DIFFERENTIATION OF HUMAN AND MURINE EMBRYONIC STEM CELLS: STUDIES ON THE COMBINED ROLES OF ADHESION MOLECULES AND GROWTH FACTORS

By

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ABSTRACT OF THE DISSERTATION

DIFFERENTIATION OF HUMAN AND MURINE EMBRYONIC STEM CELLS: STUDIES ON THE COMBINED ROLES OF ADHESION MOLECULES AND GROWTH FACTORS

By REBECCA MOORE

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The field of stem cell bioengineering can potentially revolutionize cell-based therapies for functional replacement of complex systems like the liver and nervous system. Despite significant challenges ahead, mouse and human embryonic stem (ES) cells can serve as a potential cell source for transplantation medicine, and efforts are being actively directed to guide ES cell development and maturation [1-3]. The murine ES cell model has been demonstrated to be highly organotypic based on its successful realization of specific lineages [4], but current efforts have been focused toward human ES differentiation. Despite the many research efforts, the molecular signals that can effectively promote the integration and specific differentiation of ES cells are not well characterized.

In this dissertation, I examined the molecular and microscale parameters governing the differentiation of embryonic stem cells into hepatic

and neural tissue. The goals of this study are two-fold; first, we sought to identify the nature of and presentation approaches for molecular signals that promote the liver-specific maturation of mouse and human ES cells through cell-cell adhesion molecule, E-cadherin, and growth factor stimulation. Secondly, we investigated the effects of E-cadherin on neural differentiation of human ES cells. Overall, our hypothesis is that optimal combinations of molecular growth factors and the presentation of cell adhesion molecules can provide effective tools for regenerative and reparative medicine for cell-based therapy.

ACKNOWLEDGEMENT

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TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1 Embryonic Stem Cells

There are several sources of stem cells, including early embryos, certain adult tissues, and umbilical cord blood (Figure 1.10.1). Embryonic stem (ES) cells are derived from the inner cell mass of a fertilized embryo in the blastocyst stage and are the most pluripotent of all stem cells [5, 6]. Under appropriate conditions, cultures of ES cells proliferate and self-renew indefinitely. Various methods have been developed to induce appropriate signaling molecules to stimulate differentiation of a particular specialized cell type. Mouse ES cells were derived more than 20 years ago, whereas human ES cells (hESCs) were isolated in 1998 [6]. Regardless of the source, researchers have investigated the molecular mechanisms that help maintain self-renewal and control differentiation in an effort to create cell-based regenerative therapies.

This dissertation is focused on the use of human and murine ES cells for engineering of two primary cell types: liver, and neural tissues. These areas are reviewed in brief in the following sections.

1.2 Human Embryonic Stem Cell Isolation

Human embryonic stem cells (hESCs) (Figure 1.10.2) are derived, mechanically or immunosurgically, from the polarized inner cell mass of a preimplantation-stage blastocyst [5, 6]. Pluripotent and immortal hESCs are preserved under very strict lab practices, usually in culture with embryonic or adult, somatic cells that secrete products to maintain pluripotency. Additional feeder cells have been identified to maintain hESCs in an undifferentiated state that include murine and human cell types. The Wnt signaling pathway and those associated with basic fibroblast growth factor (bFGF) and TGF-β have been identified to help maintain self-renewal [7]. Traditionally, hESCs are co-cultured with mitotically inactivated murine embryonic fibroblasts (MEFs) to form three-dimensional colonies that must be mechanically or enzymatically passaged on a weekly basis. Feeder-free and serum-free cultures have been investigated more recently to completely humanize the culture and preparation of undifferentiated hESCs. Systems that minimize human handling, increase quality control, and involve bioprocessing will address some of the key future challenges in hESC maintenance.

1.3 Tissue Engineering of the Liver

Reviews on the challenges facing functional replacement for liver cells highlight the need for identification of targeted molecular signals that induce the required hepatic phenotype from stem cells [8, 9]. The ability of healthy human liver to regenerate is steadily lost in chronic liver disease. The standard

treatment for advanced liver disease, which is the eighth leading cause of adult deaths globally, has been orthotopic liver organ transplantation, but this therapy is limited by the availability of donor tissue [10, 11]). Cell transplantation of liver parenchymal cells, hepatocytes, was shown to be an effective strategy in animal models of hepatic failure and metabolic liver diseases [12, 13]; hepatocyte transplantation has also shown to be a plausible treatment in human metabolic liver disease [14, 15]. Hepatocyte engineering continues to be an active field of investigation that has brought the key issues of cell sourcing and organoid definition to the forefront of the field of tissue engineering [16, 17]. In addition to hepatocyte-based therapies, hepatocyte cultures are widely considered as diagnostic in vitro models for pre-clinical functional and toxicogenomic screening of pharmaceutical drugs [18, 19]. However, the utility of hepatocytes in either clinical or pharmaceutical applications is limited by their availability, variability, as well as their limited proliferation and decline in hepatic functions upon extended in vitro cultures.

1.4 Tissue Engineering of the Nervous System

Neural tissue engineering is a primary focus for many researchers in an effort to implant and promote nerve regeneration and repair damage caused by injury in the peripheral and central nervous systems. Many have attempted to replicate the three types of neural tissue (brain, spinal cord and peripheral nerve), although most have agreed that the microenvironment is key to neural

tissue engineering. It is widely accepted that extracellular matrix components influence neuronal cell function, attachment and differentiation encourage certain cell phenotypes [20]. Recent studies have utilized stem cells as a source for neural injury and repair. They seek to minimize the progression of secondary injury, manipulate the inhibitory environment of the spinal cord, replace lost tissue with transplanted cells or nerve grafts and maximize the regenerative potential of endogenous progenitor cells [21]. Ultimately, there exists a need to understand the complex neurochemical and neuranatomical architecture needed to develop regenerative interventions.

1.5 Stem Cells as a Source of Hepatic-Like Cells

Embryonic stem (ES) cells afford a promising source of hepatocytes given their unlimited proliferative and pluripotent capacity [2, 22-24]. Recently, the expression of hepatocyte-associated genes has been described using in vitro differentiation of murine ES cells [25, 26], involving the formation of embryoid bodies. The major differentiating cue that has not yet been systematically exploited for ES cells arises from cell-cell interactions. There are several instances of cell-cell contact-based signaling being important for the development, morphogenesis, and phenotypic stabilization of immature or embryonic cells. For example, fetal liver cells acquired differentiated hepatic characteristics in response to soluble factors and through signals generated from cell-cell contacts [27]. Pluripotent hematopoietic stem cells seem to require heterotypic signaling from

cell-cell contact with bone marrow stromal cells (BMSCs) in order to proliferate [28, 29]. Moreover, direct contact between hepatocytes and BMSCs was shown to promote the proliferation of BMSCs by an order of magnitude [30]. Cell-cell contacts are critical for the development of ES cells during the morphologic development that accompanies embryogenesis in vivo, as well as during culture in vitro. During the culture of ES cells from aggregates called embryoid bodies (EBs), there is a loss of epithelial phenotype, called epithelial to mesenchymal transition (EMT), leading to morphologic flattening of cells and a decrease in lineage commitment. This is accompanied by a downregulation of cell-cell contacts, most notably of adhesion-based cell-cell adhesion molecules such as epithelial (E)-cadherins. Thus, the loss of E-cadherin serves as one of the driving force for epithelial cell sub-types to convert to mesenchymal cells.

1.6 Cadherin as A Major Differentiating Signal for Stem Cells

Cell-cell interactions are recognized to be of fundamental importance for embryonic development, tissue formation and differentiation [31-34]. Cadherins constitute a family of calcium-dependent, transmembrane molecules, which can mediate a wide range of cell-cell interactions and control tissue organization during development and maturation [32, 35]. The predominant cadherin of most epithelia, including liver cells, is E-cadherin (Figure 1.10.3).

Cadherins mediate cell-cell adhesion through progressive oligomerization (zippering) between cadherin dimers across two approaching

cell membrane surfaces. In mature mammalian cells, cadherin-binding causes intracellular signaling primarily via the Wnt pathway (Figure 1.10.4). Conversely, suppression of cadherin expression and binding promotes catenin-based regulation of growth or proliferation genes such as c-myc and cyclin D1 [36, 37]. Thus, the presentation of cell-based ectopic cadherin acts as a molecular switch from growth to tissue-specific differentiation pathways. In 2001, the Moghe laboratory used a hepatocyte-L929 cell co-culture and showed that the presentation of E-cadherin from a mesenchymal cell can systematically induce the differentiation of rat hepatocytes [38]. Also, the Moghe lab examined the possibility of displaying acellular cadherin fragments from artificial substrates and showed that competitive display of cadherins reduces differentiation and increase cell growth potential [39, 40] while cooperative display increases differentiation [41]. Furthermore, the Wnt/β-catenin signaling pathway was reported to play a regulatory role in the hematopoietic differentiation of murine ES cells [42]. Recent transcriptome characterization in human embryonic stem cells (hESCs) showed that the ligands for Wnt-signaling were upregulated in differentiated hESCs and consistently downregulated in undifferentiated ES cells [43]. Thus, understanding the cross-talk of the Wnt signaling pathway with other hepatotrophic signaling pathways may be integral to target the agonists/antagonists ligands for the Wnt pathway to further engineer hepatospecific differentiation in ES cells, which is one of the goals of this dissertation.

1.7 Hepatotrophic Signals for Hepatospecific Maturation of ES Cells

Largely inspired by the in vivo progression of hepatotrophic stimuli during development [44, 45], there have been extensive studies of molecular growth factors that can interact with developing liver cells, fetal liver cells, and more recently, embryonic stem cells [46-48]. A complex sequence of hepatotrophic factors were implicated in the process of murine liver development as the foregut endoderm commits to the hepatic lineage [49]. The interaction between the endoderm and cardiac-mesoderm is crucial for hepatic development [50]. This process results in the expression of two major liver-specific markers, alpha-fetoprotein (AFP) and albumin (ALB), which can be detected as early as embryonic day 8-9 (E8-E9). At this point, hepatic cells can differentiate into parenchymal hepatocytes or bile duct epithelial cells. At E10-11, hematopoietic stem cells originating from the extrahepatic organ colonize the fetal liver region and proliferate: hepatic progenitors participate in creating a conducive hematopoietic microenvironment [51]; in parallel, hematopoietic cells produce cytokines especially, oncostatin M (OSM) [52] while mesenchymal cells (non-parenchymal liver cells) produce the hepatocyte growth factor (HGF) [53]. Cells isolated from embryoid bodies similarly express receptors for EGF, HGF, and many other growth factors [54], of which only HGF and NGF have been reported to be capable of differentiating ES cells into all three embryonic germ layers (meso, endo, ectoderm). As the molecular information about the

cooperative effects of cell-cell contact based (E-cadherin) signaling and differentiation-promoting hepatotrophic factors is now emerging, these interactions can be systematically exploited to accelerate ES cell differentiation toward hepatospecific phenotype. The prevalent understanding of molecular interactions emanating from these pathways is reviewed in further detail next.

1.8 Cooperative Effects of Cadherin Signaling Pathways and Hepatotrophic Growth Factors

Hepatocyte growth factor (HGF), a mitogen and morphogen, is clearly one of the most established signals for liver development and regeneration [55]. On a molecular level, the HGF/c-met signaling pathway supports development, proliferation, scattering, and branching morphogenesis [56-58]. HGF activates c-Jun pathways in hepatocytes [59, 60], which are critical for hepatocytic development [61], and also activates SEK1 (member of the mitogen-activated protein kinase activator family) signaling pathways during hepatogenesis of the developing liver [62]. From the standpoint of its effect on differentiation, HGF was shown to induce early transition of albumin (ALB)-negative stem cells to ALB-positive hepatic precursors resembling hepatoblasts [63]. HGF-induced differentiation was terminated if the expression of CAAT/enhancer binding protein (C/EBP), a transcription factor, was inhibited, which provides insights into the mechanisms for hepatocytic specification of ES cells. The cooperative nature of signals required for hepatic maturation is best exemplified by the

significant role played by growth factors secreted from hematopoietic cells.

The most significant of these includes Oncostatin M (OSM) [27, 52, 53, 62]. Dexamathasone (DEX) is a synthetic glucocorticoid hormone promoting hepatic maturation. Notably, the effects of these factors on terminal differentiation has been noted to require additional signal(s) generated through extracellular matrix (ECM) [64]. Both DEX and OSM are potent in modulating selective phases of the maturation cascade. DEX is known to suppress AFP production (early hepatic marker) and DNA synthesis, while up-regulating albumin (mid-hepatic marker) and glucose-6-phosphatase (G6P) (late hepatic marker) production. OSM, an interleukin (IL) 6 family cytokine, has been shown to up-regulate G6P, tyrosine-amino-transferase (TAT) and glycogen accumulation. The synergistic action of DEX and OSM has found to specifically induce hepatic maturation in fetal liver E14.5 [52, 53] through induction of morphological changes, up-regulation of multiple liver-specific functions, glycogen synthesis and enhancement of homophilic cell adhesion [62, 65]. Although there is significant promise in the potential use of these maturation factors for ES cell differentiation, the exclusive application of these factors leads to a differentiation process that is inefficient and uncontrolled. Thus, an integrated application of the cross-functional signaling pathways is necessary.

It is now beginning to be appreciated that growth factors that mediate maturation act coordinately with signaling pathways emanating from other growth factors and cell-cell adhesion signaling pathways. Matsui et al [62, 65]

have shown that K-Ras regulates homophilic adhesion processes during DEX-OSM-induced hepatic development. DEX augmented the expression of E-cadherins/β-catenins, while OSM altered the sub-localization of E-cadherins/β-catenins at cell-cell contact sites. K-Ras acts as a specific downstream mediator of OSM signaling in the regulation of E-cadherin localization (enhanced homophilic adhesion) of fetal hepatocytes. This is critical since direct cell-cell contact is imperative during developmental stages in tissue organization. Studies in the Moghe laboratory had previously demonstrated that the presentation of E-cadherin significantly increased liver-specific function in adult hepatocytes through heterotypic contacts in co-culture [38] or through ectopic display of exogenous E-cadherin [41]. Further, a publication that I coauthored reported that E-cadherin expression in murine ES cells enhances and stabilizes a late maturation marker of hepatic differentiation [48]. Further work is necessary to harness the cross talk between the DEX/OSM/HGF and cadherin-based signaling pathways to more comprehensively promote ES hepatodifferentiation, a key goal for this study.

1.9 Neural hESC Differentiation Strategies

Spontaneous differentiation of hES colonies rapidly occurs *in vitro* when the system lacks preventative factors. In two-dimensional systems, spontaneous differentiation occurs at the outer borders of the colonies, at fusing colonies, or at the center core where cells begin to pile up [66]. A three-dimensional culture

system, in the form of embryoid bodies, is another way to initiate differentiation of hESCs [67]. The appearance of all three germ layers is possible in the suspension culture system. Selecting specific cell type populations can be achieved with cell-surface markers and separation techniques [68].

The process of directed differentiation is defined by induction into a specific cell type. Endogenous transcription factor activation, transcription factor transfection, growth factor supplements, or co-culture environments can accomplish this enhancement [69, 70]. The neural differentiation pathway extends from the ectodermal germ formation pathway [71], commonly considered the default development pathway for hESCs. Many investigators have used directed differentiation to induce neural lineages in the form of hESC-derived neurons [71], oligodendrocytes [72], dopaminergic neurons [73, 74], motor neurons [75], and neuroectoderm [76]. Furthermore, cardiomyocytes [77, 78], respiratory alveolar phenotyes [79], keratinocytes [80], hematopoietic progenitors [81], insulin-producing β-like cells [82], and hepatocyte-like cells [83, 84] have been developed.

In traditional two-dimensional culture systems, the process of directed neural differentiation is accomplished by supplementing the media with primary inducing growth factors or their antagonists. Within the ectoderm, various tissue types have been efficiently achieved by supplementing the media with growth factors that mimic the natural developmental pathways. For example, Noggin supplementation achieves neuroectoderm [76]; FGF2-FGF8 and sonic hedgehog

(SHH) result in forebrain and midbrain neurons [74, 85]; and bFGF, EGF and RA produce oligodendrocytes [72]. Co-culturing mouse bone marrow mesenchymal cells with hESCs achieves directed differentiation into midbrain dopamine neurons [74], while media conditioned by HepG2 liver tumor cells and supplementing serum-free media with FGF2 produces tyrosine hydrolase positive (TH^+) neurons [86].

There has been evidence that E-cadherin is involved in neural induction during gastrulation [87]. Changing E-cadherin expression has been observed in differential adhesion and cell sorting to aggregate cells into distinct groups during early development [88]. Separation and integration into developing tissue has been examined in the neural tube, neural crest and neurectoderm [89-93]. Choi et al. identified E-cadherin to be localized on the cell membrane of the ectoderm, which was further investigated by Angres et al. [94, 95]. These early experiments provided a basis for the design of E-cadherin-mediated neural induction that we further investigated in hESC differentiation.

Attempts to differentiate hESCs into any lineage have been plagued by a limited understanding of the microenvironmental cues that can effectively compensate for the addition of soluble growth and differentiation factors. Providing the hESCs with the microscale cues to promote proliferation and differentiation will allow for a more defined engineering approach to culture and scale-up of hESCs. We hypothesize that optimally defined molecular and microscale cues will compensate for the costly, time-consuming, poorly

understood hESC culture system. Regardless of the method of differentiation, the microenvironment will influence the direction and rate of specialization. The design and identification of a spatially configured and molecularly defined microenvironment can be valuable as a tool for cell-based therapies in regenerative and reparative medicine.

This thesis involves approaches for accelerated differentiation of mouse and human ESCs toward hepatic and neural lineages within optimal microenvironments that employ growth factor stimulation, paracrine and juxtacrine interactions, and a key cell adhesion molecule, E-cadherin (Figure 1.10.5). The first study uses E-cadherin and growth factor supplementation to induce hepatospecific differentiation of mouse ES cells. The second and third sets of experiments implement human ES cells and decouple the effects of E-cadherin and growth factors on differentiation. Growth factor supplementation was optimized in the third chapter to induce hepatic specification while E-cadherin was exploited in the fourth chapter to induce neural differentiation of hESCs.

1.10 Figures and Captions

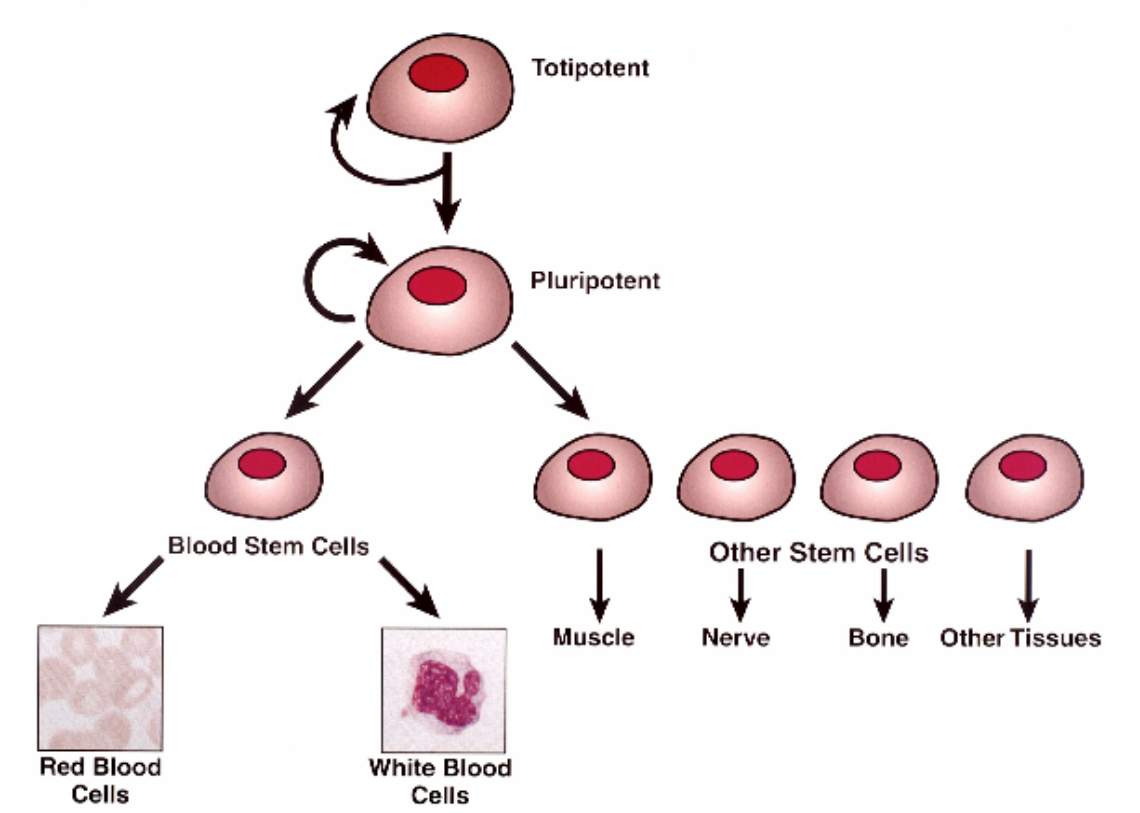

Figure 1.10.1: Hierarchy of Stem Cells

Undifferentiated stem cells have the ability to replicate indefinitely in an undifferentiated state or become specialized, under certain physiologic or experimental conditions, into a multitude of lineages [96].

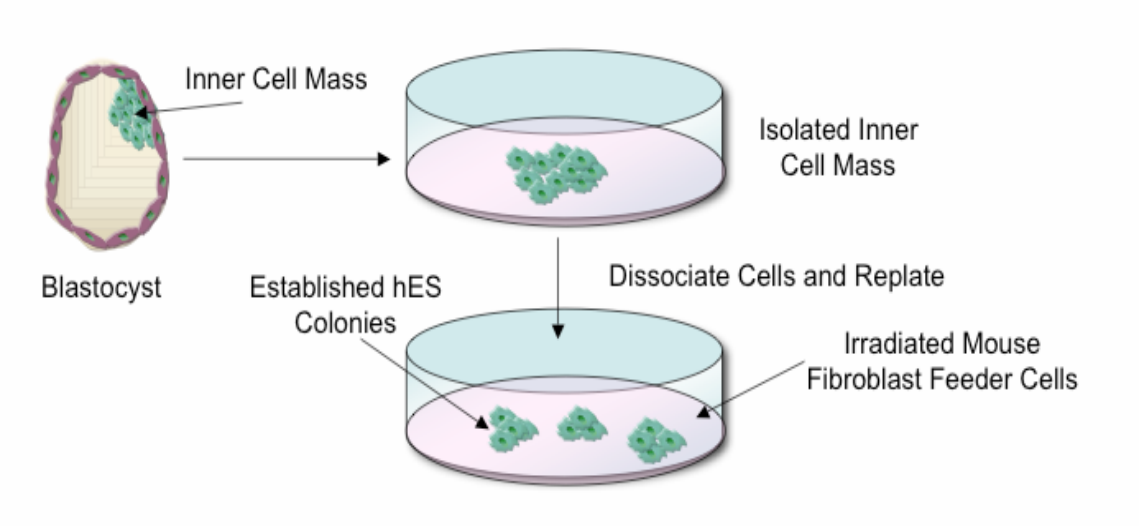

Figure 1.10.2: Derivation of hESCs

Human embryonic stem cells (hESCs) are the most pluripotent of all stem cells and are derived from embryos generated by in vitro fertilization. The fertilized egg divides and multiplies to become a blastocyst. This is composed of the outer layer, or trophoblast, that becomes the placenta and the inner cluster, or inner cell mass that becomes the embryo. The inner cell mass is mechanically isolated and transferred to a Petri dish for culture and expansion of undifferentiated hESCs.

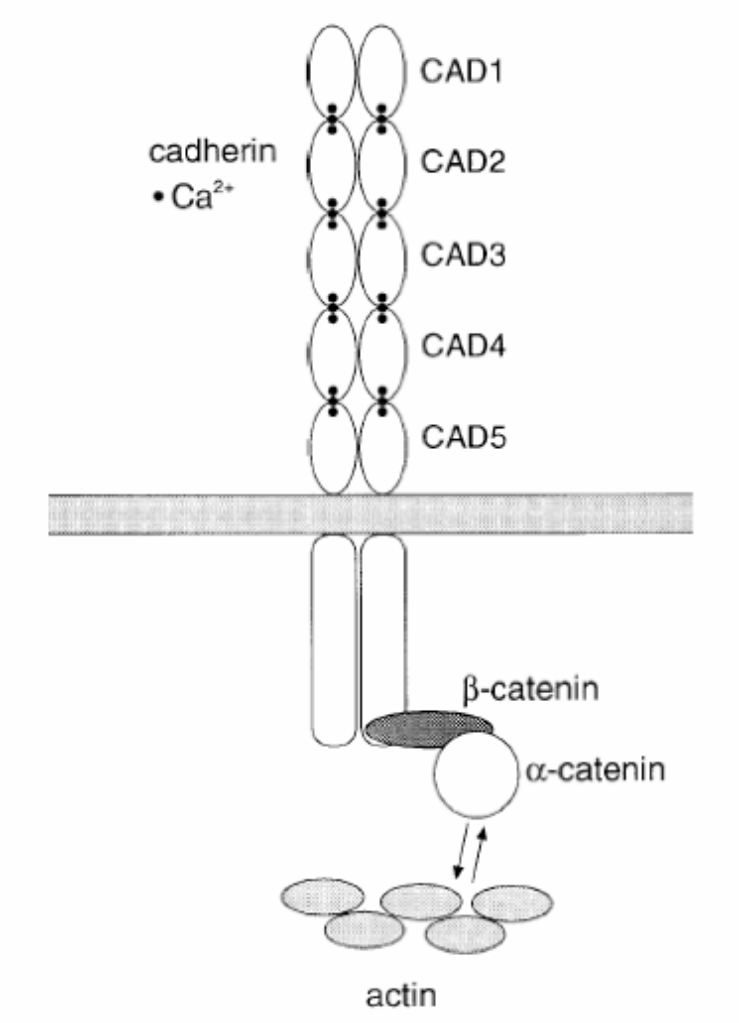

Figure 1.10.3: E-cadherin: Differentiating Signal

Epithelial (E-) cadherin is a calcium dependent transmembrane glycoprotein that mediates cell-cell adhesion through oligomerization. It controls tissue organization during development and maturation and causes intracellular signaling via the canonical Wnt signaling pathway. [97]

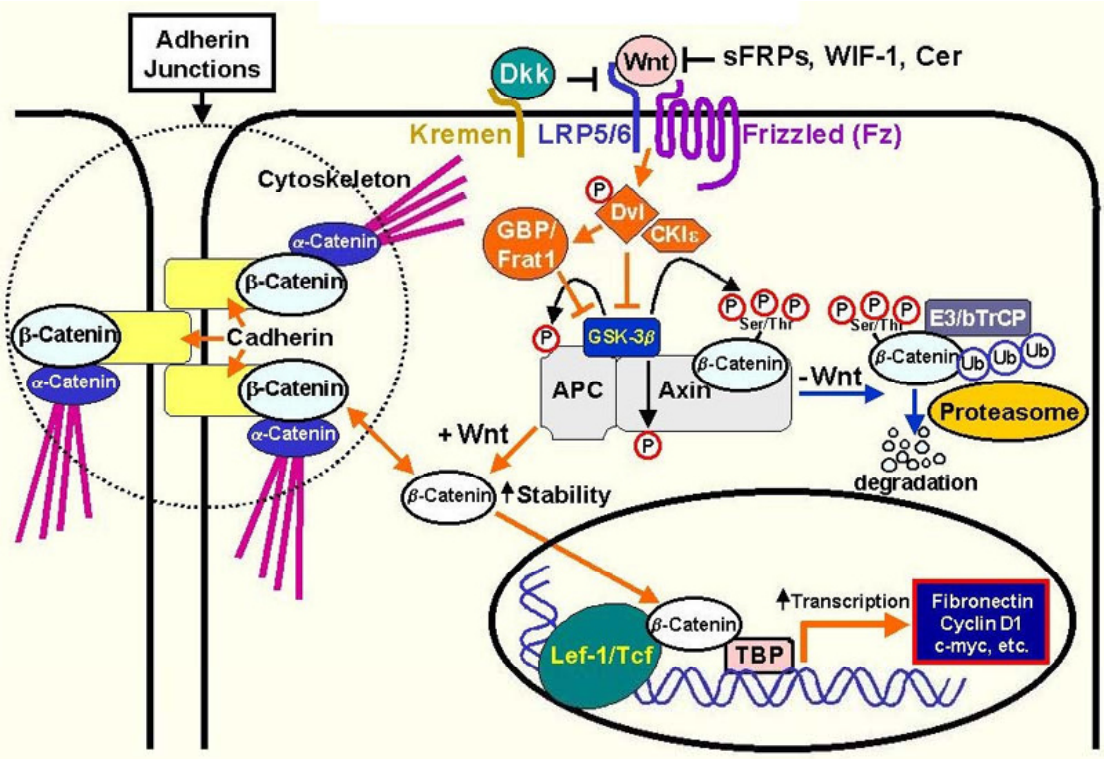

Figure 1.10.4: Diagram of Wnt Signaling Pathway

Effective cadherin engagement can cause the sequestration of a cytoplasmic protein, beta-catenin, toward the cell membrane and prevent its binding to the nuclear transcription factor, lymphocyte enhancer factor-1, LEF-1 [98, 99]. The degradation of cytosolic catenin, effected by regulatory proteins GSK-3 and Axin, is thus prevented by cadherin [100, 101].

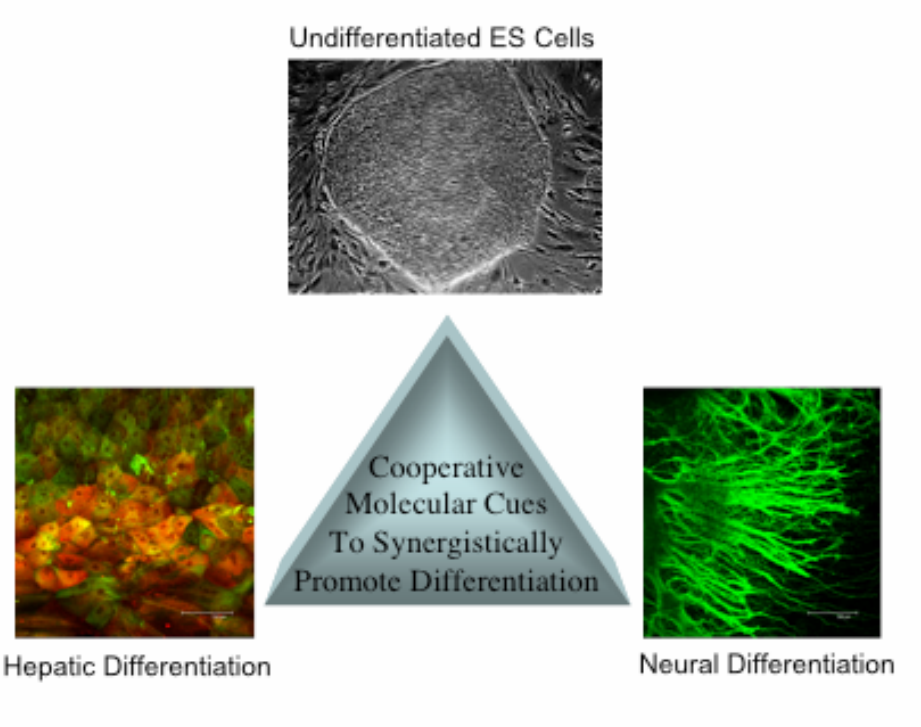

Figure 1.10.5: Schematic of Overall Research Design

The overall scope of my research incorporates the molecular and microscale cues to induce differentiation of mouse and human ES cells. Each aim probes to determine the optimal microenvironments that employ growth factor stimulation, paracrine and juxtacrine interactions, and a key cell adhesion molecule, E-cadherin.

CHAPTER 2

ENHANCED DIFFERENTIATION OF EMBRYONIC STEM CELLS USIMG CO-CULTIVATION WITH HEPATOCYTES

2.1 Abstract

We examined the effects of co-cultivated hepatocytes on the hepatospecific differentiation of murine embryonic stem (ES) cells. Utilizing an established mouse ES cell line expressing high or low levels of E-cadherin, that we have previously shown to be responsive to hepatotrophic growth factor stimulation [102], we compared co-cultures of cadherin-expressing ES (CE-ES) cells with cultured rat hepatocytes, allowing for either paracrine interactions (indirect co-cultures) or both juxtacrine and paracrine interactions (direct co-cultures, random and patterned). Hepatospecific differentiation of ES cells was evaluated in terms of hepatic-like cuboidal morphology, heightened gene expression of late maturation marker, glucose-6-phosphatase in relation to early marker, alpha-fetoprotein (AFP), and the intracellular localization of albumin. Hepatocytes co-cultured with growth factor primed CE-ES cells markedly enhanced ES cell differentiation toward the hepatic lineage, an effect that was reversed through E-cadherin blockage and inhibited in control ES cells with reduced cadherin expression. Comparison of single ES cell cultures versus co-cultures show that direct contact co-cultures of hepatocytes and CE-ES cells maximally promoted ES

cell commitment towards hepatodifferentiation, suggesting cooperative effects of cadherin-based juxtacrine and paracrine interactions. In contrast, E-cadherin deficient mouse ES (CD-ES) cells co-cultured with hepatocytes failed to show increased G6P expression, confirming the role of E-cadherin expression. To establish whether albumin expression in CE-ES cells was spatially regulated by co-cultured hepatocytes, we co-cultivated colonies of CE-ES cells around micropatterned, pre-differentiated rat hepatocytes. Albumin localization was enhanced "globally" within CE-ES cell colonies and was inhibited through E-cadherin antibody blockage in all but an interfacial band of ES cells. Thus, stem cell based cadherin presentation may be an effective tool to induce hepatotrophic differentiation by leveraging both distal/paracrine and contact/juxtacrine interactions with primary cells of the liver.

2.2 Introduction

Embryonic stem (ES) cells afford a promising source of hepatocytes given their unlimited proliferative and pluripotent differentiative capacity [103-106]. However, this is a highly inefficient and difficult process to control, and the resulting differentiated cells represent heterogeneous populations. Thus, there is a significant motivation to identify organotypic molecular cues that can rapidly differentiate ES cells into hepatic-like cells with phenotypic markers seen in adult hepatocytes. A major source of differentiating cues that remains to be systematically exploited for ES cellular engineering arises from cell-cell interactions [34, 38, 107-110].

There are several instances of cell-cell contact based signaling providing important cues for the development, morphogenesis, and phenotypic stabilization of immature or embryonic cells [111-114]. Fetal liver cells acquire differentiated hepatic characteristics in response to soluble factors in conjunction with signals generated from cell-cell contacts. Pluripotent hematopoietic stem cells (HSCs) seem to require heterotypic signaling due to cell-cell contacts with bone marrow stromal cells (BMSCs) in order to proliferate [115]. Moreover, direct contact between hepatocytes and BMSCs was shown to promote the proliferation of BMSCs by an order of magnitude [116]. The growth of HSCs exposed to BMSCs was suggested to result from direct cell-cell and cell-matrix interactions [117] and cytokine-mediated signaling [118, 119]. Mitaka and colleagues cultured small hepatocytes (Shs) with BMSCs and reported

hepatocyte proliferation was not regulated in a paracrine manner, but that direct contact was needed to enhance proliferation and differentiation [120]. This highlights the importance of direct cell-cell contact and cell-matrix interaction required for successful differentiation of ES cells into hepatic-like cells in addition to induction of hepatocyte function *in vitro* [121]. Cell-cell contacts are critical for the development of ES cells during the morphologic events that accompany embryogenesis *in vivo*, as well as during culture *in vitro*. Additionally, cell-cell interactions have been shown to play a critical role in tissue generation *in vitro* [122].

A high degree of cell-cell interactions are crucial for the ES cell differentiation process. Furthermore, stage-dependent tissues play an important role in the ES cell differentiation process [123]. Numerous studies have revealed that co-cultured ES cells have shown enhanced cell lineage differentiation. Specifically, Buttery *et al* (2001) observed enhanced differentiation of ES cells toward the osteoblast lineage through supplementing media with growth factors (ascorbic acid, beta-glycerophosphate, dexamethasone (DEX)/retinoic acid (RA)) or through co-culture with fetal murine osteoblasts [124]. Fetal osteoblasts provided a potent stimulus for osteogenic differentiation inducing a 5-fold increase in nodule number relative to ES cells cultured alone [124].

Studies comparing fetal liver-derived hematopoietic ES cells cultured using conditioned media (from adult astrocytes) to ES cells grown in direct co-culture have shown that direct cell-cell contact/interactions were necessary to

efficiently drive and induce cell differentiation [125]. Additionally, adrenal medullary chromaffin cells provided a supportive microenvironment for neural progenitor cells. Growth and differentiation of neural progenitor cells were compared to standard neural growth media, neural growth media with FGF-2, or co-cultured with bovine chromaffin cells. Survival was poor in the absence of FGF-2 whereas the chromaffin cell co-culture systems promoted robust neurospheres with enhanced mature phenotype. Consequently, the chromaffin cells provided a conducive environment for the survival and neuronal differentiation of neural progenitor cells; they provide a useful, sustained source of trophic support to improve the outcome of neural stem cell transplantation [126]. Furthermore, Schwann cell co-cultures were shown to promote the differentiation of rat embryonic neural stem cells highlighting that importance of the factors secreted by Schwann cells and more importantly, that direct cell contact needed to enhance differentiation [127].

In hepatic systems, Fair *et al* (2003) have shown hepatic differentiation induction in ES cells by co-culture with embryonic cardiac mesoderm [128]. They suggested that embryonic cardiac mesoderm activated crucial transcription factors (Sox 17alpha, HNF3beta and GATA 4) required for hepatic development and notably critical cell-cell interactions were necessary to enhance hepatic differentiation [128].

Cell-cell interactions have been reported to be important, however the key adhesion mediator, E-cadherin, involved in these interactions has not been

systematically examined. Our previous work have highlighted that E-cadherin engineered ES cells exhibited hepatospecific maturation responsiveness to hepatotrophic stimulation [102]. Furthermore, Brieva & Moghe (2001) have shown that paracrine and juxtacrine signals are important for inducing functional behavior in hepatocytes [38]. This current study aims to examine the role of E-cadherins in the differentiation of ES cells using a simple organotypic co-culture model. Our major hypothesis is that primary adult hepatocytes can induce hepatospecific maturation of primed ES cells through two signaling mechanisms related to cadherins: juxtacrine signaling (initiated through cadherin-cadherin contacts between CE-ES and hepatocytes) and paracrine signaling (distally initiated by hepatocytes; mediated by cadherin-growth factor signaling pathways). To identify the optimal co-culture configurations that maximally promote hepatodifferentiation of ES cells, we established co-cultures of rat adult hepatocytes and hepatotrophically stimulated CE-ES cells using two different co-cultures configurations: (a) indirect co-culture through insert wells to assess effects of paracrine interactions; (b) direct co-cultures to assess combined paracrine and juxtracrine interactions. Additionally, we employed micropatterned co-cultures to determine whether ES cell differentiation varies with distance from spatially organized hepatocyte cultures.

2.3 Materials and Methods

2.3.1 ES Cell Culture Conditions

Mouse embryonic stem cell lines (D3) were utilized [129]: wildtype homozygous cadherin-expressing embryonic stem (CE-ES) cells, which express high levels of E-cadherin and cadherin-deficient embryonic stem (CD-ES) cells, which are genetically modified by two rounds of homologous recombination to yield null E-cadherin ES cells. A full characterization of the CD-ES and CE-ES cells can be found in our previous publication [102]. ES cells were cultured on 0.1% (w/v) gelatin (Sigma, St. Louis, MO) coated tissue culture polystyrene plates (TCPS). Undifferentiated cells were maintained on knockout Dulbecco's modified Eagle's medium (KDMEM, Invitrogen Corp, Carlsbard, CA) with high glucose (Invitrogen Corp., Carlsbard, CA), 10% heat-inactivated fetal bovine serum (Invitrogen Corp., Carlsbard, CA), 100 U/mL penicillin/streptomycin (Cambrex, Walkersville, MD), 2mM glutamine (Biowhitaker, Walkersville, MD), 1000 U/mL leukemia inhibiting factor (LIF) ESGRO (Chemicon, USA) and 2,beta-mercaptoethanol (Invitrogen Corp., Carlsbard, CA). Cells were split every 6-7 days with 0.25% Trypsin/0.02% EDTA solution (Sigma, St. Louis, MO) and media were exchanged every other day [129].

2.3.2 Initiation of ES Cell Differentiation

The Hanging Drop method was utilized to initiate embryoid body (EB) formation [130, 131]. The EBs were allowed to form over 18 days, which elicited the outgrowth of cells of the hepatocytic lineage from the EB [102]. Briefly, hang-drop culture media consisted of Dulbecco's minimal essential medium (DMEM) (Cellgro, Herndon, VA), 20% fetal bovine serum (FBS) (Invitrogen Corp., Carlsbard, CA), 4 mM L-glutamine (Biowhitaker), and 100 U/mL Penicillin/Streptomycin (Biowhitaker). Cells were diluted to $9.9X10^4$ cells/mL; 30 μL drops were placed on the inside of a polystyrene petri dish lid spaced at least 1cm apart. To ensure gas exchange, 5 mL of basal media was placed on the bottom petri dish lid and the cells were incubated (37°C, 5% CO_2, 2 days). On day 2, the EBs were transferred to a new 100 mm petri dish and incubated for two more days. At day 4, single EBs were transferred to TCPS plates and incubated for 7 days. Media were exchanged and cultures incubated for 6 more days. At day 18 (E18), differentiating cells emanating from the EB were harvested [132] by trypsinization and plated onto adsorbed collagen (0.26mg/mL, BD Biosciences, San Diego, CA) plates. The sub-population of ES cells were cultured for 1 week either under no cocktail growth medium: basal C+H media or under DOH cocktail growth medium: DEX-OSM-HGF (dexamethasone (DEX): 10^{-7}M, oncostatin M (OSM): 10 ng/mL, hepatocyte growth factor (HGF): 20 ng/mL) (Sigma, St. Louis, MO) [133-135]. C+H culture medium is composed of DMEM containing 200 U/mL Penicillin/Streptomycin, 2

mM L-glutamine, 10% heat-inactivated fetal bovine serum (Invitrogen Corp., Carlsbard, CA), 0.5 U/mL insulin, 7 ng/mL glucagon, 7.5 μg/mL hydrocortisone and 20 ng/mL epidermal growth factor (Sigma, St. Louis, MO). In medium containing DEX, no glucagon was added as they compete for the same receptor. ES cells were grown for 1 week to prime the cells, harvested and replated under co-culture conditions in C+H media (ES cells alone, indirect or direct contact) for an additional week to mimic organotypic environments. Subsequent morphogenesis and hepatogenesis were examined.

2.3.3 Primary Adult Cell Isolation and Culture

Hepatocytes were freshly isolated from Fisher male rats (75-250g) anesthetized with a ketamine/xylazine cocktail. A modified EDTA and collagenase perfusion protocol [136, 137] was utilized. Cell suspensions were filtered through nylon meshes with 350 and 62 μm openings (Small Parts Inc., Miami Lakes, FL). Finally hepatocytes were separated via a Percoll (Sigma, St. Louis, MO) density centrifugation. All animals were maintained and handled in accordance with the Rutgers Institutional Review Board Guidelines for the Use and Care on Animals (Protocol Review Number 97-001). Cell yield and viability (typically 85-92%) were determined via trypan blue exclusion and hemocytometry. Cells were resuspended into DMEM (Cellgro, Herndon, VA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen Corp., Carlsbard, CA), epidermal growth factor (EGF, 20 ng/mL: Sigma), insulin

(18.5 μg/mL: Sigma), hydrocortisone (7.5 μg/mL: Sigma), glucagon (7 ng/mL: Sigma), 2mM L-glutamine (Cambrex BioScience, Walkersville, MD), 200 U/mL penicillin-streptomycin (Cambrex BioScience, Walkersville, MD) and gentamycin (50 μg/mL, Cambrex BioScience, Walkersville, MD); (C+H medium). Isolated hepatocytes were seeded at 2.66 X 10^4 cells/cm^2 onto adsorbed collagen (0.26 mg/mL, BD Biosciences, San Diego, CA) plates.

2.3.4 Co-cultures of Cadherin-Engineered ES Cells and Hepatocytes

Co-cultures of primary adult hepatocytes with expanded ES cells were conducted to investigate the effect of indirect (paracrine signaling) and direct (paracrine and juxtacrine signaling) contact driven cues to promote hepatodifferentiation. To conduct indirect co-culture experiments, hepatocytes were seeded on the insert wells (BD Biosciences, San Diego, CA) with 0.22 μm filters on collagen-coated membranes with the ES cells seeded below on the culture plates, identical in size and surface to the direct co-culture conditions. Hepatocytes were seeded with a volume of 400 μl/well at the same seeding densities (2.66 X 10^4 cells/cm^2) for both indirect and direct co-culture experiments. Hepatocytes were allowed to attach for 10 hrs and cell medium exchanged, after which the expanded ES cells (subjected to cocktail or no cocktail medium) were harvested and plated into the co-culture systems at a seeding density of 9.4 X 10^4 cells/cm^2.

2.3.5 Real-Time Reverse-transcriptase polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted by using an RNEasy kit (Qiagen, Valencia, CA). cDNA was synthesized from 5μL total RNA by using Superscript II first-strand synthesis with oligo dT (Promega) (48°C, 45 min, first strand synthesis; 94°C, 2 min, RT inactivation/denaturation). PCR was performed using SYBR green PCR master mix (Qiagen). Primers were synthesized for the following mouse genes as per [130]. The forward and reverse primers were located at different exons to discriminate the product from the targeted mRNA or its genomic DNA. Mouse oligonucleotide sequences are given in brackets in the order of antisense, sense primers followed by annealing temperature, cycles used for PCR and length of amplified fragment: (i) α-fetoprotein (AFP) (5'TCGTATTCCAACAGGAGG, 5' AGGCTTTTGCTTCACCAG; 55°C, 25 cycles, 173 bp); (ii) glucose-6-phosphatase (G6P) (5' CAGGACTGGTTCATCCTT, 5' GTTGCTGTAGTAGTCGGT, 55°C, 30 cycles, 206 bp); (iii) Endogenous housekeeping gene, β-actin (5' TTCCTTCTTGGGTATGGAAT, 5' GAGCAATCATCTTGATCTTC, 55°C, 20 cycles, 200 bp). The gene expression of the intermediate differentiation marker, albumin, was not included in the analysis as, similar to our previous studies, it was not consistently detectable during the onset of hepatospecific differentiation (Dasgupta et al., 2005). The localization of the gene protein product, albumin, however, does vary more consistently and was quantified (described later).

Statistical analysis was completed by single variable ANOVA followed by multiple comparison testing.

2.3.6 E-cadherin Antibody Blocking

Since CD-ES cells are inherently different from CE-ES cells even prior to co-culture, alternate control conditions were employed based on antibody blockage to determine the extent of E-cadherin mediated hepatic differentiation. Expanded CE-ES cells were pretreated with antibodies against E-cadherin prior to introduction with adult hepatocytes in co-culture to confirm that E-cadherins mediate the functional enhancement seen in direct co-culture. Primed CE-ES cells were trypsinized after 1 week of DOH treatment post EB development and exposed to rat anti-E-Cadherin (mouse) ECCD-1 IgG_{2b} monoclonal antibody (Zymed, South San Francisco, CA). A rat IgG_{2b} isotype control antibody (R&D Systems, Minneapolis, MN) was used to confirm functionality of antibody blockage. Both antibodies were prepared without preservatives and used at a concentration of 200 μg/ml. The ECCD-1 E-cadherin antibody was chosen because it recognizes E-cadherin on the surface of mouse ES cells to inhibit E-cadherin-dependent cell-cell contacts. CE-ES cell populations were incubated with the E-cadherin or control antibodies for 1 hour at 4°C. The cells were washed to remove excess antibody and reconstituted in C+H media. The antibody blocked CE-ES cells were subsequently plated with freshly isolated hepatocytes in identical concentrations to the direct co-culture, as described

above. Additionally, a non-antibody treated direct co-culture was included for direct comparison. Media was changed on day 3 and re-supplemented with antibodies to functionally block proliferating mouse ES cells. At one week in random co-culture, cells were prepared for RT-PCR, as described above. Statistical analysis was completed by ANOVA with Post-hoc Tukey's HSD method.

2.3.7 Micropatterned Co-cultures of ES cells and Hepatocytes

The fabrication of silicon wafers was carried out as per Folch (2000) [138]. Elastomeric PDMS solution (vacuum treated to remove air bubbles) was placed on the silicon wafer and cured overnight at 56ºC. The following day, the cured PDMS was carefully peeled off creating elastomeric stamp to pattern cells. The resulting elastomeric stamp provides a robust and reproducible method to micropattern freshly isolated hepatocytes. This novel application uses an elastomeric stamp to preserve cells within the channel microenvironment to pattern hepatocytes. The ability to physically entrap the cells within the feature channel (110 μm height) microenvironment allows for the cells to remain healthy and confined for subsequent cell seeding. Briefly, the stamp is soaked in ethanol for 30 minutes and allowed to dry, then soaked in bovine serum albumin (BSA) for 10 minutes to reduce cellular attachment. Hepatocytes were incubated (1 hour, 37°C) with 1 μM Cell Tracker Red (Invitrogen, Carlsbad, CA) and washed twice with C+H media to later visibly distinguish the ES cells from hepatocytes

on co-culture. Hepatocytes in media (1 mL) were seeded (4.5 X 10^6 cells/mL) onto a collagen adsorbed (0.26 mg/mL) glass bottom chamber (area = 4 cm^2). The elastomeric stamp was quickly placed atop the hepatocytes and weighed with a 50 g metal cylinder. The system was incubated at 37°C, 5% CO_2 for 48 hours. The weight and stencil were carefully removed without disturbing the patterned cells. Wells were washed 3 times with sterile PBS to remove unattached cells. (The metabolic activity of patterned hepatocytes was independently verified. Stamp-overlaid hepatocytes exhibited slightly elevated albumin secretion rate (0.6 pg/cell/d) in relation to unpatterned hepatocytes (0.4 pg/cell/d), and normal urea secretion rates (7 pg/cell/d) in relation to unpatterned hepatocytes (4.5 pg/cell/d). The mouse ES cells, primed and isolated post-DOH treatment, were then directly seeded into the chambers to allow for complementary cell attachment. ES cells were subsequently seeded at 9.4 X 10^4 cells/cm^2 around the attached hepatocytes in C+H media. Similar to the direct random co-culture, functional E-cadherin antibody blocking was conducted on the micropatterned co-cultures. Media was exchanged every day and cells were co-cultured for one week.

2.3.8 Visualization of Intracellular Albumin

ES cells were stained for the presence of intracellular protein, albumin (hepatic specific marker) to visually confirm that the cells were in fact hepatic-like. For the indirect co-culture conditions, the hepatocyte-containing insert

wells were removed immediately prior to analysis. ES cells and hepatocyte co-cultures were fixed with 4% paraformaldehyde in DPBS with Ca^{2+} and Mg^{2+} for 15 minutes at room temperature. Cells were then rapidly washed three times with DPBS and permeabilized with 0.1% Saponin (SAP, Sigma, St. Louis, MO) for 5 minutes at room temperature. After washing once with DPBS, cells were blocked with 3% (w/v) bovine serum albumin (BSA) and 1% (v/v) normal goat serum (NGS) for 30 minutes at room temperature to reduce nonspecific antibody binding [139]. Subsequently, albumin staining was attained by using FITC-conjugated mouse anti-albumin antibody (BioLegend, San Diego, CA) in SAP buffer (0.5% Saponin, 1% BSA, 0.1% Sodium Azide) for 1 hour, rocking at room temperature, at a dilution of 1:50. Finally, the cells were washed four times in DPBS and visualized using the Leica TCS.SP2 confocal microscope system (Leica Microscope, Exton, PA) at 10X (zoom 2) for random direct and indirect co-cultures and 10x for micropatterned co-cultures. Albumin staining was quantified by image process analysis (ImagePro 5.0) to quantify the mean green intensity, minus background fluorescence, for an area of interest representing only mouse ES cells, devoid of hepatocytes.

2.4 Results

2.4.1 Co-culture Treatment Altered ES Cell Morphology

The effect of media priming and co-cultivation with hepatocyes on ES cell morphogenesis was evaluated. As shown in Figure 2.8.1, beyond day 8 (post EB development and 1 week following ES cell priming), ES cells were basally cultured in C+H growth media or primed in DOH media for a week (day 15). DOH priming was investigated based on our previous work that optimized the growth factor cocktail to induce hepatocyte-like differentiation of mouse ES cells [102]. CE-ES cells appear cuboidal and cobblestone in appearance, taking on early hepatic cell morphology (Fig. 2.8.1A). In contrast, CD-ES cells exhibit very different phenotypic responses. They were elongated, well spread and displayed a fibroblast-like appearance (Fig. 2.8.1E). Priming the CE-ES cells resulted in significantly more cuboidal and cobblestone-like appearance under DOH treatment (Fig. 2.8.1B-D). In the presence of direct co-cultures, there is an enhanced degree of cobblestone appearance in CE-ES cells (Fig. 2.8.1D). Compact polyhedral cells with round nuclei are visible with well-demarcated cell-cell borders. With indirect co-cultures, the level of cobblestone appearance was not as enhanced compared to direct co-cultures (Fig. 2.8.1C). In contrast, as shown in Figure 2.8.1F-H, priming ES cells with DOH growth medium did not alter CD-ES cell morphology. This may suggest that priming CE-ES cells affects their morphology either with or without direct contact cues. However,

heterotypic cell-cell contacts seem to be important in mediating cellular response, which may be further facilitated through the presence of E-cadherins.

2.4.2 Elevated Presence of Hepatic Maturation Marker through Direct Contact Co-cultures

Expression of hepatic specific markers in the varying ES cell treatments was assessed to reveal the level of hepatic maturation. Specifically, mRNA expression of alpha-fetoprotein (AFP; early hepatic marker), glucose-6-phosphatase (G6P; late hepatic marker) and beta-actin (house-keeping gene) was examined by RT-PCR; each condition was normalized to the level of beta-actin mRNA expression. Hepatocyte-only conditions were also examined to ensure there is no cross-reactivity between rat and mouse genes (data not shown). As shown in Figure 2.8.2, normalized AFP was observed in all conditions indicating the presence of immature fetal liver cells, although these levels are not statistically significant when compared to the ES cells alone. The effect of indirect and direct co-culture in CE-ES cells was significant ($P<0.05$) for the expression of the late hepatic marker, G6P, compared to CE-ES cells cultured alone in C+H medium (no cocktail) (Fig. 2.8.2A). Interestingly, when CE-ES cells were primed in DOH medium (cocktail medium), the effect of indirect co-culture treatment appeared to be insignificant compared to CE-ES cultured alone in DOH medium. This implies that DOH priming can reach gene expression levels similar to those from hepatocyte-conditioned media from insert wells in

conjunction with results from Figure 2.8.1 showing the enhanced degree of cobblestone appearance associated with DOH priming. Further, there was enhanced expression of G6P in the direct co-culture compared to the other conditions in CE-ES cells. In contrast, there was no significant effect observable for CD-ES cells, either cultured singly or within a co-culture (Fig. 2.8.2B).

2.4.3 E-cadherin Blocking Inhibits G6P Based Hepatic Maturation in Direct Co-culture

CE-ES cells were subjected to E-cadherin antibody blocking to determine the effect of E-cadherin mediated hepatic differentiation in random co-cultures. Results from Figure 2.8.2A showed a marked increase in G6P when CE-ES cells were co-cultured with adult hepatocytes. In order to determine if E-cadherin mediated contacts were the cause of this hepatic maturation, we probed for G6P levels in E-cadherin blocked cultures. Real time RT-PCR results (Fig. 2.8.3) from the blocking experiments showed that G6P, a late hepatic marker, had statistically significant differences ($P<0.05$) when comparing the untreated, direct co-culture to the ECCD-1 E-cadherin antibody blocked co-culture. This data confirms that in random co-cultures, G6P based hepatic maturation is mediated through E-cadherin pathways. The isotype control antibody showed no statistical significance when compared to the direct random co-culture.

2.4.4 Co-cultured Hepatocytes Promoted ES Cell Morphology and Albumin Expression

To further characterize the hepatic maturation of DOH primed cultures, CD-ES and CE-ES cells were stained at day 15 (post EB 18, plus 1 week ES cell priming by DOH, plus 1 week in co-culture treatment) for intracellular albumin (liver-specific marker) to evaluate whether they were exhibiting hepatic-like behavior. The mouse intracellular albumin antibody has cross-reactivity to rat hepatocytes in our hepatocyte only controls, which is why we chose to pre-stain the hepatocytes with cell-tracker red to allow the ES cells to be distinguished from hepatocytes in all co-culture experiments. Because of this antibody cross-reactivity issue, we did not seek to detect global albumin secretion levels but instead, used immunolocalization to evaluate the relative levels of albumin protein in areas devoid of hepatocytes. In Figure 2.8.4C and 2.8.4F co-localization of intracellular albumin with cell tracker red in hepatocytes was observed. CD-ES cells showed a slight increase in intracellular albumin staining (Figure 2.8.4A-C) when introducing paracrine signaling (indirect co-culture) and the combination of paracrine and juxtacrine (direct co-culture) signaling. As shown in Figure 2.8.4D-F, intracellular albumin staining was observed in CE-ES cells alone and in indirect and direct treatments for randomly seeded hepatocyte co-cultures involving CE-ES cells. CE-ES cells alone exhibited detectable, but not prominent albumin staining. Albumin staining became more pronounced within co-cultures with more prominent and clearly visible staining in direct co-

cultures. Results show that combined juxtacrine and paracrine signaling in direct co-cultures, mediated through E-cadherin engagement, significantly increases hepatospecific differentiation.

The randomly mixed co-cultures of ES cells and hepatocytes do not allow controlled examination of albumin localization across the heterotypic interface between the two cell types. Therefore, cadherin-variant ES cells were separately seeded onto and co-cultured with primary adult hepatocytes, which were first micropatterned. Cell patterning was successfully established through utilization of elastomeric stamps (Fig. 2.8.5A-B) with red cell-tracked hepatocytes initially seeded onto the surface. The following day, the stamp was peeled off, carefully leaving patterned hepatocytes (Fig. 2.8.5C), and washed to remove unattached cells. Subsequently, cadherin-variant ES cells were seeded in co-culture. At day 2 of co-culture, hepatocytes spread out readily while ES cells proliferated. As shown in Figure 2.8.5G-I, there was an increased level of albumin staining (quantified on a confocal microscope using densitometry after subtracting background; mean green intensity = 87.62) in the cultures directly supporting E-cadherin engagement. In contrast, the cadherin deficient co-cultures show lack of albumin staining (mean green intensity = 5.81) in the ES cells (Fig. 2.8.5D-F). These results are similar to the cadherin-expressing co-cultures supplemented with an antibody used to functionally block E-cadherin engagement (mean green intensity = 8.99) (Fig. 2.8.5J-L). The images provide a direct comparison of the levels of albumin associated with cadherin-variant ES cells in micropatterned co-

cultures compared to the ES cells cultured alone or random co-culture (mean green intensity = 60.15). The micropatterning showed observable levels of albumin present in cultures with CE-ES cells and lacking in cultures with CD-ES cells. It is also worth noting that while we did not detect appreciable spatial variations in albumin expression in the CE-ES cultures away from the hepatocyte interface, the cadherin blockage elicited a heterogeneous albumin expression (44.2 intensity units in the nearly 50 mm region near the interface versus 8.99 units distal to the interface).

Results from RT-PCR and intracellular albumin staining for the key culture conditions (except micropatterned co-cultures) are summarized in Table 2.8.1. Briefly, growth factor stimulation (DOH) alone is not adequate to promote significant hepatic differentiation in ES cells cultured singly, but does induce morphological differences in terms of a cobblestone appearance. Therefore, DOH treatment was utilized in all subsequent cultures to prime the ES cells. Paracrine stimulation via indirect co-culture supports hepatic differentiation in co-cultures expressing E-cadherins, whereas cadherin-deficient co-cultures do not or minimally increase differentiation, confirmed by albumin protein expression. Direct co-cultures, capturing juxtacrine and paracrine signaling, moderately increase differentiation through albumin protein expression in cadherin-deficient cultures and significantly increase differentiation measured in terms of G6P gene expression and albumin protein expression in E-cadherin-expressing environments. Combined, these results show that E-cadherin

engagement is sufficient to promote hepatic differentiation when ES cells are optimally primed and co-cultured with differentiated hepatocytes.

2.5 Discussion

The goal of this study was to examine whether hepatocytes can promote hepatospecific differentiation in ES cells and identify the regulatory factors underlying these interactions (ES cell cadherin expression; mode of hepatotrophic interactions in terms of indirect vs. contact stimulation). Our results indicate that enhanced hepatic differentiation of ES cells requires (a) elevated E-cadherin expression, and (b) co-cultures that enable a combination of intimate hepatocyte-ES cell contacts and stimulation from metabolically active hepatocytes.

In culturing the ES cells, a modified hanging-drop protocol [130, 140] was utilized to mimic embryogenesis and expand cell growth [102]. After sub-culture at day 18 post EB (E18), ES cells were grown for one week under growth factor cocktail treatment (DEX/OSM/HGF) to help prime the cells for organotypic co-culture or in the absence of the cocktail treatment (just C+H medium). After one week of DOH treatment, ES cells were harvested and re-plated in co-culture environments and monitored for a subsequent week (2 weeks E18). CE-ES cell morphology maintained a cuboidal, cobblestone appearance which was indicative of fetal hepatic phenotype [141-143]; however, in the presence of co-culture systems, the degree of hepatic morphology was enhanced: cobblestone, compact polyhedral cells with round nuclei, well demarcated cell-cell borders and the appearance of bile canalicular networks [34]. The effect of priming the cells with DOH and C+H media did not significantly affect cell morphology in

CE-ES cells. In CD-ES cells, cell morphology was also not affected by different media treatment or by co-culture presence. CD-ES cells maintained a spread, elongated fibroblast-like appearance, which persisted throughout conditioning (cocktail/no cocktail) and co-culture condition (indirect/direct).

In probing subsequent genetic expression of ES cells, the effect of no-co-culture versus co-culture (indirect and direct) was compared between both CE-ES and CD-ES cells. Interestingly, priming CE-ES cells with DEX/OSM/HGF (DOH) was able to compensate for the effect arising from indirect co-culture treatment. There was no significant difference in gene expression (AFP/G6P) between these two conditions; however, expression of the late hepatic marker (G6P) was significantly ($P<0.05$) different when comparing indirect co-culture to the no priming condition (C+H media that primary hepatocytes are normally cultured in). Possibly the lack of priming makes the CE-ES cells less responsive, while priming ES cells compensates, to some extent, for the cues that hepatocytes secrete. Furthermore, CE-ES cells in direct co-cultures exhibited elevated expression of G6P ($P<0.05$) compared to ES cells alone (no-co-cultures). This phenomenon was further proved with the addition of E-cadherin blocking antibodies in random co-culture conditions.

To confirm that E-cadherin specifically enhanced ES cells in direct co-culture with hepatocytes, we examined the effects of blocking E-cadherin antibody on ES function through RT-PCR. The addition of a monoclonal antibody against ECCD-1 functionally blocked E-cadherin based adhesion

between ES cells and hepatocytes, resulting in diminished levels of G6P when compared to untreated random co-culture. The addition of an isotype control antibody did not have any effect on the functional enhancement. Hence, there seems to be some contact-driven cues at the heterotypic interface level that enhances hepatic differentiation/maturation. However, CD-ES cells showed no significant effect from growth factor priming or in co-culture conditions. Previous studies have all highlighted the importance of direct cell-cell contact in driving differentiated phenotype and functional cell abilities [38, 108, 144-147].

In particular, Bhatia et al (1998) [144] observed through spatially patterned co-cultures of hepatocyte and fibroblasts that a) the heterotypic interface between hepatocyte and mesenchymal cells correlates with high levels of differentiation; and b) for equivalent extent of heterotypic interfaces, differentiation increases with the number of fibroblasts, suggesting that fibroblast homotypic signaling may have a feedback on the hepatocyte environment. Furthermore, with the presence of heterotypic cell-cell contacts being established through E-cadherin engagement, other functional junction contacts may be ameliorated and/or stabilized. Studies have shown that E-cadherin is not only necessary for adherens junction formation but its adhesive activity is also critical for the assembly of other junctional complexes such as desmosomes, gap junctions and tight junctions [148, 149]. In particular, tight junction [150, 151] and gap junction [152] interactions may be augmented through the successful cell-cell E-cadherin engagement and stabilization. Tight junction (ZO-1) and gap junction

(connexins) assembly ensues with the correct establishment of cell polarity and with proper enrichment of several protein complexes at these cell junctions essential for polarity. This indicates there is an intimate relationship between junction formation and polarity [150, 153] that can effectively occur through suitable cell-cell engagement, which is mediated through E-cadherins.

The intracellular albumin localization trends of micropatterned hepatocytes and ES cells may shed some light into how the heterotypic interface may be essential in transmitting important cellular cues. The heterotypic contact may potentially be up-regulating intracellular albumin localization with CE-ES cells in direct contact with hepatocytes. Random co-cultures with hepatocytes showed moderate albumin staining, but the microstamped hepatocytes in particular increased intracellular albumin localization in CE-ES cells. Further, random co-cultures of hepatocytes with cadherin-expressing ES cells resulted in higher hepatic differentiation of ES cells compared to indirect co-cultures through paracrine-conditioned media, pointing to the role of direct cell-cell contacts between ES cells and hepatocytes.

Most of our ES cell co-cultures micropatterned with hepatocytes showed no perceptible variations in albumin localization as a function of spatial distance from the hepatocyte-ES cell interface. This indicates a more global, paracrine regulation of ES cells by hepatocytes. The microstamp-overlaid hepatocytes in our micropatterned co-cultures are metabolically somewhat more functional than monolayer cultured hepatocytes such as those we utilized in our random co-

cultures (for example, urea secretion rates; see Materials and Methods) (Berthiaume et al., 1996; Dunn et al., 1989). We believe this factor (increased paracrine and juxtacrine stimulation) may explain why the ES cells in our micropatterned co-cultures expressed greater levels of albumin (86.72 intensity units) than in random co-cultures (60.15 intensity units). It is likely that the combined paracrine and juxtacrine ES cell stimulation by such metabolically active hepatocytes may be masking the role of contact-regulation and reinforcing the global regulation of ES cell differentiation. Indeed in one condition where cadherin antibodies were incorporated into the micropatterned co-cultures, we observed the first instance of spatial heterogeneity of differentiation responsiveness. Following cadherin blockage, albumin localization in ES cells distal to the hepatocyte interface was inhibited significantly but not that in ES cells immediately adjacent to the hepatocytes, reminiscent of heterotypic spatial variation within co-cultures [34]. This points to the role of cadherin blockage on the inhibition of primarily the homotypic juxtacrine signaling among the mouse ES cells, and secondarily, cadherin-mediated juxtacrine heterotypic contacts between hepatocytes and ES cells. The antibody blockage experiments do not directly block cadherin-triggered paracrine signaling from the rat hepatocytes (as well as cadherin-mediated homotypic signaling in hepatocytes). The unblocked co-culture controls allows for E-cadherin-mediated heterotypic interactions and paracrine signaling emanating from the hepatocytes coupled with the homotypic E-cadherin-mediated interaction between mouse ES cells.

In conclusion, we report that hepatocyte co-cultures with murine embryonic stem cells promote the hepatospecific differentiation of stem cells. Three key factors promote this effect: hepatotrophic priming of stem cells; cadherin expression on stem cells; and microscale contact/proximity to hepatocytes. Identification of the molecular nature of hepatocyte-derived priming of ES cells remains a subject for future investigation. Insights from this study could be relevant to design improved hepatotrophic culture models to differentiate ES cells and yield strategies for integration of transplanted ES cells within the liver milieu for in situ stem cell based tissue engineering.

2.6 Acknowledgements

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2.7 Figures and Captions

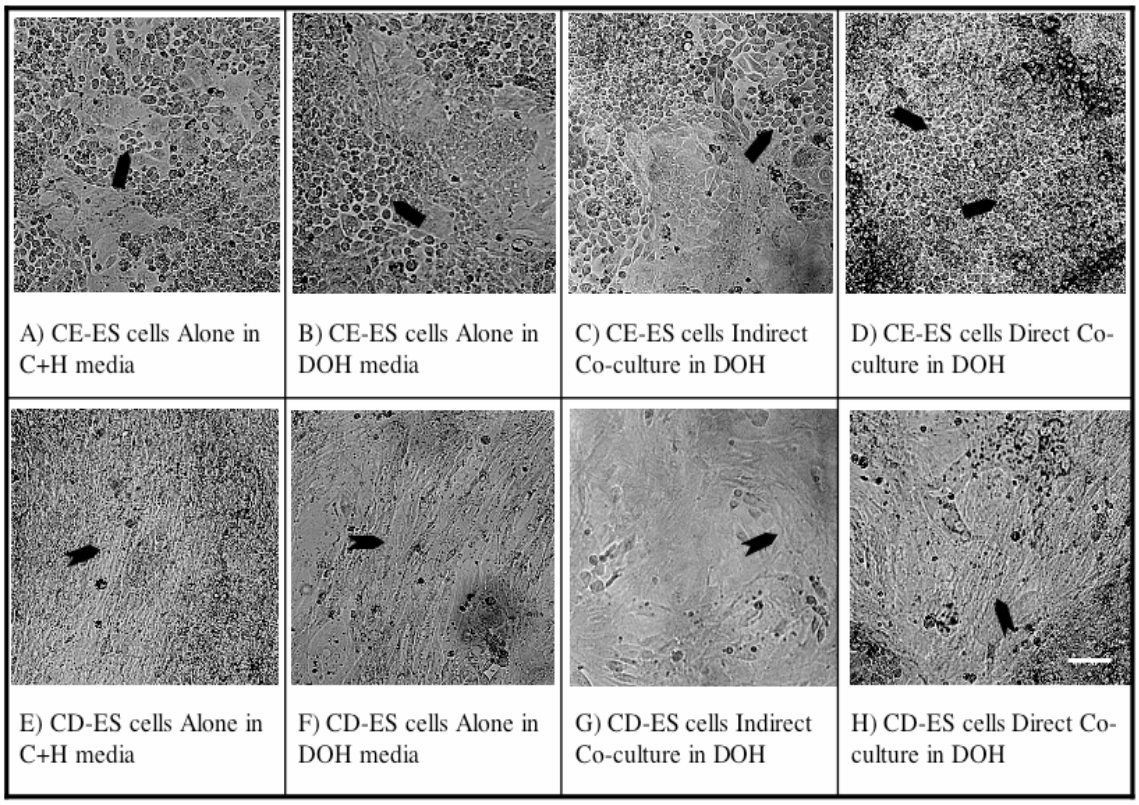

Figure 2.8.1: Morphology of Differential Cadherin Variant ES Cells in Basal and DOH Supplemented Media: Effect of Co-Culture Configuration

ES cells are cultured alone and within co-culture environments (indirect and direct) with primary adult hepatocytes at day 15. A-D) CE-ES cells, E-H) CD-ES cells. Scale bar = 100 μm. Magnification 10X (zoom 2), i.e. 2x magnification of the original image. CE-ES cells appear distinctly cuboidal, cobblestone phenotypically, whereas CD-ES cells exhibit elongated, fibrotic appearances even under co-culture conditions. CE-ES cells exhibit a greater degree of cobblestone

appearance under DOH priming. CD-ES cells continue to exhibit elongated and fibrotic phenotypes.

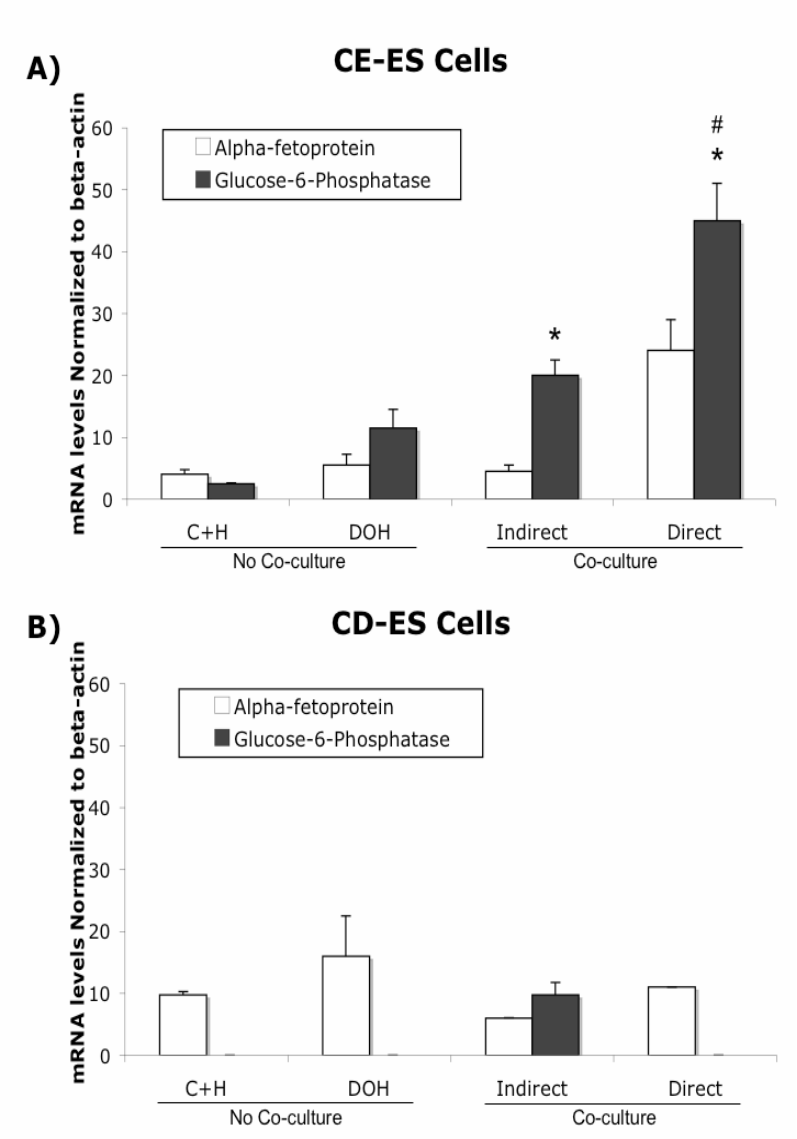

Figure 2.8.2: Expression of Hepatospecific Differentiation Markers in ES Cells: Effect of Cadherin Expression and Culture Configuration

Effect of co-culture (indirect and direct) condition on CE-ES and CD-ES cells at day 15 (post EB development plus 1 week ES priming plus 1 week in co-culture

C+H media). All cell conditions express some level of AFP indicating the presence of immature fetal liver cells. The effect of indirect and direct co-culture is significant ($P<0.05$) for G6P expression (late hepatic marker) compared to the control of CE-ES cells alone in C+H medium (no cocktail). Greater levels of G6P are present in the direct co-culture compared to the other conditions in CE-ES cells. In CD-ES cells, there seems to be no significant effect with or without the presence of co-culture. *NOTE: the asterisk denotes significance ($P<0.05$) compared to no co-culture (C+H), while # denote significance ($P<0.05$) compared to no co-culture DOH condition.*

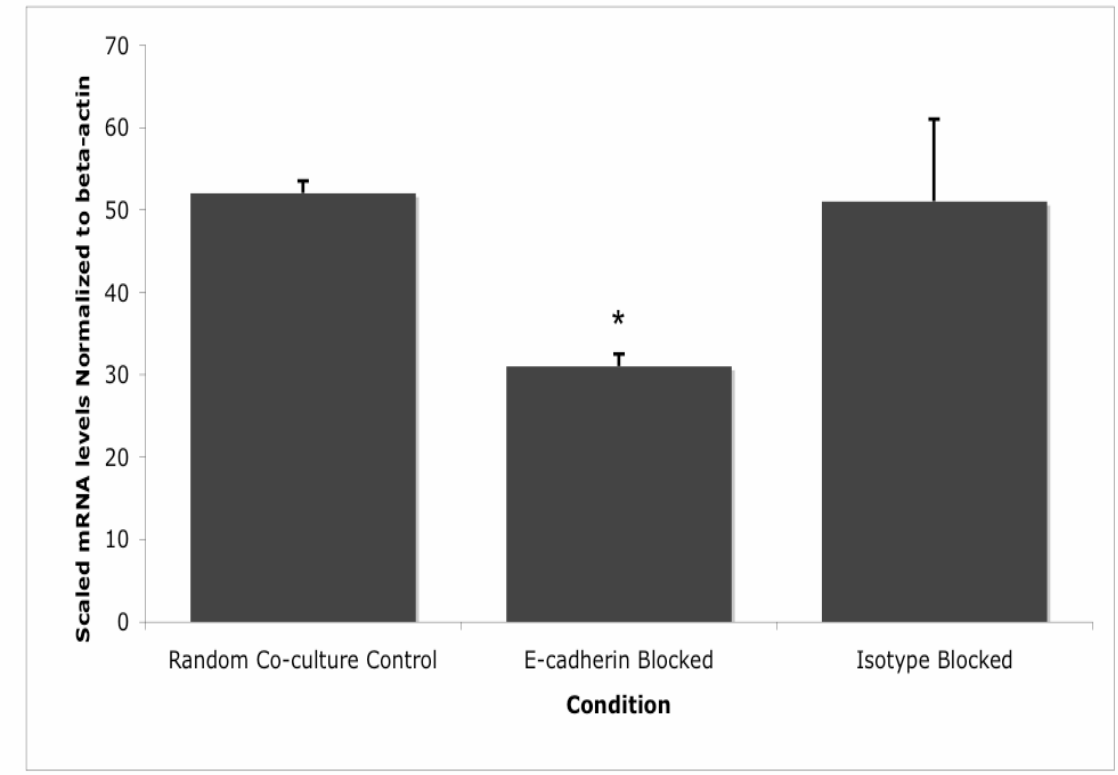

Figure 2.8.3: G6P Expression Following Antibody Blockage of E-cadherin

Effect of E-cadherin blocking in direct co-culture on CE-ES cells at day 15 (post EB development plus 1 week DOH priming plus 1 week C+H treatment with hepatocytes). The effect of ECCD-1 E-cadherin antibody blocking is significant ($P<0.05$) for G6P expression (late hepatic marker) compared to the control untreated direct co-culture. The isotype control antibody blocking is not statistically significant when compared to the random direct co-culture. *NOTE: the asterisk denotes significance ($P<0.05$) compared to random co-culture.*

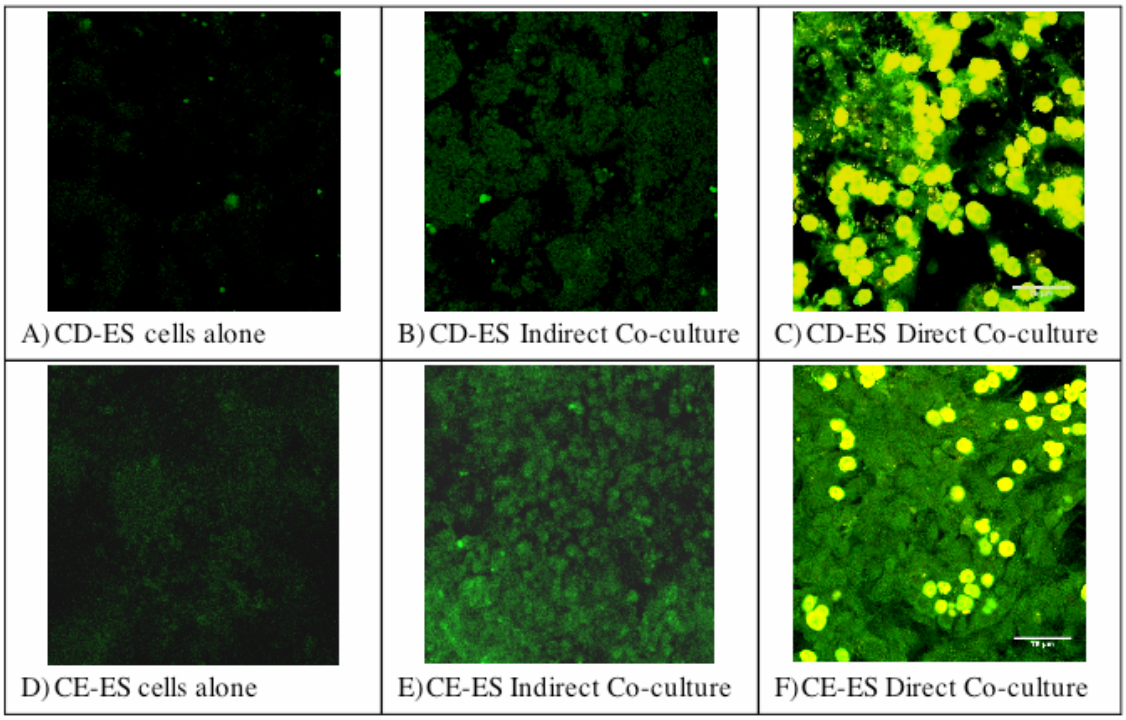

Figure 2.8.4: Intracellular Albumin Staining in Cadherin-Variant ES Cells in Single ES Cultures or Random Co-cultures with Hepatocytes

Intracellular albumin staining in cadherin-variant ES cells at day 15 (post EB development with 1 week DOH priming plus 1 week in co-culture C+H media) revealed that the degree of albumin staining increased under co-culture conditions (B-C, E-F) compared to ES cells grown alone (A, D). Furthermore, the intensity of albumin staining was most prominent under direct co-culture treatment (F) suggesting that the heterotypic interface is important in mediating cell signaling. Yellow cells in images C and F indicate hepatocytes pre-stained with cell tracker red, co-localized with albumin staining. Images were acquired under 20x, scale bar indicates 75μm.

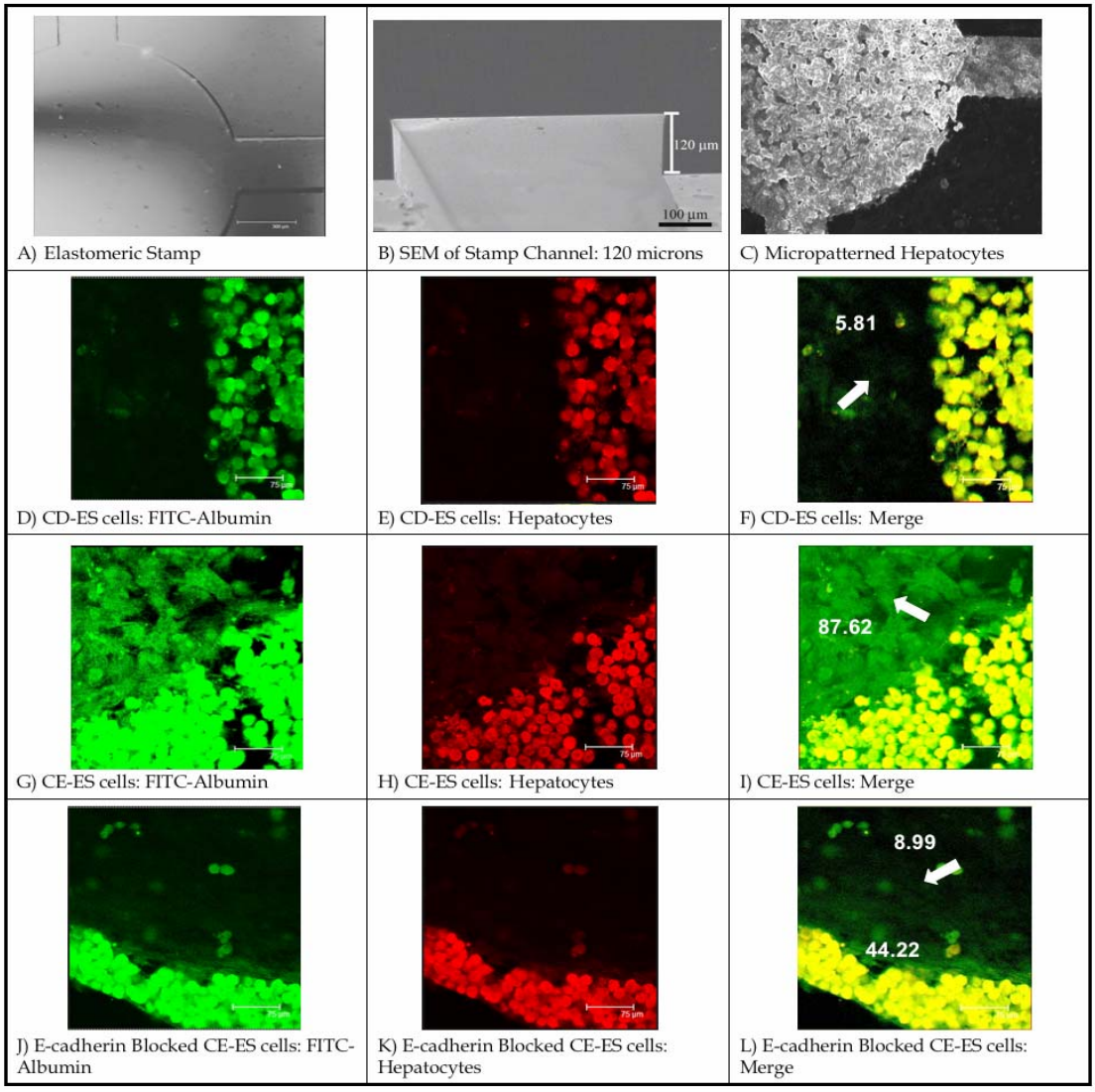

Figure 2.8.5: Micropatterned Co-cultures of ES cells with Primary Hepatocytes

Micropatterned co-cultures were established using elastomeric stamps, where ES cells (indicated with arrows) are stained with FITC-conjugated intracellular albumin and hepatocytes were pre-stained with Cell Tracker Red. A-B)

Transmitted and SEM images of elastomeric stamp. C) Image of micropatterned hepatocytes. D-L) Images of intracellular albumin staining overlayed with cell tracker red hepatocytes at day 2 of co-culture in C+H media (post EB development with 1 week DOH priming). Yellow color in merged images indicates hepatocytes, while green alone indicates mouse ES-specific albumin staining. Numbers on the images indicate mean, background-subtracted green intensity corresponding to albumin expression. Intracellular albumin staining is prominent in the co-cultures of CE-ES cells and primary rat hepatocytes (G-I). In contrast, the CD-ES cells (D-F) uniformly lack intracellular albumin staining while the E-cadherin blocked CE-ES cells (J-L) lack intracellular albumin in all regions except those near the hepatocyte interface.

	Growth Factor Stimulation		Indirect Co-culture		Direct Co-culture	
	CD-ES	CE-ES	CD-ES	CE-ES	CD-ES	CE-ES
Hepatospecific Morphology	-	+	-	++	-	+++
G6P Gene Expression	-	+	+	++	-	+++
Albumin Protein Expression	-	-	+	++	++	+++

Table 2.8.1: Summary of Hepatotrophic Stimulation of ES Cell Differentiation

The table shows a graded level of differentiation based on significance for the morphology, RT-PCR results and qualitative levels of albumin expression for immunocytochemistry. Results show that growth factor stimulation is sufficient to produce detectable levels of differentiation in cadherin expressing (CE-ES) cells. Indirect co-culture through paracrine signaling generates intermediate levels of differentiation with G6P expression and albumin protein expression in co-cultures with cadherin-expressing cells and low levels of albumin protein expression in cadherin-deficient (CD-ES) cells. The most significant levels of hepatospecific markers are seen with CE-ES cells in direct co-culture with primary hepatocytes.

CHAPTER 3

EXPEDITED GROWTH FACTOR-MEDIATED HEPATIC LINEAGE SPECIFICATION OF HUMAN EMBRYONIC STEM CELLS

3.1 Abstract

Human embryonic stem cells (hESCs) afford the potential to be a promising source of liver cells, hepatocytes, for regenerative medicine given their unlimited proliferative and pluripotent differentiative capacity. However, the inefficient embryoid body process and limited understanding of molecular signals potentiating cell-specific differentiation plague the use of hESCs as a hepatic source. In this study, we describe an efficient growth factor based process for directed differentiation of hESCs that bypasses embryoid body generation. The system involves adherent hESC culture exposure to Activin A treatment followed by incorporation of various growth factor combinations composed of dexamethasone, oncostatin M, hepatocyte growth factor, and Wnt3A. The hESC-derived hepatocyte-like cells resulting from optimal growth factor combinations exhibit characteristic hepatocyte morphology, express hepatocyte markers and possess hepatospecific functional activity. The differentiated cultures express hepatic-related genes shown by reverse transcription-polymerase chain reaction and immunofluorescence analysis revealed the co-expression of albumin/cytokeratin 18. Furthermore, the hESC-

derived hepatocyte-like cells exhibit functional hepatic characteristics, such as indocyanine green uptake and release, albumin secretion, and have inducible cytochrome P450 activity. This directed differentiation of adherent hESCs offers an efficient process to produce hepatocyte-like cells in vitro for hepatocyte differentiation studies and organotypic cultures for diagnostic and therapeutic applications.

3.2 Introduction

The field of stem cell bioengineering can potentially revolutionize cell-based therapies for functional replacement of metabolically complex tissues like that of the liver [25, 105, 154-156]. Significant challenges notwithstanding, human embryonic stem cells (hESCs) offer a promising cell source for transplantation medicine, particularly with the active efforts underway to guide embryonic stem (ES) cell development and maturation [1-3]. The ES cell model was recently demonstrated to be highly organotypic based on its successful realization of specific lineages [4], however, molecular signals that can effectively promote the integration and hepatospecific differentiation of ESCs are only now beginning to be clarified [8, 9].

The ability of healthy human liver to regenerate is steadily lost in chronic liver disease. The standard treatment for advanced liver disease, which is the eighth leading cause of adult deaths globally, has been orthotopic liver organ transplantation, but this therapy is limited by the availability of donor tissue [10, 11]. Cell transplantation of liver parenchymal cells, hepatocytes, was shown to be an effective strategy in animal models of hepatic failure and metabolic liver diseases [12, 13]; hepatocyte transplantation has also shown to be a plausible treatment in human metabolic liver disease [14, 15]. Hepatocyte engineering continues to be an active field of investigation that has brought the key issues of cell sourcing and organoid definition to the forefront of the field of tissue engineering [16, 17]. In addition to hepatocyte-based therapies, hepatocyte

cultures are widely considered as diagnostic *in vitro* models for pre-clinical functional and toxicogenomic screening of pharmaceutical drugs [18, 19]. However, the utility of hepatocytes in either clinical or pharmaceutical applications is limited by their availability, variability, as well as their limited proliferation and decline in hepatic functions upon extended *in vitro* cultures.

Embryonic stem (ES) cells afford a promising source of hepatocytes given their unlimited proliferative and pluripotent differentiative capacity [2, 22-24]. Human embryonic stem cells are derived, mechanically or immunosurgically, from the polarized inner cell mass of a preimplantation-stage blastocyst [5, 6]. Spontaneous differentiation of hES colonies rapidly occurs *in vitro* when the system lacks preventative factors, such as basic fibroblast growth factor (bFGF). In two-dimensional systems, spontaneous differentiation occurs at the outer borders of the colonies, at fusing colonies, or at the center core where cells begin to pile up [66]. A three-dimensional culture system, in the form of embryoid bodies, is another way to initiate differentiation of hES cells [67]. The appearance of all three germ layers is possible in this suspension culture system. Selecting specific cell type populations can be achieved with cell-surface markers and separation techniques, although these procedures remain difficult to scale-up and are inefficient [68]. The process of directed differentiation is defined by induction into a specific cell type, without the presence of all three germ layers and can be enhanced by endogenous transcription factor activation, transcription

factor transfection, growth factor supplements, or co-culture environments [69, 70].

Endodermal differentiation from hESCs remains a challenge due to an incomplete understanding of the differentiation process; therefore several investigators have utilized complex processes to induce hepatic-like differentiation of hESCs in both two- and three-dimension culture systems in vitro. A few successful approaches have supplemented traditional embryoid body development by growth factor treatment and the use of collagen scaffold systems [157] or were enriched by reporter gene purification by a hepatocyte-specific promoter to differentiate hESCs [83]. Treatments incorporating dimethyl sulfoxide and sodium butyrate have induced hepatocyte markers in two-dimensional cultures of hESCs [84]. Bharvand et al have proposed an interesting 28-day method that utilizes a multi-step protocol of growth factors to mediate hepatocyte-like differentiation without the use of embryoid bodies or serum [158]. Similarly, Cai et al created hepatic cells from undifferentiated hESCs through a 18-day three-stage method in serum free medium [159]. Activin A, sodium butyrate, dimethyl sulfoxide and oncostatin M were utilized to induce a differentiation process that takes up to three weeks to differentiate hESCs into hepatocyte-like cells [160]. Various groups have used a plethora of growth factors and extracellular matrixes to induce hESC hepatic differentiation [161, 162]. Each of these systems takes several days or weeks to differentiate the

hESCs, but the challenge still remains to create a direct and efficient process for directed hepatic differentiation.

Largely inspired by the *in vivo* progression of hepatotrophic stimuli during development [44, 45], there have been extensive studies of molecular growth factors that can interact with developing liver cells, fetal liver cells, and more recently, embryonic stem cells [3, 46-48, 84, 163, 164]. A complex sequence of hepatotrophic factors has been implicated in the process of liver development [49]. The interaction between the endoderm and cardiac-mesoderm is crucial for hepatic development [50]. This process results in the expression of two major liver-specific markers alpha-fetoprotein (AFP) and albumin (ALB), which can be detected as early as embryonic day 8-9 (E8-E9). Hepatic cells at this point can differentiate into parenchymal hepatocytes or bile duct epithelial cells. At E10-11, hematopoietic stem cells originating from the extrahepatic organ colonize the fetal liver region and proliferate: hepatic progenitors participate in creating a conducive hematopoietic microenvironment [51]; in parallel, hematopoietic cells produce cytokines, especially oncostatin M (OSM), [52] while mesenchymal cells (non-parenchymal liver cells) produce the hepatocyte growth factor (HGF) [53], a mitogen and morphogen, one of the key signals for liver development and regeneration [55]. The cooperative nature of signals required for hepatic maturation is best exemplified by the role played by growth factors secreted from hematopoietic cells. The most significant of these include Dexamethasone (DEX) and Oncostatin M (OSM) [27, 52, 53, 62]. DEX is known to suppress AFP

production (early hepatic marker) and DNA synthesis, while up-regulating albumin (mid-hepatic marker) and alpha-1-antitrypsin, AAT (late hepatic marker) production. OSM, an interleukin (IL) 6 family cytokine, has been shown to up-regulate AAT and HNF-4. The synergistic action of DEX and OSM has found to specifically induce hepatic maturation in fetal liver E14.5 [52, 53] through induction of morphological changes and up-regulation of multiple liver-specific functions [62, 65]. Activin A reacts through the activin/nodal signaling pathway and has been shown to induce definite endoderm differentiation [165]. The Wnt/beta-catenin signaling cascade is involved in the hepatic differentiation pathway and has been shown to induce hepatic endoderm by Wnt3A treatment [166].

Although there is significant promise in the potential use of the above maturation factors for ES cell differentiation, the exclusive application of these factors leads to a differentiation process that is inefficient and uncontrolled, thus, an integrated and phased application of these factors based on the complex cross-functional signaling pathways may be necessary. The central premise of this study is that optimal exogenous growth factor presentation to the adherent hESCs during development can be used to controllably expedite the process of directed differentiation into the hepatic lineage, bypassing the use of poorly defined embryoid body development (see Figure 1).

3.3 Materials and Methods

3.3.1 Propagation of Undifferentiated hESCs

Human embryonic stem cells (H1) were obtained from WiCell Research Institute (Madison, WI) and maintained following WiCell protocols. Briefly, cells were cultivated in gelatin-coated, six-well plates in complete media consisting of DMEM-F12 supplemented with 20% KnockOut Serum Replacer, 200 mM L-glutamine, beta-mercaptoethanol, non-essential amino acids, 4 ng/ml basic fibroblast growth factor (bFGF) and 0.12 ng/ml transforming growth factor-beta 1 (TGF-β1). Medium was changed daily. After collagenase IV treatment for 5-10 min at 37°C followed by mechanical dissociation, colonies were passaged weekly at a ratio of 1:6 on mitotically inactivated (x-irradiated, 6500 rad) mouse embryonic fibroblasts (MEFs).

3.3.2 Initiation of hESC Differentiation

Directed differentiation was accomplished by culturing the hESCs on MEFs in the presence of various growth factors over a two-week period. Human ES cells in complete media were allowed to attach overnight on the MEFs and subsequently cultured in basal media with various growth factor treatments (see Figure 1). Basal media consists of complete media without the addition of b-FGF and TGF-β. Growth factor combinations consisted of permutations of up to five components added to the basal media: dexamathasone, DEX (10^{-7} M); oncostatin M, OSM (10 ng/ml); hepatocyte growth factor, HGF (20 ng/mL); (Sigma,

Atlanta, GA), Activin A (50 ng/mL), Wnt3A (100 ng/mL) (R&D Systems, Minneapolis, MN). Colonies were allowed to grow for 1 or 2 weeks in culture with media changed daily. As a comparison culture condition (control), embryoid bodies were formed by placing dissociated colonies in an ultra-low attachment plate in the presence of basal media for 2 weeks with media changed every 2 days. Figure 1 provides an illustration of the differentiation process.

3.3.3 Immunocytochemistry of Hepatocyte-like Cells

After 1 and 2 weeks in culture with various growth factor combinations, hESCs were stained for hepatospecific markers to determine the extent of differentiation. Culture conditions were fixed with 4% paraformaldehyde in Dulbecco's phosphate buffered saline (DPBS) with Ca^{2+} and Mg^{2+} for 15 minutes at room temperature. Cells were then rapidly washed three times with DPBS and permeabilized with SAP buffer (0.5% Saponin, 1% BSA, 0.1% Sodium Azide) (Sigma, St. Louis, MO) for 30 minutes at room temperature. After washing once with DPBS, cells were blocked with 3% (w/v) bovine albumin serum (BSA) for 30 minutes at room temperature to reduce nonspecific antibody binding. Subsequently, primary antibodies for hepatic identification were incubated overnight at 4°C with the addition of stage-specific embryonic antigen-4 (SSEA-4) as a counter-stain for pluripotency. The primary antibodies, diluted in SAP buffer are as follows: alpha-fetoprotein (AFP) IgG1, 1:200 dilution (Abcam, Cambridge, MA); albumin (ALB) IgG2b, 2.5 μg/mL (Abcam); cytokeratin 18

(CK18) IgG1, 1:800 dilution (Abcam); SSEA-4 IgG3, 1:200 dilution (Millipore, Chicago, IL). Cells were washed three times with DPBS and incubated with isotype matched secondary antibodies, 1:1000 dilution in SAP buffer for 1 hour at room temperature. The secondary antibodies are as follows: AlexaFluor 488 IgG1, AlexaFluor 488 IgG2b, AlexaFluor 594 IgG3 (Invitrogen, Carlsbad, CA). Finally, the cells were washed four times in DPBS and visualized using the Leica TCS.SP2 confocal microscope system (Leica Microscope, Exton, PA).

3.3.4 RNA Extraction and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was extracted from the cell pellets using the RNEasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The high capacity cDNA reverse transcription kit and random primers (Applied Biosystems, Foster City, CA) were used to reverse transcribe total RNA to single stranded cDNA for real-time PCR. Various genes were analyzed to determine hepatospecific markers including alpha-fetoprotein (AFP), albumin (ALB), forkhead box A2 (FOXA2), transthyretin (TTR), hepatocyte nuclear factor-4 (HNF-4), alpha-1-antitrypsin (AAT), cytokeratin 18 (CK18), and beta-actin (SuperArray, Frederick, MD). Quantitative real-time PCR was performed using the SYBR Green PCR Master Mix (Qiagen) and the LightCycler System (Roche, Indianapolis, IN). cDNA samples, 1 μL in a 10 μL volume reaction, were analyzed for the gene of interest and for the housekeeping gene, beta-actin. Each

sample was run in duplicate. Statistical analysis was completed by single variable ANOVA followed by multiple comparison testing.

3.3.5 Human Albumin Secretion by Differentiated hESCs

An ELISA Quantitation kit (Bethyl Laboratories, Montgomery, TX) was used to measure human albumin levels secreted in culture medium. Samples from three separate cultures were analyzed in duplicate for each condition. ELISA plates were read at 450 nm and the resulting data analyzed with a four-parameter curve fitting computer program (KaleidaGraph). The absorbance of the basal medium was subtracted from each sample absorbance, and albumin concentration was determined from the standard curve. The final absorbance of the test samples was obtained after subtracting the zero absorbance and used to determine albumin concentrations secreted into the media. Statistical analysis was completed by ANOVA single factor analysis.

3.3.6 EROD Assay

To determine the metabolic activity of the hESC-derived hepatic-like cells, an ethoxyresorufin O-de-ethylase (EROD) assay was performed. Cytochrome P450 enzymes metabolize xenobiotic compounds with which they come in contact. After culturing the hESCs in high performance medias for 2 weeks, the EROD assay assessed the CYP1A2 activity. Briefly, cells were induced with 5 μM methylchloranthrene (Sigma) in respective medias and cultured for 24 hours.

Cells were washed with PBS and cultured with 5 μM ethoxyresorufin (Sigma) for 2 hours to initiate the EROD assay. The reaction was stopped with 20 mM NaOH and the supernatant was collected and measured on a fluorescence plate reader at 355 nm excitation and 581 nm emission. A standard curve of pure resorufin was used to calculate the activity of CYP1A2 in picomoles of resorufin formed per minute per million cells.

3.3.7 Cellular Uptake and Release of Indocyanine Green

Indocyanine green (ICG) is an inorganic anion that is used to evaluate liver function because it is non-toxic and eliminated exclusively by hepatocytes [167]. Briefly, hESC-derived hepatic-like cells were cultured for 2 weeks in high performance medias and incubated with diluted ICG (Sigma) in media (1 mg/mL) for 30 minutes at 37°C. Cultures were washed and imaged with transmitted light to determine the amount of ICG uptake, indicated by green stain. Cells were cultured for an additional 6 hours in culture media, washed and imaged again to document release of cellular ICG stain.

3.3.8 Fluorescence Activated Cell Sorting Analysis

To determine the percent population positive for albumin and CK18, cells were processed and analyzed by fluorescent-activated cell sorting (FACS) (Becton Dickinson, Franklin Lakes, NJ). The highest performance media (A_4DOH) was grown for up to 4 weeks and analyzed every other week for

albumin and CK18 expression. Briefly, single cell suspensions were used with fluorescent conjugated antibodies against human albumin and cytokeratin 18. Cells were fixed with 1% paraformaldehyde (15 min, on ice), washed twice, permeabilized with SAP buffer (30 min, on ice), washed twice, blocked with 3% BSA (30 min, on ice) and washed again twice. Cell identification was confirmed by staining with primary antibodies against human albumin (R&D Systems), 0.5 μg/ml, and human cytokeratin 18 (Millipore), dilution 1:100, for 1 hour on ice. Cells were washed twice and labeled with AlexaFluor goat anti mouse IgG (H+L) 488 (Invitrogen) for 1 hour on ice, 1:1000 dilution. Finally, cells were washed three times with PBS and analyzed with CellQuest software (BD Biosciences). MEFs alone were run with each experiment as a negative control.

3.4 Results

3.4.1 Early Hepatic-like Differentiation in Growth Factor Supplemented Cocktail

Previous studies in our laboratory have shown that the optimal growth factor cocktail for mouse ES hepatocyte-like differentiation is composed of the combination of DEX, OSM and HGF (DOH) [48]. Therefore, we examined the effects of DOH on the differentiative ability in adherent hESCs. As shown in Figure 3.7.2, there was pronounced AFP expression after one week in culture on MEFs in the presence of DOH media, whereas the H1 colony in complete media lacking DOH supplementation only had SSEA-4 staining, indicating proliferative abilities. After two weeks in culture, AFP was still present, though not as prominent, and had achieved cells with a larger cytoplasm to nuclear ratio. Our data indicates that early hepatic-like differentiation is achieved when the adherent cultures are presented with DOH supplementation. This suggests that DOH is an effective growth factor treatment for early differentiation of hESCs.

3.4.2 Time course of Genetic Expression of Hepatospecific Maturation Markers in hESCs

Additional media supplementations were added to the DOH treatment in an effort to further differentiate the hESCs in adherent cultures. Activin A and Wnt3A were added in various combinations to DOH treatment and evaluated for hepatic differentiation over a 2 week time course. An A (Activin A, ActA) placed

after DOH indicated presence of Activin A throughout culture while an A placed before DOH denotes *a priori* Activin A treatment for 4 days, removal, followed by culture in the presence of the remaining growth factors. Real time RT-PCR for an early (AFP) and mid (ALB) hepatic marker was normalized to beta-actin mRNA levels and evaluated for levels compared to basal media (Figure 3.7.3). AFP levels were higher after 1 week compared to 2 weeks indicating an increase in differentiation associated with AFP levels diminishing. Albumin levels were enhanced after 2 weeks in culture compared to 1 week indicating increased differentiation in all growth factor combinations. For each data set, the treatments that started with Activin A treatment ($A_{d0\text{-}4}$DOH and $A_{d0\text{-}4}$DOHW) showed high levels of differentiation compared to basal media.

3.4.3 hESC-Derived Hepatic Cells Exhibit Hepatocyte-Like Functions

Each growth factor combination from Figure 3.7.3 was evaluated for functional activity by albumin secretion for up to 2 weeks in adherent culture (Figure 3.7.4A). Albumin production continuously increased and reached maximal values at day 14 with the exogenous growth factor supplementation, $A_{d0\text{-}4}$DOH, resulting in the highest level of albumin secretion. Statistically significant albumin production, compared to basal media for the same day, was achieved by high performance medias, DOH, $A_{d0\text{-}4}$DOHW and $A_{d0\text{-}4}$DOH, and was further investigated for late hepatic markers. These levels are on the same

order of magnitude as fetal human hepatocytes [168], indicating that efficient hepatic differentiation is being achieved after only 14 days in adherent culture.

Hepatic metabolism was evaluated using an ethoxyresorufin O-de-ethylase (EROD) assay (Figure 3.7.4B). The hESC-derived hepatocyte-like cells were evaluated for inducible CYP1A2 activity since cytochrome P450 isoenzymes expression is indicative of hepatocellular function [169]. The data illustrates that CYP1A2 activity was elevated in all high performance medias (DOH, $A_{d0\text{-}4}$DOH and $A_{d0\text{-}4}$DOHW) and barely detectable in the control, complete media indicating that hESC-derived cells have the functional features of hepatocytes.

To confirm liver-like metabolic function, ICG uptake and release was evaluated for basal and high performance medias after 2 weeks in culture (Figure 3.7.4C). Uptake and release of ICG may me used to identify hepatocytes in ES cell differentiation models [167]. The images show ICG uptake and release 6 hours later in DOH, $A_{d0\text{-}4}$DOH and $A_{d0\text{-}4}$DOHW cultures. In contrast, the basal media did not have the capacity to take up ICG and release it later. The most pronounced uptake was achieved by DOH and $A_{d0\text{-}4}$DOH cultures indicating that hESC-derived hepatic cells have the metabolic function to rapidly uptake and excrete ICG.

3.4.4 Expression of Hepatocyte Markers in hESC-differentiated Cells

To evaluate the hepatic differentiation of hESCs in the high performance medias, hESC-differentiated cells were co-stained for hepatic markers, albumin

and cytokeratin 18, for differentiation (Figure 3.7.5A). The control, complete and basal medias, resulted in negative ALB/CK18 staining, but DOH, $A_{d0\text{-}4}$DOH and $A_{d0\text{-}4}$DOHW resulted in elevated levels of ALB/CK18. The highest level of ALB/CK18 was achieved in $A_{d0\text{-}4}$DOH media supplementation and displayed distinctively differentiated morphology, such as the emergence of binucleated cells and characteristic cuboidal phenotype.

To determine the percent of cells expressing albumin and cytokeratin 18 throughout 4 weeks of culture, flow cytometry was utilized (Figure 3.7.5B). Undifferentiated cells expressed no albumin and CK18 expression. At week 2, albumin (7.02%) begins to rise while CK18 (48.38%) is significantly upregulated. For long-term, terminal differentiation culture, at 4 weeks, 72.8% of cells are albumin positive and 72.9% are cytokeratin 18 positive. Therefore, $A_{d0\text{-}4}$DOH media supplementation is an efficient protocol to induce hepatic expression on a large population of cells.

3.4.5 Endoderm and Hepatic Specific Gene Expression of High Performers

The mRNA expression of endodermal and hepatocyte-specific genes, such as forkhead box A2 (FOXA2), transthyretin (TTR), alpha-1-antitrypsin (AAT), hepatocyte nuclear factor-4 (HNF-4) and cytokeratin 18 (CK18) was observed in the DOH, $A_{d0\text{-}4}$DOH and $A_{d0\text{-}4}$DOHW cultures during differentiation (Figure 3.7.6). Complete media showed down-regulation of each marker, indicating suppression of hepatic differentiation. Additionally, as a comparison for

traditional differentiation methods, the basal endodermal differentiation during the formation of embryoid bodies (EB) was evaluated for expression of each hepatic marker. FOXA2 and TTR were up-regulated, indicating endodermal differentiation, but were down-regulated for the hepatocyte-specific genes, AAT, HNF-4 and CK18. These results indicate that the high performance medias induce hepatic differentiation of hESCs in comparison to basal EB development, which produces limited endoderm differentiation in the absence of supplementation with exogenous growth factors.

3.5 Discussion

Methods to differentiate hESCs into hepatocytes must be direct, rapid and offer high yields, in order to be widely useful for basic research and therapeutic applications. Here we describe an efficient protocol for exogenous growth factor stimulation to induce hepatocyte-like cells from hESCs without the use of embryoid bodies. Priming hESCs with Activin A to induce endodermal differentiation, followed by treatment with select growth factors that recapitulate hepatogenesis *in vivo* result in cells with characteristic hepatocyte morphology, express hepatocyte markers and possess hepatospecific functional activity.

Pluripotent hESCs are preserved under very strict lab practices, usually in culture with embryonic or adult, somatic cells that secrete products to maintain pluripotency. Traditionally, hESCs are co-cultured with mitotically inactivated murine embryonic fibroblasts (MEFs) to form two-dimensional colonies that must be maintained on a weekly basis and supplemented with basic fibroblast growth factor (bFGF) and TGF-β to help maintain self-renewal [7]. When these factors are removed from the culture and maintained under appropriate conditions, hESCs can differentiate into the three embryonic germ layers. To induce differentiation, researchers have taken advantage of embryoid body development to mimic the environment of the peri-implantation embryo to force lineage restriction [170]. Traditional hepatic terminal differentiation results from progenitor cells isolated from the endoderm followed by exposure to the proper molecular cues. This entire process may take up to 28 days to derive EBs,

subculture the endoderm and culture with growth factors [157]. If the complex embryoid body process is bypassed, suitable growth factors will be necessary to recapitulate cues for differentiation into hepatocyte-like cells.

In our study, growth factor components for media supplementation were selected based on their published abilities to induce hepatic differentiation in embryonic stem cells. The initial growth factor selection for hepatic differentiation of hESCs stemmed from our previous work with mouse ES cells, concluding that the synergistic effects of dexamethasone, oncostatin M and hepatocyte growth factor (DOH) were optimal conditions for differentiation [48]. Dexamethasone is a synthetic glucocorticoid involved in liver gluconeogenesis [171], oncostatin M induces fetal hepatocyte maturation through the gp130 signal transducer [172], and hepatocyte growth factor supports hepatogenesis by activating the c-Jun pathways [173]. Our data suggests that the DOH combination is sufficient to induce a hepatic response from hESCs within a very short period of time. We then chose to investigate the addition of additional growth factors, Activin A and Wnt3A, to further direct differentiation associated with DOH stimulation.

Activin A has been previously shown to restrict visceral endoderm [174], induce definitive endoderm differentiation from hESCs [175-178], and was therefore assessed for incorporation with DOH treatments. Pre-treatment with Activin A for 4 days ($A_{d0\text{-}4}$DOH, $A_{d0\text{-}4}$DOHW) was compared to constant Activin A throughout the experiment (DOHA, DOHAW). Molecular analysis showed

that the effects of basal DOH treatment could be improved upon with the addition of Activin A pre-treatment, indicating that the hESCs are differentiating through a loss in AFP expression and that Activin A induces the highest levels of early hepatic differentiation. This result is consistent with previous observations of hESC liver development in which the initial Activin A treatment is an efficient signal to induce definitive endoderm differentiation. Additionally, Wnt3a was investigated for incorporation with DOH because of its known ability to induce hepatic endoderm [166]. The synergistic effect of priming the hESCs with Activin A and supplementing with DOH or DOHW induced significant hepatic differentiation compared to any single component alone (data not shown) or basal media controls. The addition of Wnt3a to DOH or ADOH provides a system that lacks preventative factors, such as bFGF, TGF-β, to support differentiation [179].

To further determine the highest inducing growth factor combinations, functional activity (albumin secretion, cellular uptake and release of indocyanine green, and inducible cytochrome P450 activity) of the hESCs was analyzed. Consistent data from several approaches to quantify hepatic differentiation concluded that DOH, $A_{d0\text{-}4}$DOH and $A_{d0\text{-}4}$DOHW treatments were deemed the highest performing combinations and were therefore further evaluated for their ability to induce differentiation. The high performance cocktails (DOH, $A_{d0\text{-}4}$DOH and $A_{d0\text{-}4}$DOHW) were characterized for their albumin (ALB) and cytokeratin 18 (CK18) staining and expresion profiles. Albumin is a classic

hepatic marker and often used in conjunction with CK18 to confirm co-expression. CK18 is perhaps the most commonly found members of the intermediate filament gene family and is indicative of hepatocyte morphology. All high performance medias showed ALB/CK18 staining, but the most striking staining associated with characteristic cuboidal morphology and the presence of double nuclei was found in the $A_{d0\text{-}4}$DOH condition, indicative of hepatic differentiation. Flow cytometric analysis of undifferentiated and $A_{d0\text{-}4}$DOH derived hESCs showed that in the presence of high performance growth factors, over 70% of the cell population is ALB and CK18 positive after 4 weeks in terminal differentiation culture. This data reveals that the directed differentiation process largely yields cells that are hepatic in nature.

To determine whether most differentiated cells with morphological and functional characteristics also have genetic profiles of hepatocyte-like cells, the hESC-derived cells were evaluated for the gene expression profiles for hepatocyte-associated markers. These hepatocyte nuclear factors are transcriptional activators for liver-specific transcripts such as albumin and transthyretin. All high performance groups and EBs have higher expression of the transcription factor, FOXA2, found in the earliest stages of definitive endoderm, compared to complete media illustrating that the EB process can induce endoderm differentiation similar to DOH, $A_{d0\text{-}4}$DOH and $A_{d0\text{-}4}$DOHW. Similar to FOXA2, transthyretin (TTR), or prealbumin, was found to be upregulated in all high performance medias and the EB condition. Further

analysis of mid-late hepatic markers provides evidence that EB development alone cannot achieve hepatic differentiation on the levels associated with the high performance medias. Mid and late hepatic markers were evaluated and showed that alpha-1-antitrypsin (AAT) was highly upregulated in the Activin A pre-treatment conditions while hepatocyte nuclear factor-4 (HNF-4) and cytokeratin 18 (CK18) were upregulated in all high performance medias, indicating that definitive endoderm restricted cells can achieve hepatocyte-like gene expression profiles with the selected growth factor regime. The insights gained from the gene expression profiles demonstrate that the high performance medias are able to up-regulate mid and late hepatic markers, whereas embryoid body differentiation is only able to induce endoderm-associated genes, supporting the idea that EB lineage restriction is an unregulated and indirect differentiation process. For this reason, growth factor supplementation is essential if attempting to direct hepatic differentiation from cells isolated from EBs [3, 83, 84, 157, 164].

In conclusion, our findings provide a simple system for further investigation into the differentiation pathways associated with hepatic lineage restriction. Here we describe an efficient process for directed differentiation of hESCs without the use of embryoid body development combined with the addition of select exogenous growth factors. Based on the emerging molecular biology of hESCs, the use of molecular growth factors in conjunction with gene-targeting approaches (for example, siRNA) may be other avenues to further

differentiate hESCs. Future studies to expand three-dimensional hESCs may also incorporate the use of three-dimensional scaffolds that can combine selected growth factor supplementation within a controlled microenvironment for scaling up cell numbers for clinical research applications.

3.6 Acknowledgements

The authors would like to thank National Science Foundation DGE 0333196 (IGERT on Biointerfaces); Rutgers University Stem Cell Core; Rutgers University Academic Excellence Fund; and The New Jersey Commission on Science and Technology (NJCST) Stem Cell Research Grant for supporting this research.

3.7 Figures and Captions

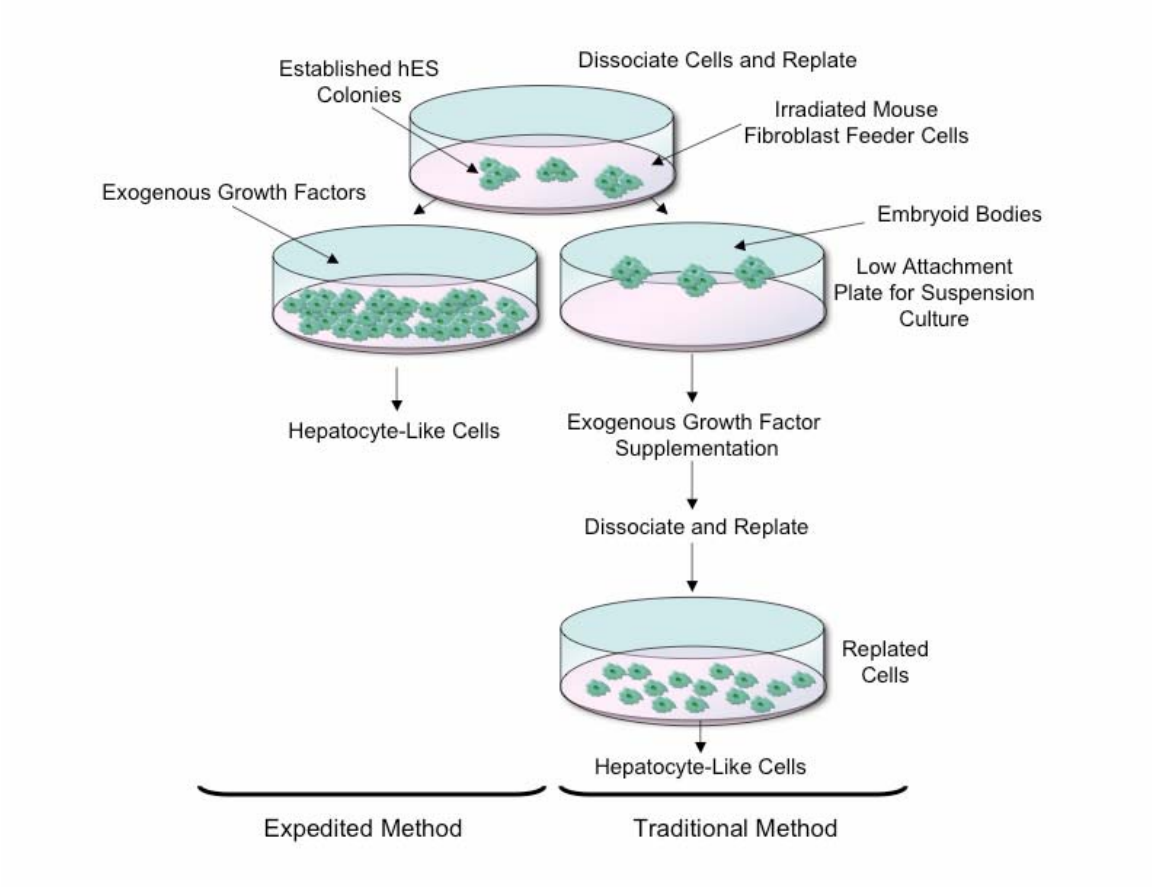

Figure 3.7.1: Schematic illustrating human embryonic stem cell (hESC) culturing and the steps initiating differentiation

Undifferentiated hESCs were cultured on mitotically inactivated mouse embryonic fibroblasts. Following weekly passaging, differentiation is initiated by the traditional method of embryoid body development or the expedited process of growth factor supplementation in adherent culture.

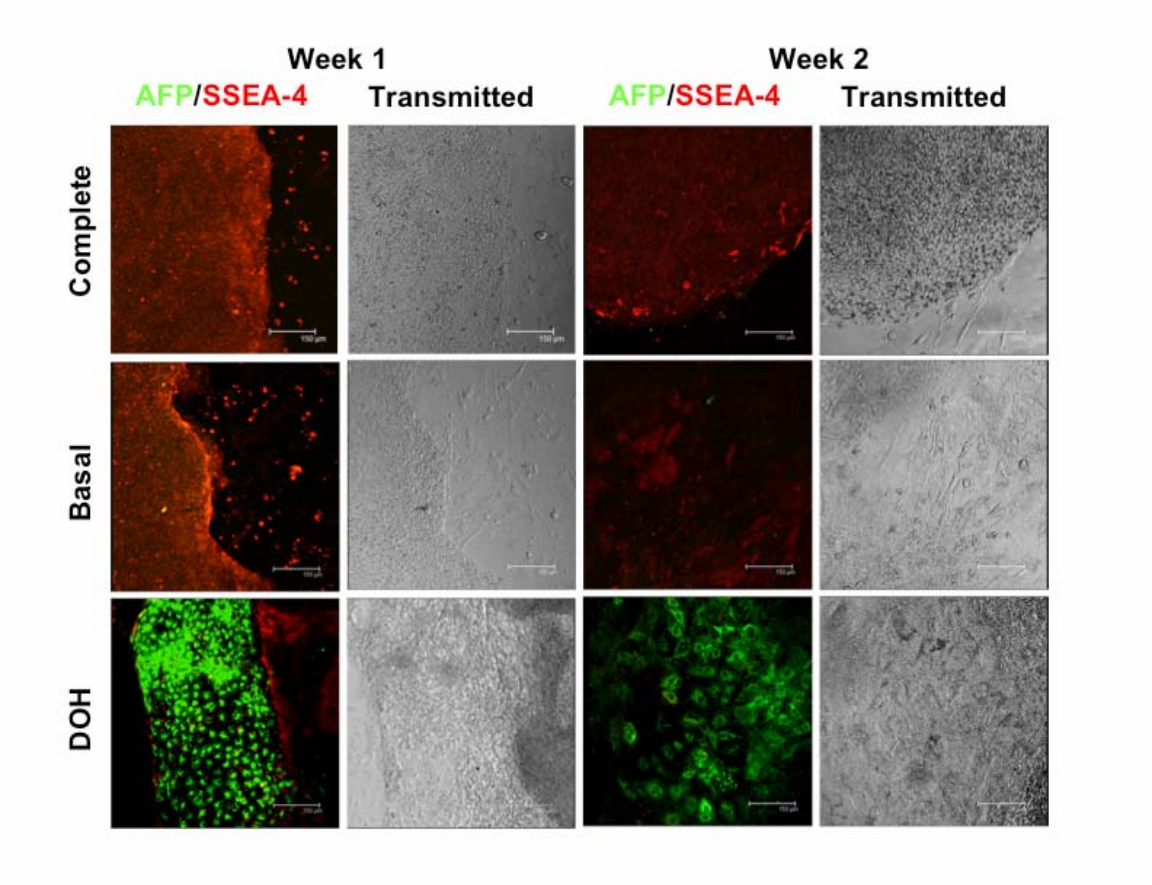

Figure 3.7.2: Early hepatic differentiation of hESCs grown on MEFs in the presence of DOH

Immunocytochemistry shows a prominent expression of AFP in the adherent culture system supplemented with dexamethasone, oncostatin M and hepatocyte growth factor (DOH) after 1 week in culture. AFP expression is down regulated after 2 weeks in culture and cells are enlarged. Cultures grown in complete and basal media show no AFP staining and pluripotency by SSEA-4 staining. Abbreviations: AFP, alpha-fetoprotein; SSEA-4, stage-specific embryonic antigen-4.

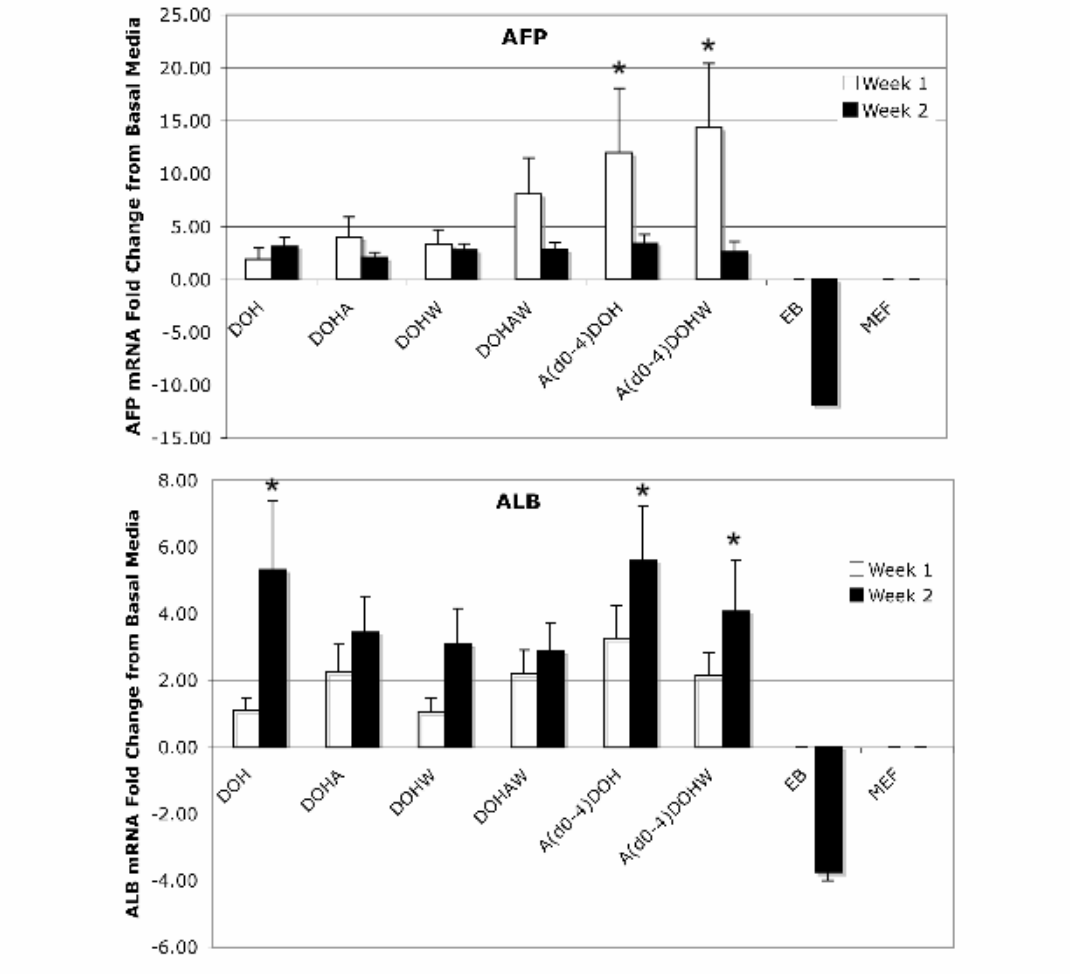

Figure 3.7.3: Progressive alteration of hESC hepatic differentiation in growth factor combinations by RT-PCR

Levels of mRNA expression were normalized to beta-actin and fold change was determined from basal media. EB and MEF conditions were grown in basal media. (* denotes $p<0.05$ compared to EB mRNA levels.) Abbreviations: AFP, alpha-fetoprotein; ALB, albumin; D, dexamethasone; O, oncostatin M; H, hepatocyte growth factor; A, Activin A; A_{d0-4}, Activin A treatment only on days 0-4; W, Wnt3a; MEF, mouse embryonic fibroblast.

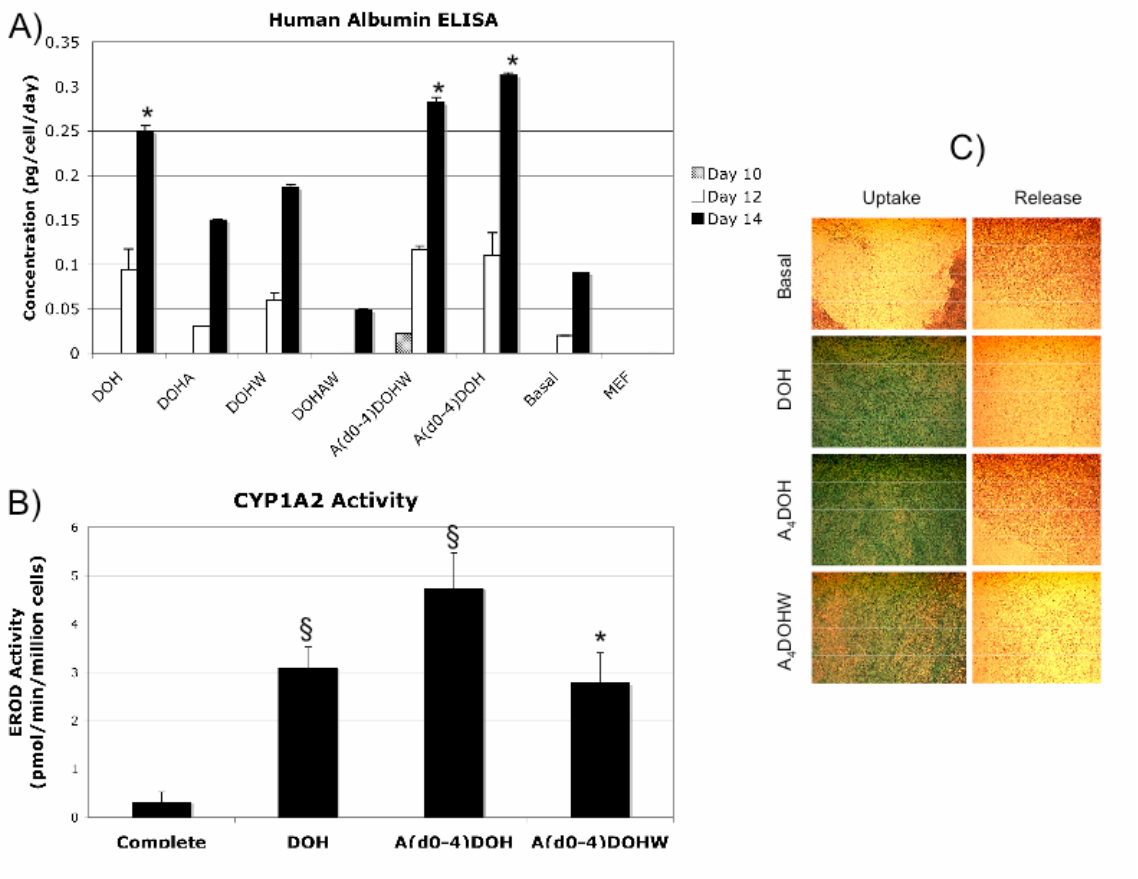

Figure 3.7.4: Functional activity of hESC-derived hepatic-like cells

(A) Human albumin ELISA analysis of growth factor combinations. All albumin concentrations were increased during differentiation with three growth factor combinations resulting in significantly more albumin when compared to basal media for the same day. These results indicated that DOH, $A_{d0\text{-}4}$DOHW and $A_{d0\text{-}4}$DOH result in the greatest level of differentiation. (* denotes $p<0.05$ compared to day 14 basal media.) (B) CYP1A2 activity in hESC-derived hepatic cells grown for 2 weeks in high performance medias, measured by EROD activity. Basal media showed minimal activity, whereas, the high performance medias had

significant activity. (* denotes $p<0.05$ compared to complete media, § denotes $p<0.01$ compared to complete media.) (C) ICG uptake and release. hESCs were grown for 2 weeks in culture in high performance medias and qualitatively evaluated for their ability to take up ICG (left column) and release it 6 hours later (right column). Abbreviations: ICG, indocyanine green; CYP, cytochrome P450; EROD, ethoxyresorufin O-de-ethylase.

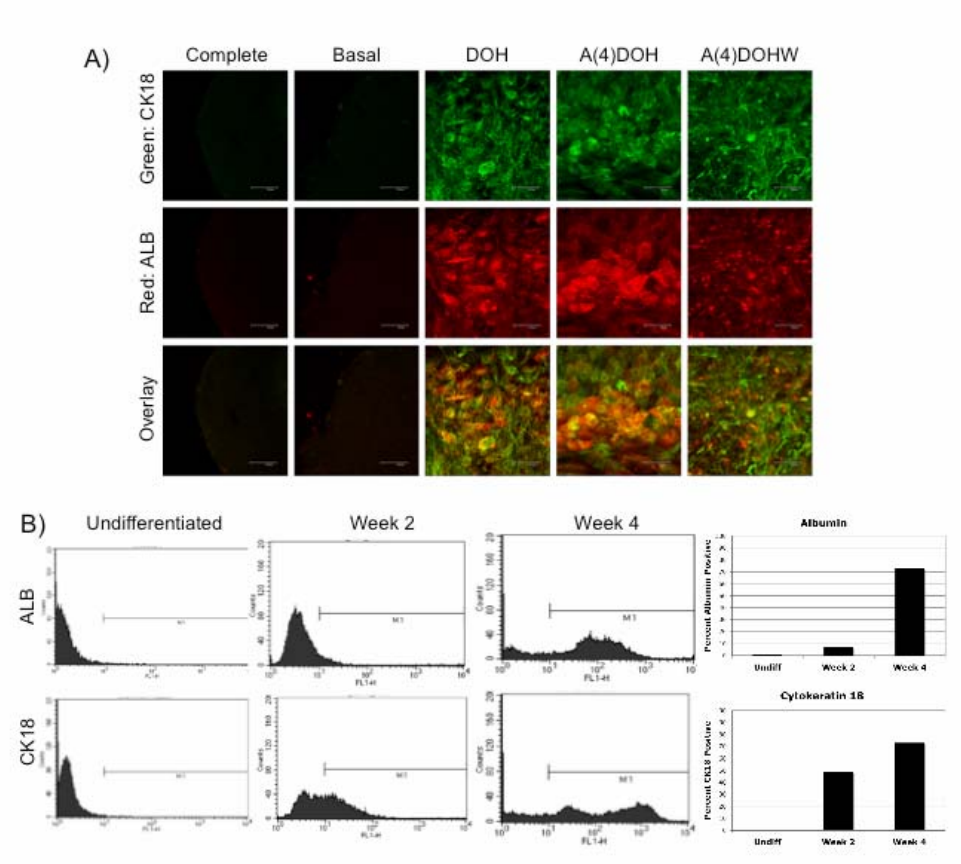

Figure 3.7.5: Representative immunofluorescent staining and flow cytometry for ALB/CK18 in hESC-derived cells

(A) Adherent cultures were grown for 2 weeks and stained with classic hepatic markers. ALB/CK18 co-expression is prominent in DOH, $A_{d0\text{-}4}$DOHW and $A_{d0\text{-}4}$DOH cultures. Undifferentiated cells in complete and basal media were negative for ALB/CK18. The ALB/CK18 positive cells in the $A_{0\text{-}4}$DOH condition had distinct cuboidal morphology and appear to be bi-nucleated, indicative of hepatocyte-like cells. (B) Raw and quantitative depiction of flow cytometric analysis for ALB and CK18 expression on undifferentiated hESCs, 2 weeks, and 4 weeks in $A_{d0\text{-}4}$DOH media. Abbreviations: ALB, albumin; CK18, cytokeratin-18.

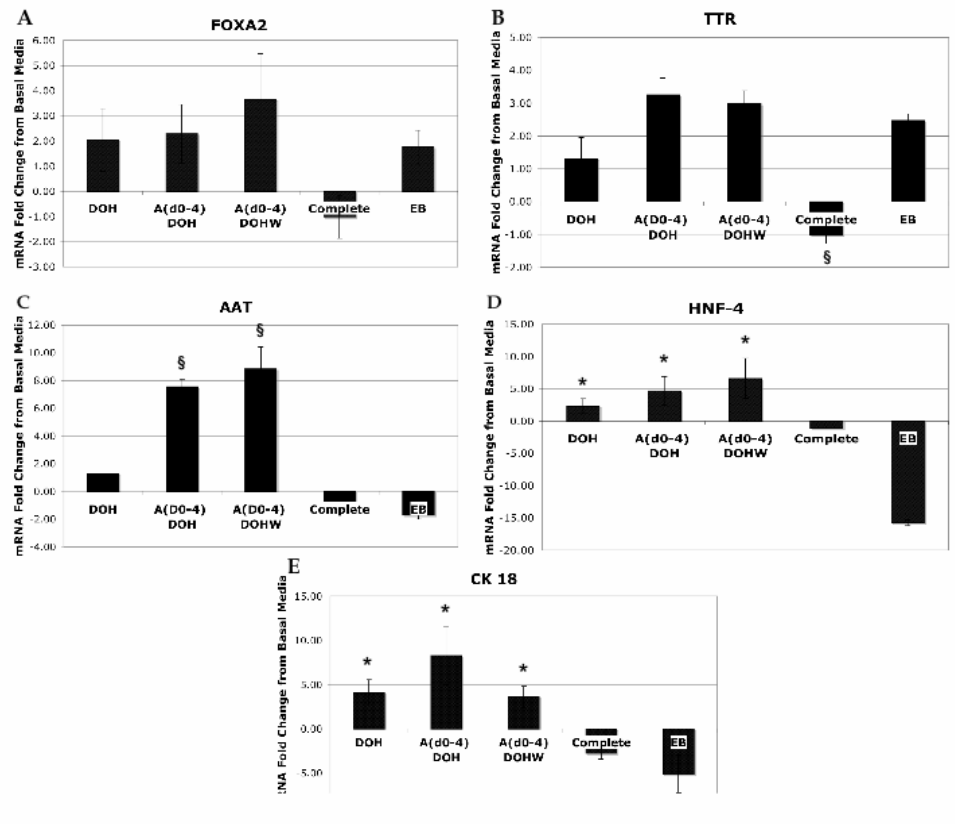

Figure 3.7.6: Real-time RT-PCR analysis of liver-specific gene expression by hESC-derived cells after 2 weeks in culture in the high performance medias

The mRNA expression of FOXA-2 (A), TTR (B), AAT (C), HNF-4 (D), and CK 18 (E) show elevated hepatic markers in DOH, ADOH and ADOHW cocktails and down-regulation in complete media. Embryoid bodies (EB) show elevated expression in the endodermal markers, FOXA-2 and TTR, but down-regulations in the late hepatic markers, AAT, HNF-4 and CK 18. Levels of mRNA expression were normalized to beta-actin and fold change was determined from basal media. (* denotes $p<0.05$ compared to EB, § denotes $p<0.01$ compared to EB.) Abbreviations: FOXA2, forkhead box A2; TTR, transthryretin; AAT, alpha-1-antitrypsin; HNF-4 hepatocyte nuclear factor-4; CK18, cytokeratin 18.

CHAPTER 4

E-CADHERIN-PRESENTING FEEDER CELLS PROMOTE NEURAL LINEAGE RESTRICTION OF HUMAN EMBRYONIC STEM CELLS

4.1 Abstract

Human embryonic stem cells (hESCs) represent a promising source of tissues of different cell lineages because of their high degree of self-renewal and their unique ability to give rise to most somatic cell lineages. In this study, we report on a new approach to differentiate hESCs into neural stem cells that can be further differentiated in neuronal restricted cells. We have rapidly and efficiently differentiated hESCs into neural stem cells by presenting the cell adhesion molecule, E-cadherin, to undifferentiated hESCs via E-cadherin transfected fibroblast monolayers. The neural restricted progenitor cells rapidly express nestin and beta-III-tubulin, but not GFAP during the one-week E-cadherin induction phase, suggesting that E-cadherin promotes rapid neuronal differentiation. Further, these cells are able to achieve enhanced neuronal differentiation with the addition of exogenous growth factors. Cadherin-induced hESCs show a loss in Oct4 and nestin expression associated with positive staining for vimentin, neurofilament and neural cell adhesion molecule. Moreover, blocking by functional E-cadherin antibody and failure of paracrine stimulation suggested that direct E-cadherin engagement is necessary to induce

neural restriction. By providing the hESCs with molecular cues to promote differentiation, we are able to utilize a specific cell-cell adhesion molecule, E-cadherin, to influence the nature and degree of neural specialization.

4.2 Introduction

Human embryonic stem cells are derived, mechanically or immunosurgically, from the polarized inner cell mass of preimplantation-stage blastocysts [5, 6]. Under appropriate conditions, cultures of ES cells proliferate and self-renew indefinitely, although various methods have been developed to induce appropriate signaling molecules to stimulate differentiation of particular specialized cell types. hESCs have often been co-cultured with mitotically inactivated murine embryonic fibroblasts (MEFs) to support the growth of two-dimensional colonies that must be mechanically or enzymatically passaged on a weekly basis. Additional feeder cells have been identified to maintain hESCs in an undifferentiated state that include murine and human cell types [180].

Spontaneous differentiation of hES colonies rapidly occurs *in vitro* when the system lacks preventative factors. In two-dimensional systems, spontaneous differentiation occurs at the outer borders of the colonies, where colonies fuse, or at the center core where cells begin to pile up [66]. A three-dimensional culture system, in the form of embryoid bodies, is another way to initiate differentiation of hESCs [67]. The appearance of all three germ layers is possible in the suspension culture system. Specific cell type populations can be selected with cell-surface markers and separation techniques [68]. The process of directed differentiation is defined by induction into a specific cell type. Endogenous transcription factor activation, transcription factor transfection, growth factor

supplements, or co-culture environments can promote cell differentiation [69, 70].

Cell-cell interactions are recognized to be of fundamental importance for embryonic development, tissue formation and differentiation [31-34]. Cadherins constitute a family of calcium-dependent, transmembrane proteins, which can mediate a wide range of cell-cell interactions and control tissue organization during development and maturation [32, 35]. The predominant cadherin of most epithelia is E-cadherin. Embryonic stem cells are genetically programmed to do one of two things. They can either differentiate to form the ectoderm, mesoderm and endoderm, or remain undifferentiated in a state that mimics the inner cell mass. Because of this, researchers have taken advantage of embryoid body development to force neural lineage restriction through neurospheres. Traditional neural terminal differentiation will result from progenitor cells isolated from the ectoderm followed by exposure to certain molecular cues.

The use of chemically defined adherent cultures has also been established for neural induction of hESCs with the goal to gain tighter control over differentiation while characterizing the effects of different molecules. Nat et al. made use of a neural induction protocol in which hESCs are cultured on poly-ornithine/laminin coated substrates or in suspension and cultured for 4 days in a neural induction media supplemented with B27 and N2, followed by 3-38 days of culture in neural proliferation media supplemented with B27, N2, and bFGF [181]. While the presence of nestin was high in the suspension and adherent

cultures (85.8% and 80.6% respectively), the expression of vimentin (1.6% and 3.5%), and beta-III-tubulin (7.4% and 9.3%) was fairly low. However, the expression of vimentin and beta-III-tubulin increases significantly by day 14. We chose to compare differentiation induced by E-cadherin with a known neural induction protocol [181] to determine the extent of neural and neuronal specification with E-cadherin-transfected fibroblasts.

The use of the cell adhesion molecule, E-cadherin, could be advantageous for neural induction of hESCs. Because E-cadherin is important for differentiation and tissue formation and is expressed very early during neural development [181], E-cadherin could play a role in neural differentiation of stem cells. Currently there are no studies that have shown the ability of E-cadherin to induce neural differentiation of hESCs. However, E-cadherin has been shown to inhibit precursor proliferation, induce premature neuronal differentiation and inhibit beta-catenin dependent signaling [182].

Our hypothesis is that E-cadherin presentation to the hESCs during development will expedite the process of directed differentiation into the neural lineage. We have examined this hypothesis by presenting to hESCs a fibroblastic feeder layer with differential levels of E-cadherin. We demonstrated that interactions of hESCs with E-cadherin-transfected cultures exhibited faster and more extensive neural induction in comparison to cultures with control feeders expressing much lower levels of E-cadherin.

4.3 Materials and Methods

4.3.1 Propagation of Undifferentiated hESCs

Human embryonic stem cells (H1 and H9) were obtained from WiCell Research Institute (Madison, WI) and maintained following WiCell protocols. Briefly, H1 cells were cultivated in gelatin-coated, six-well plates in DMEM-F12 with L-glutamine (Invitrogen, Carlsbad, CA) supplemented with 20% KnockOut Serum Replacer (Gibco, Grand Island, NY), beta-mercaptoethanol (Gibco, Grand Island, NY), non-essential amino acids (Invitrogen, Carlsbad, CA), 4 ng/ml basic fibroblast growth factor (b-FGF) (Invitrogen, Carlsbad, CA), and 0.12 ng/ml transforming growth factor-beta (TGF-β1) (Peprotech, Rocky Hill, NJ). Medium was changed every day and differentiated regions were mechanically removed as needed. After collagenase IV (Gibco, Grand Island, NY) treatment for 5-10 min at 37°C followed by mechanical dissociation, colonies were passaged weekly at a ratio of 1:6 on mitotically inactivated (x-irradiated, 6500 rad) mouse embryonic fibroblasts (MEFs). For comparison, H9 colonies were grown in feeder-free conditions on Matrigel (BD Biosciences, Franklin Lakes, NJ) coated plates in mTeSR media (Stem Cell Technologies, Vancouver, BC, Canada), changed daily. Cells were mechanically passaged every 5-7 days with 1x dispase (BD Biosciences, Franklin Lakes, NJ) in DMEM-F12 at a ratio of 1:12.

4.3.2 Initiation of hESC Differentiation

Directed differentiation was accomplished by culturing the H1 hESCs on an alternative feeder cell type that presents different levels of E-cadherin. Clonally derived L929 mouse fibroblasts were originally established by calcium phosphate coprecipitation transfection (pBATEM2 plasmid) into L cells, which normally have little cadherin [183]. L929+ fibroblasts have stable expression of mouse E-cadherin while L929- fibroblasts are untransfected. L929 fibroblasts were expanded in DMEM (Gibco, Grand Island, NY), containing 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY), penicillin, streptomycin (Lonza, Basal, Switzerland), and gentamicin (Sigma, St. Louis, MO). L929 fibroblasts transfected with E-cadherin were supplemented with G418 sulfate (Mediatech, Hemdon, VA) to help maintain the E-cadherin expression. Medium was changed every other day and passaged weekly.

In preparation for hESCs, E-cadherin-transfected L929+ or control, non-transfected L929- fibroblasts were mitotically inactivated with mitomycin C (10 μg/ml) (Sigma, St. Louis, MO), dissociated with 0.1% trypsin (Sigma, St. Louis, MO), and seeded onto gelatin coated plates at a density of 1.95×10^4 cells/cm^2. L929 fibroblasts were allowed to attach for 24 hours and washed with DMEM to remove residual serum from the feeder layer. Undifferentiated H1 hESC colonies, devoid of MEFs, were mechanically isolated and plated onto the L929s. H1 complete media was changed daily and grown for 7 days. L929 fibroblast induced H1 colonies were subcultured onto gelatin coated plates and grown in defined medias to further expand and differentiate the cells. Subculture

experiments with monolayers were conducted to determine the priming effects of L929s on the H1 cells and grown in neural proliferation media (NPM) and neural differentiation media (NDM) for 1 week each, consecutively. NPM is composed of 50% DMEM-F12 with Glutamax (Invitrogen, Carlsbad, CA), 50% neuralbasal media and supplemented with 0.5x N2, 0.5x B27 without vitamin A, and 20 ng/mL bFGF (Invitrogen, Carlsbad, CA) [181]. NDM is composed of neuralbasal media supplemented with 1x B27 without vitamin A and 10 ng/mL brain derived neurotrophic factor (R&D Systems, Minneapolis, MN) [181].

As a comparison to L929 fibroblast induced H1 cells, neural restricted progenitor cells were generated with a protocol that uses adherent differentiation and media supplementation [181]. Briefly, undifferentiated H9 hESCs were changed to neural induction media (NIM) on day 0. NIM is composed of 50% DMEM-F12 with Glutamax (Invitrogen, Carlsbad, CA), 50% neuralbasal media and supplemented with 1x N2, 1x B27 without vitamin A (Invitrogen, Carlsbad, CA) [181]. On day 1, clusters were passaged to polyornithine (15 μg/mL) (Sigma, St. Louis, MO)/laminin (20 μg/mL) (Invitrogen, Carlsbad, CA) coated plates in NIM at a density of 10-15 clusters/cm^2. On day 4, media was changed to neural proliferation media (NPM) and grown for 7 days in culture, changing media every other day. On day 24, media was changed to NDM, changing media every other day.

4.3.3 Quantification of Extracellular E-cadherin

L929 fibroblasts were stained for cell surface E-cadherin to visually confirm and quantify the levels of E-cadherin being expressed on the L929 cells and compared to MEFs. The fibroblasts were fixed with 4% paraformaldehyde in DPBS with Ca^{2+} and Mg^{2+} for 15 minutes at room temperature. Cells were then rapidly washed three times with DPBS. After washing, cells were blocked with 3% (w/v) bovine albumin serum (BSA) for 30 minutes at room temperature to reduce nonspecific antibody binding. Subsequently, E-cadherin staining was attained by using monoclonal rat anti-mouse E-cadherin primary antibody (R&D Systems, Minneapolis, MN) in PBS for 1 hour, rocking at room temperature, at a concentration of 10 μg/ml. Cells were washed twice with PBS and the goat anti-rat FITC secondary antibody (Jackson ImmunoResearch, West Grove, PA) was incubated overnight, rocking at 4°C. Finally, the cells were washed four times in DPBS and visualized using the Leica TCS.SP2 confocal microscope system (Leica Microscope, Exton, PA).

To determine quantitatively the amount of E-cadherin on the L929 fibroblasts, cells were processed and analyzed by fluorescent-activated cell sorting (FACS) (Becton Dickinson, Franklin Lakes, NJ). Briefly, cells were washed twice with HBSS with 1mM $CaCl_2$ and treated with trypsin/calcium (0.05%/2mM) and washed twice again with HBSS/$CaCl_2$. Cell identification was confirmed by staining with ECCD-1 rat anti-mouse E-cadherin (Zymed, San Fransisco, CA), 10 μg/ml, for 30 minutes on ice. Cells were washed twice and

labeled with AlexaFluor goat anti rat IgG (H+L) 488 (Invitrogen, Carlsbad, CA) for 30 minutes on ice, 1:1000 dilution. Finally, cells were washed three times with HBSS/$CaCl_2$ and analyzed with CellQuest software (Becton Dickinson, Franklin Lakes, NJ).

4.3.4 Molecular Characterization of Neural Stem Cells

After a weeklong induction by co-culturing with L929 fibroblasts, hESCs were subcultured in NPM for 1 week and switched to NDM for 1 week, and then the cells were stained for neural family markers to determine the extent of differentiation. Cultures were fixed with 4% paraformaldehyde in DPBS with Ca^{2+} and Mg^{2+} for 15 minutes at room temperature. Cells were then rapidly washed three times with DPBS and permeabilized with SAP buffer (0.5% Saponin, 1% BSA, 0.1% Sodium Azide) (Sigma, St. Louis, MO) for 30 minutes at room temperature. After washing once with DPBS, cells were blocked with 3% (w/v) bovine albumin serum (BSA) for 30 minutes at room temperature to reduce nonspecific antibody binding. Subsequently, primary antibodies for neural identification were incubated overnight at 4°C. The primary antibodies, diluted in SAP buffer are as follows: Nestin IgG1, 1:200 dilution (Millipore, Temecula, CA); beta-III-tubulin clone TUJ1 IgG2a, 1:500 dilution (Covance, Berkeley, CA); GFAP IgG2b, 1:100 dilution (Abcam, Cambridge, MA); O4 IgM, 10 μg/mL (Millipore, Temecula, CA); Neurofilament IgG1, 1:200 dilution (Sigma, St. Louis, MO); NCAM IgG1, 1:50 dilution (Santa Cruz, Santa Cruz, CA);

Vimentin IgG1, 1:20 dilution (Abcam, Cambridge, MA); Oct4 IgG1, 0.01 mg/mL (Millipore, Temecula, CA); SSEA-4 IgG3, 1:200 dilution (Millipore, Temecula, CA). Cells were washed three times with PBS and incubated with isotype matched secondary antibodies, 1:1000 dilution in SAP buffer for 1 hour at room temperature. The secondary antibodies are as follows: AlexaFluor 488 IgG1, AlexaFluor 488 IgG2a, AlexaFluor 594 IgG3, AlexaFluor 594 IgG2b, AlexaFluor 594 IgM (Invitrogen, Carlsbad, CA). Finally, the cells were washed four times in DPBS and visualized using the Leica TCS.SP2 confocal microscope system (Leica Microscope, Exton, PA).

4.3.5 Confirmation of E-cadherin-Mediated Differentiation

L929 fibroblast induced H1 cells were functionally blocked with an E-cadherin antibody to investigate whether E-cadherin mediates neural differentiation. Briefly, L929 fibroblasts were seeded on gelatin coated plates and pre-blocked with 200 μg/mL rat anti-E-cadherin (mouse) ECCD-1 antibody (Invitrogen, Carlsbad, CA) or rat IgG2b isotype control antibody (R&D Systems, Minneapolis, MN) for 24 hours. Cells were washed with DMEM-F12 to remove residual serum and undifferentiated H1 cells were mechanically passaged onto the E-cadherin blocked L929s in complete media. Media was exchanged daily and E-cadherin blocking was supplemented on day 3. Cells were fixed, stained and analyzed for neural differentiation on day 4.

To analyze whether L929 conditioned media was sufficient to induce differentiation, transwell inserts were used to physically separate the L929s from the hESCs. Briefly, L929 fibroblasts were seeded in 0.4 μm membrane transwell inserts (Costar, Corning, NY) at the same density as direct co-culture. One day later, undifferentiated H1 colonies were mechanically passaged onto gelatin coated plates in complete H1 media. L929 insert wells were introduced to the H1 culture and allowed to grow for 1 week, with media exchanged daily. The insert well was removed and H1s were fixed and stained or lysed for RT-PCR to evaluate the extent of differentiation.

4.4 Results

4.4.1 Extracellular E-cadherin Expression of Variant Feeder Cells

L929 fibroblasts were evaluated for their E-cadherin expression and ability to support hESC growth compared to MEFs (Figure 4.7.1A). Cell surface E-cadherin staining shows that MEFs and L929- fibroblasts have very little cadherin while L929 fibroblasts have high surface levels of E-cadherin. To confirm and quantify the visual E-cadherin expression, flow cytometry was utilized (Figure 4.7.1B). E-cadherin-transfected L929+ fibroblasts had a high expression of E-cadherin compared to the control, L929- fibroblasts when comparing peak channel fluorescence. Taken together, the E-cadherin transfected L929 fibroblast cell line expressed higher levels of E-cadherin than the non-transfected cultures.

4.4.2 E-cadherin Rapidly Induces Differentiation of hESCs into Neural Stem Cells

H1 colonies were cultured on monolayers of L929 fibroblasts and allowed to differentiate for 1 week. Cadherin-mediated neural induction (H1/L929+) begins as early as day 1 and is throughout the entire colony as early as day 4 (Figure 4.7.2A). Early differentiation markers were evaluated to determine the

lineage and level of specification (Figure 4.7.2B). E-cadherin-transfected co-cultures (H1/L929+) were positively stained with nestin and beta-III-tubulin, with little or no SSEA-4 present. The control, non-cadherin co-cultures (H1/L929-) were negative for all neural markers, but remained positive for SSEA-4. The nestin and beta-III-tubulin positive cells in E-cadherin-transfected cultures also begin to attain an elongated morphology as they begin to neuronally differentiate. Furthermore, the TUJ1-positive cells were counterstained with GFAP or O4 to determine that the hESCs were directed toward the neuronal lineage and not toward the glial or oligodendrocytic lineage (Figure 2C) at the one week induction phase.

To confirm that E-cadherin mediated neural induction, we evaluated a functional E-cadherin blocking antibody and physical separation of the co-culture were evaluated (Figure 4.7.3). Treatment of H1 cells grown on E-cadherin-transfected and non-transfected fibroblast monolayers with anti-E-cadherin, but not with isotype control antibodies, inhibited expression of nestin, an early neural stem cell marker, and TUJ1, a neuronal restricted marker, after 4 days in culture. The E-cadherin blocking antibody had no detectable effect on the H1 cells in co-culture with the L929 non-transfected cultures. Additionally, to rule out a paracrine-inducing effect from the L929 fibroblasts, the L929s were sequestered from hESCs via an insert well culture. Nestin and TUJ1 expression were evaluated and shown to be negative in the insert well cultures for both E-cadherin-transfected and non-transfected fibroblasts. Quantitative RT-PCR

analysis revealed that physical separation, via transwell inserts, reduced the amount of nestin and beta-III-tubulin in the E-cadherin-transfected cultures (Figure 4.7.3B). Levels of neural and neuronal expression were comparable to those of non-transfected cultures, indicating that E-cadherin engagement is required for neural stem cell differentiation.

4.4.3 hES-Derived Cells Express Neural and Neuronal Markers

Beyond using E-cadherin to promote the induction of neurally restricted precursors, we further differentiated the L929 induced hESCs with growth factors and then evaluated the cells for the expression of neural-specific markers using immunocytochemistry, qRT-PCR and morphology. Cultures that had undergone L929 fibroblast induction were subcultured in NPM and then switched to NDM with each treatment extending for one week.

Quantitative RT-PCR analysis confirmed the expression of nestin and beta-III-tubulin genes throughout induction, expansion and differentiation phases (Figure 4.7.4). All data represents fold changes from undifferentiated hESCs and are normalized to the housekeeping gene, beta-actin. The E-cadherin induced cultures show a greater than 4-fold increase in nestin expression compared to non-transfected co-cultures at week 1 induction. The nestin expression was further pronounced expansion phase (in NPM), but was down-regulated during the differentiation phase (in NDM). When compared to the neural progenitor cells (NPC), the E-cadherin-transfected co-cultures attained

levels of nestin and beta-III-tubulin that are equal or higher than the NPC. In contrast, nestin expression did not vary in the non-transfected co-cultures, further confirming an important role of E-cadherin in neural stem cell induction. Beta-III-tubulin expression steadily increased during each phase in the cadherin-induced cultures, indicating a progressive enhancement in neuronal differentiation. In contrast, non-transfected cultures were unaltered in terms of beta-III-tubulin expression throughout differentiation. Additionally, GFAP expression was evaluated throughout the E-cadherin inducing phases and remained unchanged, suggesting that E-cadherin promotes neuronal differentiation and not glial restriction (data not shown). Furthermore, the morphology of each subculture was evaluated following the differentiation phase (Figure 4.7.5). Cells subcultured from the E-cadherin-transfected fibroblasts attained an elongated, dendritic morphology, whereas the control subculture cells remained compact in morphology.

Immunocytochemical analysis of neural and neuronal associated markers revealed that the E-cadherin-transfected hESC derived cultures had an expression profile that varied throughout the 3 week culture (Figure 4.7.6). Consistent with increasing differentiation, expression of the ESC marker, Oct4 was not detected and expression of neural stem cell marker, nestin decreased over time. Remarkably, Oct4 was already undetectable one week after induction and nestin expression persisted longer but was most lost after the differentiation stage. Cadherin induced hESCs were positive for vimentin, neurofilament and

NCAM. The non-transfected fibroblast co-cultures were positive for Oct4, but negative for any neural family markers, indicating that the cells are not neurally restricted. During the differentiation phase, the Oct4 expression did begin to decrease, but this was not associated with an increase any of the neural family markers.

4.5 Discussion

In this study, we investigated the role of an alternative feeder cell type that rapidly induces neural differentiation based on E-cadherin presentation with no embryoid body formation or additional growth factor supplementation.

Several protocols have employed EB formation as a way to induce neural differentiation of hESCs in the presence of serum, retinoic acid, and bFGF [71, 75, 85, 184-186]. Typically, hESCs are mechanically or enzymatically removed from the mouse embryonic feeder layer and grown in suspension for 4-21 days with bFGF or retinoic acid to further encourage neural differentiation [71, 75, 85, 184-186]. The use of enzymatic digestion via dispase has been proposed to yield a more pure population as cells that are not committed to the neural lineage do not respond very well to dispase treatment [75, 186]. Additionally, the use of retinoic acid may promote caudalization and or motoneuron generation, thus limiting the differentiation ability of the neural progenitors [75]. After the EB culture period, the EBs are plated onto adherent substrates and cultured with various growth factors to promote specific neural differentiation into neurons or glial cells [71, 75, 85, 184-186]. In total, this process can take up to 21 days in order to generate neural precursors, neural progenitors, or terminally differentiated cells [71, 75, 85, 184-186]. Besides the time-intensive process necessary to generate neural differentiation using this method, EB formation is uncontrolled and is based on spontaneous differentiation, which leads to a mixture of various cells from the

other germ layers that have been observed even after neuroectodermal isolation [187].

The EB process yields a very heterogeneous population of cells that must be purified. In order to reduce the contamination of cells from the mesodermal and endodermal lineages during culture of hESCs in suspension, a few protocols have employed defined neural induction media versus hESC growth media. Itsykson et al. cultured hESCs as aggregates in suspension with a defined neural induction media supplemented with bFGF [184]. However, in order to eliminate the cystic structure observed in EBs and to generate a more homogenous population of cells grown in suspension, cells aggregates were treated with noggin for 3-6 weeks. The expression of mesodermal (muscle actin and smooth muscle actin) and endodermal (cytokeratin-8, CK-8) markers was essentially eliminated after 3 weeks and completely eliminated after 6 weeks in culture compared to traditionally cultured EBs and cell aggregates grown in their neural precursor media without noggin. Additionally, beta-III tubulin-positive cells only made up 37.3% of the population. While this culture method did yield a more homogenous population of neural cells, the time it takes to yield such a population is time-consuming. Also, in order to see the aforementioned effects of noggin, a high concentration (700 ng/ml) had to be used.

Co-culture of hESCs with stormal (PA6, MS5 and S2) and embryonic astrocytes is another commonly used technique to induce neural differentiation of hESCs [74, 188-192]. The co-culture of hESCs and stromal cells has been

coupled with overexpression of Wnt1 or SHH in the stromal cells [74, 189], a cocktail of growth factors such as SHH, FGF8, and BDNF [74], GDNF [188], and noggin [191] to enhance or obtain better control on terminal differentiation of the derived neural precursors. A shortcoming of stromal cell-induced neural differentiation is that typically co-culture requires anywhere from 3-4 weeks before significant neural induction is observed. In addition to the lengthy neural induction time, the neural inducing affect of stromal cells, known as stromal-derived inducing activity (SDIA), is still not very characterized. One group narrowed the possible candidates to hepatocyte growth factor, vascular endothelial growth factor, and fibroblast growth factor 7 by using microarray analysis. These growth factors had a higher fold expression in PA6 stromal cells compared to MEFs, with hepatocyte growth factor having the highest expression difference [192]. However, the growth factors alone or in combination were not able to induce dopaminergic differentiation. Stromal cell co-culture appears to be restrictive in the type of cells the neural precursors terminally differentiate into as most of the aforementioned protocols yield dopaminergic neurons [74, 188, 189, 191, 192]. Although there has been a report on the ability of hESC-PA6 co-culture to yield peripheral sensory neurons and neural crest cells [190].

Joannides et al. developed a scaleable and defined system for generating neural stem cells in a culture medium that only consisted of recombinant or human-derived products [193]. In this culture system, 16 days of culture were required for high levels of expression of nestin and Pax6 coupled with a loss of

Oct4 and SSEA-4. Following 16 days in culture, the authors found a correlation between EB size and the degree of neurogenesis; the small EBs had uniform, compact structures and where highly neurogenic, whereas larger EBs were homogenous and were not very neurogenic. While they were able to yield neural stem cells and culture them for long periods of time, the culture system is size dependent, which could be the case with all methods that make use of EBs, and makes use of expensive equipment and expensive culturing reagents. Another humanized culture system for neural differentiation of hESCs has also been reported, where the culture matrix is composed of various human-derived extracellular matrix proteins and with humanized TESR1 culture medium [194]. The protocol yielded regional specific neural precursors following treatment with retinoic acid or bFGF after 7 days in culture as shown by nestin positive and Pax6 positive cells. However, only 20% of the cells were positive for TUJ1 (beta-III-tubulin clone) after 21 days in culture indicating more time was required to yield a large population of neuronal cells.

Great efforts and successes have been achieved in developing protocols and determining and understanding how to manipulate hESCs to yield cells committed to the neural lineage. However, a method that is quick, simple and defined is still lacking for the derivation of neural stem cells or neural progenitors from hESCs.

Several studies have examined the effects of E-cadherin on the differentiated function of embryonic stem cells [102, 195, 196]. In our lab, we

have investigated how over-expressing E-cadherin in murine embryonic cells would affect hepatic differentiation in the presence of hepatotrophic growth factor stimulation [102], as well as co-culture with primary rat hepatocytes [196]. These studies demonstrated the importance of cell-cell contact on differentiation of embryonic stem cells and confirmed that there is a cooperative affect of E-cadherin based juxtacrine and paracrine interactions in hepatic differentiation [196]. The aforementioned studies suggest that E-cadherin can play an important role in the differentiation of embryonic stem cells.

There has been evidence that E-cadherin is involved in neural induction during gastrulation [87]. Changing E-cadherin expression has been observed in differential adhesion and cell sorting to aggregate cells into distinct groups during early development [88]. Separation and integration into developing tissue has been examined in the neural tube, neural crest and neurectoderm [89-93]. Choi et al. identified E-cadherin to be localized on the cell membrane of the ectoderm, which was further investigated by Angres et al. [94, 95]. These early experiments provided a basis for the design of E-cadherin-mediated neural induction that we further investigated in hESC differentiation.

The mechanism of cadherin-mediated differentiation is likely responsible for the non-linear response we found in our experiments. Low levels of cadherin elicited undetectable neural differentiation, whereas cadherin-transfected fibroblasts caused neural and neuronal differentiation. We hypothesize that there is a threshold for activation by cadherins on the differentiation of hESCs.

The formation of cadherin complexes has been studied to determine the mechanism of cadherin signaling [32, 197, 198] that may be directly related to cellular responsiveness [38, 199]. Based on the modulation hypothesis [200], local surface levels of cell adhesion molecules, as presented by L929 fibroblasts in our system, may play a role in nervous system development [201]. Changes in E-cadherin concentration presented to the hESCs cause large changes in differentiation, supporting the idea that cell adhesion molecules separate and integrated during development.

Cadherins mediate cell-cell adhesion through progressive oligomerization between cadherin dimers across two approaching cell membrane surfaces. In mature mammalian cells, cadherin binding causes intracellular signaling primarily via the Wnt pathway. Conversely, suppression of cadherin expression and binding promotes catenin-based regulation of growth or proliferation genes such as c-myc and cyclin D1 [36, 37]. Thus, the presentation of cell-based cadherin acts as a molecular switch from growth to tissue-specific differentiation pathways. Furthermore, by using E-cadherin to rapidly induce neural stem cell differentiation, we are able to make strides in completely humanizing hESC culture systems. Future experiments will examine the possibility of displaying acellular cadherin fragments from artificial three-dimension substrates to trigger the same effect. [202].

In conclusion, this research provides a strategy for rapid neural differentiation of hESCs using the presentation of a cell adhesion ligand, E-

cadherin. Culture conditions are identical to the traditional hES cultures with the exception of exchanging E-cadherin-transfected cells (L929 fibroblasts) in lieu of mouse embryonic fibroblasts. The novelty of the research lies in the presentation of E-cadherin to the hESC cultures via an alternative feeder cell type. The genetic, morphological, and expression profiles confirm that E-cadherin induces neuronally restricted cells. Specifically, through the incorporation of E-cadherin, we are able to induce neuronal restricted precursor differentiation, determined by nestin-, NCAM-, vimentin-, neurofilament-, and beta-III-tubulin-positive cells. Insights gleaned from this study could be potentially useful for priming strategies of transplantable hESCs such that cues within a spinal cord injury can augment neuronal differentiation in situ.

4.6 Acknowledgements

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4.7 Figures and Captions

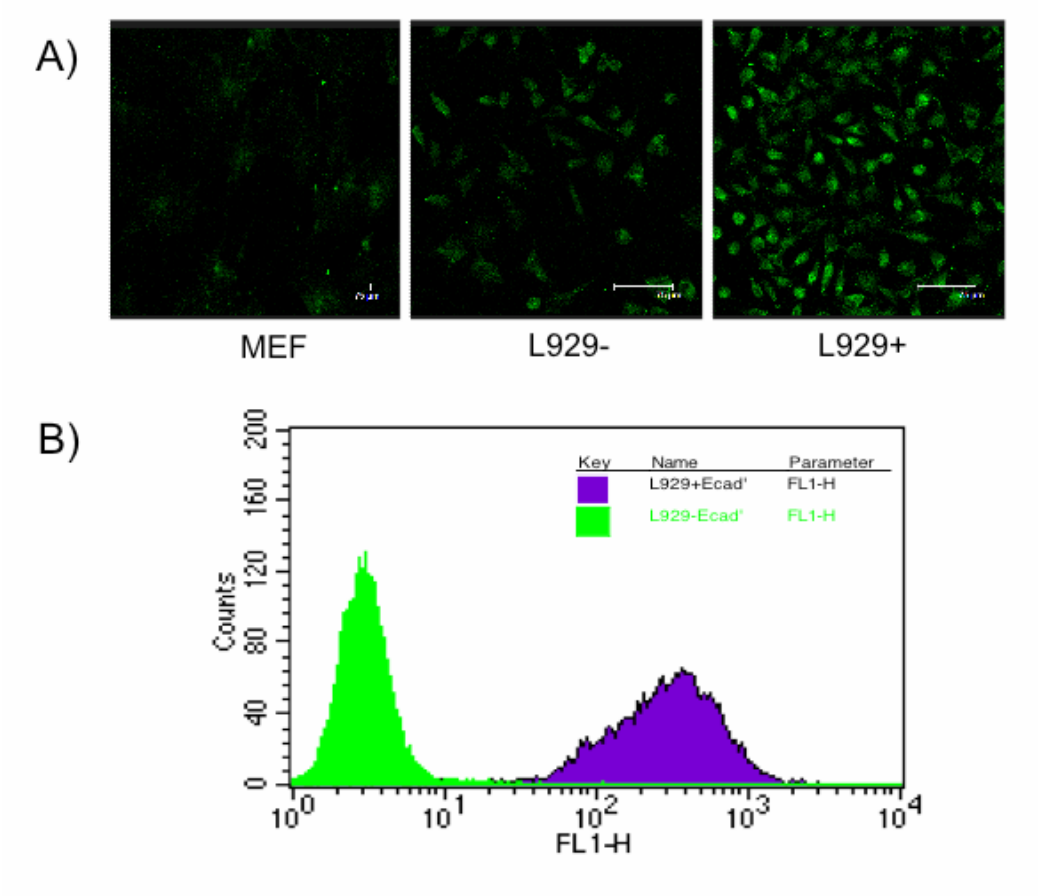

Figure 4.7.1: Characterization of E-cadherin variant L929 fibroblasts

L929 fibroblasts were evaluated for their E-cadherin expression and compared to traditional MEFs. A) Immunofluorescence for extracellular E-cadherin expression revealed that L929- fibroblasts had very little E-cadherin on the surface, similar to MEFs. L929+ fibroblasts had high expression of E-cadherin. B) Flow cytometry of the L929 fibroblasts confirm that cadherin-deficient, L929- fibroblasts (green) lack E-cadherin expression and that cadherin-presenting, L929+ fibroblasts (purple) have high levels of E-cadherin. Abbreviation: MEF, mouse embryonic fibroblast.

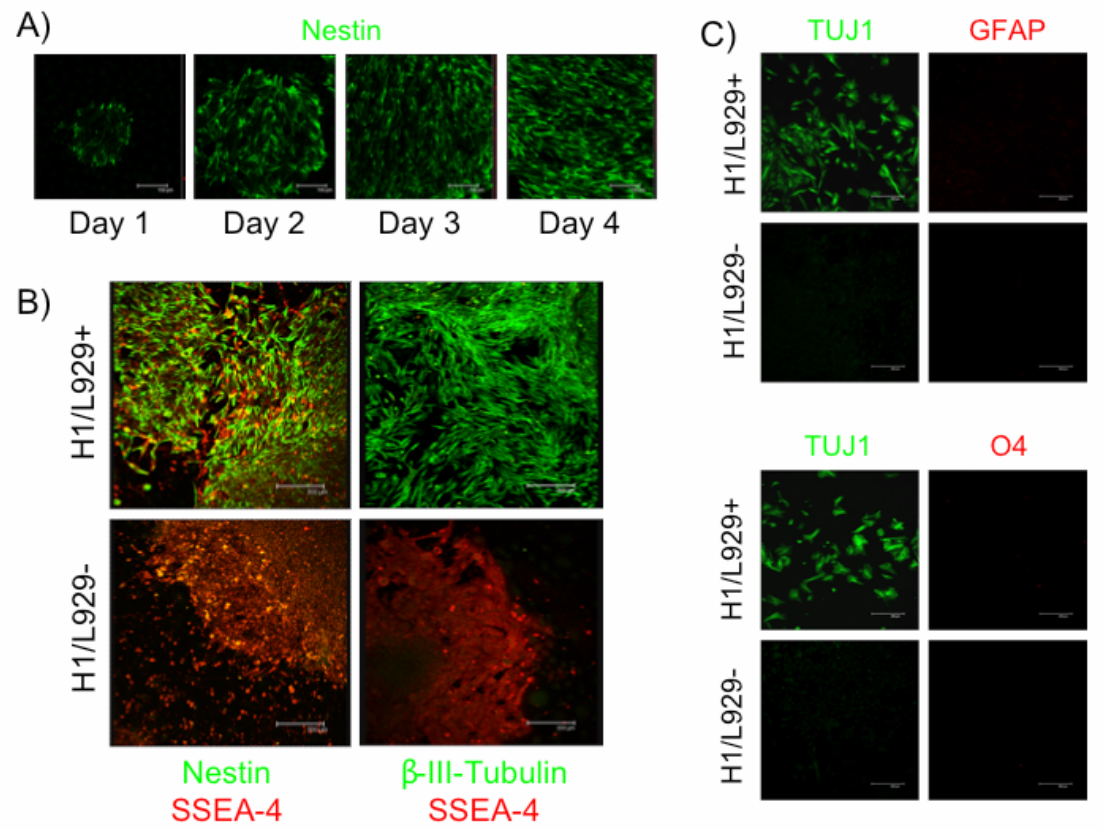

Figure 4.7.2: E-cadherin rapidly induces neural and neuronal differentiation

hESCs were evaluated for A) neural (nestin) and neuronal (beta-III-tubulin), B) glial (GFAP) and oligodendrocyte (O4) expression after 1 week in culture with cadherin-presenting (L929+) or cadherin deficient (L929-) fibroblasts. Nestin (green) and beta-III-tubulin (green) expression was evident on the H1/L929+ cultures and negative in the H1/L929- cultures. SSEA-4 (red) expression was only detected on the cadherin-deficient cultures. Furthermore, cells stained positive for beta-III-tubulin TUJ1 clone (green) were negative for glial GFAP (red) and oligodendrocyte O4 (red) expression indicating that E-cadherin rapidly induces neuronally restricted progenitor cells. Abbreviations: GFAP, glial

fibrillary acidic protein; O4, oligodendrocyte marker; SSEA-4, stage-specific embryonic antigen-4.

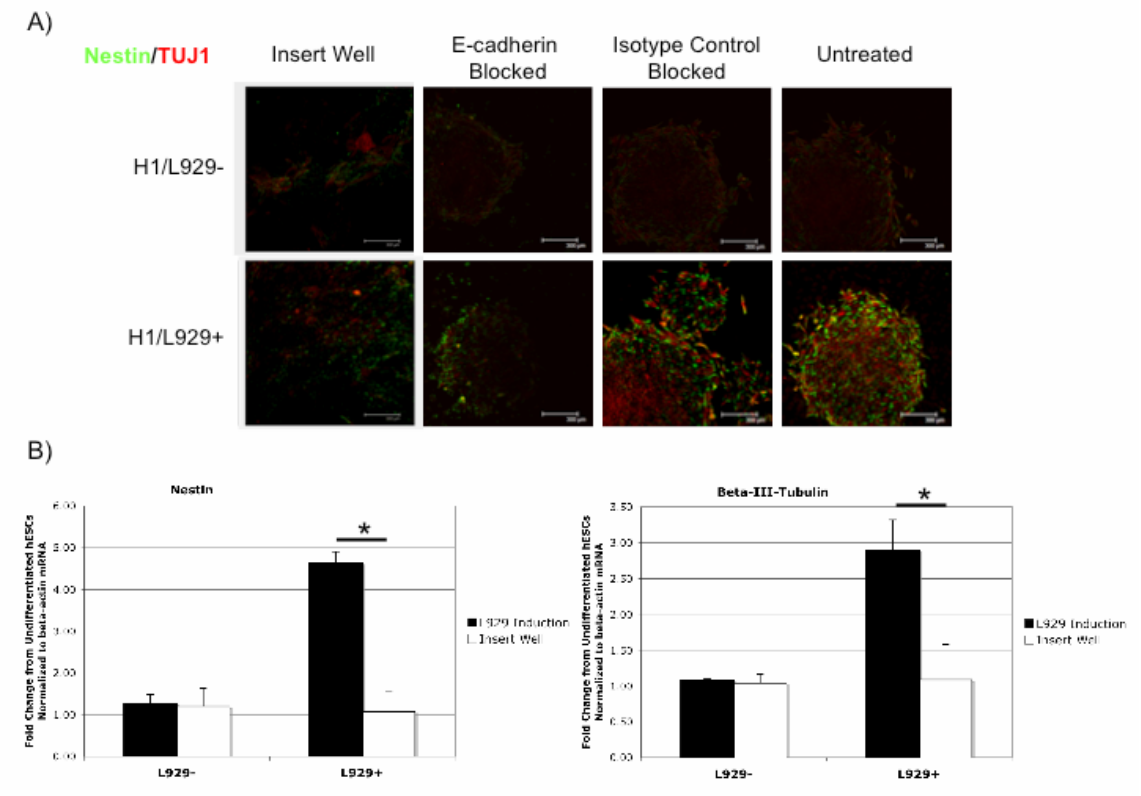

Figure 4.7.3: E-cadherin engagement required for neural differentiation

hESCS were evaluated for neural differentiation when E-cadherin engagement was disrupted. A) H1s were stained with nestin (green) and TUJ1 (red) to determine the extent of neural differentiation when cells were physically removed from the fibroblasts via insert wells (first column), functionally blocked with E-cadherin antibody (second column), blocked with isotype control antibody (third column), or left untreated (forth column). Cultures that impeded direct E-cadherin cell-cell contact were negative for nestin/TUJ1. B) Insert well cultures were quantitatively evaluated with RT-PCR to further confirm that interrupting E-cadherin engagement results in nestin and beta-III-tubulin levels similar to cadherin-deficient cultures. Levels of mRNA expression were

normalized to beta-actin and fold change was determined from undifferentiated H1 cells (* denotes $p<0.05$.)

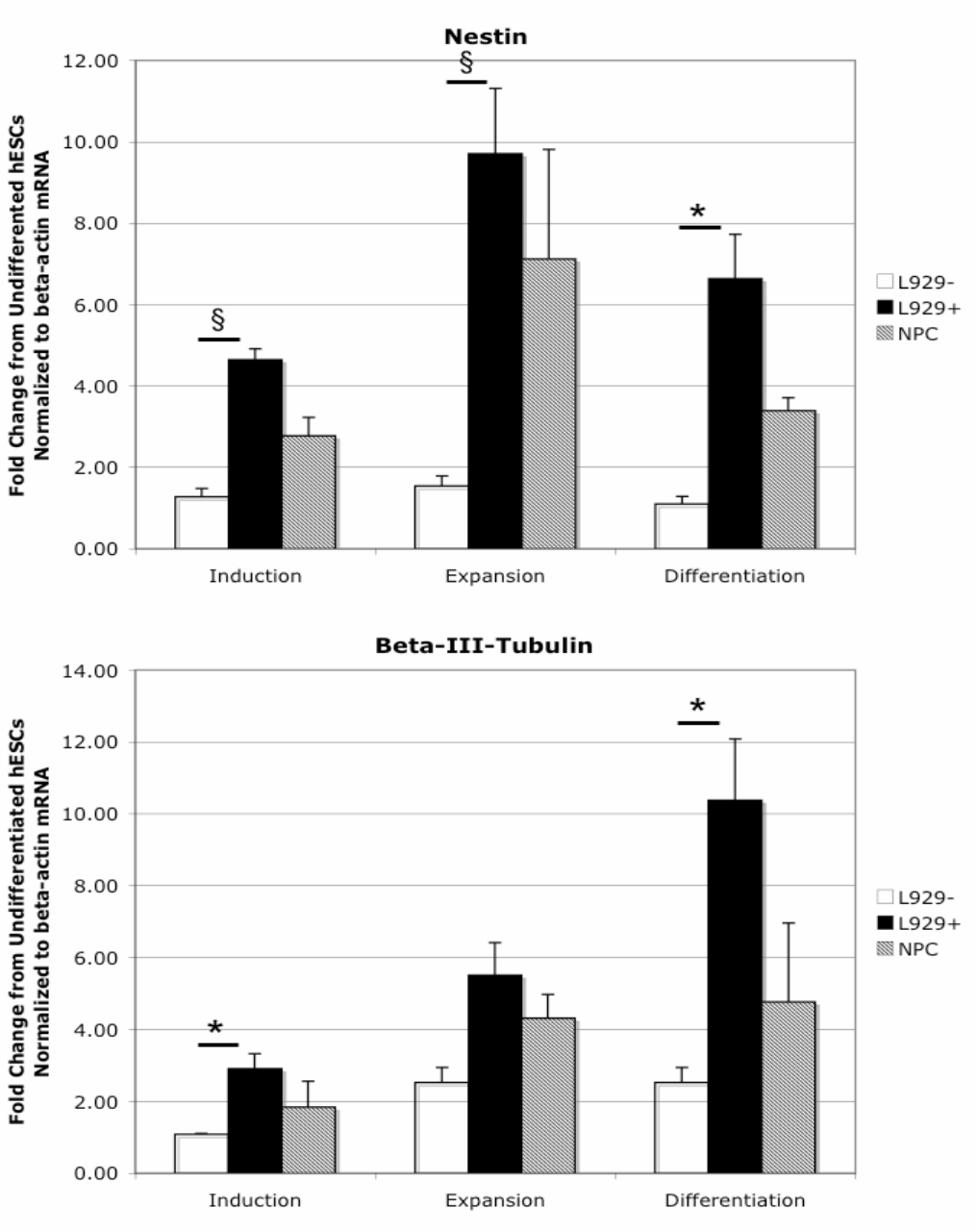

Figure 4.7.4: Progressive alteration of hESC neural differentiation in three phases

Cadherin-presenting (L929+) and cadherin deficient (L929-) induced H1 cells were compared to neural restricted progenitor cells (NPC) [181]. Each phase

(induction, expansion and differentiation) was evaluated for nestin and beta-III-tubulin expression. Levels of mRNA expression were normalized to beta-actin and fold change was determined from undifferentiated cells. In all phases, L929+ cultures achieved greater levels of differentiation compared to NPCs while L929- cultures were unaffected. (* denotes $p<0.05$, § denotes $p<0.01$.)

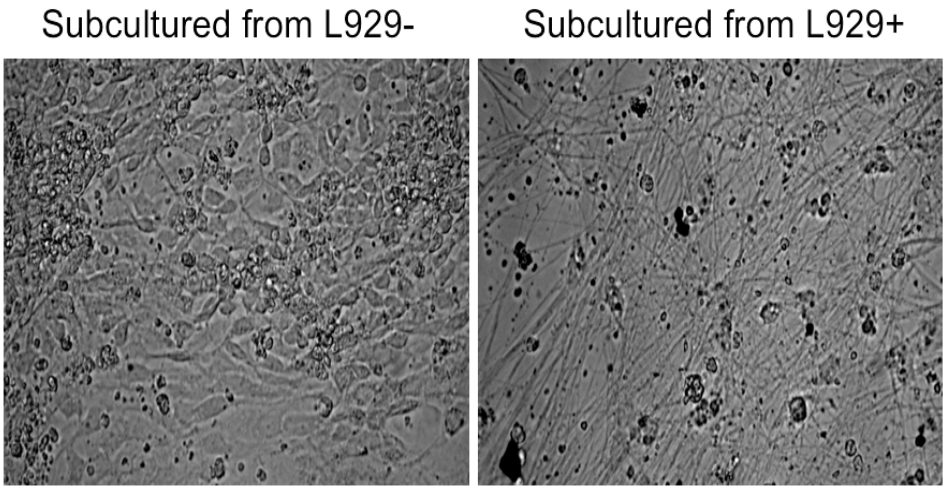

Figure 4.7.5: Morphology of neuronally derived hESCs following growth factor stimulation

Morphology of cultures was evaluated following L929 induction, NPM expansion and NDM differentiation phases. Cells that were subcultured from cadherin deficient fibroblasts retained a compact, densely packed morphology, whereas cells from cadherin-presenting fibroblasts resulted in elongated, dendritic morphology, indicative of neurons.

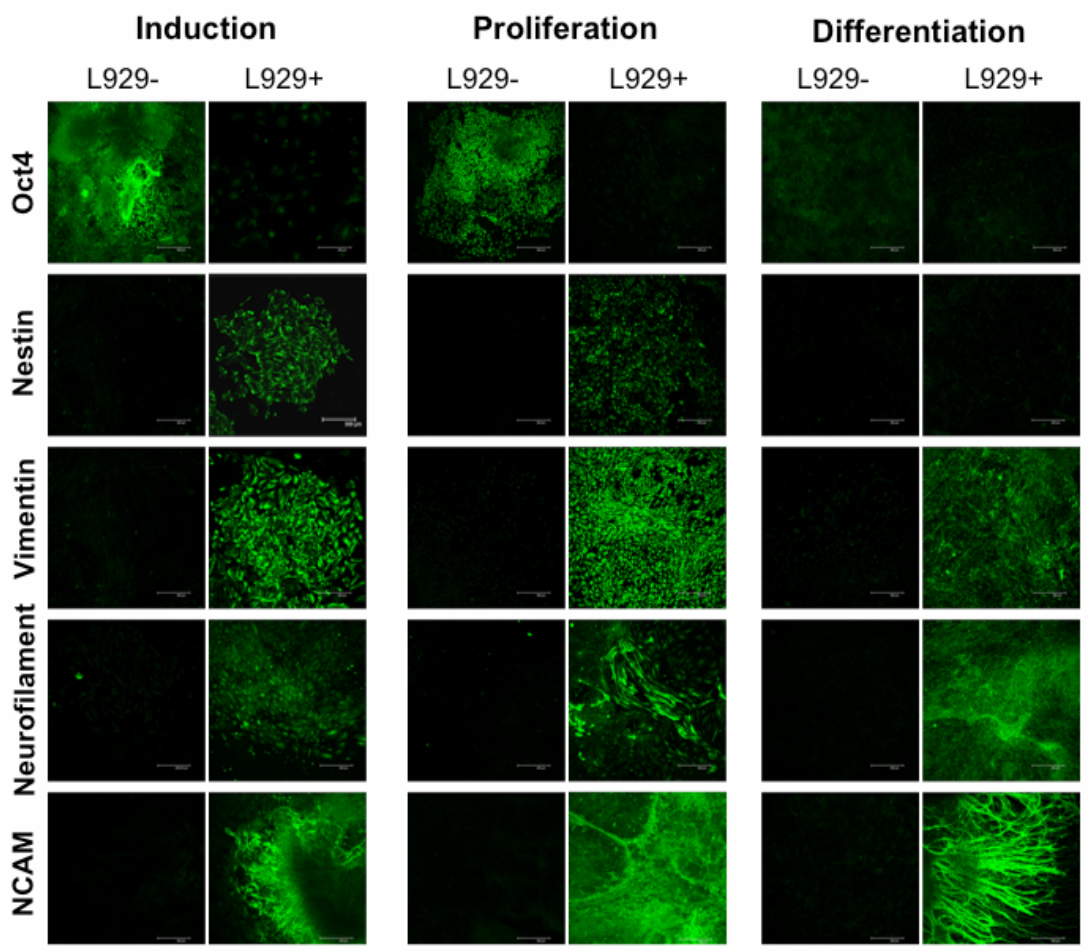

Figure 4.7.6: Immunocytostaining of neural family markers

Various markers to evaluate the extent of neural differentiation were evaluated during all three phases. Oct4 expression was not detected in L929+ cultures, but L929- cultures showed Oct4 expression detectable in the first 2 phases, but waning in the differentiation phase. Nestin expression declines over time in the L929+ cultures and is associated with positive vimentin, neurofilament and neural cell adhesion molecule (NCAM) expression throughout differentiation indicating that E-cadherin induction results in neural differentiation of hESCs. All neural markers are negative in the L929- cultures regardless of the phase.

CHAPTER 5

RESEARCH SUMMARY AND FUTURE DIRECTIONS

5.1 Research Summary

This thesis focused on studies of molecular and microscale cues to differentiate embryonic stem cells. The molecular cues involved in the directed differentiation of human and mouse embryonic cells into different lineages remains to be elucidated, though interactions between cells are critical to the maintenance of cell function in culture [202]. As a result, innovative culture systems have become more widely employed to explore the effects of spatial growth, spatial contact inhibition, transport phenomena, and cell–cell communication. Here, we examined three approaches to induce directed differentiation of ES cells. First, we investigated the effects of co-cultured hepatocytes to induce hepatospecific differentiation of mouse ES cells that were genetically engineered to present E-cadherin. Secondly, we created an efficient growth factor process to directly differentiate hESCs into hepatic-like cells that bypasses embryoid body development. Lastly, we discovered a novel strategy to differentiate hESCs into neural stem cells that can be further differentiated into neuronal restricted cells by presenting E-cadherin to the surface.

A primary conclusion of this work is that E-cadherin can be used as a basis to engineer cellular differentiation into hepatic and neural lineages, when

combined with growth factor stimulation. The role of E-cadherin expression was shown to be an effective tool to induce hepatotrophic differentiation when combining paracrine and juxtacrine interactions with primary hepatocytes. Direct cell-cell contact, mediated through E-cadherin engagement, drives differentiated cell phenotype and functional cell abilities. The factors that promote directed differentiation are growth factor priming of stem cells, cadherin presentation and microscale cues.

We chose to switch from the mouse ES system to human ES system and decouple the E-cadherin/growth factor stimulation effects. First, we determined the optimal growth factor stimulation needed to induce hepatotrophic differentiation of hESCs. We were able to bypass the EB process and incorporated identical mouse ES growth factor stimulation (DOH) with the addition of activin A pre-treatment. The hESC-derived hepatocyte-like cells resulting from optimal growth factor combinations ($A_{0\text{-}4}$DOH) exhibit characteristic hepatocyte morphology, express hepatocyte markers and possess hepatospecific functional activity. Secondly, we investigated the effect of E-cadherin on hESC differentiation. To our surprise, we were able to rapidly and efficiently differentiate hESCs into neural stem cells by presenting undifferentiated hESCs with E-cadherin via E-cadherin expressing fibroblasts. Furthermore, these cells are able to achieve neuronal differentiation with the addition of exogenous growth factors.

5.2 Future Directions

5.2.1 Three-dimensional systems

The future of stem cell engineering will lie in multi-dimensional systems that can present all of the necessary cues to induce differentiation. There has been recent evidence of the success of three-dimensional systems for ES cell proliferation and differentiation. Studies using mouse ES cells have demonstrated an up-regulation of gene expression involved in hematopoietic differentiation in a three-dimensional culture system compared to a traditional two-dimensional culture system. Furthermore, cells grown in a three-dimensional system displayed increased cell adhesion and ECM production [203]. Other groups have also been able to optimize conditions for neuronal differentiation using embryoid bodies embedded in a fibrin scaffold [204].

While mouse models have been employed for investigating differentiation into numerous lineages including adipocytes [205], neurons and astrocytes [204], and hepatocytes [206], few attempts have been made to create three-dimensional culture systems for hESCs, indicating a need to draw parallels between the two systems. Numerous scaffolds have been characterized to find a potential biocompatible scaffold that would support hESC survival, proliferation and differentiation. Various chemical compositions have been explored to optimize a polymer scaffold surface chemistry that would allow for effective integration and survival of hESCs [207]. Additionally, protein-derived meshes or scaffolds have

been developed to support mesenchymal stem cell (MSC) adhesion, growth, and differentiation [208].

5.2.2 External Cues

In order to control and regulate the differentiation of ES cells, several external cues are required such as growth factors, transcription factors, as well as cell adhesion molecules [209-212]. Additionally, the mechanical properties of the growth substrate will also have an impact on cellular behavior [213]. The control and regulation of cellular adhesion, growth and cellular function can be modulated significantly by presenting and controlling the biological signals at the nanoscale level [214-216]. Building upon the result that E-cadherin can induce neural differentiation of hESCs, it would be necessary to present acellular E-cadherin to the surface of feeder free H9 hESCs, either through conjugation to nanoparticles or microbeads. It is unlikely that E-cadherin alone would be sufficient to induce neuronal differentiation; therefore, it is necessary to determine the exact components the L929 fibroblasts contribute to the microenvironment. Also, cadherin clustering may be a way to further augment the differentiation process associated with cell signaling pathways.

Exogenous growth factor supplementation was heavily used in this work, but the list is not exhausted. We chose to use growth factors that were optimal for mouse hepatic differentiation to determine if there were parallels to the hESC system. We only chose to investigate the addition of 2 growth factors, but other signals associated with the Wnt/beta-catenin pathway, such as Activin/Nodal

and BMP, will likely play a role in differentiation. These signals should be exploited with pulsing techniques to determine the optimal growth factor regime to induce differentiation.

The degree of cell-substrate interactions and cell-cell contact establishment may cooperatively influence the differentiation of progressive cell generations in ES cultures. The combined techniques that allow cooperative interactions between ECM molecules, growth factor stimulation, cell-cell and cell-surface interactions in a three dimensional culture system will create a system where the selection of particular ECM molecules and growth factors can trigger a given differentiation pathway. This will be dependent on the cell type desired and in system that resembles a three dimensional native ECM more so than a 2D culture system. Finally, this system could provide a flexible platform that can be used in the differentiation of multiple cell lineages using temporal and spatial matrix/growth factor interactions. The hESCs, once differentiated and integrated within the microscale scaffolds, may be used as a differentiated tissue-equivalent for future implantation or pharmacologic analysis.

REFERENCES

1. Choi D, O.H., Chang UJ, Koo SK, Jiang JX, Hwang SY, Lee JD, Yeoh GC, Shin HS, Lee JS, Oh B, *In vivo differentiation of mouse embryonic stem cells into hepatocytes.* Cell Transplant, 2002. **11**(4): p. 359-68.

2. Hu, A., J. Cai, Q. Zheng, X. He, Y. Shan, Y. Pan, G. Zeng, A. Hong, Y. Dai, and L. Li, *High-ratio differentiation of embryonic stem cells into hepatocytes in vitro.* Liver Int., 2004. **24**(3): p. 237-45.

3. Shirahashi H, W.J., Yamamoto N, Catana A, Wege H, Wager B, Okita K, Zern MA, *Differentiation of human and mouse embryonic stem cells along a hepatocyte lineage.* Cell Transplant, 2004. **13**(3): p. 197-211.

4. Kubo, A., K. Shinozaki, J.M. Shannon, V. Kouskoff, M. Kennedy, S. Woo, H.J. Fehling, and G. Keller, *Development of definitive endoderm from embryonic stem cells in culture.* Development, 2004. **131**(7): p. 1651-62.

5. Reubinoff, B.E., M.F. Pera, C.Y. Fong, A. Trounson, and A. Bongso, *Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro.* Nat Biotechnol, 2000. **18**(4): p. 399-404.

6. Thomson, J.A., J. Itskovitz-Eldor, S.S. Shapiro, M.A. Waknitz, J.J. Swiergiel, V.S. Marshall, and J.M. Jones, *Embryonic stem cell lines derived from human blastocysts.* Science, 1998. **282**(5391): p. 1145-7.

7. Sato, N., L. Meijer, L. Skaltsounis, P. Greengard, and A.H. Brivanlou, *Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor.* Nat Med, 2004. **10**(1): p. 55-63.

8. Selden, C. and H. Hodgson, *Cellular therapies for liver replacement.* Transpl Immunol, 2004. **12**(3-4): p. 273-288.

9. Strain, A.J. and J.M. Neuberger, *A Bioartificial liver -- State of the Art.* Science, 2002. **295**: p. 1005-1009.

10. Harper, A.M. and J.D. Rosendale, *The UNOS OPTN waiting list and donor registry.* Clin Transplant., 1996. **10**: p. 69-90.

11. Susick, R., N. Moss, H. Kubota, E. Lecluyse, G. Hamilton, T. Luntz, J. Ludlow, J. Fair, D. Gerber, K. Bergstrand, J. White, A. Bruce, O. Drury, S. Gupta, and L.M. Reid, *Hepatic Progenitors and Strategies for Liver Cell Therapies.* Ann. N.Y.Acad. Sci., 2001. **944**: p. 398-419.

12. Kobayashi, N., T. Fujiwara, K.A. Westerman, Y. Inoue, M. Sakaguchi, H. Noguchi, M. Miyazaki, J. Cai, N. Tanaka, I.J. Fox, and P. Leboulch, *Prevention of acute liver failure in rats with reversibly immortalized human hepatocytes.* Science, 2000. **287**: p. 1258-1262.

13. Strom, S., J. Chowdhury, and I. Fox, *Hepatocyte transplantation for the treatment of human disease.* Semin. Liver Dis., 1999. **19**: p. 39-48.

14. Malhi, H. and S. Gupta, *Hepatocyte transplantation: new horizons and challenges.* J. Hepatobiliary Pancreat Surg, 2001. **8**: p. 40-50.

15. Fox, I., J. Chowdhury, S.S. Kaufman, T.C. Goertzen, N.R. Chowdhury, P.I. Warkentin, K. Dorko, B.V. Sauter, and Stron, *Treatment of the Crigler-Najjar syndrome type I with hepatocyte transplantation.* N. Engl. J. Med., 1998. **338**: p. 1422-1426.

16. Griffith, L.G. and G. Naughton, *Tissue Engineering-- Current challenges and expanding opportunities.* Science, 2002. **295**: p. 1009-1014.

17. Allen, J.W. and S.N. Bhatia, *Engineering Liver Therapies for the Future.* Tissue Engineering, 2002. **8**(5): p. 725-737.

18. Harries, H.M., S.T. Fletcher, C.M. Duggan, and V.A. Baker, *The use of genomics technology to investigate gene expression changes in cultured human liver cells.* Toxicology in Vitro, 2001. **15**: p. 399-405.

19. Modriansky, M., J. Ulrichova, P. Bachleda, P. Anzenbacher, E. Anzenbacherova, D. Walterova, and V. Simanek, *Human hepatocyte--a model for toxicological studies. Functional and biochemical characterization.* General Physiol. & Biophysics., 2000. **19**: p. 223-235.

20. Bellamkonda R, A.P., *Review: Tissue engineering in the nervous system.* biotechnol Bioeng, 1994. **43**(7): p. 543-54.

21. Garbossa, D.F., M.; Fronda, C.; Benevello, C.; Muraca, G.; Ducati, A.; Vercelli, A., *New strategies for repairing the injured spinal cord: the role of stem cells.* Neurological Research, 2006. **28**(5): p. 500-505.

22. Chinzei, R., Tanaka, Y., Shimizu-Saito, K. et al., *Embyroid-body cell derived from a mouse embryonic stem cell lines show differentiation into functional hepatocytes.* Hepatology, 2002. **36**: p. 22-29.

23. Jochheim, A., Hillemann, T., Kania, G., Scharf, J., Attaran, M., Manns, MP., Wobus, AM., Ott, M., *Quantitative gene expression profiling reveals a fetal*

hepatic phenotype of murine ES-derived hepatocytes. Int. J. Dev. Biol., 2004. **48**: p. 23-29.

24. Kania, G., Blyszczuk, P., Jochheim, A., Ott, M., Wobus, AM., *Generation of glycogen- and albumin-producing hepatocyte-like cells from embryonic stem cells.* Biol Chem, 2004. **385**(10): p. 943-53.

25. Miyashita, H., A. Suzuki, K. Fukao, H. Nakauchi, and H. Taniguchi, *Evidence for hepatocyte differentiation from embryonic stem cells in vitro.* Cell Transplant, 2002. **11**(5): p. 429-34.

26. Jones, E., Tosh, D., Wilson, DI., Lindsay, S., Forrester, LM., *Hepatic Differentiation of Murine Embryonic Stem Cells.* Exp Cell Res, 2002. **272**: p. 15-22.

27. Kojima, N., Kinoshita, T., Kamiya, A., Nakamura, K., Nakashima, K., Taga, T., Miyajima, A., *Cell Density-Dependent Regulation of Hepatic Development by a gp130-Independent Pathway.* Biochemical and Biophysical Research Communications, 2000. **277**: p. 152-158.

28. Issaad, C., Croisille, L., Katz, A., Vainchenker, W., Coulombel, L., *A murine stromal cell line allows the proliferation of very primitive human CD34++/CD38- progenitor cells in long-term cultures and semisolid assays.* Blood, 1993. **81**: p. 2916-2924.

29. Nakamura, Y., Ando, K., Chargui, J., Kawada, H., Sato, T., Tsuji, T., Hotta, T., Kato, S., *Ex vivo generation of CD34(+) from CD34(-) hematopoietic cells.* Blood, 1999. **94**: p. 4053-4059.

30. Mizuguchi, T., Hui, T., Palm, K., Sugiyama, N., Mitaka, T., Demetriou, AA., Rozga, J., *Enchanced Proliferation and Differentiation of Rat Hepatocytes Cultured with Bone Marrow Stromal Cells.* J Cell Physio, 2001. **189**: p. 106-119.

31. Takeichi, M., *Cadherin cell adhesion receptors as a morphogenetic regulator.* Science, 1991. **251**: p. 1451-1455.

32. Steinberg, M.S. and P.M. McNutt, *Cadherins and their connections: adhesion junctions have broader functions.* Curr Opin Cell Biol, 1999. **11**(5): p. 554-60.

33. Omelchenko, T., E. Fetisova, O. Ivanova, E.M. Bonder, H. Feder, J.M. Vasiliev, and I.M. Gelfand, *Contact interactions between epitheliocytes and fibroblasts: Formation of heterotypic cadherin-containing adhesion sites is accompanied by local cytoskeletal reorganization.* Proc. Natl. Acad. Sci. USA, 2001. **98**: p. 8632-8637.

34. Bhatia, S.N., U.J. Balis, M.L. Yarmush, and M. Toner, *Effect of cell-cell interactions in preservation of cellular phenotype: cocultivation of hepatocytes and nonparenchymal cells.* Faseb J, 1999. **13**(14): p. 1883-900.

35. Vleminckx, K. and R. Kemler, *Cadherins and tissue formation: integrating adhesion and signaling.* Bioessays, 1999. **21**(3): p. 211-20.

36. Shtutman, M., J. Zhurinsky, I. Simcha, C. Albanese, M. D'Amico, R. Pestell, and A. Ben-Ze'ev, *The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway.* Proc Natl Acad Sci U S A, 1999. **96**(10): p. 5522-7.

37. Barker, N., P.J. Morin, and H. Clevers, *The Yin-Yang of TCF/beta-catenin signaling.* Adv Cancer Res, 2000. **77**: p. 1-24.

38. Brieva, T. and P. Moghe, *Functional engineering of hepatocytes via heterotypic presentation of a homoadhesive molecule, E-cadherin.* Biotechnology and Bioengineering, 2001. **76**: p. 295.

39. Brieva, T. and P. Moghe, *Engineering the Hepatocyte Differentiation/Proliferation Balance by Acellular Cadherin Micropresentation.* Tissue Engineering, 2004. **10**: p. 553-564.

40. Brieva, T. and P.V. Moghe, *Exogenous Cadherin Microdisplay can Interfere with Endogenous Signaling and Reprogram Gene Expression in Cultured Hepatocytes.* Biotechnol. Bioeng., 2004. **85**: p. 359-369.

41. Semler, E.J., A. Dasgupta, and P.V. Moghe, *Cytomimetic engineering of hepatocyte morphogenesis and function by substrate-induced presentation of acellular E-cadherin.* Tissue Engineering, 2005. **In Press**.

42. Feng, Z., A.S. Srivastava, R.A. Mishra, and E. Carriera, *A regulatory role of Wnt signaling pathway in the hematopoietic differentiation of murine embryonic stem cells.* Biochemical and Biophysical Research Communications, 2004. **324**: p. 1333-1339.

43. Brandenberger, R., H. Wei, S. Zhang, S. Lei, J. Murage, G.J. Fisk, Y. Li, C. Xu, R. Fang, K. Guegler, M.S. Rao, R. Mandalam, J. Lebkowski, and L.W. Stanton, *Transcriptome characterization elucidates signaling networks that control human ES growth and differentiation.* Nature Biotechnology, 2004. **22**: p. 707-716.

44. Duncan, S., *Transcriptional Regulation of Liver Development.* Developmental Dynamics, 2000. **219**: p. 131-142.

45. Duncan, S., *Mechanisms controlling early development of the liver.* Mechanisms of Development, 2003. **120**: p. 19-33.

46. Rossi, J., Dunn, NR., Hogan, BLM>, Zaret, KS., *Distinct mesodermal signals, including BMPs from the septum trnsversum mesenchyme, are required combination for hepatogenesis.* Genes & Development, 2001. **15**: p. 1998-2009.

47. Michalopoulos, G., Bowen, WC., Mule, K., Luo, J., *HGF-, EGF-, and dexamethasone-induced gene expression patterns during formation of tissue in hepatic organoid cultures.* Gene Expr, 2003. **11**(2): p. 55-75.

48. Dasgupta, A., R. Hughey, P. Lancin, L. Larue, and P. Moghe, *E-cadherin synergistically induces hepatospecific phenotype and maturation of embryonic stem cells in conjunction with hepatotrophic factors.* Biotechnol. Bioeng., 2005. **92**(3): p. 257-66.

49. Zaret, K.S., *Hepatocyte differentiation: from the endoderm and beyond.* Curr Opin Genet Dev, 2001. **11**: p. 568-574.

50. Cascio, S., Zaret, KS., *Hepatocyte differentiation initiates during endodermal-mesenchymal interactions prior to liver formation.* Development, 1991. **113**(1): p. 217-25.

51. Mukouyama, Y., Hara, T., Xu, M., Tamura, K., Donovan, PJ., Kim, H., Kogo, H., Tsuji, K., Nakahata, T., Miyajima, A., *In vitro expansion of murine multipotential hematopoietic progenitors from the embryonic aorta-gonad-mesonephros region.* Immunity, 1998. **8**(1): p. 105-14.

52. Kamiya, A., T. Kinoshita, and A. Miyajima, *Oncostatin M and hepatocyte growth factor induce hepatic maturation via distinct signaling pathways.* FEBS Letts., 2001. **492**: p. 90-94.

53. Kamiya, A., T. Kinoshita, Y. Ito, T. Matsui, Y. Morikawa, E. Senba, K. Nakashima, T. Taga, K. Yoshida, T. Kishimoto, and A. Miyajima, *Fetal liver development requires a paracrine action of oncostatin M through the gp130 signal transducer.* EMBO Journal, 1998. **18**: p. 2127-2136.

54. Schuldiner, M., O. Yanuka, J. Itskovitz-Eldor, D.A. Melton, and N. Benvenisty, *Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells.* Proc Natl Acad Sci, 2000. **97**: p. 11307-12.

55. Huh, C.G., V.M. Factor, A. Sanchez, K. Uchida, E.A. Conner, and S.S. Thorgeirsson, *Hepatocyte growth factor/c-met signaling pathway is required for efficient liver regeneration and repair.* Proc Natl Acad Sci, 2004. **101**: p. 4477-4482.

56. Birchmeier, C. and E. Gherardi, *Developmental roles of HGF/SF and its receptor, c-Met tyrosine kinase.* Trends in Cell Biol., 1998. **8**: p. 404-410.

57. Michalopoulos, G., K. and M.C. DeFrances, *Liver Regeneration.* Science, 1997. **276**: p. 60-66.

58. Zhang, Y.W. and G.F. Woude, *HGF/SF-met signaling in the control of branching morphogenesis and invasion.* J. Cell. Biochem., 2003. **88**: p. 408-417.

59. Auer, K., Contessa, J., Brenz-Verca, S., Pirola, L., Rusconi, S., Cooper, G., Abo, A., Wymann, MP., Davis, RJ., Birrer, M., Dent. P., *The Ras/Rac1/Cdc42/SEK/JNK/c-Jun Cascade is a Key Pathway by Which Agonists Stimulate DNA Synthesis in Primary Cultures of Rat Hepatocytes.* Mol. Biol. Cell, 1998. **9**: p. 561-573.

60. Gomez-Lechon, M., Guillen, I., Ponsoda, X., Fabra, R., Trullenque, R., Nakamura, T., Castell, JV., *Cell Cycle Progression Proteins (Cyclins), Oncogene Expression, and Signal Transduction During the Proliferative Response of Human Hepatocytes to Hepatocyte Growth Factor.* Hepatology, 1996. **23**: p. 1012-1019.

61. Behrens, S., Sibilia, M., David, JP., Mohle-Steinlein, U., Tronche, F., Schutz, G., Wagner, EF., *Impaired postnatal hepatocyte proliferation and liver regeneration in mice lacking c-jun in the liver.* EMBO Journal, 2002. **21**: p. 1782-1790.

62. Kinoshita, T., Miyajima, A., *Cytokine regulation of liver development.* Biochimica et Biophysica Acta, 2002. **1592**: p. 303-312.

63. Suzuki, A., A. Iwama, H. Miyashita, H. Nakauchi, and H. Taniguchi, *Role for growth factors and extracellular matrix in controlling differentiation of prospectively isolated hepatic stem cells.* Development, 2003. **130**: p. 2513-24.

64. Kamiya, A., Kojima, N., Kinoshita, T., Sakai, Y., Miyajima, A., *Maturation of Fetal Hepatocytes In Vitro by Extracellular Matrices and Oncostatin M: Induction of Tryptopha Oxygenase.* Hepatology, 2002. **35**: p. 1351-1359.

65. Matsui, T., T. Kinoshita, Y. Morikawa, K. Tohya, M. Katsuki, Y. Ito, A. Kamiya, and A. Miyajima, *K-Ras mediates cytokine-induced formation of E-cadherin-based adherens junctions during liver development.* EMBO Journal, 2002. **21**: p. 1021-1030.

66. Sathananthan, H., M. Pera, and A. Trounson, *The fine structure of human embryonic stem cells.* Reprod Biomed Online, 2002. **4**(1): p. 56-61.

67. Itskovitz-Eldor, J., M. Schuldiner, D. Karsenti, A. Eden, O. Yanuka, M. Amit, H. Soreq, and N. Benvenisty, *Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers.* Mol Med, 2000. **6**(2): p. 88-95.

68. Levenberg, S., J.S. Golub, M. Amit, J. Itskovitz-Eldor, and R. Langer, *Endothelial cells derived from human embryonic stem cells.* Proc Natl Acad Sci U S A, 2002. **99**(7): p. 4391-6.

69. Pera, M.F. and A.O. Trounson, *Human embryonic stem cells: prospects for development.* Development, 2004. **131**(22): p. 5515-25.

70. Trounson, A., *Derivation characteristics and perspectives for mammalian pluripotential stem cells.* Reprod Fertil Dev, 2005. **17**(1-2): p. 135-41.

71. Carpenter, M.K., M.S. Inokuma, J. Denham, T. Mujtaba, C.P. Chiu, and M.S. Rao, *Enrichment of neurons and neural precursors from human embryonic stem cells.* Exp Neurol, 2001. **172**(2): p. 383-97.

72. Nistor, G.I., M.O. Totoiu, N. Haque, M.K. Carpenter, and H.S. Keirstead, *Human embryonic stem cells differentiate into oligodendrocytes in high purity and myelinate after spinal cord transplantation.* Glia, 2005. **49**(3): p. 385-96.

73. Park, S., K.S. Lee, Y.J. Lee, H.A. Shin, H.Y. Cho, K.C. Wang, Y.S. Kim, H.T. Lee, K.S. Chung, E.Y. Kim, and J. Lim, *Generation of dopaminergic neurons in vitro from human embryonic stem cells treated with neurotrophic factors.* Neurosci Lett, 2004. **359**(1-2): p. 99-103.

74. Perrier, A.L., V. Tabar, T. Barberi, M.E. Rubio, J. Bruses, N. Topf, N.L. Harrison, and L. Studer, *Derivation of midbrain dopamine neurons from human embryonic stem cells.* Proc Natl Acad Sci U S A, 2004. **101**(34): p. 12543-8.

75. Li, X.J., Z.W. Du, E.D. Zarnowska, M. Pankratz, L.O. Hansen, R.A. Pearce, and S.C. Zhang, *Specification of motoneurons from human embryonic stem cells.* Nat Biotechnol, 2005. **23**(2): p. 215-21.

76. Pera, M.F., J. Andrade, S. Houssami, B. Reubinoff, A. Trounson, E.G. Stanley, D. Ward-van Oostwaard, and C. Mummery, *Regulation of human embryonic stem cell differentiation by BMP-2 and its antagonist noggin.* J Cell Sci, 2004. **117**(Pt 7): p. 1269-80.

77. Mummery, C., D. Ward, C.E. van den Brink, S.D. Bird, P.A. Doevendans, T. Opthof, A. Brutel de la Riviere, L. Tertoolen, M. van der Heyden, and

M. Pera, *Cardiomyocyte differentiation of mouse and human embryonic stem cells.* J Anat, 2002. **200**(Pt 3): p. 233-42.

78. Mummery, C., D. Ward-van Oostwaard, P. Doevendans, R. Spijker, S. van den Brink, R. Hassink, M. van der Heyden, T. Opthof, M. Pera, A.B. de la Riviere, R. Passier, and L. Tertoolen, *Differentiation of human embryonic stem cells to cardiomyocytes: role of coculture with visceral endoderm-like cells.* Circulation, 2003. **107**(21): p. 2733-40.

79. Denham, M., T.J. Cole, and R. Mollard, *Embryonic stem cells form glandular structures and express surfactant protein C following culture with dissociated fetal respiratory tissue.* Am J Physiol Lung Cell Mol Physiol, 2006. **290**(6): p. L1210-5.

80. Green, H., K. Easley, and S. Iuchi, *Marker succession during the development of keratinocytes from cultured human embryonic stem cells.* Proc Natl Acad Sci U S A, 2003. **100**(26): p. 15625-30.

81. Kaufman, D.S., E.T. Hanson, R.L. Lewis, R. Auerbach, and J.A. Thomson, *Hematopoietic colony-forming cells derived from human embryonic stem cells.* Proc Natl Acad Sci U S A, 2001. **98**(19): p. 10716-21.

82. Brolen, G.K., N. Heins, J. Edsbagge, and H. Semb, *Signals from the embryonic mouse pancreas induce differentiation of human embryonic stem cells into insulin-producing beta-cell-like cells.* Diabetes, 2005. **54**(10): p. 2867-74.

83. Lavon, N., O. Yanuka, and N. Benvenisty, *Differentiation and isolation of hepatic-like cells from human embryonic stem cells.* Differentiation, 2004. **72**(5): p. 230-8.

84. Rambhatla, L., C.P. Chiu, P. Kundu, Y. Peng, and M.K. Carpenter, *Generation of hepatocyte-like cells from human embryonic stem cells.* Cell Transplant, 2003. **12**(1): p. 1-11.

85. Yan, Y., D. Yang, E.D. Zarnowska, Z. Du, B. Werbel, C. Valliere, R.A. Pearce, J.A. Thomson, and S.C. Zhang, *Directed differentiation of dopaminergic neuronal subtypes from human embryonic stem cells.* Stem Cells, 2005. **23**(6): p. 781-90.

86. Schulz, T.C., S.A. Noggle, G.M. Palmarini, D.A. Weiler, I.G. Lyons, K.A. Pensa, A.C. Meedeniya, B.P. Davidson, N.A. Lambert, and B.G. Condie, *Differentiation of human embryonic stem cells to dopaminergic neurons in serum-free suspension culture.* Stem Cells, 2004. **22**(7): p. 1218-38.

87. Broders F, T.J., *Contribution of cadherins to directional cell migration and histogenesis in Xenopus embryos.* Cell Adhes Commun., 1995. **3**(5): p. 419-40.

88. Takeichi, M., *Cadherins: a molecular family important in selective cell-cell adhesion.* Annu Rev Biochem, 1990. **59**: p. 237-52.

89. Aoyama H, D.A., Thiery JP., *Cell adhesion mechanisms in gangliogenesis studied in avian embryo and in a model system.* Cell Differ., 1985. **17**(4): p. 247-60.

90. Crossin KL, C.C., Edelman GM., *Expression sequences of cell adhesion molecules.* Proc Natl Acad Sci U S A, 1985. **82**(20): p. 6942-6.

91. Hatta K, T.M., *Expression of N-cadherin adhesion molecules associated with early morphogenetic events in chick development.* Nature, 1986. **320**(6061): p. 447-9.

92. Hatta K, T.S., Fujisawa H, Takeichi M., *Spatial and temporal expression pattern of N-cadherin cell adhesion molecules correlated with morphogenetic processes of chicken embryos.* Dev Biol, 1987. **120**(1): p. 215-27.

93. Thiery JP, D.A., Grumet M, Edelma, *Initial appearance and regional distribution of the neuron-glia cell adhesion molecule in the chick embryo.* J Cell Bio, 1985. **100**(2): p. 442-56.

94. Angres B, M.A., Kellermann J, Hausen P., *Differential expression of two cadherins in Xenopus laevis.* Development, 1991. **111**(3): p. 829-44.

95. Choi YS, G.B., *Expression of cell adhesion molecule E-cadherin in Xenopus embryos begins at gastrulation and predominates in the ectoderm.* J Cell Bio, 1989. **108**(6): p. 2449-58.

96. Health, N.I.o., *Stem Cell Basics.* In Stem Cell Information, 2008.

97. Alattia JR, K.H., Ikura M, *Structural view of cadherin-mediated cell-cell adhesion.* Cell Mol Life Sci, 1999. **55**(3): p. 359-67.

98. Gumbiner, B.M., *Signal transduction of beta-catenin.* Curr Opin Cell Biol, 1995. **7**(5): p. 634-40.

99. Orsulic, S., O. Huber, H. Aberle, S. Arnold, and R. Kemler, *E-cadherin binding prevents beta-catenin nuclear localization and beta-catenin/LEF-1-mediated transactivation.* J Cell Sci, 1999. **112**(Pt 8): p. 1237-45.

100. Howard, J.C., V.M. Varallo, D.C. Ross, J.H. Roth, K.J. Faber, B. Alman, and B.S. Gan, *Beta-catenin Mediated Wnt Signaling as a Marker for Characterization of Human Bone Marrow-Derived Connective Tissue Progenitor Cells.* BMC Musculoskelet Disord., 2003. **4**: p. 16.

101. Aberle, H., A. Bauer, J. Stappert, A. Kispert, and R. Kemler, *E-cadherin binding prevents beta-catenin nuclear localization and beta-catenin/LEF-1-mediated transactivation.* J. Cell Sci., 1999. **112**: p. 1237-1245.

102. Dasgupta, A., R. Hughey, P. Lancin, L. Larue, and P.V. Moghe, *E-cadherin synergistically induces hepatospecific phenotype and maturation of embryonic stem cells in conjunction with hepatotrophic factors.* Biotechnol Bioeng, 2005. **92**(3): p. 257-66.

103. Fuchs, E., Segre, JA., *Stem cells: a new lease on life.* Cell, 2000. **100**(1): p. 143-55.

104. Gonzalez-Reyes, A., *Stem Cells, niches and cadherins: a view from Drosophila.* J Cell Sci, 2003. **116**: p. 949-954.

105. McKay, R., *Stem cells - Hype and Hope.* Nature, 2000. **406**: p. 361-364.

106. Orkin, S.H., *Stem cell alchemy.* Nat Med, 2000. **6**(11): p. 1212-3.

107. Bhatia, S.N., U.J. Balis, M.L. Yarmush, and M. Toner, *Probing heterotypic cell interactions: hepatocyte function in microfabricated co-cultures.* J Biomater Sci Polym Ed, 1998. **9**(11): p. 1137-60.

108. Ball, S.G., A.C. Shuttleworth, and C.M. Kielty, *Direct cell contact influences bone marrow mesenchymal stem cell fate.* Int J Biochem Cell Biol, 2004. **36**(4): p. 714-27.

109. Kii, I., N. Amizuka, J. Shimomura, Y. Saga, and A. Kudo, *Cell-cell interaction mediated by cadherin-11 directly regulates the differentiation of mesenchymal cells into the cells of the osteo-lineage and the chondro-lineage.* J Bone Miner Res, 2004. **19**(11): p. 1840-9.

110. Liu, N., M. Lu, X. Tian, and Z. Han, *Molecular mechanisms involved in self-renewal and pluripotency of embryonic stem cells.* J Cell Physiol, 2007. **211**(2): p. 279-86.

111. Rudy-Reil, D. and J. Lough, *Avian precardiac endoderm/mesoderm induces cardiac myocyte differentiation in murine embryonic stem cells.* Circ Res, 2004. **94**(12): p. e107-16.

112. Sui, Y., T. Clarke, and J.S. Khillan, *Limb bud progenitor cells induce differentiation of pluripotent embryonic stem cells into chondrogenic lineage.* Differentiation, 2003. **71**(9-10): p. 578-85.

113. Lamy, I., A. Corlu, and C. Guguen-Guillouzo, *[Differentiation of hepatic and hematopoietic stem cells: study of liver regulation protein (LRP)].* Ann Pharm Fr, 2000. **58**(4): p. 260-5.

114. Soto-Gutierrez, A., N. Navarro-Alvarez, D. Zhao, J.D. Rivas-Carrillo, J. Lebkowski, N. Tanaka, I.J. Fox, and N. Kobayashi, *Differentiation of mouse embryonic stem cells to hepatocyte-like cells by co-culture with human liver nonparenchymal cell lines.* Nat Protoc, 2007. **2**(2): p. 347-56.

115. Nakamura, Y., K. Ando, J. Chargui, H. Kawada, T. Sato, T. Tsuji, T. Hotta, and S. Kato, *Ex vivo generation of CD34(+) cells from CD34(-) hematopoietic cells.* Blood, 1999. **94**(12): p. 4053-9.

116. Mizuguchi, T., T. Hui, K. Palm, N. Sugiyama, T. Mitaka, A.A. Demetriou, and J. Rozga, *Enhanced proliferation and differentiation of rat hepatocytes cultured with bone marrow stromal cells.* J Cell Physiol, 2001. **189**(1): p. 106-19.

117. Koller, M.R., I. Manchel, and B.O. Palsson, *Importance of parenchymal:stromal cell ratio for the ex vivo reconstitution of human hematopoiesis.* Stem Cells, 1997. **15**(4): p. 305-13.

118. Prockop, D.J., *Marrow stromal cells as stem cells for nonhematopoietic tissues.* Science, 1997. **276**(5309): p. 71-4.

119. Weimar, I.S., C. Voermans, J.H. Bourhis, N. Miranda, P.C. van den Berk, T. Nakamura, G.C. de Gast, and W.R. Gerritsen, *Hepatocyte growth factor/scatter factor (HGF/SF) affects proliferation and migration of myeloid leukemic cells.* Leukemia, 1998. **12**(8): p. 1195-203.

120. Mitaka, T., F. Sato, T. Mizuguchi, T. Yokono, and Y. Mochizuki, *Reconstruction of hepatic organoid by rat small hepatocytes and hepatic nonparenchymal cells.* Hepatology, 1999. **29**(1): p. 111-25.

121. Kamiya, A., N. Kojima, T. Kinoshita, Y. Sakai, and A. Miyaijma, *Maturation of fetal hepatocytes in vitro by extracellular matrices and oncostatin M: induction of tryptophan oxygenase.* Hepatology, 2002. **35**(6): p. 1351-9.

122. Albrecht, D.R., G.H. Underhill, T.B. Wassermann, R.L. Sah, and S.N. Bhatia, *Probing the role of multicellular organization in three-dimensional microenvironments.* Nat Methods, 2006. **3**(5): p. 369-75.

123. Yoshimizu, T., M. Obinata, and Y. Matsui, *Stage-specific tissue and cell interactions play key roles in mouse germ cell specification.* Development, 2001. **128**(4): p. 481-90.

124. Buttery, L.D., S. Bourne, J.D. Xynos, H. Wood, F.J. Hughes, S.P. Hughes, V. Episkopou, and J.M. Polak, *Differentiation of osteoblasts and in vitro bone formation from murine embryonic stem cells.* Tissue Eng, 2001. **7**(1): p. 89-99.

125. Hao, H., Zhao, J, Thomas, RL, Parker, GC, Lyman, WD., *Fetal human hematopoietic stem cells can differentiate sequentially into neural stem cells and then astrocytes in vitro.* J Hematother Stem Cell Res, 2003. **12**(1): p. 23-32.

126. Schumm, M.A., D.A. Castellanos, B.R. Frydel, and J. Sagen, *Enhanced viability and neuronal differentiation of neural progenitors by chromaffin cell co-culture.* Brain Res Dev Brain Res, 2002. **137**(2): p. 115-25.

127. Wan, H., Y.H. An, M.Z. Sun, Y.Z. Zhang, and Z.C. Wang, *Schwann cells transplantation promoted and the repair of brain stem injury in rats.* Biomed Environ Sci, 2003. **16**(3): p. 212-8.

128. Fair, J.H., B.A. Cairns, M. Lapaglia, J. Wang, A.A. Meyer, H. Kim, S. Hatada, O. Smithies, and L. Pevny, *Induction of hepatic differentiation in embryonic stem cells by co-culture with embryonic cardiac mesoderm.* Surgery, 2003. **134**(2): p. 189-96.

129. Larue, L., C. Antos, S. Butz, O. Huber, V. Delmas, M. Dominis, and R. Kemler, *A role for cadherins in tissue formation.* Development, 1996. **122**(10): p. 3185-94.

130. Hamazaki, T., Y. Iiboshi, M. Oka, P.J. Papst, A.M. Meacham, L.I. Zon, and N. Terada, *Hepatic maturation in differentiating embryonic stem cells in vitro.* FEBS Lett, 2001. **497**(1): p. 15-9.

131. Metzger, J.M., W.I. Lin, and L.C. Samuelson, *Transition in cardiac contractile sensitivity to calcium during the in vitro differentiation of mouse embryonic stem cells.* J Cell Biol, 1994. **126**(3): p. 701-11.

132. Hu, A.B., J.Y. Cai, Q.C. Zheng, X.Q. He, Y. Shan, Y.L. Pan, G.C. Zeng, A. Hong, Y. Dai, and L.S. Li, *High-ratio differentiation of embryonic stem cells into hepatocytes in vitro.* Liver Int, 2004. **24**(3): p. 237-45.

133. Kamiya, A., T. Kinoshita, Y. Ito, T. Matsui, Y. Morikawa, E. Senba, K. Nakashima, T. Taga, K. Yoshida, T. Kishimoto, and A. Miyajima, *Fetal liver development requires a paracrine action of oncostatin M through the gp130 signal transducer.* Embo J, 1999. **18**(8): p. 2127-36.

134. Kamiya, A., T. Kinoshita, and A. Miyajima, *Oncostatin M and hepatocyte growth factor induce hepatic maturation via distinct signaling pathways.* FEBS Lett, 2001. **492**(1-2): p. 90-4.

135. Kinoshita, T. and A. Miyajima, *Cytokine regulation of liver development.* Biochim Biophys Acta, 2002. **1592**(3): p. 303-12.

136. Berhiaume, F., Tompkins, RG., Yarmush, ML., ed., *Tissue Engineering Methods and Protocols*. Tissue Engineering Methods and Protocols. Vol. 18. 1999, Totowa: Humana Press. 447-456.

137. Seglen, P.O., *Preparation of isolated rat liver cells.* Methods Cell Biol, 1976. **13**: p. 29-83.

138. Folch, A., B.H. Jo, O. Hurtado, D.J. Beebe, and M. Toner, *Microfabricated elastomeric stencils for micropatterning cell cultures.* J Biomed Mater Res, 2000. **52**(2): p. 346-53.

139. Maguire, T., E. Novik, R. Schloss, and M. Yarmush, *Alginate-PLL microencapsulation: effect on the differentiation of embryonic stem cells into hepatocytes.* Biotechnol Bioeng, 2006. **93**(3): p. 581-91.

140. Dang, S.M., M. Kyba, R. Perlingeiro, G.Q. Daley, and P.W. Zandstra, *Efficiency of embryoid body formation and hematopoietic development from embryonic stem cells in different culture systems.* Biotechnol Bioeng, 2002. **78**(4): p. 442-53.

141. Kuai, X.L., X.Q. Cong, X.L. Li, and S.D. Xiao, *Generation of hepatocytes from cultured mouse embryonic stem cells.* Liver Transpl, 2003. **9**(10): p. 1094-9.

142. He, Z.P., W.Q. Tan, Y.F. Tang, and M.F. Feng, *Differentiation of putative hepatic stem cells derived from adult rats into mature hepatocytes in the presence of epidermal growth factor and hepatocyte growth factor.* Differentiation, 2003. **71**(4-5): p. 281-90.

143. Hu, A., J. Cai, Q. Zheng, X. He, Y. Pan, and L. Li, *Hepatic differentiation from embryonic stem cells in vitro.* Chin Med J (Engl), 2003. **116**(12): p. 1893-7.

144. Bhatia, S.N., U.J. Balis, M.L. Yarmush, and M. Toner, *Microfabrication of hepatocyte/fibroblast co-cultures: role of homotypic cell interactions.* Biotechnol Prog, 1998. **14**(3): p. 378-87.

145. Bhatia, S.N., M.L. Yarmush, and M. Toner, *Controlling cell interactions by micropatterning in co-cultures: hepatocytes and 3T3 fibroblasts.* J Biomed Mater Res, 1997. **34**(2): p. 189-99.

146. Kamei, N., Y. Oishi, N. Tanaka, O. Ishida, Y. Fujiwara, and M. Ochi, *Neural progenitor cells promote corticospinal axon growth in organotypic co-cultures.* Neuroreport, 2004. **15**(17): p. 2579-83.

147. Abouelfetouh, A., Kondoh, T, Ehara, K, Kohmura, E., *Morphological differentiation of bone marrow stromal cells into neuron-like cells after co-culture with hippocampal slice.* Brain Research, 2004. **1029**(1): p. 114-9.

148. Watabe, M., A. Nagafuchi, S. Tsukita, and M. Takeichi, *Induction of polarized cell-cell association and retardation of growth by activation of the E-cadherin-catenin adhesion system in a dispersed carcinoma line.* J Cell Biol, 1994. **127**(1): p. 247-56.

149. Gumbiner, B.M., *Cell adhesion: the molecular basis of tissue architecture and morphogenesis.* Cell, 1996. **84**(3): p. 345-57.

150. Tunggal, J., Helfrich, I, Schmitz, A, Schwarz, H, Gunzel, D, Fromm, M, Kemle, R, Krieg, T, Niessen, CM., *E-cadherin is essential for in vivo epidermal barrier function by regulating tight junctions.* Embo J, 2005. **24**(6): p. 1146-56.

151. Loreal, O., F. Levavasseur, C. Fromaget, D. Gros, A. Guillouzo, and B. Clement, *Cooperation of Ito cells and hepatocytes in the deposition of an extracellular matrix in vitro.* Am J Pathol, 1993. **143**(2): p. 538-44.

152. Mesnil, M., J.M. Fraslin, C. Piccoli, H. Yamasaki, and C. Guguen-Guillouzo, *Cell contact but not junctional communication (dye coupling) with biliary epithelial cells is required for hepatocytes to maintain differentiated functions.* Exp Cell Res, 1987. **173**(2): p. 524-33.

153. Nelson, W.J., *Adaptation of core mechanisms to generate cell polarity.* Nature, 2003. **422**(6933): p. 766-74.

154. Mitaka, T., *Reconstruction of the hepatic organoid by hepatic stem cells.* J. Hepatobiliary Pancreat Surg, 2002. **9**: p. 697-703.

155. Daley, G., *Prospects for stem cell therapeutics: myths and medicines.* Curr Opin. Genet. Dev., 2002. **12**: p. 607-613.

156. Fair, J., Cairns, BA., LaPaglia, M., Wang, J., Meyer, AA., Kim, H., Hatada, S., Smithies, O., Pevny, L., *Induction of hepatic differentiation in embryonic stem cells by co-culture with embryonic cardiac mesoderm.* Surgery, 2003. **134**(2).

157. Baharvand, H., S.M. Hashemi, S. Kazemi Ashtiani, and A. Farrokhi, *Differentiation of human embryonic stem cells into hepatocytes in 2D and 3D culture systems in vitro.* Int J Dev Biol, 2006. **50**(7): p. 645-52.

158. Baharvand H, H.S., Shahsavan M, *Differentiation of human embryonic stem cells into functional hepatocyte-like cells in a serum-free adherent culture condition.* Differentiation, 2007. **Epub ahead of print**.

159. Cai J, Z.Y., Liu Y, Ye F, Song Z, Qin H, Meng S, Chen Y, Zhou R, Song X, Guo Y, Ding M, Deng H., *Directed differentiation of human embryonic stem cells into functional hepatic cells.* Hepatology, 2007. **45**(5): p. 1229-39.

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

161. Ishii T, F.K., Yasuchika K, Adachi K, Kawase E, Suemori H, Nakatsuji N, Ikai I, Uemoto S., *Effects of extracellular matrixes and growth factors on the hepatic differentiation of human embryonic stem cells.* Am J Physiol Gasrointest Liver Physiol., 2008. **295**(2): p. G313-21.

162. Agarwal S, H.K., Lanza R, *Efficient differentiation of functional hepatocytes from human embryonic stem cells.* Stem Cells, 2008. **26**(5): p. 1117-27.

163. Levenberg, S., N.F. Huang, E. Lavik, A.B. Rogers, J. Itskovitz-Eldor, and R. Langer, *Differentiation of human embryonic stem cells on three-dimensional polymer scaffolds.* Proceedings of the National Academy of Sciences of the United States of America, 2003. **100**(22): p. 12741-12746.

164. Schwartz RE, L.J., Painschab MS, Hu WS, Verfaillie CM, Kaufman DS, *Defined conditions for development of functional hepatic cells from human embryonic stem cells.* Stem Cells Dev., 2005. **14**(6): p. 643-55.

165. McLean AB, D.A.K., Jones KL, Krishnamoorthy M, Kulik MJ, Reynolds DM, Sheppard AM, Liu H, Xu Y, Baetge EE, Dalton S, *Activin a efficiently specifies definitive endoderm from human embryonic stem cells only when phosphatidylinositol 3-kinase signaling is suppressed.* Stem Cells, 2007. **25**(1): p. 29-38.

166. Fletcher J, C.W., Samuel K, Black JR, Hannoun Z, Currie IS, Terrace JD, Payne C, Filippi C, Newsome P, Forbes SJ, Ross JA, Iredale JP, Hay DC., *The Inhibitory Role of Stromal Cell Mesenchyme on Human Embryonic Stem*

Cell Hepatocyte Differentiation is Overcome by Wnt3a Treatment. Cloning Stem Cells, 2008. **Epub ahead of print**.

167. Yamada T, Y.M., Kanda S, Kato Y, Nakajima Y, Ishizaka S, Tsunoda Y., *In vitro differentiation of embryonic stem cells into hepatocyte-like cells identified by cellular uptake of indocyanine green.* Stem Cells, 2002. **20**(2): p. 165-54.

168. Leclerc, E., Y. Sakai, and T. Fujii, *Microfluidic PDMS (polydimethylsiloxane) bioreactor for large-scale culture of hepatocytes.* Biotechnol Prog, 2004. **20**(3): p. 750-5.

169. Ek M, S.T., Küppers-Munther B, Edsbagge J, Andersson TB, Björquist P, Cotgreave I, Jernström B, Ingelman-Sundberg M, Johansson *Expression of drug metabolizing enzymes in hepatocyte-like cells derived from human embryonic stem cells.* Biochem Pharmacol, 2007. **74**(3): p. 496-503.

170. Wang Z, L.H., Wang YC, Cong XQ, *Human embryonic stem cells and liver diseases: from basic research to future clinical application.* J Dig Dis., 2008. **9**(1): p. 14-9.

171. Lavon N, B.N., *Study of hepatocyte differentiation using embryonic stem cells.* J Cell Biochem., 2005. **96**(6): p. 1193-202.

172. Kamiya A, K.T., Ito Y, Matsui T, Morikawa Y, Senba E, Nakashima K, Taga T, Yoshida K, Kishimoto T, Miyajima *Fetal liver development requires a paracrine action of oncostatin M through the gp130 signal transducer.* EMBO J., 1999. **18**(8): p. 2127-36.

173. Hay DC, Z.D., Ross A, Mandalam R, Lebkowski J, Cui W., *Direct differentiation of human embryonic stem cells to hepatocyte-like cells exhibiting functional activities.* Cloning Stem Cells, 2007. **9**(1): p. 51-62.

174. Yasunaga M, T.S., Torikai-Nishikawa S, Nakano Y, Okada M, Jakt LM, Nishikawa S, Chiba T, Era T, Nishikawa S., *Induction and monitoring of definitive and visceral endoderm differentiation of mouse ES cells.* Nat Biotechnol, 2005. **23**(12): p. 1542-50.

175. Jiang J, A.M., Lu K, Eshpeter A, Korbutt G, Fisk G, Majumdar AS., *Generation of insulin-producing islet-like clusters from human embryonic stem cells.* Stem Cells, 2007. **25**(8): p. 1940-53.

176. D'Amour KA, A.A., Eliazer S, Kelly OG, Kroon E, Baetge EE., *Efficient differentiation of human embryonic stem cells to definitive endoderm.* Nat Biotechnol, 2005. **23**(12): p. 1534-41.

177. Kubo A, S.K., Shannon JM, Kouskoff V, Kennedy M, Woo S, Fehling HJ, Keller G., *Development of definitive endoderm from embryonic stem cells in culture.* Development, 2004. **131**(7): p. 1651-62.

178. Chen Y, S.-G.A., Navarro-Alvarez N, Rivas-Carrillo JD, Yamatsuji T, Shirakawa Y, Tanaka N, Basma H, Fox IJ, Kobayashi N, *Instant hepatic differentiation of human embryonic stem cells using activin A and a deleted variant of HGF.* Cell Transplant, 2006. **15**(10): p. 865-71.

179. Dravid G, Y.Z., Hammond H, Chen G, Pyle A, Donovan P, Yu X, Cheng L., *Defining the role of Wnt/beta-catenin signaling in the survival, proliferation, and self-renewal of human embryonic stem cells.* Stem Cells, 2005. **23**(10): p. 1489-501.

180. Biswas A, H.R., *Embryonic stem cells.* Stem Cells Dev, 2007. **16**(2): p. 213-22.

181. Nat, R., M. Nilbratt, S. Narkilahti, B. Winblad, O. Hovatta, and A. Nordberg, *Neurogenic neuroepithelial and radial glial cells generated from six human embryonic stem cell lines in serum-free suspension and adherent cultures.* Glia, 2007. **55**(4): p. 385-99.

182. Noles, S.R. and A. Chenn, *Cadherin inhibition of beta-catenin signaling regulates the proliferation and differentiation of neural precursor cells.* Mol Cell Neurosci, 2007. **35**(4): p. 549-58.

183. Nagafuchi, A. and M. Takeichi, *Cell binding function of E-cadherin is regulated by the cytoplasmic domain.* Embo J, 1988. **7**(12): p. 3679-84.

184. Itsykson, P., N. Ilouz, T. Turetsky, R.S. Goldstein, M.F. Pera, I. Fishbein, M. Segal, and B.E. Reubinoff, *Derivation of neural precursors from human embryonic stem cells in the presence of noggin.* Mol Cell Neurosci, 2005. **30**(1): p. 24-36.

185. Schuldiner, M., R. Eiges, A. Eden, O. Yanuka, J. Itskovitz-Eldor, R.S. Goldstein, and N. Benvenisty, *Induced neuronal differentiation of human embryonic stem cells.* Brain Res, 2001. **913**(2): p. 201-5.

186. Zhang, S.C., M. Wernig, I.D. Duncan, O. Brustle, and J.A. Thomson, *In vitro differentiation of transplantable neural precursors from human embryonic stem cells.* Nat Biotechnol, 2001. **19**(12): p. 1129-33.

187. Reubinoff, B.E., P. Itsykson, T. Turetsky, M.F. Pera, E. Reinhartz, A. Itzik, and T. Ben-Hur, *Neural progenitors from human embryonic stem cells.* Nat Biotechnol, 2001. **19**(12): p. 1134-40.

188. Buytaert-Hoefen, K.A., E. Alvarez, and C.R. Freed, *Generation of tyrosine hydroxylase positive neurons from human embryonic stem cells after coculture with cellular substrates and exposure to GDNF.* Stem Cells, 2004. **22**(5): p. 669-74.

189. Hong, S., U.J. Kang, O. Isacson, and K.S. Kim, *Neural precursors derived from human embryonic stem cells maintain long-term proliferation without losing the potential to differentiate into all three neural lineages, including dopaminergic neurons.* J Neurochem, 2008. **104**(2): p. 316-24.

190. Pomp, O., I. Brokhman, L. Ziegler, M. Almog, A. Korngreen, M. Tavian, and R.S. Goldstein, *PA6-induced human embryonic stem cell-derived neurospheres: a new source of human peripheral sensory neurons and neural crest cells.* Brain Res, 2008.

191. Sonntag, K.C., J. Pruszak, T. Yoshizaki, J. van Arensbergen, R. Sanchez-Pernaute, and O. Isacson, *Enhanced yield of neuroepithelial precursors and midbrain-like dopaminergic neurons from human embryonic stem cells using the bone morphogenic protein antagonist noggin.* Stem Cells, 2007. **25**(2): p. 411-8.

192. Zeng, X., J. Cai, J. Chen, Y. Luo, Z.B. You, E. Fotter, Y. Wang, B. Harvey, T. Miura, C. Backman, G.J. Chen, M.S. Rao, and W.J. Freed, *Dopaminergic differentiation of human embryonic stem cells.* Stem Cells, 2004. **22**(6): p. 925-40.

193. Joannides, A.J., C. Fiore-Heriche, A.A. Battersby, P. Athauda-Arachchi, I.A. Bouhon, L. Williams, K. Westmore, P.J. Kemp, A. Compston, N.D. Allen, and S. Chandran, *A scaleable and defined system for generating neural stem cells from human embryonic stem cells.* Stem Cells, 2007. **25**(3): p. 731-7.

194. Erceg, S., S. Lainez, M. Ronaghi, P. Stojkovic, M.A. Perez-Arago, V. Moreno-Manzano, R. Moreno-Palanques, R. Planells-Cases, and M. Stojkovic, *Differentiation of human embryonic stem cells to regional specific neural precursors in chemically defined medium conditions.* PLoS ONE, 2008. **3**(5): p. e2122.

195. Brieva, T.A. and P.V. Moghe, *Functional engineering of hepatocytes via heterocellular presentation of a homoadhesive molecule, E-cadherin.* Biotechnol Bioeng, 2001. **76**(4): p. 295-302.

196. Moore, R.N., A. Dasgupta, N. Rajaei, M.L. Yarmush, M. Toner, L. Larue, and P.V. Moghe, *Enhanced differentiation of embryonic stem cells using co-cultivation with hepatocytes.* Biotechnol Bioeng, 2008.

197. Goichberg, P. and B. Geiger, *Direct involvement of N-cadherin-mediated signaling in muscle differentiation.* Mol Biol Cell, 1998. **9**(11): p. 3119-31.

198. Owens, D.W., V.G. Brunton, E.K. Parkinson, and M.C. Frame, *E-cadherin at the cell periphery is a determinant of keratinocyte differentiation in vitro.* Biochem Biophys Res Commun, 2000. **269**(2): p. 369-76.

199. Starbuck, C. and D.A. Lauffenburger, *Mathematical model for the effects of epidermal growth factor receptor trafficking dynamics on fibroblast proliferation responses.* Biotechnol Prog, 1992. **8**(2): p. 132-43.

200. GM., E., *Cell adhesion molecules.* Science, 1983. **219**(4584): p. 450-7.

201. Hoffman S, E.G., *Kinetics of homophilic binding by embryonic and adult forms of the neural cell adhesion molecule.* Proc Natl Acad Sci U S A, 1983. **80**(18): p. 5762-6.

202. Mueller-Klieser, W., *Three-dimensional cell cultures: from molecular mechanisms to clinical applications.* Am J Physiol, 1997. **273**(4 Pt 1): p. C1109-23.

203. Liu, H., J. Lin, and K. Roy, *Effect of 3D scaffold and dynamic culture condition on the global gene expression profile of mouse embryonic stem cells.* Biomaterials, 2006. **27**(36): p. 5978-89.

204. Willerth, S.M., K.J. Arendas, D.I. Gottlieb, and S.E. Sakiyama-Elbert, *Optimization of fibrin scaffolds for differentiation of murine embryonic stem cells into neural lineage cells.* Biomaterials, 2006. **27**(36): p. 5990-6003.

205. Kang, X., Y. Xie, H.M. Powell, L. James Lee, M.A. Belury, J.J. Lannutti, and D.A. Kniss, *Adipogenesis of murine embryonic stem cells in a three-dimensional culture system using electrospun polymer scaffolds.* Biomaterials, 2007. **28**(3): p. 450-8.

206. Imamura, T., L. Cui, R. Teng, K. Johkura, Y. Okouchi, K. Asanuma, N. Ogiwara, and K. Sasaki, *Embryonic stem cell-derived embryoid bodies in three-dimensional culture system form hepatocyte-like cells in vitro and in vivo.* Tissue Eng, 2004. **10**(11-12): p. 1716-24.

207. Levenberg, S., J.A. Burdick, T. Kraehenbuehl, and R. Langer, *Neurotrophin-induced differentiation of human embryonic stem cells on three-dimensional polymeric scaffolds.* Tissue Eng, 2005. **11**(3-4): p. 506-12.

208. Shih, Y.R., C.N. Chen, S.W. Tsai, Y.J. Wang, and O.K. Lee, *Growth of mesenchymal stem cells on electrospun type I collagen nanofibers.* Stem Cells, 2006. **24**(11): p. 2391-7.

209. Stanworth, S.J. and A.C. Newland, *Stem cells: progress in research and edging towards the clinical setting.* Clin Med, 2001. **1**(5): p. 378-82.

210. Zoldan, J. and S. Levenberg, *Engineering three-dimensional tissue structures using stem cells.* Methods Enzymol, 2006. **420**: p. 381-91.

211. Xu, C., *Characterization and evaluation of human embryonic stem cells.* Methods Enzymol, 2006. **420**: p. 18-37.

212. Cohen, S., L. Leshanski, and J. Itskovitz-Eldor, *Tissue engineering using human embryonic stem cells.* Methods Enzymol, 2006. **420**: p. 303-15.

213. Semler, E.J. and P.V. Moghe, *Engineering hepatocyte functional fate through growth factor dynamics: the role of cell morphologic priming.* Biotechnol Bioeng, 2001. **75**(5): p. 510-20.

214. Maheshwari, G., G. Brown, D.A. Lauffenburger, A. Wells, and L.G. Griffith, *Cell adhesion and motility depend on nanoscale RGD clustering.* J Cell Sci, 2000. **113 (Pt 10)**: p. 1677-86.

215. Lee, K.Y., E. Alsberg, S. Hsiong, W. Comisar, J. Linderman, R. Ziff, and D. Mooney, *Nanoscale Adhesion Ligand Organization Regulates Osteoblast Proliferation and Differentiation.* Nano Lett., 2004. **4**(8): p. 1501-1506.

216. Koo, L.Y., D.J. Irvine, A.M. Mayes, D.A. Lauffenburger, and L.G. Griffith, *Co-regulation of cell adhesion by nanoscale RGD organization and mechanical stimulus.* J Cell Sci, 2002. **115**(Pt 7): p. 1423-33.

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Moore R., Dasgupta A., Rajaei N., Yarmush M., Toner M., Larue L., Moghe P.V. Enhanced differentiation of embryonic stem cells using co-cultivation with hepatocytes. *Biotechnol. Bioeng*. 2008 June 2. (Epub ahead of print)