiation – including hepatic nuclear factor 1 alpha (HNF1α), forkhead box A1 (FOXA1), hepatic nuclear factor 4 alpha (HNF4α), and CCAAT/enhancer binding protein alpha (C/EBPα) – we observed that expression of CAR is greatly enhanced in the differentiating cell population, to levels approaching those observed in cultures of primary adult human hepatocytes. This enhanced CAR expression is likely due to activation of the glucocorticoid receptor (GR) by the synthetic glucocorticoid dexamethasone in our culture conditions, as a GR response element has been mapped ~4.4 kb upstream of the CAR transcription start site [7]. While members of the nuclear receptor (NR)

superfamily to which CAR belongs are known to play important roles in both the maintenance of pluripotency in hESCs and the induction of cell lineage commitment [8], aside from the role of HNF4α as a master regulator of hepatogenesis [9], little is known about expression levels and potential roles for other members of the NR superfamily in this process. Two recent investigations, however, corroborate our findings that CAR is expressed highly during the early phases of hepatogenesis. The first study profiled NR expression levels in undifferentiated ESCs and during embryoid body differentiation and found that CAR is expressed at increasing levels in hESC undergoing early embryoid body differentiation [10] while the second study observed CAR expression in hepatic-like cells generated from hESCs using a 30-day differentiation protocol [11]. While available data suggest that CAR is expressed relatively highly in fetal liver tissue [12], we found that CAR is robustly and consistently expressed in fetal liver tissue obtained from donors of a range of gestational ages, at levels even higher than those of cultures of adult primary human hepatocytes.

While the aforementioned studies examined expression levels of CAR during induction of hepatic differentiation, none evaluated how this expression correlates with function. Our investigations show that CAR expression is necessary (although not sufficient) for maximal induction of hepatic differentiation, as CAR over-expression considerably enhances the differentiation character of the hESC-derived hepatic-like cells and siRNA-mediated reduction in CAR mRNA levels attenuates it. Interestingly, PXR – the nuclear receptor exhibiting the most sequence homology to CAR – is not expressed during hepatic differentiation, is minimal in human fetal liver tissue, and PXR over-expression in hESCs induced to differentiate along a hepatic lineage does not result in increased expression of hepatic marker genes.

Despite CAR's original characterization as a 'xenosensor,' emerging evidence points to roles for this NR in a diverse array of physiological processes as well. Through regulation of genes involved in hepatic biotransformation and disposition, CAR also plays a role in the metabolism of steroids [13], heme/bilirubin [13], thyroid hormone [14], and cholesterol/bile acids [15;16]. CAR has also been implicated in energy metabolism and lipid homeostasis, making it a potential therapeutic target for the treatment of diabetes and obesity [17]. Other genes subject to regulation by CAR include those involved in the innate immunity response [18], lipid peroxidation [19], inhibition of apoptosis [20], and cell proliferation [21]. Results presented herein expand our understanding of this versatile NR by defining an additional role for CAR as a regulator of human hepatic differentiation and liver development.

Because CAR-null mice exhibit no obvious morphological abnormalities [22], either functional redundancy during hepatogenesis is such that mechanisms are in place to compensate for the lack of CAR function or, more likely, CAR regulates hepatogenesis in a species-specific manner. Indeed, the authors of a recent review summarizing advances in the study of the roles of NRs in 'stemness' and early cell lineage commitment in a variety of species note significant interspecies differences in the expression profiles and roles of the 48 members of the NR superfamily in the maintenance of pluripotency and differentiation [10]. The aforementioned study profiling NR expression levels in undifferentiated ESCs and during embryoid body differentiation further supports this hypothesis, as results show that while CAR is increasingly expressed during embryoid body differentiation in both human ESC lines analyzed, expression of CAR in mouse ESCs is not detectable at any developmental stage [10]. Microarray expression data from panels of human and mouse tissue, publicly available through the UCSC Genome Browser website [23;24], confirm that while CAR is expressed robustly in human fetal liver tissue, expression in the mouse embryo is minimal. Taken together, these data imply that CAR's function in liver specification may indeed be species-specific.

The mechanism by which CAR enhances human hepatic differentiation remains speculative. As CAR over-expression or opposing siRNA-mediated reduction in mRNA in the differentiating cells results in concomitant changes in expression of the hepatic-enriched transcription factors C/EBPα, HNF1α, HNF4α, and FOXA1, perhaps CAR's effects on hepatogenesis are mediated through control of these integral hepatic regulators. However, an analysis of 3 kb sections of the upstream regulatory regions of these genes using the NHR-Scan program [25] revealed no CAR-binding direct repeat (DR)-4 elements. While this does not preclude the possibility that CAR directly regulates one or more of these genes, as CAR may bind to a DR-4 located further upstream or bind to a different regulatory element, it seems more likely the CAR's effects on these genes is mediated indirectly. Indeed, while CAR regulates expression of hepatic metabolic and transport genes directly by binding to regulatory elements in the promoters of these genes and activating transcription, many of its effects on lipid and energy metabolism are mediated indirectly. For example, CAR inhibits gluconeogenesis by acting as a co-repressor to down-regulate forkhead box O1 (FOXO1) target genes such as the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK) [26] and by competing with the positive regulator HNF4α for binding to the co-activators glutamate receptor-interacting protein 1 (GRIP-1) and peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α) and the DR-1 response element upstream of key genes in glucose metabolism [27]. Thus, additional studies are necessary to determine the nature of the mechanism through which CAR enhances hepatogenesis.

5.3 Future Directions

Ability of the hESC-derived hepatic-like cells to restore liver function in vivo

In our studies the acquisition of hepatic function of the hESC-derived hepatic-like cell population was demonstrated by the cells' capacity to store glycogen and to transport anionic compounds *in vitro*. An evaluation of the ability of the hepatic-like cells to restore liver function *in vivo* when transplanted into a mouse model of liver dysfunction will be an important next step in proving the potential of these cells for use in a therapeutic context. To this end, the hepatic-like cells may be injected into the spleens of immune-deficient non-obese diabetic/severe combined immune deficient (NOD/SCID) mice pre-treated with retrorsine – to prevent proliferation of native hepatocytes – and subjected to 50% partial hepatectomy [28]. The ability of the cells to home to and engraft in the liver may be monitored non-invasively by the cells' expression of GFP under control of a constitutive promoter and introduced virally prior to transplantation. Serum levels of human albumin, α-1-antitrypsin, transferrin, and transthyretin may be measured to determine whether the transplanted human hepatic-like cells are indeed functioning in the *in vivo* context.

Global analysis of CAR's role in human hepatic development

Microarray and chromatin immunoprecipitation-on-chip (ChIP-on-chip) analyses may be employed to gain a more comprehensive understanding of CAR's role in hepatic differentiation and to lend insight into the identify of CAR-regulated genes that may be mediating this process. Microarray analysis will facilitate the comparison of global gene expression profiles of human fetal liver cells at various stages of development and hESCs +/- CAR undergoing hepatogenesis using our defined hepatic differentiation protocol. Comparing the gene expression profiles of human and mouse fetal liver and during the human and mouse hepatogenesis process may lend insight into species-specific differences in CAR's role in hepatic specification. In order to gain a better understanding of the core transcriptional regulatory circuitry of specific cell types recent studies by the Young group have ChIP-on-chip analysis to map the promoter occupancy of six integral hepatic transcription factors in human hepatocytes [29] and the

three key 'stemness' transcription factors in hESCs [30]. Similar analyses may be undertaken to profile global binding of CAR to DNA elements in hESCS. Data from multiple ChIP-on-chip analyses comparing differential promoter occupancy of CAR throughout hepatic differentiation may be integrated and mapped to define cross-regulatory transcriptional interactions that control hepatic differentiation.

Role(s) of the CAR splice variants in human hepatogenesis

We and others have identified a number of CAR variants including those produced by the use of alternative translation start sites [31;32] and/or by altered splicing of the pre-mRNA transcript [31;33-35]. At least two of these variants, CAR2 (generated by the use of alternative splice acceptor site in intron 6 leading to a 12 nucleotide insertion in the receptor's LBD) and CAR3 (generated by the use of alternative splice acceptor site in intron 7 leading to a 15 nucleotide insertion in the receptor's LBD), are highly expressed in human liver and exhibit unique biological functions compared to the reference form of the receptor (CAR1) [33-37]. Thus far our work analyzing the regulatory networks governing hepatic specification has focused only on the role of total CAR in this process, i.e. discriminatory assessments between the splice variants were not undertaken. It is quite probable that the observed hepatogenic effects are actually mediated predominantly by CAR1, CAR2, or CAR3. Alternatively, the variants may contribute to hepatic specification through distinct mechanisms. To address these questions, expression levels of CAR1, CAR2, and CAR3 may be controlled throughout the hepatic differentiation process by siRNA-mediated reduction in mRNA levels – siRNAs targeted to CAR2's 12 nucleotide insertion, CAR3's 15 nucleotide insertion, or, in the case of CAR1, spanning the insertion site(s) – or phosphorodiamidate morpholino oligomers (PMOs) which are short, single-stranded, neutral chemical species that ablate protein expression by binding to translation initiation sites in the pre-mRNA sequence and blocking ribosomal assembly or, of particular interest for our future studies, by altering splicing through binding to splice effector sites [38]. Together with the total CAR-deficient hepatic-like cells developed in this study, CAR1-, CAR2-, and CAR3- deficient cells may be used as models to better elucidate the roles of these receptors in human hepatogenesis.

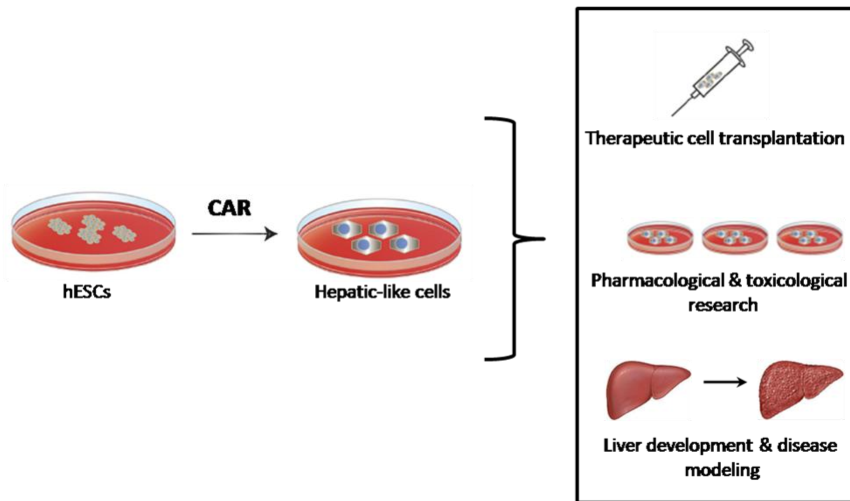

Figure 5-1. Potential uses for hepatic-like cells derived from hESCs. This work defines a novel approach by which hepatic-like cells may be generated from hESCs and identifies a role for CAR in the human liver specification process. HESC-derived hepatic cells may provide an alternative source of cells for transplantation to ameliorate liver dysfunction, for pharmacological and toxicological research, and to model hepatogenesis and liver disease processes. *Adapted from Dalgetty et al. Progress and future challenges in stem cell-derived liver technologies. Am J Physiol Gastrointest Liver Physiol 2009; 297:G241–G248 with permission of publisher. Copyright 2009, The American Physiological Society.*

5.4 Impact

In conclusion, this work defines a straightforward, easily reproducible process by which a theoretically limitless reserve of hepatic-like cells may be generated from hESCs for a variety of purposes. When combined with other hepatic differentiation methodologies, this approach may provide the supplementary factors needed to generate fully functional hepatocytes from human embryonic stem cells and ultimately provide a valuable resource of cells for therapeutic transplantation to ameliorate liver dysfunction, as hESC-derived hepatic-like cells effectively circumvent the current caveats limiting this therapy such as the paucity of donor tissue, variability in quality of available tissue, and risk of an adverse immune response in the recipient. Pharmacological and toxicological research efforts, which utilize hepatocytes to model liver metabolism and responses to xenobiotic exposure, are also impeded by the limited availability of quality tissue and thus could benefit greatly from a model system such as this. Finally, this hepatic differentiation protocol serves as a model of hepatic specification, a paradigm which we effectively employed to identify a heretofore unknown role for CAR as a mediator of human liver development (Figure 5-1).

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