STEM CELL ENGINEERING OF THE ENDODERM: APPROACHES TO CONTROLLING
ENDODERM INDUCTION AND DIFFERENTIATION FROM EMBRYONIC STEM CELLS

by

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ABSTRACT OF THE DISSERTATION

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Thesis Supervisor:
Dr. Martin L. Yarmush, MD, PhD

Embryonic stem (ES) cell technology holds promise for curing innumerable human ailments. However, studies of the endoderm germ layer and its derivatives (liver, pancreas, and lung) are lacking. The overall objective of this thesis is to elucidate the factors that influence endoderm induction and differentiation from ES cells. To improve results in aggregate culture, a microfabricated PDMS (polydimethylsiloxane) stencil was engineered using standard soft lithography techniques used to control ES cells. Precise control over initial aggregate size was obtained, varying the initial aggregate size from 100-500 µm. Analysis of the cells by RT-PCR (Reverse Transcriptase Polymerase Chain Reaction) demonstrated endoderm on Day 10 and hepatocyte-like cells on Day 20, but a mixed population was present. To further enhance endoderm induction, a coculture system was developed. The culture of ES cells on top of collagen-sandwiched mature rat hepatocytes, resulted in a rapid proliferation into a 95% positive endoderm progenitor population by Day 10. Late stage differentiation of these cells and placement in a extracorporeal device to support liver failure in rats resulted in enhanced (50%) survival.

To further understand endoderm induction, a simpler culture system was developed. The culture of ES cells on fibronectin-coated collagen gels resulted in an endoderm fraction of 53% by Day 10 that remained committed upon in vivo implantation. Treatment with activin, an important TGFβ superfamily soluble factor, caused an 80% decrease in the endoderm fraction, while follistatin, an activin inhibitor with unknown function, increased the endoderm fraction to 78%. The activin treated population delayed the induction of endoderm by preventing differentiation of its transient precursors, the epiblast and mesendoderm. Differentiation of activin treated cells to Day 24 resulted in a two-fold reduction in hepatic gene expression and three-fold reduction in hepatic protein expression of when compared to follistatin-
treated cells. Subcutaneous transplantation of activin-treated cells resulted in generated a heterogeneous teratoma-like mass, suggesting these cells were primitive. In summary, factors that influence endoderm induction include initial size of aggregate, coculture environment, extracellular matrix, and soluble factors. Two new tools for evaluating clinical applications include in vivo implantation and placement in an extracorporeal device.
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CHAPTER 1: EMBRYONIC STEM CELLS AND EARLY DEVELOPMENT

1.1 Potential of regenerative medicine

An era of regenerative medicine is believed to be forthcoming. This area of research encompasses numerous advances which will likely revolutionize the manner in which medical conditions are prevented, diagnosed, and treated. In doing so, this field promises to augment the current biotechnology industry through economic benefit, while at the same time providing more efficient, cheaper, prolonged, and effective therapies for patients. The fundamental concept in regeneration is that the augmentation of healing of damaged tissues or injured tissues can result in improved health benefits. However significant biotechnological infrastructure has to be developed in order to establish and sustain this area of medicine.

1.2 Stem cells for cellular therapy, gene therapy, tissue engineering, and biopharmaceuticals

Stem cell technologies are a central technology in the development of a series of technologies, some of which are critical for the development of regenerative medicine. These include cellular and gene therapy, tissue engineering, biopharmaceuticals, and cloning.

Cellular therapy, the use of cells as therapeutic agents, is a promising form of therapy in which cells will be either exogenously delivered or endogenously activated. Historically, the most frequent type of cell used clinically has been hematopoietic stem cells which have been used in transplantation protocols for clinical conditions which require reconstitution of function of the bone marrow (Cao, Wagers et al. 2004).

Another promising therapy currently being used in clinical trials is immunotherapy. In this case, the immune system can be engineered to respond to tumor antigens by delivering either expanded autologous T-Cells or dendritic cells which induce a host response (Colombo and Piconese 2007). The development of cellular therapies for other types of diseases is in its infancy. Obviously, stem cells can contain modified genes, hence making them carriers, or “vectors” for gene therapy (Conrad, Gupta et al. 2007). Moreover, by incorporating the gene into a renewable cell that can also differentiate, the gene can be replaced continually within a patient. Thus, the self-renewing capability of stem cells makes them an attractive target as a source of cells for cell therapy as well as gene therapy.

Tissue engineering is an interdisciplinary field which involves the fabrication and use of tissues for replacing body parts which may be undergoing failure or degeneration. The main paradigm for products is cells are isolated from adult tissues and then added to a biomaterials-based construct, which is then
implanted in the host. For example, current skin products include Dermagraft, Transcyte, Apligraft, and Orcel (MacNeil 2007). For tissue engineering devices, isolation of cells is typically energy intensive and a shortage of cells themselves is believed to be a rate limiting step for the production of tissue engineered devices. While there are other challenges to integrating the cells into biomaterial constructs and eventually into patients, the development and characterization of stem cell technologies is likely to impact the field of tissue engineering.

Biopharmaceuticals are a growing portion of the biotechnology market in which biological products are used therapeutically. Some examples of these include genetically engineered proteins, such as monoclonal antibodies. Many glycosylated proteins can not be produced in bacteria due to ineffective protein folding or glycosylation. As a result, mammalian cell lines, such as CHO cells, are typically transfected with genetically engineered proteins, and then purified. However, cell lines have been transformed or are altered in important ways. Stem cells differentiated into a particular cell type could be used for producing a particular protein which might be secreted by the particular cell type, with or without genetic modification. Recently, this approach has been taken. Parietal endoderm cells, which are developmentally important cells which line the extraembryonic tissues, have been used as a source of production of an extracellular matrix protein (Notarianni and Flechon 2001). Another pharmaceutical application of stem cells would be in drug discovery, drug screening and development. Patient-specific stem cells (adult or embryonic) could be engineered into tissues (like liver, skin, gut epithelial cells) and used in drug screening applications in which new drug targets are found.

1.3 Stem cells-overview

Stem cells are a unique group of cells because they retain the ability to self-renew while giving rise to daughter cells that have restricted potency. Stem cells are known to be present in multicellular organisms both in mature organisms and during development. The term stem cell was coined initially EB Wilson (1896) in studies of the germ line of the parasitic nematode worm, Ascaris megalocephala. Studies of the origins of invertebrates, especially marine species, indicate that all somatic cells are derived from one single cell, termed the 4d blastomere. On the other hand, in Drosphilla (multilineage invertebrate) and all vertebrates, somatic cells and germ cells originate from several cells rather than just one. This implies that
most tissues are polyclonal (Marshak et al 2001). Tissues are believed to be composed of two subpopulations of cells, stem cells and terminally differentiated cells. However, kinetic studies indicates the presence of an intermediate cell, the transit amplifying cell, which is believed to limit the amount of divisions that the stem cells need to make during the lifetime of the organism (Marshak et al 2001).

1.3.1. Adult Stem Cells

The paradigm of stem cells and stem cell differentiation originally was founded based on Hematopoietic Stem Cells (HSC), first discovered in the 1960’s (Weissman, Anderson et al. 2001). These cells were found harbored in fractional quantities amongst mature cells in bone marrow and eventually were isolated, expanded, and shown to reconstitute the entire myeloid, lymphoid, and erythroid lineages when transplanted into an irradiated adult organism whose bone marrow has been ablated. Consequently, HSC cells served as a basic model for adult stem cells, and methods for expansion, isolation, controlling differentiation were developed (Weissman, Anderson et al. 2001). In general, adult stem cells are believed to reside within tissues in small numbers, and contribute to tissue homeostasis during normal tissue turnover or when reconstitution of the adult tissue is required such as during injury or pathological loss of tissue. Stem cells have now been discovered to exist in many major tissues including the heart, the pancreas, and the brain. Countless examples of other types of stem cells exist, including male and female germ cells, embryonal carcinoma cells, trophoblast stem cells, umbilical cord stem cells, placental stem cells, mesenchymal stem cells, epidermal stem cells, neural stem cells, cardiac stem cells, liver, pancreatic, and intestinal stem cells. Boosting endogenous stem cell production and function is a viable therapeutic option. However, the extrinsic and intrinsic factors which govern cell fate decisions are just beginning to be understood. These extrinsic factors compose the microenvironment, or niche that is required to control stem cells. The intrinsic factors may consist of internal aspects of the cell, such as transcription factor networks or signaling networks which are poorly understood.

Factors which limit the use of adult stem cells include the inability to isolate and expand stem cells in large scale quantities for clinical use. Typically, adult stem cells are located in a small amount and reside deep in tissues (especially resident stem cells). These cells are typically present in regulated microenvironments, or “niches,” and changes in the niche results in external cues that initiate cellular
processes such as proliferation and or differentiation (Moore and Lemischka 2006). The niches which control these adult stem cells are just beginning to be unraveled. Furthermore, establishing the environments in vitro that are required to proliferate and/or differentiate these cells is a daunting challenge which has not yet been solved.

1.3.2 Embryonic Stem Cells

Embryonic Stem Cell (ES) technology has enormous potential for gene, cell or tissue engineering therapies for ameliorating widespread health problems such as Heart Disease, Diabetes, Stroke, and Neurodegenerative Diseases (Wobus and Boheler 2005). ES cells’ unlimited potential is reflected in their pluripotency in the presence of LIF (Leukemia Inhibitory Factor), capacity for unlimited self-renewal, contribution to all major cell lineages when transplanted into normal mouse embryos, and differentiation of major cell lineages in vitro (Wobus and Boheler 2005). However, precise control over differentiation has not yet been achieved, and as a result cell populations of high yield and high purity can not yet be engineered. Major concerns for ES cells before becoming cell therapies include: 1) Concerns about Genetic Alteration 2) Tumorigenesis 3) Purification and Lineage Selection 4) Tissue-Specific Integration and Function 5) Immunogenecity and Graft Rejection (Wobus and Boheler 2005).

Embryonic stem (ES) cells were initially described (Evans and Kaufman 1981; Martin 1981) as a pluripotent cell population derived from the inner cell mass of the mouse blastocyst. Recently, ES cells have also been derived from the blastocysts of various animals, ranging from rat to cow. Their uniqueness lies in their ability to be maintained and expanded for extended periods of times, suggesting that mechanisms of cellular aging are altered. Importantly, these cells are not transformed, as are cell lines. After in vitro culture, upon transplantation, these cells contribute to tissues of all three germ layers, as well as the germ cells, indicating they are pluripotent in vivo. When these ES cells are implanted ectopically, they give rise to all three germ layers. Chimeric mice can be produced because these cells can be genetically modified and transplanted into the blastocyst and then reimplanted into pseudopregnant mice. Importantly, basic studies of in vitro differentiation indicated that these cells also give rise to the three germ layers. However, differentiation in ES cells differs from in vivo development in many important ways. Table 1.1 highlights differences between these two cases.
1.3.3 Embryonal Carcinoma cells

Fundamental work with teratocarcinoma cells paved the way for future development of embryonic stem cells. The undifferentiated component of teratocarcinoma is described as embryonal carcinoma cells (Kleinsmith and Pierce 1964). Interestingly they are often capable of multiligneage differentiation. These cells could be reintroduced into the embryo and in some cases give rise to the germ layers, chimeric fetuses, and live offspring. These cells do not give rise to tumors, and as a result they are not transformed or oncogenic. However serial transfer in vivo of these cells alters the structure and function of the genome results in aneuploidy, which results in loss of differentiation potential. Nonetheless, initial work with these cells laid the groundwork for derivation of ES cells.

1.3.4 Classic derivation of Mouse ES cells

Mitotically inactivated feeder cells were found to support embryonal carcinoma cell lines. These were then used in conjunction with the culture of mouse blastocyst (Evans and Kaufman 1981; Martin 1981) to develop pluripotent cells lines known as embryonic stem cells. The derivation of ES cells lines has been shown to be strain dependent, with 129 inbred mice and C57BL/6 strains giving rise to ES cell lines, while other strains have had difficulty doing so (Smith A. et al). Human ES cells were first derived in 1998 from embryos that were obtained from in-vitro fertilization (IVF) clinics, which store embryos that can be used to treat infertility. These clinics have approximately 400,000 embryos in storage just in the United States, and about 2.8% of will likely be discarded (Smith A, et al) This fact itself has raised ethical concerns and debate regarding the federal funding for ES cell research.

1.3.5 Other Sources of Pluripotent Cells; Pluripotency, Reprogramming, and Cloning

The establishment of ES cell lines has led to studies regarding the pluripotency of ES cells, as well as to determining if ES cells can be derived using other techniques. If pluripotency is well understood then other approaches can be used to derive ES cells. Studies of pluripotency of ES cells have focused on genetic and epigenetic events. Oct4, Nanog, and Sox2 have been identified as transcription factors that regulate pluripotency (Boyer and Jaenisch 2006). Polycomb proteins provide a further layer of regulation by suppressing the expression of genes that promote differentiation (Lee, Jenner et al. 2006). These factors are
believed to establish and maintain pluripotent states by repressing transcriptional factors important for lineage differentiation. Self renewal is maintained by the interaction of Leukemia Inhibitory Factor (LIF) with the STAT3 pathway. Epigenetic control of pluripotency is important because heritable changes in gene expression need to be established. ES cells themselves demonstrate a permissive like euchromatin with acetylated histone modifications and increased accessibility. Lineage specification is typified by a decrease in acetylation and increase in heterochromatin formation (Jaenisch, Hochedlinger et al. 2004).

Other sources of ES cells include somatic cell reprogramming via nuclear transfer or cell fusion, and parthenogenesis. Nuclear transfer was initially developed in 1952 (Briggs and King) to test nuclear potency. Nuclei from frog blastomeres were transplanted into enucleated frog oocytes and shown to generate early cleavage embryos. Somatic cell nuclear transfer involves removing the nucleus from a somatic cell, like the skin cell, and then injecting this into an enucleated oocyte. The metaphase spindle is removed from a metaphase II-arrested oocyte. Then the resulting cell is reactivated, and this resulting reconstructed diploid embryo undergoes division into the blastocyst, and embryonic stem cells can be derived from it (Jaenisch, Hochedlinger et al. 2004). The blastocyst can also be transferred to a pseudopregnant mouse. This technique, which has been used for cloning, has its difficulties, because cloning efficiency decreases when the differentiated state of the cell is increased. Nevertheless, breakthroughs in cloning in 1986-1989 led to cloned lambs, cattles, and pigs, when the transplanted nuclei were transferred from blastomeres. The animal “Dolly” represented the first animal cloned from an adult cell. These experiments were repeated by using a two-step procedure in which nuclear transfer of lymphocytes was done, followed by generation of embryonic stem cells followed by tetraploid complementation and generation of monoclonal mice (Jaenisch, Hochedlinger et al. 2004). Other than differences in techniques and oocyte physiology between species and within cells of a particular species, problems in epigenetic reprogramming of the donor nuclei are believed to be central to problems in the efficiency of cloning.

While animal cloning has had recent successes, therapeutic cloning has also been demonstrated. ES cells were produced by nuclear transfer of somatic cells from an immunodeficient mouse. This defect in immunodeficiency was repaired by homologous recombination, and then these repaired ES cells were then differentiated into hematopoietic precursors and transplanted into the donor mice. Normal, functional
lymphocytes were found to be present in the transplant recipient, thus curing the animal of the immunodeficiency (Rideout, Hochedlinger et al. 2002). This highlights the derivation of ES cells using nuclear transfer, and the use of this in cell and gene therapy protocol. The prospect of deriving patient-specific human ES cells from nuclear transfer remains a possibility and one that is actively being investigated.

Other techniques for generating reprogrammed cells include cell fusion, reprogramming via cellular extracts, and genetic engineering. Reprogramming via cell fusion between ES cells and fibroblasts resulted in activation of pluripotent genes in the recipient nucleus (Cowan, Atienza et al. 2005). However, the cell fusion event is extremely inefficient, and this technique leads to doubling of the nuclei and a tetraploid cell. Reprogramming using cellular extracts has been used to reprogram fibroblasts into T cells, which allowed kinetic and biochemical analysis of reprogramming (Hakelien, Landsverk et al. 2002). However, how the functional extent of reprogramming was not shown. Reprogramming by altering the culture conditions is also a possibility, and culture conditions have been shown to promote pluripotent cell types, such as ES cells, germ cells, etc. Reprogramming by genetic engineering of embryonic and adult fibroblasts has shown that expression of Oct4, Sox2, c-Myc, and Klf4 were able to reprogram human embryonic fibroblasts into human embryonic stem cells (Takahashi, Tanabe et al. 2007).

Not only somatic cells, but also germ cells can be reprogrammed into pluripotent cells. Past studies have shown that primordial germ cells isolated from midgestation embryos could form experimentally induced teratomas, and these cells could be cultured to form embryonic germ cells. These cells in turn had a similar phenotype to ES cells in terms of the ability to contribute to the germline chimeras (Donovan and de Miguel 2003). Recent studies have indicated, however, spermatogonial stem cells (SSC) can give rise to ES cell-like colonies with ES cell markers (Guan, Nayernia et al. 2006). These cells were termed multipotent adult germline stem cells, and were found to give rise to all three germ layers in vitro. These cells were also capable of germline transmission when injected into blastocyst. However, these cells could not generate live offspring when using tetraploid blastocyst complementation, probably because they lack both maternal and paternal chromosome contributions.

Parthenogenesis is a normal biological process by which the oocyte can be spontaneously activated and initiate embryonic development (Kim, Lerou et al. 2007). These oocytes can also be activated
in vitro, during the early postimplantation stage, and when they are parthenogenetic blastocysts, they can give rise to pluripotent cells. Interestingly, pluripotent cell lines derived from parthenogenetic process is not genetically identical to the donor nucleus. These cells would be expected to display haploidentity to the original oocyte. As a result, at the MHC locus, these cells would be expected to be homozygous and may be rejected upon cell transplantation by a process known as hybrid resistance. However, this has recently been addressed by developing techniques to derive cells from oocytes that are inhibited at meiosis I or a meiosis II, where MHC heterozygosity can be present either due to lack of parental segregation or crossing over. These cells could contribute to multiple lineages in vivo and in vitro but could not support development of blastocyst in tetralecomplementation assay.

1.4 Early in vivo differentiation and analogies to early ES cell differentiation

Differentiation in vivo is a highly organized, spatially and temporally regulated, dynamic process which results in the mature organism. Importantly, the process of development is an evolutionarily conserved process, which suggests that the differentiation of ES cells can be understood by understanding early development, and this has been confirmed by many recent studies (Niwa, Miyazaki et al. 2000; Gadue, Huber et al. 2006; Tam and Loebel 2007).

1.4.1 Cell surface markers during early ES cell development

Although difficult to identify, cell surface markers are a powerful tool used by stem cell biologists to purify a cell of interest from a heterogeneous cell population. Based on the work of Ling and Neben et al., traditional germ cell and hematopoietic markers were used to characterize the cell surface of ES cells. Early approaches to control ES cell differentiation relied primarily on cell sorting (Ling and Neben, 1997). Although the hematopoietic system has cell surface markers which clearly delineate early and late progenitor cells, it is more difficult to separate out early cell populations derived from ES cells. In general, embryonic stem cell populations do not have known cell surface markers that correspond to specific early populations. Important cell surface markers believed to be present during early development are shown in Table 1.2. A notable marker includes SSEA-1, which is present in primitive cells but downregulated during
early differentiation. However, more development of new cell surface markers will be needed to make these valuable tools for ES cell differentiation.

1.4.2 Zygote to preimplantation blastocyst

Early differentiation can be characterized as the transition from the zygote to the morula, the preimplantation blastocyst, the postimplantation blastocyst, the gastrula, and then the fetus. Initially, the oocyte is released from the ovary and fertilized in the oviduct. Next, a series of slow, specialized, asynchronous, cleavages occur. At the 8-cell stage these cells undergo compaction, with clustering of cells and active gap junction formation. The 16-cell morula contains outer cells that give rise to trophoectoderm cells and inner cells give to the inner cell mass cells. At the 64-cell stage the differences in these two layers are clear. The trophoblast cells secrete fluid into the morula during a process called cavitation, to create the blastocyst. Implantation occurs as a result of trophoblast cells attaching to the uterine wall through ECM proteins secreted by the uterus and integrin receptors on the trophoblast. Once attached, the trophoblast secretes proteases, such as collagenase, to bury the blastocyst within the uterine wall (Robb and Tam 2004).

1.4.3 Postimplantation blastocyst to gastrula

The tissues which allow absorption of maternal nutrients into the embryo then initiate development. A representation of the early populations during development, and their direct interactions with each other is shown in Figure 1.1, adopted form (Tam and Loebel 2007). The chorion is the fetal organ which absorbs nutrients, and is derived from embryonic trophoblast cells and mesodermal cells from the inner cell mass (Robb and Tam 2004). The chorion induces the decidua from the uterine wall, which is rich in maternal blood vessels and allows exchange of nutrients. Implantation itself causes the inner cell mass to form the primitive endoderm or the hypoblast, and the remaining inner cells are referred to the epiblast (Robb and Tam 2004). The primitive endoderm gives rise to extraembryonic structures only, such as visceral and parietal endoderm, while the epiblast is a key cell type because it gives rise to all embryonic structures, as well as extraembryonic mesoderm. Thus most in vitro protocols with ES cells are interested in the derivatives of the epiblast.
Immediately after postimplantation, the mouse embryo changes in dramatically in size, about a 40-fold increase. The embryo takes on the shape of two layers in the shape of a cup, with the outer visceral endoderm and the inner epiblast. At this point, the embryo has well-delineated embryonic and extra-embryonic layers and proximal-distal and dorso-ventral axis (Robb and Tam 2004). During gastrulation, there are a set of defined morphogenetic movements, coupled with proliferation and differentiation to convert to the three layers. Gastrulation is initiated by recruiting epiblast cells to the transient embryonic structure known as the primitive streak, which also delineates the anterior-posterior (AP) axis. The epiblast cells undergo an epithelial-mesenchymal transition, ingress between layers and either form mesoderm or definitive endoderm. By the end of gastrulation, the cell layers have formed in proximity of each other which allows inductive interactions between cell layers critical for specification of particular lineages (Robb and Tam 2004).

1.4.4 The importance of organizers during gastrulation

The organizer is a term which refers to the ability of specialized cells to direct gastrulation. The tissue is of critical importance in ES cells because: 1) transcription factors that are associated with specific germ layers, such as neurectoderm, mesoderm, mesendoderm or endoderm, are present in organizer tissue 2) the organizers secrete numerous soluble signaling factors and their inhibitors, which may disrupt or result in counterintuitive or confounding in vitro results 3) The organizer takes part in early embryonic patterning, which causes distinguishable changes in transcription factors based on location (anterior-posterior) and is generally considered an irreversible process (Robb and Tam 2004). If organizer-like cells are present in ES cell cultures, they could effect measurements of differentiation.

The organizer has two biological properties: the induction upon implantation of neural and mesoderm differentiation of host tissues, and the patterning of the induced tissues into embryonic structures with polarity reminiscent of the three primary axes of a embryo (Robb and Tam 2004). The concept of an embryonic organizer was first introduced in the 1920s from studies on amphibian development. These studies identified a region on the dorsal side of the amphibian embryo, known as Spemann’s organizer, which when transplanted to an ectopic site on the ventral side of a host embryo was able to induce a second body axis (Harland and Gerhart 1997; Davidson and Tam 2000). Amazingly, this organizer was able to
recruit host tissues to form a complete axis, including a head, with appropriate Dorso-ventral (D–V), Anterior-posterior (A–P,) and Left-Right (L–R) patterning. In the mouse, the organizing center or node is a distinct group of cells located at the anterior end of the primitive streak (Davidson and Tam 2000). The node establishes the L–R axis, and cells emanating from the node form the axial mesodermal structures, the prechordal plate mesoderm and notochord, that signal D–V patterning in the embryo. Transplantation of the mouse node to an ectopic site can induce a secondary body axis. This secondary axis, however, lacks head structures, which suggests that the organizer is separated into two distinct entities, the node or trunk organizer and the head organizer. The anterior visceral endoderm, a specialized structure of the visceral endoderm, is believed to be the mouse head organizer (Jin, Harpal et al. 2001). Both the anterior visceral endoderm (AVE) and the node not only share the same function but also share common transcription factor expression, such as Lim and Otx2, and secrete antagonists to major signaling pathways.

1.4.5 Regionalization of the gastrula

Regionalization of cell fate and tissue patterning are the fundamental components of the “body plan” during embryonic development. This body plan, blueprint is composed of progenitor cells across all x, y, and z axes, at various stages and with various levels of specification (Tam and Behringer 1997). This developmental state is achieved by proper specification of progenitors and axes, and coordination of cell and tissue movements.

Recent findings show that inductive interactions between extra-embryonic and embryonic tissues are crucial for lineage specification and embryonic patterning, and these interactions are mediated principally by Wnt and TGFβ signaling activity (Tam and Loebel 2007). The extraembryonic ectoderm, the dorsal visceral endoderm, and the anterior visceral endoderm all have important roles in tissue patterning. Reciprocal interactions between epiblast and extraembryonic ectoderm maintain signals for formation of the primitive streak.

1.4.6 Soluble factor network during early development

An example of the complexity of tissue patterning and soluble factors is the following, adapted from Tam et al 2007. The epiblast’s nodal activity and nodal antagonists secreted by the anterior visceral endoderm
are necessary for anterior posterior patterning of the epiblast. Wnt3 signaling through β-Catenin and LRP5 and LRP6 (Low density lipoprotein receptor-related protein) maintains nodal signaling in the epiblast. At the same time tissues like anterior visceral endoderm secrete antagonists for posteriorizing signals such as Nodal and Wnt. In the extraembryonic ectoderm the nodal precursor protein activates BMP4, in addition the TGFβ convertase enzymes Furin and Pcsk6. These convertases process nodal protein. A graded signaling activity, mediated by nodal, GDF3, and Cripto, and antagonized by Lefty1 and CER1 from the Distal Visceral Endoderm upregulates expression of mesendodermal-related genes in the proximal epiblast. BMP4 from the extraembryonic ectoderm activates a regulatory pathway involving Wnt3, which then activates Brachyury expression. Expression of FGF’s is downstream of Brachyury expression and is enhanced by the nodal precursor protein (Tam and Loebel 2007).

In summary, patterning of the primitive streak is also accompanied by complex gradients of BMP, Fgf, and Nodal across the embryo. The anterior most region of the primitive streak gives rise to the node axial mesoderm and the definitive endoderm, the anterior third gives rise to the paraxial mesoderm, the middle region gives rise to the lateral plate mesoderm, and the posterior progenitor gives rise to the extraembryonic mesoderm. Cells that do not form traverse the primitive streak give rise to the ectoderm.

1.5 REFERENCES


Figure 1.1. A summary of early cell populations and their interactions in vivo (adopted from Tam et al 2007). Here, the development of the embryo is shown with time from blastocyst formation to gastrula formation. The epiblast gives rise to only embryonic tissues. All other tissues in theory can be derived from embryonic stem cells. Visceral endoderm and extraembryonic ectoderm provide key signals at all levels of early development. The key populations by day 6.5 are the primitive streak, definitive endoderm, and mesoderm.
### 1.7 TABLES

<table>
<thead>
<tr>
<th>In Vivo</th>
<th>In Vitro</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Development of cell mass analogous to blastocyst</td>
<td>yes</td>
<td>Embryoid body formation mimics blastocyst, with cavitation internally</td>
</tr>
<tr>
<td>Primitive Streak Formation and AP-DV Axes</td>
<td>no</td>
<td>Some genes present in Primitive Streak have been identified in ES cells, but no actual streak or cell migratory event has been identified</td>
</tr>
<tr>
<td>Time-Dependent activation of key genes during development</td>
<td>yes</td>
<td>Has been show in a few studies that in vitro gene progression, roughly recapitulates in vivo genes</td>
</tr>
<tr>
<td>Inductive forces of other cell populations (epithelial-mesenchymal interactions)</td>
<td>no</td>
<td>No role for induction can be identified due to lack of control over populations of cells, although visceral endoderm believed to induce gastrulation</td>
</tr>
<tr>
<td>Spatial and Temporal Regulated Microenvironments (Gradients of Key Growth Factors or necessity of sequential steps)</td>
<td>no</td>
<td>Key factors like BMP, Notch, Wnt, and FGF, have been implicated in cocktails but precise roles have not been defined in ES cells</td>
</tr>
<tr>
<td>Defined embryonic versus extraembryonic endoderm formation</td>
<td>no</td>
<td>Visceral endoderm and definitive endoderm have been identified but not distinguished in EB cultures</td>
</tr>
<tr>
<td>Defined formation of cell layers with cell layer movement, folding, and compartmentalization</td>
<td>no</td>
<td>Separate layers of germ layers, highly preserved in vivo, not identified in EB.</td>
</tr>
<tr>
<td>Fixed locations and microenvironments with known cell types</td>
<td>no</td>
<td>No spatial control over cells once EB is plated</td>
</tr>
<tr>
<td>Unique developmental programs for each germ layer resulting in one specific cell type of interest in adult organism</td>
<td>no</td>
<td>Evidence that mixed developmental signals result in mixed developmental programs being activated within a cell or cell population</td>
</tr>
<tr>
<td>Controlled size of relative cell populations</td>
<td>no</td>
<td>Differential Cell Kinetics in vitro in ES cell cultures</td>
</tr>
</tbody>
</table>

Table 1.1 Large differences between in vivo and in vitro ES cell development
<table>
<thead>
<tr>
<th>Marker</th>
<th>Cell Type</th>
<th>Structure/Function</th>
<th>Expression in Mouse ES Cells</th>
<th>Kinetic Information</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSEA-1</td>
<td>ES Cells, Inner Cell Mass</td>
<td>The Lewis x epitope, an a(1,3)-fucosylated glycan,Galb1-4(Fuca1-3)GlcNAcb1-R, type II poly lactosamine lacto-series glycolipid</td>
<td>yes, demonstrated to be present early d4-5 and late d12</td>
<td>Varies 55 % at d0 to about 30% at d6 to 10% at d12</td>
<td>Ling and Neben '97 and others</td>
</tr>
<tr>
<td>SSEA-3</td>
<td>Early ES cell, Visceral Endoderm</td>
<td>An extended globo-series glycosphingolipid. Involved in morula compaction in F9 EC cells and signaling due to carb-carb interactions.</td>
<td>yes, down regulated with differentiation, ?Visceral endoderm</td>
<td>?</td>
<td>Krupnick, JG</td>
</tr>
<tr>
<td>Forssman</td>
<td>Inner Cell Mass preimplantation /Embryonic Endoderm (d 6.5)</td>
<td>A Globo-series of glycolipids. Binds Inner cell Mass prior to implantation, in postimplantation stains embryonic endoderm. Function unknown.</td>
<td>yes</td>
<td>75 % at d0 to 25 % by d12 in ES cell suspension culture. Gradually decreases</td>
<td></td>
</tr>
<tr>
<td>Flk1</td>
<td>Mesoderm (Lateral Plate), Hematopoietic progenitor</td>
<td>Fetal liver kinase 1 (Flk-1)/VEGF receptor 27 (VEGFR2) is one of the receptors of VEGF. Involved in the development of Endo.</td>
<td>yes</td>
<td>In mouse embryos, Flk1 expression in the presumptive mesodermal yolk sac as early as E7.</td>
<td>Yun Shin Chung 2002</td>
</tr>
<tr>
<td>FGFR1</td>
<td>Endoderm</td>
<td>FGF is family of GF and GF receptors. Require Heparin Sulfate PG or other embryoglycans for binding implicated</td>
<td>Yes, present on ES cells and knockout studies present.</td>
<td>Unknown</td>
<td>Esner M 2002</td>
</tr>
<tr>
<td>HGFR (C-met)</td>
<td>Hepatoblast, Endothelial, Hematopoietic</td>
<td>The c-Met is a receptor tyrosine kinase. Hepatocyte growth factor (HGF) is a mesenchymal derived growth factor</td>
<td></td>
<td>In P19 Mouse EC cells, D7-14 plated aggregates studied.</td>
<td>Yang, Xiu-Ming and Park, Morag'93</td>
</tr>
<tr>
<td>Thy1.2 (CD90)</td>
<td>Thymus, thymocytes, fibroblast, bone marrow</td>
<td>GPI-anchored membrane glycoprotein of the IgG family involved in signaling</td>
<td>yes initially at 25% of cell population, down by day 6 and up to 20 % by d12</td>
<td></td>
<td>Parekh 1987, Ling and Neben '97</td>
</tr>
<tr>
<td>CD34</td>
<td>Mesoderm, Endothelial Cells, Hematopoietic Progenitors</td>
<td>Neuraminidase sensitive(sialomucin) epitope on hematopoietic progenitors, no known function</td>
<td></td>
<td>Mesoderm, Endothelial, Hematopoietic precursors. CD34-null ES cells showed a significant</td>
<td>Cheng '96</td>
</tr>
<tr>
<td>Ckit</td>
<td>Hematopoietic and ES cells</td>
<td>Transmembrane tyrosine kinase receptor, a protooncogene that binds to Steel Factor, a soluble ligand.</td>
<td>yes</td>
<td>50% day 0 to 75 % by day 12</td>
<td>Ling and Neben 1997</td>
</tr>
</tbody>
</table>

Table 1.2 Cell surface markers during early development
CHAPTER 2:
CONCEPTS AND TECHNIQUES IN ENDODERM INDUCTION AND PATTERNING

2.1 Introduction

Here, concepts regarding in vivo endoderm development, with the goal of applying these concepts to ES cell cultures, are presented. Molecular aspects of endoderm induction, recent insights into endoderm gene discovery, and fundamental concepts in endoderm patterning are all reviewed. Lastly, techniques used to study early development, which also will have applicability to understanding embryonic stem cells, are presented.

2.2 Overview—endoderm induction and patterning

Regarding endoderm and the complex organs which arise from it, there are many recent reviews, yet there is a lack of information when compared to mesoderm or ectoderm germ layers. Some pitfalls in mouse endoderm studies thus far, as pointed out by Wells, et al. (1999) are: 1) Mouse endoderm cells are small in number and difficult to study 2) Genes that are markers of early endoderm are scarce 3) Embryonic endoderm is difficult to obtain 4) Favorable culture conditions have not been established and 5) No endodermal cell lines exist. Endoderm has been the most difficult germ layer to form in vitro thus far. This suggests that current strategies are limited by the ability to induce and pattern endoderm, and thus these two critical steps may be rate limiting steps in differentiation toward endodermally-derived organs (Wells and Melton 1999).

The definitive endoderm gives rise to critical organs which specialize in respiration, metabolism, and endocrine function. The differentiation of endoderm in the context of organogenesis, will give rise to epithelial linings of the gastrointestinal including the oropharynx, thymus, thyroid, trachea, lower respiratory system, upper and lower gastrointestinal system, liver, pancreas, prostate and bladder. Numerous types of epithelium are present in these cases, including squamous, cuboidal, columnar, and transitional. These epithelial cells provide a barrier between the outside and inside world, provide transport of nutrients, ions, and gases between the outside and the inside, or between the blood and the organ.

Endoderm induction is the process by which the initial embryonic precursor, the epiblast, gives rise to mesendoderm which then gives rise to endoderm. This complex process takes place in the context of
gastrulation, or germ layer formation, and is regulated by soluble factors and transcription factors.

Specialized sets of molecules, called organizers, also have key roles in endoderm induction (see Chapter 1). The first step after endoderm induction is called endoderm patterning, during which the endoderm begins to differentiate to varying extents along the anterior-posterior axis of the embryo. This process is also regulated by soluble factors and transcription factors. Each portion of the foregut is in contact with slightly different portions of mesenchyme, which are believed to result in reciprocal interactions that can govern organogenesis. Defective endoderm patterning itself is believed to be responsible for numerous congenital diseases of the gut, which are the most common type of congenital anomalies. Figure 2.1 demonstrates key lineage decisions during endoderm induction. Figure 2.2 demonstrates key lineages during endoderm patterning. Understanding, modeling and controlling endoderm differentiation will also be useful for understanding the molecular basis for congenital defects. These congenital diseases are an important cause of morbidity and mortality and treatments are typically treated surgically, and there are no curative therapies for these congenital diseases.

**2.3 Relevance to adult stem cells and cancer**

The endoderm derived organs are highly specialized, and each tissue type is believed to harbor adult stem cells. The intestinal stem cell is one of the most well understood stem cells, and has been a useful model experimental system (Holmberg, Genander et al. 2006). In the intestine, epithelial stem cells reside at or near the bottom of crypts along the intestinal lumen at the base of the villus. Members of the BMP and hedgehog families direct the positioning of the crypts. Wnt proteins present at the bottom of the crypts induce nuclear translocation of β-catenin, and synergistically with the Notch pathway, β-catenin maintains intestinal stem cells and controls their differentiation. The role for adult stem cell populations in epithelial tissue homeostasis is other endodermal organs has recently been reviewed, including Liver (Dahlke, Popp et al. 2004), Lung (Kim, Jackson et al. 2005), and Pancreas (Zhang, Kritzik et al. 2005). This suggests that studying endoderm induction and patterning from ES cells will also result in furthering of knowledge of adult stem cells and vice versa.

Cancer stem cells have recently received increased attention (Dalerba, Cho et al. 2007) In many tumors, fetal proteins or proteins in earlier development are activated, (Yao, Dong et al. 2007), indicating
that these cells may take on the characteristics of a more immature cell type. The best example of this is alphafetoprotein (AFP), which is a secreted protein, and is used as a clinical marker for numerous epithelial malignancies, such as colon cancer. This suggests that knowledge about induction and differentiation of endoderm in vitro could be used to understand tumors of endodermal organs. This includes tumors of the bladder, prostate, lung, and pancreas, which have enormous morbidity and mortality.

2.4 The role of regulatory transcription factors in understanding early development

2.4.1 Overview and significance

Since development is tightly controlled, transcriptional control of early development is important in shaping the early cell populations that either interact with or are precursors to endoderm. Evolutionarily conserved, master developmental transcription factors are believed to form networks that are conserved across organisms and govern early endoderm development. These transcription factors are typically used as markers for early ES cell populations and/or endoderm (see Chapter 3-6). Their role in development is studied by either gene knockout or overexpression, and careful analysis of the mutant phenotype. Here, concepts of endoderm induction, and major contributors to early endoderm development, are reviewed. The knockout phenotypes of major epiblast, mesendodermal, and endodermal transcription factors are described in detail in Table 2.1.

2.4.2 Role for regulatory transcription factors in control of differentiation

Although there are no known cell surface markers of endoderm, recent research in endoderm development has pointed towards key regulatory transcription factors (TF) which are conserved amongst various species which may be involved in induction, maintenance, and patterning of the endoderm. Mouse knockout studies of Brachyury, GATA4/6, Sox 17, Foxa2, (Kimelman and Griffin 2000; Hallonet, Kaestner et al. 2002; Kanai-Azuma, Kanai et al. 2002; Loose and Patient 2004) combined with RNA in situ studies during early development, have clearly demonstrated that these regulatory TF’s are present in the endoderm germ layer and are critical for its induction, maintenance, and/or differentiation. Interestingly, these four molecules are conserved throughout many invertebrates and all vertebrates and are shown to be involved in endoderm development. Two more recent molecules also found to be critical for mouse endoderm
development/differentiation include FTF (Fetoprotein Transcription Factor) and HNF1β (Tcf2) (Barbacci, Reber et al. 1999; Rausa, Galarneau et al. 1999). The TF evaluated in detail includes several important epiblast, mesendodermal, or endodermal transcription factors, including Foxa2, Brachyury, Goosecoid, Lim1, Otx2, Evx1, Tbx6, FoxH1, Sox17.

Shivdasani et al 2004 has reviewed molecular control of endoderm development and the several functions they may have. These TF have a role in: 1) Initiating regional expression of essential soluble ligands 2) Sequentially activating a transcriptional cascade that confers endodermal identity at the expense of alternate cell fates 3) Activating stage-specific endodermal genes while repressing genes associated with other germ layers. 4) Generating a machinery to respond to growth and differentiation signals and 5) Establishing domains of chromatin configuration that prime cells for subsequent high-level expression of differentiation products (Shivdasani 2002).

In general, regulatory TF functions are an essential part of a gene regulatory mechanism. The regulatory mechanism is highly complex, involving multiple layers of temporal and spatial feedback control loops and dynamic chromatin modeling (Zhou and Ly 2003). A pre-initiation complex can assemble at the promoter/start site which includes numerous proteins and RNA Polymerase II. While basal transcription may or may not occur, stimulated transcription requires TF that have DNA binding domains, and transaactivating domains which interact with other TF. In addition, coregulators and corepressors which bind to TF but not to DNA help facilitate and regulate transcription. Lastly, chromatin remodeling and covalent modification, and epigenetic regulation (methylation) are another level of control of gene regulation (Zhou and Ly 2003).

2.4.3 Role for regulatory transcription factors in Embryonic Stem cells

Several studies in ES cells have shown that manipulating the concentration of a TF can affect the lineages that form from that cell. This approach was first attempted for endoderm differentiation in ES cells in Levinson-Dushnik et al. Foxa2 (HNF3β) was overexpressed which resulted in increased markers of endodermal derivatives like Lung and Liver (Levinson-Dushnik and Benvenisty 1997). Zhao et al. (2002) studied differentiation of ES cells in which Sox2 overexpression led to a highly enriched population of neuroepithelial precursor cells. In addition, regulatory TF have been shown to respond to other TF and
direct differentiation with their precise concentrations. The transcription factor Brachyury is altered in a concentration-dependent manner in the presence of BMP4 and results in mesodermal formation from ES cells (Johansson and Wiles 1995). Conditional up or down-regulation of the essential POU homeodomain protein Oct4 results in modulation of lineage allocation. Intermediate levels of Oct4 maintains pluripotency, while high levels induce mesoderm and primitive endoderm-like cells while low levels induce trophoblast-like cells (Hay, Sutherland et al. 2004). Clearly, a proper understanding of endoderm development from ES cells in terms of regulatory transcription factors will likely facilitate the discovery of new molecular diagnostics, and augment the development of stem cell-based therapeutics for gene, cell, and tissue therapies.

If control of regulatory transcription factors can lead to control of differentiation, than the cis-regulatory modules of these TF, which include the promoter and the enhancer regions and control the output of a particular TF, are critical determinants of the outcome of development. Thus knowledge of how regulatory TF negatively and positively regulate the cis-regulatory regions of other TF is fundamental to understanding how ES differentiation proceeds.

2.4.4 Endoderm circuits of regulatory TF’s in sea urchin

In vivo developmental studies Sea Urchin have shed light on important principles which represent possible mechanisms of controlling endoderm differentiation in ES cells (Bolouri and Davidson 2002). These organisms have been studied rigorously to point where a fate map of every cell in every field has been developed. Studies regarding Sea Urchin development have allowed expansion of knowledge regarding the roles of not just regulatory TF. In addition, a regulatory TF network that has been constructed that models specification of the mesoderm and endoderm from mesendoderm. This network was constructed on the basis of large scale perturbation experiments by numerous investigators which utilize mRNA, antisense, pathway blockers, and genetically engineered mutants. The data from all these studies was pooled in order to design the network. The genes which were identified as having responses underwent further sequence analysis for identifying binding sites on their promoter/enhancer of the TF which might have caused a perturbation. Interestingly, the endodermal TF’s from all the major familes, such as Brachyury, Fox, HNF1β(Tcf), GATA, and Sox are all key components of the regulatory network. When the network is
viewed with time, one can see how commitment occurs; an initial stimulus is propagated and stabilized through positive feedback loops as well as expanded into other control elements. A putative regulatory network based on the literature in mouse, that may be applicable in stem cells, is shown in Figure 2.3. Details of the network are explained below Figure 2.3.

2.5 Gene screening/discovery for endoderm induction and patterning

2.5.1 Background and significance

The ability to create therapeutically useful cells from ES cells lies in the ability to differentiate endoderm cells and compare them to known endoderm genes. However, very little is known in fact about the specific genes that define endoderm, other than genes and proteins discovered through classic genetic mutation. The recent enthusiasm for stem cell differentiation methodologies and the clinical potential for these cells have heightened the need for better tools and a further understanding of normal embryonic development. Whether ES cells have the capacity to differentiate into cell types that closely resemble early embryonic populations such as definitive endoderm is unknown in part because the embryonic equivalents have not been sufficiently characterized (Sherwood, Jitianu et al. 2007). This clearly can act as a roadblock in terms of developing therapeutically useful cells.

Here we review several recent studies which attempt to discover and identify new markers in endoderm progenitor cells. Most of these techniques rely on array based approaches, using cutting edge genomics techniques.

2.5.2 Endoderm discovery through DNA microarray based approaches

2.5.2.1 Separation of germ layers and spatial/temporal analysis

One approach to identification of new markers is to use DNA arrays, which are useful for large scale gene identification. Since endoderm is scarce and difficult to isolate from surrounding tissues, a limiting problem in using DNA arrays is to obtain pure or enriched populations prior to study. Gu and Wells et al. developed techniques to purify germ layers (endoderm, ectoderm, mesoderm) and pool these germ layer isolates from mouse embryos at various stages of endoderm and pancreas development. Isolated RNA from germ layers were compared to their opposing germ layers, as well as to later stage tissues; however, somite stage was not controlled for in this study, as it has been shown that at a particular
time point there can be a variety of somite stage embryos. Nevertheless, this technique allowed for identification of spatial and temporal differences in gene regulation, by comparing endoderm to later times in development (temporal) as well as to other germ layers at the same time (spatial). This is important because in ES cell cultures, both kinetic and spatial data (markers that confer particular locations in vivo) may be dysregulated. Amplified RNA samples were hybridized to the Affymetrix microarrays (12,000 genes) and the data was analyzed using GeneSpring and Resolver clustering analysis software. E7.5 endoderm expressed 193 genes or expression sequence tags (EST) (out of the ~12,000 on the microarray) at greater than threefold higher levels than cells at later stages of pancreas development. These include 25 growth factors or other signaling-related molecules and 44 TF or other nuclear proteins. Many of these factors were previously implicated in embryonic pattern formation. For example, endoderm expresses molecules involved in TGFβ signaling superfamily molecules, including Nodal, cerberus 1, and follistatin and the Wnt antagonist dickkopf, and endoderm-expressed transcription factors including Cdx1, Hesx1, Irx3, Gata3, Mespl and Sox17. In addition, new signaling pathways were identified in endoderm and pancreatic development, including the C-Kit ligand, Edg2 (G-protein coupled receptor) and Epha2 (Eph receptor A2). Spatial analysis of gene expression, that is endoderm compared to ectoderm and mesoderm, was also accomplished. 203 transcripts were greater than threefold enriched in endoderm, while 262 were enriched in the mesoderm plus ectoderm. Several genes such as Hes1 (hairy enhancer of split), Klf5 (kruppel-like factor 5), EYA2 and Six1 were enriched in endoderm. Additionally, there were several signaling molecule genes that were more abundantly expressed in endoderm, as compared to mesoderm + ectoderm, including Wnt11, IgfII, chordin, cerberus 1 (Cer1) and growth factors such as, Fgfbp1 and Igfbp5. In mesoderm, genes cellular RAbp2, Follistatin, Gbx2, Hoxa1, Zic 2 were found upregulated in the mesoderm/ectoderm mixture (Gu, Wells et al. 2004).

Moore-Scott, and Opoka et al. 2007 have advanced these studies further. Using the data from the arrays, in situ hybridization analyses was done to identify genes that are expressed in discrete Anterior-Posterior (A-P) domains of the gastrula stage endoderm and in the developing fetal gut. Three genes that are expressed in a region of endoderm overlying the node, Tmprss2, NM_029639, and Dsp, were studied. Importantly, these endoderm cells map to the midgut domain, and markers for this domain of endoderm have not previously been identified. Furthermore, two genes were studied which are expressed in future
hindgut endoderm, Klf5 and Epha2. Interestingly, the expression of these five genes persists in restricted to posterior domains of the midgut and hindgut up to Day 9-10.5, suggesting that these genes predict gut tube domains.

2.5.2.2 Refined array approach by separation of germ layers and extraembryonic structures

Another recent array based study (Sherwood, Jitianu et al. 2007) was focused on establishing an endoderm signature and differentiating between definitive and visceral endoderm. In this study, gene expression profiling was combined with cell surface marker identification and sorting in order to obtain pure populations of E8.25 definitive and extraembryonic endoderm as well as non-endodermal tissues. The cell surface of isolated embryonic cells with the highest expressors of Sox 17-venus (venus being similar to GFP) cells was characterized using new antibodies and lectins. A monoclonal antibody, G8.8, that recognizes the cell adhesion protein EpCAM (Farr et al., 1991) was found to identify EpCAM, which remains strongly expressed in most endodermal cells until at least d12.5. Thus, EpCAM allows isolation of endoderm at and after d8.25. For isolation of endoderm at stages earlier than d8.25, lectin soybean bound to endoderm at d7.5 and colocalizes with EpCAM(hi) endoderm at d8.25 and d9.5. To distinguish between visceral and definitive endoderm, EpCAM cells were again screened for cell surface markers. Dolichos biflorus lectin (DBA) and SSEA-4 were found to be markers for visceral endoderm. This study established new approaches for isolating definite endoderm from d8.25 whole embryos. The populations included anEpCAM(hi)DBA(−)SSEA-4(−) for definitive endoderm and EpCAM(hi) DBA(+)SSEA-4(+) as visceral endoderm.

The ability to isolate definitive and visceral endoderm as well as mesoderm and neuroectoderm/notochord permitted global gene expression analysis of these tissues. Thus, microarray analysis was performed on d8.25 (2–8 somites) embryonic and extraembryonic tissues using three different isolation techniques. These approaches included endoderm obtained from whole embryo microdissection, isolation of Sox 17 positive cells from whole embryo, and dissected and sorted extraembryonic EpCAM(hi) DBA(+) visceral endoderm, extraembryonic EpCAM(−) DBA(−) yolk sac mesoderm, embryonic EpCAM(hi)DBA(−) definitive endoderm, embryonic EpCAM(lo)DBA(−) neuroectoderm/ and mesoderm. In all cases, 3–6 biological replicates of amplified cDNA were hybridized to hybridized to Affymetrix
Mouse Genome 430 2.0 GeneChips which has a capacity of 39,000 transcripts. Results using microarrays contrasted with those reported in vitro studies. Interestingly, genes used to identify endoderm, such as Mixl1, Gsc, T, Cer1, and Hhex, Hhex (Kubo, Shinozaki et al. 2004; D'Amour, Agulnick et al. 2005; Tada, Era et al. 2005; Yasunaga, Tada et al. 2005) were not found to be expressed at higher levels in E8.25 definitive endoderm than in non-endodermal tissues. Furthermore, CXCR4, reported as an in vitro cell surface marker for definitive endoderm (D'Amour, Agulnick et al. 2005; Yasunaga, Tada et al. 2005) was expressed equally strongly in definitive endoderm, neuroectoderm/notochord and mesoderm. In contrast, its utility appears to be in distinguishing embryonic and extraembryonic tissues and not in recognizing endoderm.

The profiling of visceral and definitive endoderm resulted in more interesting data. Only four transcription factors were expressed >2-fold higher in definitive endoderm and >3-fold higher in visceral endoderm than any other comparison tissue. These transcription factors were Sox17, FoxA1, Ripk4 and 5730467H21Rik (unknown). Transcription factors expressed exclusively in either definitive or visceral endoderm were also analyzed, and ten transcription factors expressed >2-fold higher in definitive endoderm than in any other tissue all have known expression patterns at d8.25. These TF are involved in components of pharyngeal endoderm organogenesis, and none of these genes is expressed throughout the endoderm. Four uncharacterized TF are expressed >3-fold higher in visceral endoderm than in all other tissues (Irf5, Nfatc2, Npas2, Vdr). Other visceral endoderm enriched TF included Hnf4a Tcf2, Cited1, and FoxA3.

TF that were expressed in other tissues, but varied significantly between visceral and definitive endoderm were 12 homeobox family transcription factors (HoxA1, HoxA3, HoxA9, HoxB1, HoxB2, HoxB3, HoxB9, HoxC4, HoxC8, HoxD1, HoxD8, HoxD9) and members of other homeobox families involved in embryonic patterning such as paired (Pax1, Pax3, Pax6, Pax8, Pax9), distal-less (Dlx2), Iroquois related (Irx2, Irx3, Irx5), sine oculis-related (Six1, Six3) and SRY-box containing (Sox9, Sox11, Sox21). TF expressed >3-fold higher in visceral than definitive endoderm but also expressed in non-endodermal tissues included one homeobox transcription factor (HoxB8) and other TF such as Gata4, Gata6, Lhx1, and Sox7 that are implicated in endoderm formation in lower vertebrates. Plasma membrane proteins were also analyzed. Plasma membrane-localized genes upregulated >3-fold in visceral endoderm as compared to all other tissues were Icam1, Ceacam1, Dpp4, CD38 and Timd2.
Sherwood et. al. then designed an endodermal signature based on genes overexpressed in definitive endoderm versus non-endodermal tissues and not excluding visceral endoderm expression. Thirty-one genes that were enriched >2-fold in dissected definitive endoderm, and sorted into Sox17Venus (hi) endoderm and in embryonic EpCAM (hi)DBA(−) definitive endoderm as compared to all other nonendodermal tissues, were chosen for further examination as “endoderm signature” genes. Four of the genes were already presented as endoderm enriched genes. The remaining 27 genes were analyzed, and it was found that 16 of these genes (5730521E12Rik, AnxA4, Bnipl, Cacna1b, Cdep1, Cldn8, Clic6, Dsg2, Krt2-7, Npnt, Rab15, Rbm35a, Ripk4, Sh3gl2, St14, and Tmprss2) were highly enriched in endoderm by in situ hybridization, antibody staining, and qPCR, while (Spink3 and Tmem30b) were enriched by two of three techniques. Other candidates were not present at all times (5730467H21Rik, Crb3, Dpp4, Ell3, Gpgrc5c, Prss8). Thus, a total of 22 genes, enriched in definitive and visceral endoderm can collectively be used as an “endoderm signature.”

2.5.2.3 Conclusion
Gene discovery approaches have been critical not only for discovering new genes but also in questioning markers used in current ES cell protocols for endoderm induction. This reflects how little is known about endoderm induction and the transcription factors and other molecules that are differentially expressed within it.

2.5.3 Endoderm gene discovery by high throughput functional screening
In this study, Chiao and Baker et al. used a high-throughput method in which >60,000 mouse gastrula cDNA clones from mouse cDNA libraries were injected into Frog embryo (Xenopus.) The phenotypes were then screened, which resulted in identification of new pathways that regulate mesoderm or endoderm. This technique is feasible because the Xenopus embryo can uptake RNA/DNA without any limitations. Pools of cDNA were selected in a high throughput fashion in order to obtain single active clones which reproduced phenotype. In this study two separate mouse cDNA libraries (stages d6.5 and 7.5) were arrayed on 384-well plates, and pools of 96 were generated from this. Next functional screening was accomplished by examining effects of overexpression within the presumptive mesoderm/endoderm or ectoderm. To
check for the ability to induce endoderm or mesoderm, three Xenopus markers, XBra (Brachyury), Xsox17 (Sox 17), or endodermin (edd), were used. Morphological screening was also done based on changes in cell cycle or patterning, and these were assumed to be due to high doses.

The results were that eighty-two pools (11.5%) had reproducible phenotypic effects on either endoderm and mesoderm (55) or general morphology (27). From these, 23 single active clones were isolated by mesoderm and/or endoderm activities and 19 were isolated by general morphology changes. These clones were sequenced and their identity revealed by BLAST searches (Altschul et al. 1990). Eight molecules, Mixl1, Hspa8, Kif22, Fzr1/Cdh1, Tcfeb, Habp2, Otx2, and Foxa2 resulted in ectopic induction of endoderm. Four molecules were identified that led to the ectopic formation of both endodermal markers—Kif22, Mixl1, Hspa8, and Fzr1/Cdh1 while four were specific for induction of edd-Tcfeb, Habp2, Otx2, and Foxa2. None were capable of inducing Xsox17 without edd in the whole embryo, consistent with a known model for the regulatory network in which edd is downstream of Xsox17. Three molecules, Tcfeb, Otx2, and Foxa2, that induce edd but not Xsox17, strongly suggest that they act downstream of Xsox17. Interestingly, molecules that disrupt mesoderm or endoderm, which could eventually be used to repress cell fate, were identified. These included Rsk4, Cks2, Lefty2, cyclin F, D7Ssb2, and geminin. Lefty2, geminin, and D7Ssb2 inhibited both Xbra and edd expression, while cyclin F, Cks2, and Rsk4 inhibited only Xbra. β-catenin, RhoA, D7Ssb2, Fgf8a, Wnt3a, Bmp4, and cyclin F resulted in specific developmental phenotypes.

In conclusion, this approach provided a valuable tool by applying a large number of genes in a high throughput fashion, and identified novel activators and repressors of endoderm. The next step would be to identify these in actually mouse models rather than Xenopus models. The most interesting aspect was that molecules involved in cell growth or cell cycle directly effected endoderm induction, suggesting that perhaps the endoderm regulatory network is associated with the cell cycle in an unique an unknown way.

2.5.4 Endoderm gene discovery by serial analyses of gene expression (SAGE)

2.5.4.1 Introduction

Hou et al (2007) recently used Serial Analysis of Gene Expression (SAGE) for endoderm gene discovery. SAGE is an alternative approach to DNA array and functional cDNA screening which provides quantitative
gene expression profile information. Compared with microarrays, SAGE permits the identification of novel transcripts and the data can be easily shared among investigators and compared across different experiments and tissues (Hou, Charters et al. 2007). The overall approach was use longSAGE libraries, obtain tagged sequences, and compare these sequences to other known libraries, bioinformatics criteria and the mouse genome. Then these candidate genes were functionally validated by qPCR and or in situ hybridization. The SAGE sampling depth (~100,000 tags per library) in this study yields gene detection sensitivity approximately equivalent to that of fluorescence-based microarray approaches, and is thus sufficient for detection of abundant and moderately abundant transcripts but is likely insufficient for reliable detection of rare transcripts. Sequencing SAGE libraries to a greater depth can prevent this problem.

2.5.4.2 Details of approach using SAGE of endoderm libraries

Hou et al. generated and analyzed three mouse definitive endoderm longSAGE libraries. A list of candidate genes enriched for expression in endoderm was compiled through comparisons within these three endoderm libraries and against 133 mouse longSAGE libraries generated by the Mouse Atlas of Gene Expression Project [32,33]. Sixty nine percent of these candidate genes showed previously uncharacterized expression in restricted tissues, including definitive endoderm, after further whole mount in situ hybridization validation.

Hou et al. created libraries using early whole endoderm library (SM108), foregut (including notochord), and hindgut (including notochord) libraries respectively (SM107 and SM112). A total of 322,208 tags were sequenced from these three longSAGE endoderm libraries. Next unique tag-sequences were identified and the corresponding genes were identified by comparison to transcript databases (Refseq, MGC and Ensembl). Remaining unmatched tag-sequences were then mapped to Ensembl gene units, which were extracted from the Ensembl database, and tags were eventually mapped to the mouse genome. The quality of the endoderm libraries were then assayed using known endoderm enriched genes. For identification of foregut endoderm genes, a cross-comparison between the two libraries (SM107 and SM112, respectively) was performed, based on criteria regarding transcript type and number, thus allowing identification of top 25 highly expressed tags in foregut and top 20 in hindgut libraries. These genes were
then subject to qPCR or whole mount in situ hybridization. Seven of these genes, Trh, Otx2, Prrx2, Tbx1, Cyp26a1, Hoxb6, and Cdx1 were expressed in other tissues as well as endoderm at the early somite stage. One of the genes, Pyy, was exclusively expressed in the foregut endoderm. To increase the efficiency of identification of novel endoderm genes, the Mouse Atlas of Gene Expression Project database was used, which contains 133 libraries from different tissues and stages of development. The tag sequences were then compared to the longSAGE libraries and positive searches for sequences that had limited expression in as few libraries as possible were identified. Nine(9) genes exhibited high tissue-specificity since they were present in only a few libraries. To identify genes expressed in the definitive endoderm, a constraint based technique was used. The constraints were a higher T/L ratio representing the total number of Mouse Atlas SAGE libraries in which a tag-sequence was present (L), and the total number of times that a tag-sequence was found in the three pooled endoderm libraries (T). All genes that were confirmed in vivo in endoderm or other tissues were then classified into groups. Importantly, two genes identified, Pyy and 5730521E12Rik, showed exclusive definitive endoderm expression at early stages of endoderm patterning. In addition to Pyy, 5730521E12Rik exhibited exclusive expression in the definitive endoderm at early somite stages in our analysis and was restricted to the midgut.

2.5.4.3 Conclusion

In this case, serial analysis of gene expression (SAGE) was able to identify potential new markers in endoderm. This technique was dependent upon the availability of mouse endoderm libraries. Regarding ES cells, one possible application would be to compare genes obtained through DNA microarray in endoderm studies and compare to known mouse endoderm libraries. Another approach would be to create libraries based on endoderm induction from ES cells and compare those to either in vitro libraries or to in vivo libraries. The goal in this case would be to identify differences between in vitro endoderm and in vivo endoderm.

2.5.5 Discovery of control by endoderm regulatory networks

2.5.5.1 Introduction
The advent of microarray technology and bioinformatics has provided a powerful tool to determine the transcriptional regulatory circuits that control complex developmental processes. The regulatory networks that guide these processes depend on an array of transcription factors (TF). Thus, how these TF control development is not well understood. For example, some regulatory TF are activated transiently, while others are more persistent during early development. Here, a study by Gaudet et al uses computational approaches to attempt to answer these questions with applications to gut development in C. Elegans.

2.5.5.2 Approach

Focusing of the pha-4/FoxA gene in C. Elegans, the authors (Gaudet, Muttumu et al. 2004) first propose a general principle based on previous studies. The hypothesis is that high affinity sites for cis-regulatory elements that Pha-4 bind to, are typically found in promoters of genes expressed early in development, whereas low-affinity sites are restricted to late promoters (Gaudet and Mango 2002). Genes activated at later stages at high affinity promoters, which occurs for Pha-4, are due to combinatorial effects with other TF.

The approach was to first identify candidate pharyngeal genes by microarray analysis and then subdivide these genes into clusters based on their initiation of gene expression. From the list of these genes, a list of candidate promoters was obtained. These were searched for short sequences enriched from early or late clusters. These sequences were then tested for enhancer or repressor activity in vivo using both natural and synthetic promoters. To identify candidate genes, the investigators used two mutant phenotypes in which effects on pha-4 were either significantly higher or significantly lower than wild type, resulting in a differential expression level of approximately 100 fold. These were then classified into early or late gene expression groups based on in vivo data, the Nematode Expression Pattern Database, and GFP expression. The list was narrowed to 37 early expression, 34 late expression genes, and 43 genes that were indeterminate. Interestingly, the early genes were found to be enriched for transcription factors, whereas the late set was found to be enriched for muscle and cytoskeletal proteins.

The cis-regulatory regions of the candidate genes were then examined looking for conserved sequences within either 500bp or 500-1000bp of the start site. Next, the “Improbizer expectation maximization algorithm” was used to search pharyngeal promoters for potential regulatory elements.
Improbizer detects short sequences that are over-represented within a cohort of genes. Motifs that were common to Pha4 and negative control gene sets were eliminated. Using this approach, nine candidate sequences were identified. Of these five were associated with either the early or late data sets. Early motifs occurred nearly twice as frequently for early expression genes than for late expression genes. Late motifs occurred two to three fold times more than in late gene cluster than in the early gene cluster.

Next, the positive effects of these regulatory elements were tested by determining whether a motif was sufficient to activate gene expression with a heterologous basal promoter. Knockout phenotypes were then measured by using site-directed mutagenesis to inactivate a motif within a native pharyngeal promoter and examine whether it was necessary for expression. These studies then identified that these elements combined with Pha4 to positively or negatively regulate gene expression either at early or late phases of development. Interestingly, combination of elements observed in natural promoters suggests that a gene with a high-affinity PHA-4 site and an early element will be expressed early in development, while a gene with a high-affinity PHA-4 site and a late element will be expressed later in development. The authors suggest that a cis-regulatory motif combined with the PHA-4 binding site establishes a regulatory network that can account for the timing of activation of at least half of C. elegans pharyngeal genes.

2.5.5.3 Conclusion

The authors took extensive experimental and computational approach to identifying novel cis-regulatory regions or regulatory elements that contribute to temporal gene expression during endoderm formation. Amazingly, the cis-regulatory motifs discovered here, combined with the PHA-4 binding site, establish a regulatory network that can account for the timing of activation of at least half of C. elegans pharyngeal genes. The test of new candidate regulatory regions included: 1) testing whether the regulatory elements were necessary for expression of native pharyngeal genes 2) determining whether they were sufficient for pharyngeal expression from synthetic promoters and 3) genome-wide searches based on these elements. With a good model for endoderm induction from ES cells, and good mouse genome data, this type of approach could be used for determining the cis-regulatory gene network in endoderm.
2.6 Endoderm patterning

2.6.1 Introduction

Endoderm patterning is the process by which endoderm progenitors cells are specified into particular tissue types. This process is extremely relevant clinically, and defective endoderm patterning can be viewed as the cause of all major congenital gastrointestinal abnormalities. Endoderm patterning in vivo occurs by exposure to adjacent tissues, such as mesenchyme, and the factors that they secrete. Also, patterning occurs when critical transcription factors are activated in particular domains, thereby specifying their fate but restricting their potency. The ability to differentiate endoderm progenitor cells can also be stated as the ability to pattern endoderm progenitors in vitro. The process of endoderm patterning appears to be a rate limiting step in vitro differentiation protocols of ES cells, based on the difficulty in differentiating endoderm from embryonic stem cells.

2.6.2 Endoderm patterning

After endoderm induction, the next step in the sequential endoderm differentiation process is called endodermal “patterning.” This occurs when the gut tube receives instructive signals to form regionalized “domains.” These tissue domains induce formation of primary buds which eventually form the major organs. It is this step where the largest amount of lineage diversification occurs. Developmental biologists have observed and identified that certain genes or transcription factors (TF) are expressed in different domains of tissue after endodermal tissue patterning. For example, in patterning of the foregut endoderm, markers for Liver, Lung, and Pancreas are apparent prior to major morphologic changes and bud formation. (Wells and Melton 2000). For Liver, the albumin gene is the first gene activated (Ang, Wierda et al. 1993) This process represents commitment to a certain lineage and occurs rapidly, within a matter of two days in mice.

Two parameters that govern endoderm patterning are the state of the endoderm progenitor cell itself and the state of its environment. The state of the endoderm progenitor is characterized by its multipotency, as well as potential differences in this multipotency along the anterior-posterior axis of the embryo. Since the regulatory transcription factor network is believed to confer the multipotentiality of the cells and the competency of the cells to receive instructive signals, the state of the regulatory transcription
factor network becomes fundamental to understanding how to control differentiation. These changes in network are believed to define different domains of endoderm (Yoshitomi and Zaret 2004).

2.6.3 Anterior-posterior markers in endoderm patterning

Although the endoderm appears morphologically homogeneous, differences exist between anterior and posterior. Importantly, anterior endoderm expresses several markers not expressed in posterior endoderm, including cerberus (Bouwmeester, Kim et al. 1996), Otx2 (Rhinn, Dierich et al. 1998) and Hesx1 ((Thomas and Beddington 1996). In contrast, posterior endoderm has a higher cell division rate and expresses intestinal fatty acid binding protein (IFABP; (Green, Cohn et al. 1992)) and Cdx2 (Beck, Erler et al. 1995). This implies that these markers and or transcription factors determine anterior-posterior (A-P) differences.

A major change in reversibility occurs during these phases. Specifically, d7.5 endoderm is not yet irreversibly determined (Gu, Wells et al. 2004) whereas d8.5 endoderm, a day later, has received local patterning instructions that have made the cells more determined. The level of determination can be measured by the ability to “re-pattern” endoderm by changing its relative position in the embryo. Unlike d7.5 endoderm, d8.5 endoderm is no longer competent to express more anterior (pancreatic) markers, suggesting a determination event has occurred. Coincident with early gut tube patterning is a morphological differentiation of endoderm, where the cuboidal-type endoderm of the E7.5 embryo begins to morphologically differentiate into a columnar epithelium, which will eventually line the respiratory and digestive tracts.

2.6.4 Mechanisms of patterning

Recent studies suggest that adjacent germ layers, mesoderm and ectoderm, are also patterned during development, and that these may indeed secrete soluble factors to pattern endoderm (Wells and Melton 2000; Serls, Doherty et al. 2005). For example, anterior endoderm contacts notochord precursor cells and ectoderm fated to become head. Posterior endoderm, however, is in close association with the node, lateral mesoderm, and primitive streak. Importantly, this has important implication for ES cell cultures. If other germ layers are required to differentiate or pattern endoderm, than by creating pure populations of endoderm form ES cells, one may hinder endoderm patterning. Furthermore, differentiating mixed
populations from ES cells may result in defective patterning, since the local interactions can not be
controlled.

Although the molecules that pattern endoderm are undetermined, anterior ectoderm, node and
primitive streak express a variety of signaling molecules (Beddington and Smith 1993). Recently,
preliminary evidence implicates the involvement of early mesoderm and ectoderm in patterning d7.5 mouse
endoderm. These studies have identified that the adjacent germ layers provide soluble, spatio-temporal
specific signals that induce a pattern in endoderm (Wells and Melton 2000). Moreover, explant studies
indicate that posterior endoderm appears to be patterned by the growth factor FGF4, which appears to act in
a concentration dependent manner.

Classic genetic approaches have been used, for example, to determine the functions of the
hedgehog gene family in digestive system development. This family of molecules is implied strongly in gut
development disorders, because genetic studies have demonstrated defect similar to real congenital diseases
(Ramalho-Santos, Melton et al. 2000) Targeted inactivation Sonic hedgehog (Shh) gene in mouse results in
a numerous of malformations, including tracheoesophageal fistulae, duodenal stenosis, intestinal
transformation of the stomach, abnormal innervation and anorectal anomalies. Indian hedgehog (Ihh)
mutants show reduced epithelial stem cell proliferation and features of Hirschsprung disease. Both Shh and
Ihh mutant mice display reduced smooth muscle, gut malrotation, and annular pancreas. In conclusion, the
Hh pathway plays an important role not only during gut development but also in the homeostasis of the
adult human gut epithelium. The Hh pathway remains an important area of research, since it may help us
better understand the molecular (patho)physiology of GI epithelial differentiation of the rapidly
regeneration adult human GI tract.

2.6.5 Explant culture as a tool for studying endoderm patterning

Endoderm explant cultures have been critical to developing these ideas. Initial studies by (Ledouarin 1964)
developed the concept that cardiac mesoderm cocultured with foregut endoderm resulted in induction of
hepatic genes. Gualdi and Zaret et al. have continued these studies in mouse. When endoderm is cultured
for 2 days from mouse at the 2-6 somite stage, the cells remain uncommitted towards hepatogenesis. When
endoderm explants were cultured alone, they assumed a default pancreatic fate. However, when cardiac
mesoderm was cocultured with endoderm hepatic fate would be specified in the endoderm cells (Gualdi, Bossard et al. 1996). Looking at this in more detail, cells that were closest to the cardiac mesoderm displayed albumin activation, whereas cells slightly farther away express Pdx1 gene. Further studies demonstrated that this was because these cells were indeed bipotential in cell fate. Furthermore, the cells would grow more vigorously, similar to the growth that appears in the liver bud.

Mesodermal signals have also been shown to be inhibitory to endoderm induction, using endoderm explant studies. Dorsal endoderm cultured alone is able to activate the albumin gene. Furthermore, it was found that the dorsal posterior mesoderm and dorsal ectoderm actually inhibit the activation of the albumin gene in explanted endoderm, when cultured in tissue recombination studies (Bossard and Zaret 2000). The mechanism by which the endoderm was irreversibly unable to activate endoderm was found to be due to a loss of binding of Foxa2 (HNF3) to DNA, around day 13.5.

Further studies have shown that endoderm in explants can be patterned along the lung lineage. Interestingly, high doses of FGF 2, higher than that required for albumin activation, have been shown to activate Nk2.1 in explant cultures of endoderm with cardiac mesoderm (Serls, Doherty et al. 2005). These signals act through FGF1 and FGF4 receptors. Interestingly, NKx2.1 expression was seen slightly later (9-9.5 days), and double positive cells (Nkx2.1 and Albumin) were identified. FGFR4 upregulation coincided with the establishment of lung precursor cell fate.

These studies suggested that the mesodermal signals are also critical to endoderm induction. After the endoderm is formed, mesoderm interacts with endoderm to result in organ bud formation and organogenesis. For example, during liver development, the septum transversum mesenchyme, the cardiac mesoderm, and angioblasts all deliver critical soluble that trigger endoderm differentiation towards liver. This is related to the fact that the anterior endoderm and the posterior endoderm are in direct contact with different mesodermal environments. Interestingly, pulmonary mesenchyme, intestinal mesenchyme, and pancreatic mesenchyme have all been shown to have critical and specific effects on differentiation of embryonic stem cells.
Transcription factors in gut patterning

Transcriptional control of cell fate within the gut tube has been well studied. The caudal homologues (Cdx1, 2, 4) are similarly implicated in patterning and cell differentiation in the gut (Chawengsaksophak, de Graaff et al. 2004). Cdx2, as mentioned above, is expressed in posterior structures that include endoderm and gut. Mutations in Cdx2 in humans and mice result in loss of gut growth control and formation of colon tumors (Chawengsaksophak, de Graaff et al. 2004). Pdx1 is a homeobox gene expressed early in the posterior foregut and midgut and later in the pancreatic islets of Langerhans (Ohlsson, Karlsson et al. 1993). Mice and humans that lack Pdx1 function fail to develop a pancreas. Furthermore, humans that carry one null allele of PDX1 often develop diabetes (Habener and Stoffers 1998).

The Anterior-Posterior and Dorsal-Ventral expression of many genes including the Hox cluster, Pax, Nkx, bHLH, HNF, and nuclear receptor genes suggests a role in establishment of gut tube pattern. For example, the anterior gut tube, which gives rise to several organs including thyroid, parathyroid, thymus, esophagus, and lungs, expresses several Hoxb genes, such as Nkx2. Nkx 2.1, Pax 8 and Pax 9 (Wells and Melton 1999). The homeobox transcription factors play important roles in a variety of processes, including anterior–posterior patterning, left–right development, intestinal villus formation, epithelial differentiation, development of the liver and pancreas, and formation of the enteric nervous system. Hox genes are believed to function in regional specification of GI morphology and function, and their expression within the mesoderm defines the borders of the mid- and hindgut (de Santa Barbara, van den Brink et al. 2002). Mutations of Hox genes have significant consequences for gut development and cell specification. For example, ectopic expression of Hoxd13 in the chick midgut mesoderm results in induction of a hindgut/large intestine morphology of the midgut epithelium. Hoxa5, which is expressed in the mesenchyme of the foregut and midgut, is required for normal differentiation of gastric epithelial cells and maturation of the small intestinal epithelium (de Santa Barbara, van den Brink et al. 2002).

Numerous transcription factors are also expressed in the domain of the gut that gives rise to the stomach, pancreas, and duodenum, including Pdx1, Pax 4 and 6, Nkx 2.2, Isl-1, and NeuroD. The lung expresses Nkx2.1 and Gli2/Gli3 amongst others, and deletion of Nkx 2.1, or double knockout Gli2/3 mice resulted in mice lacking a thyroid gland and showing impaired lung morphogenesis. Similarly, Pax 9-deficient mice have global defects such as absence of thymus, parathyroid glands, and ultimobranchial
bodies, all of which derive from the pharyngeal pouches (Wells and Melton 1999). In contrast, loss of Pax 8 results in a specific deletion of follicular cells of the thyroid gland. Transcription factor genes expressed in fore and midgut include Pdx1, Pax 4, Pax 6, Neuro D, and Nkx2.2, and mice that lack these genes show several phenotypes in the stomach, pancreas, and duodenum. Loss of Pdx1 results in mice that lack a pancreas, and certain endocrine cell types in the stomach and duodenum. In contrast, absence of either Pax 4, Pax 6, or Nkx2.2 results in loss of specific populations of hormone-producing (endocrine) cell types in the pancreas and duodenum. Specifically, deletion of Pax 4 or Nkx2.2 results in loss of insulin-producing cells, whereas deletion of Pax 6 results in loss of glucagon-producing cells in the pancreas (Wells and Melton 1999)

2.6.7 Congenital diseases of gut—molecular mechanisms important for patterning

Gut malformations are the most common human congenital anomalies and cause substantial perinatal morbidity and mortality, and these were recently reviewed (de Santa Barbara, van den Brink et al. 2002). Recent advances in developmental biology have suggested molecular mechanisms that regulate development of the digestive system. Congenital gut diseases include biliary atresia, tracheoesophageal fistula, malrotation, hypertrophic pyloric stenosis, and anal atresia. With known molecular mechanisms, this suggests that in the future genotyping, followed by use of gene therapy or autologous cell therapy could be used as therapeutic approaches for congenital diseases of the gut. Importantly, many molecules participating in these processes have been identified, including signaling molecules and their receptors, extracellular matrix proteins, proteins involved in cell polarity, and transcription factors. Reciprocal interactions between adjacent tissue compartments, such as epithelium, mesenchyme, and enteric neurons and their precursors, play important roles in development, function, and pathophysiology of the digestive system.

2.7 In vivo techniques for studying endoderm in vivo

2.7.1 Overview and significance

Endoderm studies from ES cells have been in general lacking. This may be due, in part, to a lack of understanding or application of the critical processes that occur during endoderm induction in vivo. Here
we review developmental biology techniques and concepts important in understanding endoderm induction, with an eye towards applying these techniques towards in vitro studies and controlling in vitro differentiation.

2.7.2 RNA is situ hybridization
Identification of markers of a particular tissue, and understanding the molecular mechanisms and events of the endoderm induction and patterning is critical for the development of technologies regarding ES cells. The primary method used for gene identification is RNA in situ hybridization using a labeled probe. Since RNA has been detected prior to protein formation, data based on the expression kinetics of particular marker may differ between genes and proteins. Furthermore, probe design is a critical parameter. Since regulatory transcription factors often have more than one stage specific enhancer, the length and target of the probe becomes critical parameters. RNA in situ hybridization, can be accomplished on whole mount or sectioned tissue. Although the technique is not quantitative and can be tedious, whole mount in situ hybridization using a labeled RNA probe is a fundamental technique for understanding spatio-temporal localization of genes of interest. However, this technique is limited by RNA degradation, issues limited to fixation and specificity of detection.

2.7.3 Immunofluorescence
Immunofluorescence using antibodies to a transcription factor or marker of interest is another standard technique used for identifying proteins of interest. This technique can be amplified using labeled secondary antibodies to the tissue of interest. One problem is that transcription factors are low in abundance, and proteins that are at low levels are hard to detect. As a result, in these cases, the sensitivity of the antibody is critical. Second, cross reactivity can occur leading to false positives.

2.7.4 In vivo cell labeling
Labeling specific cells of interest is critical to spatiotemporal identification of particular cells of interest. Genetically modifying cells by obtaining transgenic or knock-in animals is a critical technique for identifying cell of interest. Knock-in techniques have been established over the past twenty years. These
techniques rely on genetically modifying ES cells and then re-introducing these cells into blastocysts, implanting these into pregnant mice, and giving rise to offspring, some of which have transmitted the gene to the germline. If appropriate animals are mated, homozygote or heterozygote can be recovered. Lac Z is a typical gene used as a reporter, however it can accumulate in cells and may demonstrate delayed staining. As a result, short half-life GFP offers is a more attractive alternative.

2.7.5 Assessment of gene function

To understand functions of proteins during development, or to understand functions of the regulatory regions of these proteins, techniques using cloned DNA and modification of this DNA are used. For example, mutated proteins, via truncation or domain swapping, can be designed and placed under the control of a heterologous promoter or can be knocked in to a particular locus of interest. The result is the ability to evaluate a protein’s function on development when overexpressed, knocked out, or modified. Conditional mutation has been shown to be a critical technology for understanding endoderm development. Using this technology, the Cre-recombinase protein is placed under the control of a tissue specific promoter. This approach has been used extensively in the development of the lung, using Cre-recombinase under the control of the surfactant protein C (Sfptc) promoter (Whitsett and Tichelaar 1999).

2.7.6 Assessment of effect of developmental changes

In mutant mice, developmental changes can take place variably in different tissues. Gross morphology and viability of offspring are initial observations. Analysis of pups at a particular day results in assessment of viability and gross morphology. Stage specific analysis requires recovery from the uterus and separation of embryos. Embryos can be removed, extraembryonic tissues can be separated from embryonic structures, and these can be staged and classified. For example, differing somite stages may be present at the same time period, but classification by somite stage can lead to better results. These tissues can then be genotyped, culture in vitro, or fixed for further tissue analysis.

2.7.7 Tetraploid complementation and ES cell chimera formation
One of the problems with studying mutant embryos, especially with studying endoderm, is that the genes that effect endoderm induction are not the same as the genes that effect visceral endoderm. Since visceral endoderm patterns the epiblast during development, the entire developmental program is affected. Thus any technique that allows the ability of extraembryonic structures to form normally, while evaluating mutations in the embryonic structures would be useful. Furthermore, when mutations are evaluated, it is difficult to tell whether the mutation resulted in an autonomous effect on differentiation or a nonautonomous effect.

Two techniques are available to deal with these problems. The first is known as tetraploid complementation. In this assay, a tetraploid blastocyst is generated. When ES cells are introduced into this blastocyst, and implanted into pseudopregnant mice, it gives rise to a normal embryo. It turns out that the embryonic portion of the mouse is the portion complemented by ES cells, and thus how mutations effect embryonic structures can be evaluated using this technique. The solution to evaluating whether cells generate autonomous or non-autonomous effects involves first generating labeled ES cells and then implanting them into blastocysts. For example it is known that ES cells can be derived from the mouse blastocyst, and when reintroduced into the mouse blastocyst they can contribute to embryonic tissues of the mice.

In the second technique one can create a chimeras by reintroducing labeled, genetically modified ES cells back into the developing blastocyst and following how they develop. Once a mutant mouse is created, whether the animal is viable or not, an ES cell can be generated. This can then be genetically modified with a label and then can be reintroduced into the mouse blastocyst. If the cells give rise to autonomous effects, then the cells will migrate into places where the endoderm normally is, leading to defective embryo. If the mutation is non-autonomous, then the cells will migrate into other compartments, such as mesoderm or ectoderm derivatives. At times, cells can migrate into both sites, indicating both autonomous and non-autonomous effects.

2.7.8 Conclusion

Several important techniques in evaluating development are reviewed. Each approach has strengths and weaknesses which have been highlighted. However, these techniques help shed light on how information in developmental biology is developed, and how this can be used to shape information about ES cells.
2.8 Summary and Thesis Objectives

Chapters 1 and 2 focus on the early differentiation of embryonic stem cells as well as critical factors in endoderm induction and patterning. Also presented are novel approaches to gene and pathway discovery in endoderm progenitor cells, which was highlighted since little is still known about these endoderm progenitor cells. Below, the overall objectives of this thesis are now presented.

2.8.1 A framework for analyzing early differentiation

The first objective of this thesis is to establish an analytical framework for measuring early differentiation. This framework will consist of the particular techniques to measure differentiation and the particular molecular targets or genes that will be used to measure differentiation. The major technique chosen to measure differentiation is Reverse Transcriptase Polymerase Chain Reaction (RT-PCR), which is a sensitive technique for measuring the relative quantity of a RNA transcript for a particular gene of interest. This technique is complemented with flow cytometry and immunofluorescence, which can be used to sensitively detect proteins. The second important issue is: which genes should be selected as markers for populations? The particular class of markers to be chosen will be transcription factors. Certain developmental transcription factors are lineage-specific and are transcriptionally regulated. The appropriateness of these transcription factors will be determined by examining the in vivo developmental expression patterns presented in the developmental biology literature. This implies that the presence or absence of a particular gene will be related to the presence or absence of a particular lineage within a population. This analytical framework is presented throughout Chapters 3-6.

2.8.2 New culture configurations that promote endoderm induction and endoderm-enriched populations

The next major objective will be use established or develop new culture systems that enhance endoderm induction. The overall goal will be to obtain an enriched population endoderm progenitor cells. Here we will loosely define enrichment as greater than 50% of the total cell fraction. Current literature reports that
endoderm can be induced at a fraction of roughly 25% of the population (Kubo and Keller 2004, Yasunaga 2005). Here, we will take advantage of the framework established in objective one.

2.8.3 Microenvironmental factors that effect endoderm induction

Objective three will be to clearly identify factors that effect endoderm induction and the pathway to definitive endoderm induction. Since little is known about endoderm progenitors and what influences their induction, a broad category of changes in the microenvironment. These will include, not any particular order: 1) Extracellular matrix 2) Soluble factors 3) Cell-cell interactions, including homotypic and heterotypic (coculture) 4) Medium conditions 5) Serum conditions. Importantly, each of these categories may have different effects depending on the overall culture configuration, and individual effects may be difficult to discern. Nevertheless, here we will attempt to examine as fundamentally as possible, how factors in each category affect endoderm induction.

An important point will be to ensure that an appropriate lineage is present for endoderm induction. Definitive endoderm, which gives rise to embryonic derivatives, must arise first from epiblast and then mesendoderm in sequential order. Thus, by examining the kinetics of markers for these populations, one can identify the precursors and claim that lineage pathway is correct. Visceral endoderm, which shares similar genes to definitive endoderm, arises prior to epiblast formation, and does not form all the appropriate products of endoderm, such as pancreas, liver, etc.

2.8.4 Approaches for endoderm patterning/differentiation

One of the ultimate objectives from endoderm induction will be to differentiate these cells into an enriched or pure population. Endoderm first forms foregut, midgut, and then hindgut. Each of these areas or domains is patterned differently. Thus, to prove that the endoderm progenitor cells can differentiate, gene expression of important patterning markers, or lineage-specific transcription factors, will be measured using RT-PCR. Different culture configurations and different microenvironmental conditions will be assessed to enhance endoderm differentiation into particular lineages, such as liver or gut.
2.8.5 In vivo differentiation and clinical applications

An important aspect that is beginning to be addressed in the literature is the utility of these cells in either cellular therapy or tissue engineering types of strategies. Thus, the fifth objective will be to understand if these endoderm progenitor cells or their derivatives are applicable in these settings.

One of the problems with endoderm studies thus far is the inability to differentiate cells in vivo. One reason to differentiate cells in vivo is that it is not known whether early progenitor cells or late committed cells will be the cell of choice for therapies. Another aspect is that it is not known whether in vitro differentiation can ever result in terminally differentiated cells, and thus in vivo differentiation may provide an alternative to in vitro differentiation. In this scenario, cells would be implanted, differentiated, and then recovered in vivo. Lastly, in vivo differentiation may reveal novel insights how morphogenesis occurs, and how tissue architecture is established from progenitor cells, without the need for scaffolding.

Cell based devices are one type of therapy in which stem cells will potentially be useful. The ability of differentiated endoderm progenitors to function in a clinical model will also be evaluated and strengthen the case that these cells have functional capabilities.
2.9 REFERENCES


Figure 2.1. Lineage relationships in endoderm induction
This figure demonstrates current dogma on how germ layers form from embryonic stem cells with time. Each circle represents a transient cell that is labeled below by its name. Above or below each lineage are transcription factors or markers that delineate that particular cell. The figure starts on the left and proceeds to the right. The time scale is in days, and the total length is approximately 6.5 days in mice.
Figure 2.2. Mature tissue derivatives of endoderm
This figure demonstrates epithelial tissues in the gastrointestinal tract (left) and respiratory tract (right). This figure does not include endocrine derivatives (thyroid and parathyroid glands) and genitourinary derivatives of endoderm (bladder and prostate).
Figure 2.3. Putative genome-wide transcription factor network during endoderm differentiation

This figure demonstrates a regulatory transcription factor network for mouse embryonic stem cells, based on the work of Davidson et al (2000). Each node has a control element on which an arrow is attached. The control element represents the input and the arrow represents the output. The inputs to the control element represent transcription factors binding to the promoter element. The output represents the translated protein (ie. after both transcription and translation). Each node has a name beneath it that delineates the particular transcription factor of interest (in all cases except fgf5 and alb). The network proceeds down as time goes on, depicted by the arrow on the right. Each level of transcription factors represent those specific for a particular lineage. The epiblast transcription factors/markers are Oct4, Otx2, Fgf5. The mesendoderm transcription factors are Lhx1, Brachyury, Goosecoid, and Foxa2. The endoderm transcription factors are Foxa2, Sox17, GATA4, HNF1b, HNF6.

The bottom arrow represents space along the gut tube from anterior to posterior. The transcription factors/markers listed above the arrow represent early transcription factors that “pattern” a particular domain, Nkx2.1(Lung, Thyroid), Hex(Liver), Alb(Liver), Pdx1 (Pancreas). In this case, the initiation and maintenance of the endodermal transcription factor network promotes activation of early patterning transcription factors that pattern the endoderm.
### 2.11 TABLES

<table>
<thead>
<tr>
<th>Transcription Factor Name</th>
<th>Endodermal Developmental Expression</th>
<th>Knockout Phenotype</th>
<th>Cis-regulatory region</th>
<th>Genes activated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foxa2(HNF2b)</td>
<td>Primitive Streak, Mesendoderm, Endoderm, Neural Plate</td>
<td>1) Knockout- no axial structures, defective gastrulation 2) conditional- Nestin Driven - arrests differentiation 3) Foxa3 driven- no Liver(Foxa1 and Foxa2 knockout) 4) Adult- Liver conditional knockout has no effect of Liver differentiation markers</td>
<td>HNF6, HNF1α, IRF-1, UF1, Foxa2, C/EBP</td>
<td>TTR, Alb, AFP, Apo B, Apo AI, Transferrin, ILGF, BP, α1AT, Cholesterol 7 hydroxylase, PEPCK, PFK-2, Aldolase B, p450, complement C, HepB, Pdx, Foxa1, Hnf4αm HNF1α, HNF1b, Proglucagon, (Gluc. Response Elements). Glucokinase,</td>
</tr>
<tr>
<td>Brachyury</td>
<td>Primitive Streak/ Mesendoderm</td>
<td>no axial structures, disrupts gastrulation</td>
<td>β-Catenin/TCF(in ES cells)-Wnt pathway is activator in ES cells</td>
<td></td>
</tr>
<tr>
<td>Foxh1</td>
<td>Primitive Streak/Endoderm</td>
<td>no axial structures, disrupts gastrulation</td>
<td>Activin Response Elements(ARE)</td>
<td></td>
</tr>
<tr>
<td>Sox17</td>
<td>Definitive Endoderm, when patterned goes to mid and hindgut but not foregut</td>
<td>depleted endoderm</td>
<td>In Xenopus Explant- Hnf1β, HNF6, Foxa1, Foxa2, No Pdx in knockouts,</td>
<td></td>
</tr>
<tr>
<td>FTF(Fetal Transcription Factor)</td>
<td>Definitive Endoderm</td>
<td>No visceral endoderm, disrupted differentiation</td>
<td>GATA 4/5/6, Retinoic Acid Element, Pdx(in Pancreas)</td>
<td>AFP, HNF3b, HNF4a, Hnf1α</td>
</tr>
<tr>
<td>HNF1β(Tcf2)</td>
<td>Primitive Streak, Endoderm</td>
<td>No visceral endoderm, disrupted differentiation, no ventral and diminished dorsal pancreas in rescued tetraploid embryos</td>
<td>AFP, Alpha1 Anti-Trypsin, Albumin, HNF1α(direct or indirect), Foxa3, (not GATA4 not GATA6), HNF6,</td>
<td></td>
</tr>
<tr>
<td>GATA4</td>
<td>Primitive Streak, Endoderm</td>
<td>No visceral endoderm, disrupted differentiation</td>
<td>In Xenopus, Activated by Activin/Nodal</td>
<td>Xenopus- Induces Sox17, HNF1β, Foxa2, Shh(in GATA 4-/- Gut). Parietal, Pit, and Neck Cells have regulated proteins</td>
</tr>
<tr>
<td>GATA6</td>
<td>Primitive Streak, Endoderm, Lung, Liver, Mid and Hindgut</td>
<td>1) No visceral endoderm, disrupted differentiation 2) Conditional Inactivation- No Liver</td>
<td>In Xenopus, Activated by Activin/Nodal, Has Gut and Cardiac Specific Enhancer. In Mice- GATA, Cdx, Oct1, Retinoic Acid Inducible</td>
<td>Xenopus- Induces Sox17, HNF1β, Foxa2. GATA6 direct activator of the first 2. Mouse(ES, Visceral Endoderm)- GATA4, HNF4, AFP</td>
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Table 2.1 Regulatory transcription factors in endoderm progenitor cells
CHAPTER 3:
MICROENVIRONMENTAL EFFECTS ON THE INDUCTION AND DIFFERENTIATION OF ENDODERM FROM EMBRYONIC STEM CELLS

3.1 INTRODUCTION

Embryonic stem cells-derived endoderm progenitors offer a remarkable potential for the treatment of major diseases effecting the pancreas, liver, lungs, bladder, and prostate (Wells and Melton 1999; Oottamasathien, Wang et al. 2007). Endoderm progenitors would also be useful for the study of congenital diseases (de Santa Barbara, van den Brink et al. 2002), gene discovery, toxicology screening, and drug development (Wobus and Boheler 2005). Current knowledge regarding the development of the definitive endoderm, one of three major germ layers, is derived from in vivo fate mapping (Gardner and Rossant 1979; Poelmann 1981; Lawson and Pedersen 1987), mouse genetics (Shivdasani 2002) and in vitro tissue explants (Gualdi, Bossard et al. 1996). These studies indicate that the endoderm is derived from the inner cell mass through the differentiation of the transient epiblast and mesendoderm (anterior primitive streak) populations (Tam and Behringer 1997). Each of these populations expresses unique markers or transcription factors. For example, the epiblast expresses Oct4 and Fgf5, mesendoderm expresses Brachyury and Foxa2, (Ang, Wierda et al. 1993) and endoderm expresses Foxa2 and Sox 17 (Kanai-Azuma, Kanai et al. 2002) (Abe, Niwa et al. 1996). Studies of endoderm derivatives have lagged behind other germ layers due to a lack of understanding of factors which control induction and maintenance. Preliminary studies as well as current literature with ES cell cultures in a specialized differentiation medium indicate that critical parameters for endoderm differentiation include medium type and components, culture configuration, and extracellular matrix.

Medium has been shown to be a critical factor endoderm induction. Kubo et al. demonstrated that a decreased serum resulted in enhanced endoderm induction based on enhanced brachyury expression using a brachyury-GFP ES cell line. This particular protocol relied on initially using serum (15% FBS) for 2.5 days, followed by a change to a serum-free environment, composed of knockout serum replacement on day 2.5. The data indicated that at high serum levels there was higher brachyury present (80%) on day 4. However, most of this mesendoderm was converted to mesoderm, not endoderm. When serum was
removed from the medium, the brachyury fraction went down, but the amount converted to endoderm was enhanced. Studies by Tada et al. using a Goosecoid-GFP cell line in monolayer culture, demonstrated that mesendoderm increased from 2.1 % to 65 % on day 4 by changing from serum to serum free medium and adding activin at 10 ng/ml. However, the effects of serum alone were not measured, and the serum free medium was proprietary. When serum free medium and activin were used in the embryoid body configuration, Tada et al. reported a 21% mesendoderm positive fraction. Human ES cell studies (D'Amour, Agulnick et al. 2005) have also demonstrated the serum free medium promotes mesendoderm induction (approximately 80% brachyury positive cells) and endoderm induction in monolayer culture. Taken together, reduced serum appears to enhance endoderm formation, by either enhancing mesendoderm formation or by enhancing conversion from mesendoderm to endoderm, in both embryoid body and monolayer culture systems.

Culture configuration is a key parameter for controlling embryoid body differentiation. Embryoid Bodies (EB) are heterogeneous in size, known to aggregate (Dang, Kyba et al. 2002), may have mass transfer limitations, and are tedious to produce and break down to into individual cells. Moreover, in the EB, it is difficult to identify the morphology of a particular cell type of interest. However, since ES cells are known to secrete factors in an autocrine manner, ES cells in EB are likely exposed to these factors, potentially at high concentrations. Furthermore, the compact geometry of EB suggests that there are a high number of cellular interactions for each cell. Lastly, EB may allow cell migration, which in vivo is coupled to early differentiation and morphogenesis. Monolayer cultures, on the other hand, are useful because they allow identification of morphological characteristics of particular cell types and because any added growth factors will likely be uniformly distributed in culture, eliminating major mass transfer limitations. However, cells may not have the same level of cell-cell interactions, and may not be exposed to high concentrations of autocrine factors as they may be within the EB. The discrepancies between these two systems are large, and therefore it may be difficult comparing data from published work when the protocols used are either exclusively EB or monolayer.
Extracellular matrix has also been shown to be a critical component for endoderm induction, but is not as well studied as activin or serum components. In vivo, the endoderm sits upon a basement membrane composed of collagen Type IV, Laminin amongst other molecules (Wells and Melton 1999). The effect of a particular extracellular matrix molecule (ECM) component on endoderm induction is poorly understood. Epithelial maintenance, an important component of endoderm induction, has been shown to be effected ECM components. Epithelium in endoderm has been shown to transition between squamous, cuboidal, and columnar phenotypes (Wells and Melton 1999). While this has not yet been shown in vitro, the ECM molecule Hensin has been shown to modulate this transition from transition of ES cells of visceral, but not definitive endoderm, from squamous to columnar epithelium (Takito and Al-Awqati 2004). ECM has also been shown to effect endoderm cell fate in vitro. Yasunaga et al 2005, using the Gooscoid-GFP cell line and adding a Sox 17-CD4 reporting locus, demonstrated that high cell densities, on gelatin or fibronectin coated dishes, promote visceral endoderm formation (Goosecoid (-) and Sox 17(+). However, using collagen IV coated dishes and activin (10ng/ml), double positive (Goosecoid and Sox17) cells were obtained which were termed definitive endoderm.

Here, gene expression studies as well as immunofluorescence studies were undertaken to identify the effects of these parameters on differentiation towards an endodermal lineage.

3.2 MATERIALS AND METHODS

3.2.1 Reagents

Fetal Bovine Serum (FBS), Dulbecco’s modified Eagle medium (DMEM), Iscove’s modified Dulbecco’s Medium (IMDM), Knockout DMEM (KO-DMEM), Knockout Serum Replacement (KO-SR), penicillin, streptomycin, knockout serum, knockout DMEM, bovine gelatin, and dispase were obtained from Invitrogen Life Technologies (Carlsbad, CA). Human Fibronectin was purchased from BD Biosciences (San Jose, CA, USA). Hydrocortisone was obtained from Pharmacia (Kalamazoo, MI). Glucagon and Insulin were purchased from Eli-Lilly (Indianapolis, IN). Immunofluorescence grade paraformaldehyde was purchased from Electron Microscope Sciences (Hatfield, PA). Rabbit anti-mouse Foxa2 antibody was purchased from R&D Systems (Minneapolis, MN). Goat anti-mouse Sox17 was purchased from Santa Cruz (Santa Cruz, CA). For immunofluorescence studies, normal donkey serum and secondary F(ab)2 antibody
fragments, ML grade, were obtained from Jackson Immunoresearch (Bar Harbor, ME). Unless otherwise noted, all other chemicals, growth factors, and solutions were purchased from Sigma-Aldrich Chemicals.

3.2.2 Embryonic stem cell culture
Undifferentiated mouse D3-ES cells (ATCC) were cultured on 0.2% gelatin-coated tissue culture T75 flasks, at low dilutions of 1:50, such that small colonies were maintained. Medium was changed daily. Experiments were carried out with cells at passage 30. Undifferentiated mES cells were cultured in Knockout DMEM supplemented with 15% Knockout serum, L-Glutamine (4 mM), Penicillin(100 U/ml), Streptomycin (100 U/ml), Gentamicin (10 mg/ml), ESGRO (1,000 U/ml), and 2-Mercaptoethanol (0.1 mM). Proliferating ES cell cultures were maintained in a 5% CO₂-humidified incubator at 37°C.

3.2.3 Embryoid body differentiation
A simple differentiation method was developed utilizing Embryoid Bodies (EB) which was generated using the Hanging Drop (HD) Technique (Hamazaki 2001). To generate HD EB, ES cells were diluted in IMDM + 20% FBS medium (see above) at a concentration of 1000 cells/ml. The cover of a non-treated 100mm bacteriologic dish was inverted and 30µl of the cell mixture using a Multipipetter for approximately 50 HD per dish, with 15 ml of PBS placed in the dish for humidification. These HD were then incubated for 2.5 days at 37°C and 5% CO₂ Prior to harvesting EB’s, P-60mm dishes were incubated with 0.1% Pluronic/HBSS for at least 1h at 37°C. After incubation, dishes were washed once with PBS. On day 2.5 days, the EB’s were harvested. Each dish containing HD was removed and the cover was inverted. Warm PBS was used to gently wash off all of the EB’s. The EB’s were washed once in PBS and allowed to settle. After a second washing step, the EB’s were then placed in either in IMDM + 20% FBS, or IMDM +Knockout Serum Replacement,. Approximately 200 EB’s were then placed in each P60 dish and cultured in suspension. Cultures of EB’s in suspension were carried out until day 14 in suspension.

3.2.4 Fibronectin-coated collagen gels
Type I collagen stock solution was prepared from rat tail tendon as described by Dunn et al (Dunn, Yarmush et al. 1989). Collagen gelling solution was prepared by mixing nine parts of collagen stock (1.25
mg/ml) with one part of 10 x DMEM on ice. The collagen gelling solution was then added at a volume of 250 μl/ well (66 μl/cm²), spread evenly on the dish and incubated at 37°C for 30 min for gel formation. Fibronectin dissolved in PBS was then added on top of the gel, at a concentration at 3.8 μg/well (1 μg/cm²) and the tissue culture plates were incubated for 1h at 37°C to insure ensure fibronectin adsorption on collagen gel.

3.2.5 Mouse embryonic stem (mES) cell differentiation

Mouse embryonic stem (mES) cells were directly seeded at a density of 2 x 10⁴ cells/well (5.2 x 10³ cells/cm²) onto fibronectin-coated collagen gels. Basal differentiation medium consisted of high glucose DMEM supplemented with 10% Fetal Bovine Serum, Glutamine (100 U/ml), Penicillin (100 U/ml), Streptomycin (100 U/ml), Gentamycin (10 mg/ml). For growth factor studies, the medium was augmented with activin (20 or 50 ng/ml) or follistatin (100 ng/ml). Medium was changed every 2 days of culture. Differentiating ES cell cultures were maintained in a 10% CO₂-humidified incubator at 37°C. Embryoid Body (EB) cultures were formed by culturing mES cells using the hanging drop method. ES cells were resuspended in differentiation medium and spotted as 30 μl drops on an inverted lid of a 100 mm dish at a concentration of 30,000 cells/ml. Dishes were incubated at 37°C for 48h in PBS. EB’s were recovered and matured in suspension for 48h and then transferred to a 12 well plate.

3.2.6 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

For each experimental condition, cell lysates were generated using Gaunidinium Isothiocyanate-based solution (Clonetech) and stored at -80°C for later use. RNA isolation was conducted using the manufacturer’s instructions for the Nucleospin II RNA Kit (Clonetech). RNA gels were run using 2% Agarose (RNAase free) to ensure that RNA was intact prior to RT-PCR. One-step gene-specific RT-PCR (Qiagen) was performed for gene expression analysis and resolved on a 2% agarose gel. Each reaction represents 10 micrograms of total RNA per condition. Cycle number was 30 for all genes unless otherwise specified. Cycling conditions were 55°C, 30 sec, 94°C, 30 sec, and 72°C 30 sec, with a ten minute extension at 72°C. Thermal cycling was done using the Mastercycler Epigradient X (Eppendorf) with 96 well plates
Gels were imaged using fluorescent gel scanner, the Fluor-S Multi-Imager (BioRad) and captured using Multi-analyst software. Primers were as follows:

**B-Actin** (F) 5’-GAGGGAAATCGTGCGTGA-3’;
**B-Actin** (R) 5’-CCAAAGGAAGGGCTGGAA-3’;
**Oct4** (F) 5’-GAAAGCCGACAACAATGA-3’;
**Oct4** (R) 5’-CAAGCTGATTGGCGAATGT-3’;
**Fgf5** (F) 5’-GTTCAGCAGTCCGAGCAA-3’;
**Fgf5** (R) 5’-TAGGCACAGCAGGGATG-3’;
**Foxa2** (F) 5’-ACACGCAAACCTCCCTAC-3’;
**Foxa2** (R) 5’-GGGCACCTTGAGAAAGCA-3’;
**Sox17** (F) 5’-ATCAACCAGCCCACTGA-3’;
**Sox17** (R) 5’-TCGGCAACCGTTCAATG-3’;
**Gata1** (F) 5’-CACCATCAGGTCACAGG-3’;
**Gata1** (R) 5’-TGAGGGCAGGTAGAGTGC-3’;
**Foxf1** (F) 5’-CGTGTGTGATGTGGAGGT-3’;
**Foxf1** (R) 5’-CTCCGTGGCTGGTTTCA-3’;
**Runx2** (F) 5’-TTCAGACCAGCAGCAGCCTC-3’;
**Runx2** (R) 5’-GCCGCCCAACAGACTCAT-3’;
**Pax6** (F) 5’-TGCCCTTCCATCTTTGCT-3’;
**Pax6** (R) 5’-CCATCTTCTGCGTGGGTTG-3’;

### 3.2.7 Immunofluorescence

Samples were washed three times with PBS, and fixed in 4% EM-grade paraformaldehyde for 10 min at room temperature. Samples were then washed with PBS and were permeabilized for 15 min with 0.1% Triton X-100, blocked for 30 min with 1% bovine serum albumin, 5% donkey serum at room temperature followed by staining with primary antibodies at overnight at 4°C. The dilutions of primary antibodies used were Foxa2 (1:100), Sox 17 (1:100) and albumin (1:500). After additional washes with PBS, samples were stained with fluorescently tagged secondary antibodies at a dilution of 1:500, for 45 min at room
temperature and washed three times with PBS. Cells were then imaged using phase contrast and fluorescent microscopy (Ziess) and captured using AxioVert software.

3.2.8 Flow cytometry

Samples were washed three times with cold PBS, kept on ice, and fixed in 1% EM-grade paraformaldehyde for 15 min at 4°C. Samples were then washed with ice cold PBS and were permeabilized for 15 min with 0.1% Triton X-100 while cells were vortexing, and then washed twice with cold PBS. Cells were blocked for 30 min with 1% bovine serum albumin, 5% donkey serum at room temperature for 30 min. Primary antibodies diluted in blocking buffer (above) were then added for 20 min at room temperature, at the same dilution as for immunofluorescence. After additional washes with ice cold PBS, samples were stained with fluorescently tagged secondary antibodies, at the same dilution as immunofluorescence, for 45 min at room temperature, and then washed twice again. Flow cytometry was conducted on a Coulter Epics Altra Flow Cytometer (Beckman Coulter) and raw data were captured using Expo 32 Multicomponent software.

3.3 RESULTS

3.3.1 Embryoid body formation and differentiation

Embryoid bodies (EB) give rise to all three germ layers including give rise to ectoderm, endoderm, and mesoderm. To generate endoderm we first generated embryoid bodies using the hanging drop technique (HD) and measured endoderm gene expression by RT-PCR. The morphology of EB’s in suspension is shown on day 6 (Figure 3.1). Distinct EB’s formed, at times they aggregated, and normally they were heterogenous in size, ranging from 200-to 1000 µm. EB’s were differentiated in serum for 2.5 days and then kept in serum up to day 10 or transferred to serum-free medium (with 15% Knockout serum replacement) for 10 days. To estimate the amount of endoderm gene expression, we measured Foxa2 expression on day 6. In both serum containing and serum free conditions, we detected faint to none endoderm gene expression (Figure 3.2).
3.3.2 Epithelial cell induction in monolayer culture on fibronectin coated collagen gels

To enhance endoderm induction from embryonic stem cells, we attempted to use monolayer culture systems, similar to those used previously for mesendoderm and endoderm induction (Yasunaga 2005). Prior to monolayer cultures, when using embryoid bodies (EB), we had observed that EB’s on day 6 could be trypsinized and replated, but that the attachment efficiency was low. We observed that fibronectin coating improved ES cell attachment after harvest from the EB (data not shown). At the same time, we knew that collagen gels promote epithelial phenotypes, but we observed poor attachment to collagen gels. Therefore, we added fibronectin to collagen gels and we achieved enhanced attachment onto collagen gels.

We compared the morphology of ES cells on collagen only, fibronectin only, and fibronectin coated collagen gels (Figure 3.3). Here, the medium used was a specialized hepatic differentiation medium, which contained hydrocortisone, insulin, glucagon, and epidermal growth factor (EGF). Insulin and EGF had been shown to be important for endoderm induction (Zandstra 2004). On collagen gels without fibronectin coating, we had poor attachment and as a result, ES cells stayed in suspension and formed large aggregates or embryoid bodies. When ES cells were attached to fibronectin, we observed elongated cells reminiscent of primitive ES cells (Figure 3.3). When we cultured ES cells on fibronectin-coated collagen gels, we observed small clusters and outgrowths of ES cells that had a characteristic cuboidal morphology. These cells expressed characteristic genes and proteins (see below). At a collagen gel seeding density of 250µl per well of 12 well plate (52 µl/cm²), we observed cuboidal like cells. However, when we increased gel thickness we observed less endoderm induction, and ES cells remained clustered (data not shown).

3.3.3 Temporal maintenance of endoderm gene expression maintenance

To test whether endoderm could be maintained for long times in culture, we cultured ES cells on fibronectin-coated collagen gels for extended periods, up to 31 days. We observed very little changes in morphology and gene expression when we tested these cells long term. Endoderm and mesoderm gene expression did not change significantly between days 10 and day 17 as shown (Figure 3.4). This indicated to us that endoderm could be maintained, once induced on collagen gels.

3.3.4 Effects of medium changes on endoderm gene expression maintenance
We wanted to determine the mechanism of enhanced endoderm gene expression on collagen gels. We first suspected that medium conditions that effect metabolism may have a potent effects on differentiation. For example, Foxa2 regulates genes such as glucagon in the liver during fasting states (Zhang and Kaestner 2005) and has been shown to be excluded from the nucleus in animal models of hyperinsulinemia (Wang and Wollheim 2005).

Knowing that Foxa2 and the endodermal cell also are believed have evolutionarily conserved genes that regulate and are regulated by carbohydrate metabolism, (Zhang and Kaestner 2005), we took the C+H medium and separated it into a fraction that was more promoting glycogenolysis and gluconeogenesis (glucagon and hydrocortisone) and a fraction that was promoting more glycogen production and carbohydrate storage (insulin). However, we observed no major changes in endoderm gene expression when we tested these two conditions (Figure 3.5). Furthermore, we tested the case where we compared the C+H medium to simple DMEM and observed no changes in endoderm gene expression (Figure 3.6).

Serum has been shown to have profound effects on endoderm induction (Kubo 2004, Yasunaga 2005). These studies demonstrated that enhanced endoderm induction was present when serum was decreased. We decreased the serum fraction to 5% or 1% FBS and a serum-free formulation which contained 15% Knockout (KO) serum. The morphology of the culture demonstrated characteristic epithelial cells in the 5% and 1% FBS conditions (Figure 3.7). However, in the serum-free condition, no cell attachment after day 2 was seen, which culminated in no cell growth, and inability to analyze gene expression profiles of the cells (Figure 3.7). The gene expression profiles of the primitive gene marker Fgf5, and the 1%, 5%, and 10% FBS demonstrate that Fgf5, a primitive marker, is increased with a decrease in serum concentration (Figure 3.8). Thus Fgf5 expression is lowest in the 10% FBS condition, indicating these cells are differentiated, but is increased at 5% and increased even more at 1%. This indicates first that endoderm is induced in all cases at day 10. However, reducing the serum causes less cells to differentiate, thus increasing the Fgf5 concentration. Thus, in this culture system, reducing serum causes a shift to primitive populations. We also found that reducing serum reduced the normal expansion of endoderm progenitor cells seen on collagen gels coated with fibronectin (data not shown). The normal order of magnitude expansion of endoderm progenitor cells is demonstrated through phase contrast images at different time points (Figure 3.9).
3.3.5 Culture of ES cells on collagen gels selectively induces endoderm gene and protein expression

The culture of epithelial cells on collagen gels was previously shown to promote epithelial polarity and function (O'Brien, Zegers et al. 2002). In order to determine the ability of the collagen gel culture to induce an endodermal phenotype we cultured mouse ES cells at 5,000 cells/cm² seeding density on fibronectin-coated collagen gels, in the presence of 10% serum. By day 10, the cells acquired a characteristic epithelial morphologically with bright cell borders reminiscent of endoderm-like cells (Notarianni and Flechon 2001; Hisatomi, Okumura et al. 2004; Tada, Era et al. 2005) (Figure 3.10). In contrast, mouse ES cells cultured on tissue culture plastic coated with a thin layer of collagen and fibronectin formed a morphologically mixed population with numerous spindle-like cells in addition to the epithelial population. Embryoid body culture (EB) outgrowths demonstrate a clear heterogeneous morphology by day 10 (Figure 3.10). We note that mES cells cultured in serum free media, as described by Kubo et al. 2004, failed to adhere or proliferate when cultured on fibronectin-coated collagen-gels (data not shown).

To determine if the observed morphologies correlated with particular genetic phenotypes, we studied the day 10 populations using RT-PCR. Day 10 mouse embryonic RNA was used for a positive control for all primers. We detected endodermal (Foxa2 and Sox17) but not mesodermal (Foxf1, Runx2, GATA1) (Tang, Liu et al. 2001; Kim, Zhou et al. 2005; Schroeder, Jensen et al. 2005) genes in all three conditions (Figure 3.11). As expected, only the EB condition expressed transcripts for the ectodermal gene Ascl1 (Britz, Mattar et al. 2006). To determine if the collagen gel-cultured cells with epithelial-like morphology expressed endoderm-specific markers, we performed immunofluorescence staining for the major endoderm transcription factors Foxa2 and Sox17. We found that nearly all cells with epithelial-like morphology expressed both Foxa2 and Sox 17 (Figure 3.12). To determine the relative fraction of these endoderm-like cells, we used flow cytometry, demonstrating that approximately 53% of the cells were positive for Foxa2 by day 10 of culture (Figure 3.13).

3.3.6 Endoderm-like cells are Oct4-GFP negative and clustered cells remain Oct4 GFP positive

When ES cells were cultured on collagen gels coated with fibronectin, this results in endoderm like cells surrounding cell clusters. Oct4 is a transcription factor whose downregulation coincide with endoderm induction into mesoderm and then endoderm induction. To examine whether the endoderm outgrowths on
collagen gels are Oct4 positive or Oct4 negative, we utilized Oct4-GFP cell line which has GFP knocked in two one allele at the Oct4 gene locus. In all cases, Oct4 GFP cells that were positive at early culture periods were also present at later culture periods (Figure 3.14). All cells that had an endoderm-like morphology were Oct4 negative, while all primitive appearing clusters were Oct4 positive and remained Oct 4 positive for the culture periods tested (10 days).

3.4 DISCUSSION

The induction of endoderm in vivo and in vitro is a complex and dynamic process. Therefore the generation of a simple ES cell culture system that could address fundamental questions, without the need for complex medium formulation or cell sorting is a major goal in the field. Our work demonstrates that an endoderm-like cell population can be induced by culture on fibronectin-coated collagen gel, without the use of activin, complex serum free medium, or serial cell sorting, which were previously thought to be essential for endoderm induction (Kubo, Shinozaki et al. 2004; Yasunaga, Tada et al. 2005).

Although little is known about endoderm, serum conditions have been shown to be an important factor. Exposure of embryoid bodies (EB) to serum and exposure or ES cells in monolayer have been shown to inhibit mesendoderm and hence endoderm induction. However, these results conflict directly with results suggesting 20% FBS can support hepatic induction in embryoid body cultures (Hamazaki). When serum was varied in embryoid bodies, we found decreasing serum from 20% to 10% enhanced endoderm induction (Figure 3.2 and Figure 3.10). When serum was varied even more, in fibronectin-coated collagen cultures from 10% to 1%, primitive populations, marked by enhanced Fgf5, were enhanced. Importantly, in these studies, serum-free systems using Knockout Serum (KO) Replacement did not enhance endoderm in EB’s and did not promote endoderm formation in monolayer collagen cultures. This data suggests the complex nature of serum effects, and suggests that each stage of differentiation may require differing serum concentrations, and different components of serum. Furthermore, future protocols will need to follow Good Manufacturing Practice (GMP), so no animal products can be used if stem cells are used for human therapies.

The effects of extracellular matrix (ECM) have also been unclear regarding endoderm induction. Collagen gels have been to maintain, but not induce both ES-derived neural stem cells (Lin and Isacson
2006), and ES-derived endothelial cells (McCloskey, Gilroy et al. 2005). Our studies demonstrated for the first time that fibronectin-coated collagen gels both induced and maintained endoderm progenitor cells. We observed what appeared to be a biomechanical effect on endoderm outgrowth. Decreasing gel thickness increased endoderm outgrowth, but when a thin coating was used endoderm progenitor cells were not maintained. While we did not analyze gene expression profiles, our data suggests that endoderm induction on collagen gels may be under biophysical control. How biomechanical signaling effects endoderm induction, and stage specific differentiation remains to be determined.

Cell sorting is the main approach for generating endoderm progenitor cells. Current studies of endoderm induction rely on GFP-based on labeling of mesendoderm transcription factors such as Brachyury and Goosecoid. In later papers these cell lines were modified with cell surface marker CD4 under the control region of regulatory transcription factors Foxa2 (Brachyury) and Sox17 (Goosecoid). This type of approach requires that cells be double sorted. Different cell surface markers for mesendoderm or endoderm, including E-Cadherin, PDGFrα, and CXCR4 have all shown to be allow endoderm enrichment. While our study did not sort by GFP based techniques or cell surface markers, our system still achieved appreciable amounts of endoderm induction compared to other studies. Rather than sorting out our population of interest, we were able to identify favorable conditions by which epiblast cells form mesendoderm, and mesendoderm gives rise to endoderm. Combining these approaches should lead to furthering an understanding of endoderm induction.

3.5 REFERENCES


3.5 FIGURES

Figure 3.1. Phase contrast image (10x) of embryoid bodies derived from embryonic stem cells. Morphologic presentation of day 6 embryoid body cultures made by hanging drop (HD) technique cultured in non-tissue culture treated 60mm dishes.

Figure 3.2. Gene expression of endoderm (Foxa2) expression in embryoid body culture under various medium conditions. Foxa2 (endoderm) gene expression measured by RT-PCR (30 cycles) of embryonic stem cells cultured for 6 days in EB configuration. Day 0 ES cell RNA was used as a negative control. Medium conditions include IMDM + 20% FBS, IMDM + 15% KO serum replacement, IMDM + 15% KO serum + Activin 5ng/ml, IMDM + 15% KO Serum + Activin 50ng/ml.
Figure 3.3. Effects of culture configuration/extracellular matrix on morphology of embryonic stem cells. Phase contrast images (10x) of day 4 ES cells cultured on collagen gels only (250µl collagen /well of 12 well plate), tissue culture treated 12-well plate coated with fibronectin, or collagen gels coated with fibronectin (250µl collagen /well of 12 well plate). Medium was C+H medium (with 10% FBS).

Figure 3.4. Long term endoderm gene expression on collagen gels coated with fibronectin. Germ layer gene expression measured by RT-PCR (30 cycles) of embryonic stem cells cultured on Day 10 or Day 17 in gel culture, Endoderm (Foxa2 and Sox 17), Mesoderm (Foxf1, Runx2, GATA1), and Neuroectoderm (Pax6) were used to assess germ layers. Medium was C+H medium (with 10% FBS).
Figure 3.5. Effects of subdividing C+H medium on endoderm induction

Endoderm (Foxa2 and Sox17) gene expression measured by RT-PCR (30 cycles) of embryonic stem cells cultured on Day 10 gel culture. Medium was C+H medium (with 10% FBS), or C+H medium subdivided into two components; One that might support gluconeogenesis (DMEM + 10% FBS + Glucagon + Hydrocortisone) or one that might support glycogen production (DMEM + 10% FBS + Insulin).
Figure 3.6. Effects of basal medium on endoderm gene expression on day 10
Germ layer gene expression measured by RT-PCR (30 cycles) of embryonic stem cells cultured on Day 10 gel culture. Medium was C+H medium (with 10% FBS), or DMEM + 10% FBS. Endoderm (Foxa2 and Sox17), Mesoderm (Foxf1, Runx2, GATA1), and Neuroectoderm (Pax6) were used to assess germ layers.
Figure 3.7. Effect of serum-free and low serum conditions on morphology of ES cells cultured on fibronectin-coated collagen gels. (a) Phase contrast comparison (10x) of day 10 embryonic stem cells cultured on collagen gels coated with fibronectin (gel culture). Medium types included DMEM + 1% FBS, DMEM + 5% FBS, DMEM + 15% KO Serum.

Figure 3.8. Effect of low serum conditions on the differentiation of ES cells cultured on fibronectin-coated collagen gels. Endoderm (Foxa2 and Sox 17) and primitive (Fgf5) gene expression measured by RT-PCR (30 cycles) of embryonic stem cells cultured on Day 10 on fibronectin coated collagen gels. Medium types included DMEM + 1% FBS, DMEM + 5% FBS, DMEM + 10% FBS Serum.
Figure 3.9. Morphological depiction of kinetics of endoderm induction. Phase contrast images (10x) of day 1, day 3, day 5, and day 7 ES cells cultured on fibronectin coated collagen gels. Medium was DMEM medium with 10% FBS.

Figure 3.10. Morphology of embryonic stem cells cultured on collagen gels. (a) Morphologic comparison of day 10 embryonic stem cells cultured on collagen gels coated with fibronectin (gel culture), tissue culture treated 12-well plate coated with collagen and fibronectin (collagen coat), and embryoid bodies plated on tissue culture plastic (embryoid bodies). Medium was DMEM plus 10% FBS. Bar = 100µm.
Figure 3.11. Gene expression in gel culture compared to controls
Germ layer gene expression measured by RT-PCR (30 cycles) of embryonic stem cells cultured on Day 10 in gel culture, collagen coat, and embryoid body configurations. Day 10 embryonic RNA (see methods) was used as a positive control. Endoderm (Foxa2 and Sox 17), Mesoderm (Foxf1, Runx2, GATA1), and Neuroectoderm (Ascl1) were used to assess germ layers.
Figure 3.12. Endoderm protein expression in gel culture. Immunofluorescence images of nuclear staining (DAPI) and either Foxa2 (red), or Sox17 (green), staining of day 10 embryonic stem cells cultured in gel culture. Medium was DMEM + 10% FBS.
Figure 3.13. Fraction of endoderm in gel culture. Flow cytometric analysis and dotplot images of IgG or Anti-mouse Foxa2 stained day 10 ES cells cultured on collagen gels, plotted against arbitrary fluorescence. Medium was DMEM + 10% FBS.
Figure 3.14. Oct4-GFP fluorescence distribution in gel culture. Phase (10x) and fluorescent micrographs of Oct4+GFP ES cells cultured in gel culture. Both day 4 and day 10 were examined. Medium was DMEM + 10% FBS.
CHAPTER 4:
GERM LAYER CONTROL OF EMBRYONIC STEM CELL DIFFERENTIATION VIA MICROFABRICATED STENCILS

4.1 INTRODUCTION

Embryonic stem (ES) cells promise to be useful therapeutically due to their ability to self-renew and differentiate into derivatives of all three major germ layers. (Smith 1998; Solter and Gearhart 1999; Reubinoff, Pera et al. 2000). Currently, methods are available to differentiate ES cells into ectoderm, (Reubinoff, Itsykson et al. 2001; Schuldiner, Eiges et al. 2001; Zhang, Wernig et al. 2001) mesoderm, (Bigas, Martin et al. 1995; Nishikawa, Nishikawa et al. 1998; Kaufman, Hanson et al. 2001) and endoderm (Hamazaki and Terada 2003) derivatives. During differentiation, ES cells form aggregates. In in vitro culture, ES cell aggregates, termed embryoid bodies (EBs), can be typically made by using the ‘hanging drop’ method (Keller 1995). Once these aggregates form, the cells continue to proliferate and differentiate, recapitulating early development and germ layer formation. Extensive studies have been performed using differentiation directed by growth factors (Morali, Jouneau et al. 2000). However, ES cells can also spontaneously form aggregates and differentiate without exogenous growth factors. This suggests that cell–cell interactions induce differentiation, most likely by mimicking the natural microenvironment and releasing autocrine factors (Koller and Papoutsakis 1995). The question of how these aggregates affect differentiation is poorly understood. For example, when ES cells are differentiated as single cells in a monolayer culture, differentiation into the hematopoietic lineage is repressed, (Bautch, Stanford et al. 1996) and differentiation into osteoblasts is dominant. (Karp, Ferreira et al. 2006) Also, when ES cells are differentiated as EBs, the EBs with an initial number of cells of 1000 showed more hematopoietic differentiation, indicating ES cell differentiation may depend on the extent of initial cell–cell interaction (Ng, Davis et al. 2005).

Microfabrication may offer advantages for studying ES cell biology because it can provide control of the cellular microenvironment. Reports have shown that microfabrication is effective in providing a high level of control over heterotypic cell–cell contact, (Bhatia, Balis et al. 1999) cell geometry, (Chen, Mrksich et al. 1997) cell differentiation (Flaim, Chien et al. 2005), and size of EB (Khademhosseini, Langer et al.
The majority of these studies were based on extracellular matrix (ECM) patterning or geometric constraint, such as a well, which later constrained differentiation and proliferation of ES cells. In contrast, the microfabricated stencil technique provides a homogeneous surface without constraint on cells, (Folch, Jo et al. 2000) thereby allowing cell outgrowth, and can be useful for studying ES cell differentiation.

In this study, we investigated the effect of the size of ES cell aggregates on differentiation through the use of a microfabricated stencil technique. Using these microfabricated stencils, we controlled the initial sizes of the ES cell aggregate between 100 and 500 µm. We cultured them to investigate their differentiation without physical constraint. After 20 days of culture, we performed gene and protein expression studies. The results demonstrated that germ layer formation varied with the initial size of the aggregate. Among the aggregate sizes we tested, the aggregates with 300 µm diameter showed gene and protein expression profiles similar to EBs formed by the ‘‘hanging drop’’ technique with approximately 1000 cells and approximately 300 µm in diameter. This study indicates that initial aggregate size is a critical factor in ES cell differentiation.

4.2 MATERIALS AND METHODS

4.2.1 Cell culture and differentiation

Culture medium to maintain the ES cells in an undifferentiated state consisted of Knockout D-MEM supplemented with 15% (v/v) Knockout Serum Replacement (Invitrogen, Carlsbad, CA), 4 mM L-glutamine (Cambrex, Walkersville, MD), 100 mg /mL penicillin–streptomycin (Invitrogen), 10³ U/mL leukemia inhibitory factor (LIF) (Chemicon, Temecula, CA), and 10 mg/mL gentamicin (Invitrogen), and 0.1 mM 2-mercaptoethanol (Sigma, St. Louis, MO). The tissue culture plates (T75) were coated with 0.1% gelatin (Sigma). ESD3 cells (ATCC, Manassas, VA) and ES-R1 (Oct4-GFP, provided by Dr A. Nagy, Mount Sinai Hospital, Toronto, Ontario, Canada) were cultured in the non-differentiating medium at 37 °C, with medium changed daily. When the ES cells on the tissue culture plates were 80% confluent, they were detached using trypsin/EDTA (0.1%/1 mM), and transferred to new plates. Differentiation culture medium consisted of Iscove’s Modified Dulbecco’s Medium (Gibco, Gaithersburg, MD) supplemented with 20% (v/v) fetal bovine serum (Gibco, Gaithersburg, MD), 4 mM L-glutamine (Cambrex), 100 U/mL penicillin–streptomycin (Invitrogen), and 0.01 mg/ mL gentamicin (Gibco).
For hanging drop EB formation, approximately 1000 ES cells were suspended in 30 mL differentiation medium without LIF. Using a micro-multi-channel pipette, the arrays of the micro drops were pipetted onto the lid of a culture dish. The lid was inverted and placed on the culture dish, which contained 5 mL differentiation medium to prevent the drops from evaporating. After 2 days, the ES cells in the drops aggregated and formed EBs, which were, then, transferred to a bacterial culture dish and cultured for another 2 days for proliferation. Thereafter, the EBs were transferred onto a collagen (0.25 mg/mL) coated culture dish (P60), and differentiated under controlled conditions. The differentiation protocol by Hamazaki and Terada et al. toward a hepatic lineage was used for both hanging drop technique and the micropatterned EB culture. On day 8, medium was replaced with medium containing fibroblast growth factor (100 ng/mL) (MP Biomedicals, Aurora, OH). On day 11, medium was replaced with medium containing hepatocyte growth factor (20 ng/mL) (MP Biomedicals, Aurora, OH). On day 14, medium was replaced with medium containing oncostatin-M (10 ng/mL) (Sigma, St. Louis, MO), dexamethasone (1027 M) (Sigma, St. Louis, MO) and ITS (insulin 5 mg/mL, transferrin 5 mg/ml, selenious acid 5 ng/mL) (BD Biosciences, San Jose, CA).

4.2.2 ES cell patterning using PDMS stencils and cell seeding

The shadow mask was designed to produce a center-center spacing between aggregates of 5 mm. This was to eliminate the possibility of cell interactions between the aggregates. The number of aggregates in an array was 25. Photosensitive epoxy (SU-8, Microchem, Newton, MA) was spincoated onto the silicon wafers which had been cleaned with oxygen plasma. The thickness of SU-8 was approximately 100 nm. The wafers were then soft baked at 65 uC for 5–20 min, followed by pre-baking at 100 uC for 20–90 min, depending on the thickness of the SU-8. The wafers were exposed to UV light (360 nm wave length) through the shadow mask, followed by post-exposure baking at 100 ºC for 10–20 min. The SU-8 patterns on the substrates were developed in SU-8 developer (Microchem, Newton, MA) for 10 min and rinsed with IPA (isopropyl alcohol) three times. After drying the substrates using nitrogen gas, they were overexposed to UV-light without a mask and baked at 150 ºC for 1 h. Polydimethylsiloxane (PDMS) (Sylgard 184, Dow Corning, Midland, MI) was mixed with curing agent (10 : 1 ratio), and poured on the wafers after degassing bubbles entrapped in the PDMS mixture. On the applied PDMS, a polyacetate film was placed
and clamped with an aluminium block, making the plastic film contact the top surface of the SU-8. The PDMS and aluminium block were heated to 90°C for curing for 12 h. The next day, the PDMS stencil was detached, trimmed and sterilized in ethanol for 20 min for cell patterning (Fig. 4.1). Rat tail collagen (type I) diluted in water (0.25 mg/mL) was applied and incubated overnight on tissue culture grade dishes (60 mm). The excess collagen water mixture was aspirated, and dried in a laminar flow hood. The PDMS stencil was then attached to the culture dish surface (Fig. 4.1). ES cells were seeded at a concentration of 0.5 x 10^6 cells mL in 2 mL culture medium with the stencil attached. Cells immediately began to aggregate, even as early as one hour after seeding. Detaching the stencil on day 2 after seeding ES cells allowed for stable aggregate formation (Fig. 4.2/4.3). If the stencil was detached after day 2, the cells proliferated and continued to grow up vertically to the height of the stencil itself, and the detachment of the stencil became difficult without disrupting the pattern.

After the stencil was removed on day 2, the average diameters of the micropatterned aggregates and EBs were measured by Sigmascan Pro image software (Jandel Scientific, CA). Once the stencil was removed, the ES cells would migrate out of the aggregates and form outgrowths. The phase contrast images demonstrate that these cells can form outgrowths, and the aggregates remained for 18 days of culture with uniform spacing and at uniform size. Compared to other studies (Hamazaki et al.), this protocol does not need a suspension stage in the formation of aggregates. For monolayer culture, ES cells were seeded in collagen-coated culture dishes (60 mm) at a concentration as low as 1000 cells/mL in 2 mL of culture medium to prevent cell–cell interaction.

4.2.3 Immunofluorescence assay
Primary antibodies for intracellular staining were rabbit antib- III tubulin (Chemicon, Temecula, CA), mouse anti-Muscle specific actin (Lab Vision, Fremont, CA), and goat anti-AFP (Santa Cruz Biotechnology, Santa Cruz, CA) with 1: 200 dilution. Secondary antibodies were anti-goat, rabbit, or mouse IgG conjugated with FITC or Cy3 (MP Biomedicals, Aurora, OH). The samples were washed twice with phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde in PBS at room temperature for 30 min. After incubation, the dishes were washed twice in PBS, followed by adding 0.2% Triton X-100 in PBS to permeabilize cells for intracellular staining. After a 5 min incubation at room temperature, the cells
were washed twice in PBS and resuspended in blocking buffer (PBS/20% FBS/0.05% Triton X-100) to block nonspecific antibody binding, and then incubated for 60 min at room temperature. The primary antibodies were added and incubated for 2 h at room temperature. After washing twice in blocking solution, cultures were incubated with the secondary antibody FITC or Cy3-conjugated rabbit, mouse, or goat IgG for 60 min at room temperature, and washed twice at room temperature. In some cases, cells were counterstained with 49,6-diamidino-2-phenylindole (DAPI) (Invitrogen, Carlsbad, CA) for nuclear staining. The immunofluorescence images were obtained by using a Zeiss Microscope. Since the central core of the aggregates and EBs showed non-specific staining, the images were therefore obtained from the peripheral area.

4.2.3 Functional analysis (albumin and urea assays)

The culture medium samples were collected for analysis of albumin and urea content. The albumin content was determined by enzyme-linked immunosorbent assay (ELISA) using purified mouse albumin and a peroxidase-conjugated antibody (Bethyl Laboratory, Montgomery, TX, USA). Urea content was determined with a commercially available kit (StanBio Laboratory, Boerne, TX, USA). Standard curves were generated using purified mouse albumin or urea dissolved in culture medium. Absorbances were measured with a Thermomax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

4.2.4 Reverse transcription Polymerase Chain Reaction (RT-PCR)

Differentiated cells were collected by using trypsin/EDTA and cell scraper. Typically, the number of the collected cells was more than $10^6$. RNA isolation was performed using the Nucleospin II RNA kit according to manufacturer’s instructions. Isolated RNA typically demonstrated a ratio of A260/ A280 $\geq 1.8$. One-Step RT-PCR kit from Qiagen (Qiagen, Valencia, CA) was used for PCR. For each RNA isolation, RNA gel was performed. Reaction conditions for all genes were based on the manufacturer’s instructions. Primers were designed using the Primer 3 Software available on the Web (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3 www. cgi). RNA (10 ng) was added for each reaction condition, and primer concentrations were calculated to be 0.6 nM. The primers are listed in Table 1. Cycling conditions for three-step cycling were: denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C
for 1 min. The number of cycles varied between 30 and 35, so as not to saturate the reaction. A final extension time of 10 min at 72 °C was also performed. Following PCR, samples were run on 2% agarose gel, and labeled with ethidium bromide solution. The gels were imaged using a Fluor-X Multiimager (Bio-Rad Laboratories, Hercules, CA).

4.3 RESULTS

4.3.1 Microfabricated stencils control initial aggregate size

In order to develop a tool to form uniform sized ES cell aggregates, we employed two techniques. The two techniques were surface modification and stencil placement. When surface modification was used as a technique to pattern the substrate, ES cells attached but did not form large uniform aggregates (data not shown). Furthermore, these aggregates did not form outgrowths. To surmount these problems, a microfabricated PDMS stencil was constructed and placed into a collagen type I coated 100 mm dish (Figure 4.1.) The entire process of cell stencil placement, ES cell seeding, and stencil removal is shown in Figure 4.2. Detaching the stencil at two days allowed for stable aggregate formation. At approximately day 6, the ES cells began to migrate out of the aggregates and form outgrowths. Compared to the hanging drop EBs (SD = ±65.8 µm, N = 8), the patterned aggregates showed much less variation in size (SD = ±15.3 µm, N = 24) on day 2. In terms of the aggregate size, the 300 µm diameter aggregates were similar to the EBs made using the hanging drop technique (Figure 4.3).

Figure 4.3 demonstrates temporal changes in aggregate size on days 2 and 6. A high ES cells at a density (0.5x10⁶/ml) was seeded onto the dish with the stencil attached, and cells immediately began to aggregate, even as early as an hour after seeding, as seen in Figure 4.3. Furthermore, phase images demonstrate that these cells can form outgrowths, and the aggregates stayed stable by day 18 of culture. As can be seen, aggregates can be made at uniform spacing and at uniform size. These results suggest that aggregate-like structures consisting of ES cells can be designed at uniform sizes, and produce outgrowths. Stencils appear to be a simple, reproducible, and much simpler to use than hanging drop techniques.
4.3.2 Germ layer formation in embryoid body versus monolayer culture

Using the microfabricated stencil technique as a tool to study differentiation, we varied the diameter of the holes in the stencil to investigate the effect of the initial size of the aggregate on late stages of differentiation of three germ layers. The rationale for this was that since ES cells secrete FGF, Activins, Wnt (Tam and Loebel 2007), and since these molecules are morphogens which form gradients and help promote specification of specific cell fates, then different sized aggregates would have different gradients of specific growth factors. Secondly, since different sized EB’s likely have different microenvironments based on oxygen diffusion and nutrient transport, this would suggest that different sized aggregates have varying microenvironments from a metabolic point of view. The island sizes chosen were 100 μm, 300 μm, and 500 μm, which allowed a five fold change in the diameter of the island. To compare differentiation in this system to controls, we used both an EB control as well as a sparsely seeded condition in which no aggregate was present. For measuring the differentiation of germ layers, we used RT-PCR.

To establish baseline conditions of germ layer expression, we investigated and compared the fate of the ES cells in EBs and single ES cells plated in a monolayer configuration by using PCR and immunofluorescence assays to assess germ layer differentiation. Here we are comparing monolayer and EB conditions across all figures. In the EB culture, transcripts for mesodermal (Fig. 4.4), ectodermal (Fig. 4.6), and endodermal (Fig. 4.8) genes were present. For the monolayer condition, transcripts for mesoderm (Figure 4.6) and endoderm were present (Figure 4.8), but no transcript for ectoderm (Figure 4.4) was present. This PCR result was consistent with immunofluorescence image data, showing that muscle-specific actin (mesoderm) (Fig. 4.5), β-III tubulin (neuroectoderm) (Fig. 4.7) and AFP (endoderm) (Fig. 4.9) were positive for EBs. For the monolayer condition, the immunofluorescence data showed that AFP was expressed in a significant fraction of the cells, muscle-specific actin was expressed in small clusters, and β-III tubulin was expressed very weakly. These results show that EBs and monolayer cultures were not only morphologically different, but also had differentiated differently over time.

4.3.3 Mesodermal differentiation in micropatterned aggregates

To measure mesodermal differentiation in the micropatterned ES cell aggregates, we measured the presence of major regulatory transcription factors, Nkx2.5 (cardiac mesoderm), Foxf1 (mesenchymal
mesoderm) and GATA1 (hematopoietic and endothelial). Foxf1 was not detected in ES cells patterned in the 100 µm aggregates but was detected in ES cells patterned in 300 µm and 500 µm aggregates (Fig. 4.4). This supports the fact that mesenchymal phenotypes varied with the size of the aggregate. GATA1 expression was low but similar in the 100 and 300 µm aggregates, while higher in the 500 µm aggregates, indicating that hemangioblast and/or hematopoietic induction was favored in the larger aggregates. However, both Foxf1 and GATA1 expression in the aggregates was lower than in the monolayer culture (Figure 4.4). Interestingly, a significant difference was seen in the cardiac mesoderm marker Nkx2.5. This marker was strongly expressed in the 300 µm case with decreased expression in the 500 µm case, and was very weakly expressed in the 100 µm case. Thus, mesenchymal phenotypes as well as cardiac mesoderm marker Nkx2.5 were favored in the 300 µm and 500 µm aggregates, while the mesodermal phenotypes were downregulated in the 100 µm aggregates. Immunofluorescence staining demonstrated that muscle-specific actin was positive and showed a similar trend as Nkx2.5 gene expression (Figure 4.5). Taken together, these results showed that mesenchymal and hematopoietic mesoderm were favored in the monolayer culture and the larger initial aggregate size, while cardiac mesoderm was favored in the 300 and 500 µm aggregates.

4.3.4 Neural differentiation in micropatterned aggregates

It has been shown that multiple bHLH genes play a critical role in the regulation of neural stem cell differentiation (Kageyama, Ohtsuka et al. 2005). In order to measure neural differentiation, we focused on three critical transcription factors that represent early neural commitment and/or early differentiation. For example, it is known that HES genes regulate maintenance of neural stem cells, while Ascl1 and Ngn promote neurogenesis (Mattar, Britz et al. 2004) Thus, we chose to measure Ascl1, which is a proneural transcription factor that specifies cortical neurons. In addition, we also measured Ngn2, which specifies neural progenitors with glutaminergic receptors (Kageyama, Ohtsuka et al. 2005). The hanging drop EBs expressed HES and Ascl1, while Ngn2 was less expressed (Figure 4.6). For the 100 µm aggregates, HES1 was expressed strongly, while Ngn2 and Ascl1 were expressed less, when compared to the hanging drop EBs, respectively (Figure 4.6). At the intermediate aggregate size of 300 µm, all three markers were clearly
expressed. ES cells in 500 µm aggregates did not express any neural markers, similar to ES cells cultured in a monolayer.

### 4.3.5 Endodermal differentiation in micropatterned aggregates

The endoderm gives rise to many lineages, and thus far it has been the most difficult germ layer to induce and differentiate. In this case we chose to expand the endoderm lineage using a known protocol, initially reported by Hamazaki and Terada 2001. The major factors agreed upon include Foxa2 and Sox 17 (Kubo, Shinozaki et al. 2004) which are regulatory transcription factors coexpressed in definitive endoderm. The visceral endoderm, a derivative of extraembryonic endoderm, which many times can also be generated in endoderm differentiation protocols, and also coexpresses Foxa2 and Sox17, was measured using Sox7 expression (Tada, Era et al. 2005). We chose to measure the extent of liver differentiation by measuring Foxa2, AFP and Albumin (Alb) gene expression, which when coexpressed, signifies activation of the liver program in vivo. When we varied the size of initial aggregate and measured markers for endoderm, Foxa2 was positive for all sizes, suggesting that early endoderm-like progenitors were present (Figure 4.8).

However, AFP, a marker for late endodermal and early hepatic expression, was significantly lower in 100 µm aggregates compared to 300 µm or 500 µm aggregates, suggesting that these cells had not undergone endodermal differentiation even by day 20 (Figure 4.8). Alb, a marker for commitment to the hepatic lineage, was only expressed in the 300 µm aggregates and not in the 100 µm and weakly expressed in the 500 µm aggregates. This suggested that only the 300 µm aggregate promoted hepatic differentiation.

Immunofluorescent staining demonstrated that AFP expression was weak in ES cells cultured in monolayers or 100 µm aggregates, while strong in 300 and 500 µm aggregates, and hanging drop EBs (Figure 4.9). These results demonstrated that the extent of endoderm differentiation varied from the least at 100 µm, to intermediate at 500 µm, and most at 300 µm, indicating that there was an optimal aggregate size for endoderm differentiation.

### 4.3.6 Differentiation kinetics of micropatterned aggregates compared to embryoid bodies

Screening of the germ layer markers while varying the size of initial aggregate demonstrated that the size of the micropatterned aggregates could contribute to germ layer formation. To further assess how well
micropatterned aggregates differentiated compared to EBs made using the hanging drop technique, we examined both the kinetics of gene expression as well as a functional assay. We focused analysis to endoderm induction and differentiation, using the Hamazaki protocol. We also chose the aggregate condition of 300 µm because its size and gene expression profile closely mimicked those of the EB. In addition to Foxa2, AFP and Alb which were previously measured, we also compared Sox 17 which is a regulatory transcription factor coexpressed in definitive endoderm, and Sox7 which is expressed in the visceral endoderm. Gene expression studies indicated very similar kinetic profiles for the EB and the 300 µm aggregate. Fig. 4.10 show the gene expression profiles of endoderm and mesoderm markers on culture days 10 and 20. As can be seen, transcripts for Foxa2, Sox17, Sox7, and AFP were expressed in both the EB and the micropatterned aggregates on day 10. On day 10, Alb was not expressed in the EB or the micropatterned aggregate conditions. This suggests that both conditions produced the same amount of endoderm progenitor cells. By day 20, there was an up regulation of Alb in both the EB and the micropatterned 300 µm aggregate conditions. This indicated that the endoderm had been specified towards liver. Hamazaki and Terada et al reported that hepatic differentiation was observed using hanging drop EBs and urea synthesis was detected. We also examined urea secreted in the medium, which is specific for hepatocyte-like cells and has been used as functional markers in hepatocyte studies. The EB and the micropatterned aggregates demonstrated similar urea function. The secreted values were 6.75 and 6.27 mg/mL on day 11, 4.45 and 5.86 mg/mL on day 14, and 5.69 and 5.41 mg/mL on day 16 of culture, respectively. In summary, the studies of gene expression kinetics and the functional studies suggest that similar rates of differentiation occurred in both EBs and the 300 µm aggregates, suggesting that the “normal” mechanisms of differentiation due to aggregation were present in the micropatterned 300 µm aggregates.

4.4 DISCUSSION

ES cell differentiation has been known to depend on many different conditions, such as ECM, (Flaim et al 2004) growth factors (Miller, Dinsio et al. 2004; Nakayama, Momoki-Soga et al. 2004),(Faloon, Arentson et al. 2000),(Morali, Jouneau et al. 2000),(Kuai, Cong et al. 2003; Hu, Cai et al. 2004; Kania, Blyszczuk et al. 2004), local oxygen concentration and cell-cell interactions. The ability to control the microenvironment
and thus control differentiation has been challenging. In this study, we focused on using microfabrication process to control the initial aggregate size. Our data demonstrates that this can be an important factor for governing lineage selection during ES cell differentiation. Traditional techniques such as such as hanging-drop and suspension culture (Keller 1995) have several problems which have not yet been addressed. These can be grouped as having variation in size, variation in plating efficiency, fragility of EB’s, as well as aggregation of embryoid bodies (Dang, Kyba et al. 2002). In addition, because EB’s in normal cultures are not addressable because one cannot control adhesion of a certain number of EB’s to a specific location.

This study illustrates that a variety of problems can be solved by using microfabricated stencils. EB’s were reproducible, addressable, stable, did not aggregate. The technique was easy to use such that beyond basic tissue culture training, no additional training would be needed to master the technique of using stencils. The stencil was made of PDMS which has the opposite electrostatic charge to polystyrene culture dish, and can be attached directly on a pure or ECM-coated culture dish without any surface treatment. The technique was better suited for embryonic stem cell cultures because standard surface treatment in our case promoted aggregation but did not allow outgrowths to form. The stencils were recyclable which would allow re-use.

Using the microfabricated stencils, we studied the initial size effect on differentiation, and found that the initial size of the aggregate is one of decisive factors in differentiation. In this sense we uncovered new biology of early differentiation, using this enabling technology. Our data suggests that large aggregate sizes (500µm) appeared to behave similar to the monolayer case, while our intermediate size (300µm) appeared to be similar to the embryoid body controls. However, these studies were limited to only a few sizes, to specific time points, and to particular culture conditions (Hamazaki et al.). Our studies suggest that these stencils can be used in the future, together with other technologies, such as microcarrier based technology, microencapsulation technology, biomimetic biomaterials technology, or other high throughput techniques, to further control spatial and temporal information during early ES cell differentiation for in vitro and large scale applications.

4.5 REFERENCES

Figure 4.1. Microfabricated Stencils for ES cell seeding. SEM (scanning electron microscope) images of microfabricated stencils of 100, 300, 500 mm diameter. The thickness of the stencils was kept uniform at approximately 100 mm.
Figure 4.2. **Cell seeding onto microfabricated stencils.** Schematic for patterning ES cells. The PDMS stencil was attached on a collagen-coated glass, and ES cells were seeded with $0.5 \times 10^6$ cells/mL in 2 mL. Initially, ES cells were seeded in each well as a confluent monolayer. By day 2, the ES cells proliferated and formed an array of aggregates. When the PDMS stencil was detached, the aggregates remained attached on the collagen-coated surface.
Figure 4.3. Morphology of ES cell culture in control and microfabricated conditions. Phase-contrast photomicrographs of patterned ES cell aggregates on Day 2 and Day 6. Above: Monolayer and Hanging Drop (HD) conditions. Below: Diameter of stencil varied from 100µm to 500µm. Bar = 100µm.
Figure 4.4. Mesodermal gene expression after long term culture in control and micropatterned stencil conditions. Mesoderm (Foxf1, GATA1, Nkx2.5) gene expression measured by RT-PCR (30 cycles) of embryonic stem cells cultured for 20 days. Day 0 ES cell RNA was used as a negative control. Differentiation was staged using the Hamazaki protocol.
Figure 4.5. Immunofluorescence for mesoderm (Muscle-specific antigen) after long term culture in control and micropatterned stencil conditions. Phase contrast images and nuclear DAPI and Muscle specific antigen staining in all control and micropatterned stencil conditions. Differentiation was staged using the Hamazaki protocol. Bar equals 100 µm.
Figure 4.6. Neural gene expression after long term culture in control and micropatterned stencil conditions. Neural (Ngn2, Hes1, Ascl1) gene expression measured by RT-PCR (30 cycles) of embryonic stem cells cultured for 20 days. Day 0 ES cell RNA was used as a negative control. Differentiation was staged using the Hamazaki protocol.
Figure 4.7. Immunofluorescence for Neural (βIII-tubulin) after long term culture in control and micropatterned stencil conditions. Phase contrast images and nuclear DAPI and β-tubulin staining in all control and micropatterned stencil conditions. Differentiation was staged using the Hamazaki protocol. Bar equals 100 µm.
Figure 4.8. Endodermal gene expression after long term culture in control and micropatterned stencil conditions. Endodermal (Foxa2, AFP, Alb) gene expression measured by RT-PCR (30 cycles) of embryonic stem cells cultured for 20 days. Day 0 ES cell RNA was used as a negative control. Differentiation was staged using the Hamazaki protocol.
Figure 4.9. Immunofluorescence for Endoderm (Alpha-fetoprotein) after long term culture in control and micropatterned stencil conditions. Phase contrast images and nuclear DAPI and Alphafetoprotein staining in all control and micropatterned stencil conditions. Differentiation was staged using the Hamazaki protocol. Bar equals 100 µm.
Figure 4.10. Figure 4.8. Kinetics of endodermal gene expression in control and micropatterned stencil (300µm) conditions. Endodermal (Foxa2, Sox7, AFP, Alb, Sox17) gene expression measured by RT-PCR (30 cycles) of embryonic stem cells cultured for 20 days in control and micropatterned (300µm). Day 0 ES cell RNA was used as a negative control. Differentiation was staged using the Hamazaki protocol.
Figure 4.11. Kinetics of urea secretion in control and micropatterned stencil conditions. Urea secretion, a functional measure of liver differentiation, was measured using a standardized absorbance assay (see methods), compared to controls, and plotted as μg/ml. Mean and standard deviation from n=3 samples for Hanging Drop and 300µm conditions.
CHAPTER 5:
ACTIVIN ALTERS THE KINETICS OF ENDODERM INDUCTION OF COLLAGEN GEL CULTURES OF EMBRYONIC STEM CELLS

5.1 ABSTRACT
Embryonic stem cell-derived endoderm is critical for the development of cellular therapies for the treatment of disease such as diabetes, liver cirrhosis, or pulmonary emphysema. Here, we describe a novel approach to induce endoderm from mouse embryonic stem cells (mES) using fibronectin-coated collagen gels. This technique results in a homogenous endoderm-like cell population, demonstrating endoderm-specific gene and protein expression, which remains committed following in vivo transplantation. In this system, activin, normally an endoderm inducer caused an 80% decrease in the Foxa2 positive endoderm fraction, while follistatin increased the Foxa2 positive endoderm fraction to 78%. Our work suggests that activin delays the induction of endoderm through it transient precursors, the epiblast and mesendoderm. Long term differentiation, displays a two-fold reduction in hepatic gene expression and three-fold reduction in hepatic protein expression of activin-treated cells compared to follistatin-treated cells. Moreover, subcutaneous transplantation of activin-treated cells in a syngeneic mouse generated a heterogeneous teratoma-like mass, suggesting these were a more primitive population. In contrast, follistatin-treated cells resulted in an encapsulated epithelial-like mass, suggesting these cells remained committed to the endoderm lineage. In conclusion, we demonstrate a novel technique to induce the direct differentiation of endoderm from mES cells without cell sorting. In addition, our work suggests a new role for activin in induction of the precursors to endoderm, and a new endoderm-enrichment technique using follistatin.

5.2 INTRODUCTION
Embryonic stem cells-derived endoderm progenitors offer a remarkable potential for the treatment of major diseases effecting the pancreas, liver, lungs, bladder, and prostate (Wells and Melton 1999; Oottamasathien, Wang et al. 2007). Endoderm progenitors would also be useful for the study of congenital diseases (de Santa Barbara, van den Brink et al. 2002), gene discovery, toxicology screening, and drug development (Wobus and Boheler 2005). Current knowledge regarding the development of the definitive
endoderm, one of three major germ layers, is derived from in vivo fate mapping (Gardner and Rossant 1979; Poelmann 1981; Lawson and Pedersen 1987), mouse genetics (Shivdasani 2002) and in vitro tissue explants (Gualdi, Bossard et al. 1996). These studies indicate that the endoderm is derived from the inner cell mass through the differentiation of the transient epiblast and mesendoderm (anterior primitive streak) populations (Tam and Behringer 1997). Each of these populations express unique markers or transcription factors. For example, the epiblast expresses Oct4 and Fgf5, mesendoderm expresses Brachyury and Foxa2, (Ang, Wierda et al. 1993) and endoderm expresses Foxa2 and Sox 17 (Kanai-Azuma, Kanai et al. 2002) (Abe, Niwa et al. 1996). The ability to identify and control these transient precursor populations is a major goal of the field (Zaret 1999; Yasunaga, Tada et al. 2005).

Endoderm induction has been shown to be controlled by soluble factors such as activin-nodal-TGFβ, BMP, and Wnt (Zhou, Sasaki et al. 1993; Albano and Smith 1994; Conlon, Lyons et al. 1994; Winnier, Blessing et al. 1995; Waldrip, Bikoff et al. 1998; Beppu, Kawabata et al. 2000). Recent studies demonstrated that definitive endoderm can be derived from a mesendoderm precursor, using serum-free medium, activin, and serial cell sorting (Kubo, Shinozaki et al. 2004; Yasunaga, Tada et al. 2005). Activin, which binds to the same receptor as nodal (Chang, Brown et al. 2002) has been shown to generate different tissues as a function of concentration in both Xenopus and mouse embryonic stem (mES) cell studies, and has therefore been classified as a potent developmental morphogen (Okabayashi and Asashima 2003). However, recent studies suggest that while activin enhanced endoderm in embryoid body (EB) cultures, it failed to do so in monolayer culture (Sherwood, Jitianu et al. 2007). Furthermore, in human ES cells, activin-nodal signaling was shown to inhibit ES cell differentiation, rather than induce endoderm. (Beattie, Lopez et al. 2005; James, Levine et al. 2005; Vallier, Alexander et al. 2005; Ogawa, Saito et al. 2007) A similar mechanism was shown to inhibit the differentiation of inner cell mass cells in ex vivo mouse blastocyst cultures (James, Levine et al. 2005) suggesting a complex role of activin-nodal signaling in ES cell differentiation. The role of follistatin plays in vitro studies is unclear (Albano, Arkell et al. 1994). However, recent studies suggest that follistatin has an important role during liver regeneration (Rodgarkia-Dara, Vejda et al. 2006) and pancreas differentiation (Miralles, Czernichow et al. 1998).

Studies thus far have relied on multiple cell sorting, complex serum-free formulations, and multiple growth factors for the differentiation of endoderm. Here, we describe a simple cell culture system
which induces the differentiation of mES cells toward endoderm in the presence of serum without cell sorting, growth factors, or hormones. The endoderm-like cell population was positive for endoderm specific markers Foxa2 and Sox17 and negative for major mesodermal and ectodermal markers by day 10 of culture. Surprisingly, activin caused a dose-dependent decrease in the expression endoderm markers, while inducing the expression epiblast and mesendoderm markers, such as Brachyury and Fgf5. On the other hand, the activin-inhibitor follistatin increased the Foxa2 positive endoderm fraction to 78.4%, without altering the expression kinetics. Long term gene and protein expression studies indicated that activin-treated cells had reduced hepatic differentiation potential compared to follistatin and controls. In vivo differentiation of activin-treated cells in a syngeneic mouse model generated a heterogeneous, teratoma-like mass, suggesting a primitive population, while follistatin-treated cells generated an encapsulated epithelial-like mass, similar to control. In conclusion, these studies demonstrate a novel technique to induce an endodermal cell population in vitro and suggest an intriguing role of activin in endoderm development.

5.3 METHODS

5.3.1 Reagents

Fetal Bovine Serum (FBS), Dulbecco’s modified Eagle medium (DMEM), penicillin, streptomycin, knockout serum, knockout DMEM, bovine gelatin, and dispase were obtained from Invitrogen Life Technologies (Carlsbad, CA). Human Fibronectin was purchased from BD Biosciences (San Jose, CA, USA). Hydrocortisone was obtained from Pharmacia (Kalamazoo, MI). Glucagon and Insulin were purchased from Eli-Lilly (Indianapolis, IN). Oncostatin M, Human Bone Morphogenic Protein-2 (BMP2), Hepatocyte Growth Factor (HGF), Human Activin, Mouse Follistatin, were purchased from R&D Systems (Minneapolis, MN). ESGRO (Recombinant Leukemia Inhibitory Growth Factor) was purchased Chemicon (Temecula, CA). Immunofluorescence grade paraformaldehyde was purchased from Electron Microscope Sciences (Hatfield, PA). Rabbit anti-mouse Foxa2 antibody was purchased from R&D Systems (Minneapolis, MN). Goat anti-mouse Sox17 was purchased from Santa Cruz (Santa Cruz, CA). Goat anti-mouse Albumin was purchased from ICN Pharmaceuticals (Aurora, OH). For immunofluorescence studies, normal donkey serum and secondary F(ab)2 antibody fragments, ML grade, were obtained from Jackson
IMMUNORESEARCH (Bar Harbor, ME). Unless otherwise noted, all other chemicals, growth factors, and solutions were purchased from Sigma-Aldrich Chemicals.

5.3.2 Embryonic stem cell culture

Undifferentiated mouse D3-ES cells (ATCC) were cultured on 0.2% gelatin-coated tissue culture T75 flasks, at low dilutions of 1:50, such that small colonies were maintained. Medium was changed daily. Experiments were carried out with cells at passage 30. Undifferentiated mES cells were cultured in Knockout DMEM supplemented with 15% Knockout serum, L-Glutamine (4 mM), Penicillin(100 U/ml), Streptomycin (100 U/ml), Gentamicin (10 mg/ml), ESGRO (1,000 U/ml), and 2-Mercaptoethanol (0.1 mM). Proliferating ES cell cultures were maintained in a 5% CO₂-humidified incubator at 37°C.

5.3.3 Fibroblast cell culture

NIH 3T3 mouse fibroblasts (ATCC) were cultured at 1:10 dilution on tissue culture-treated T75 flasks in high glucose DMEM containing 10% FBS, Penicillin (100 U/ml), Streptomycin (10 mg/ml), Gentamicin (1,000 U/ml) and medium was changed every 2 days.

5.3.4 Fibronectin-coated collagen gels

Type I collagen stock solution was prepared from rat tail tendon as described by Dunn et al. (Dunn, Yarmush et al. 1989). Collagen gelling solution was prepared by mixing nine parts of collagen stock (1.25 mg/ml) with one part of 10 x DMEM on ice. The collagen gelling solution was then added at a volume of 250 μl/well (66 μl/cm²), spread evenly on the dish and incubated at 37°C for 30 min for gel formation. Fibronectin dissolved in PBS was then added on top of the gel, at a concentration at 3.8 μg/well (1 μg/cm²) and the tissue culture plates were incubated for 1h at 37°C to insure ensure fibronectin adsorption on collagen gel.

5.3.5 Mouse embryonic stem (mES) cell differentiation

Mouse embryonic stem (mES) cells were directly seeded at a density of 2 x 10⁴ cells/well (5.2 x 10³ cells/cm²) onto fibronectin-coated collagen gels. Basal differentiation medium consisted of high glucose
DMEM supplemented with 10% Fetal Bovine Serum, Glutamine (100 U/ml), Penicillin (100 U/ml), Streptomycin (100 U/ml), Gentamycin (10 mg/ml). For growth factor studies, the medium was augmented with activin (20 or 50 ng/ml) or follistatin (100 ng/ml). Medium was changed every 2 days of culture. Differentiating ES cell cultures were maintained in a 10% CO₂-humidified incubator at 37°C.

Embryoid Body (EB) cultures were formed by culturing mES cells using the hanging drop method. ES cells were resuspended in differentiation medium and spotted as 30 μl drops on an inverted lid of a 100 mm dish at a concentration of 30,000 cells/ml. Dishes were incubated at 37°C for 48h in PBS. EB’s were recovered and matured in suspension for 48h and then transferred to a 12 well plate.

5.3.6 Late stage in vitro ES cell maturation

Following 10 days of culture, the endoderm-like cells were harvested from gel culture using dispase digestion (1 mg/ml), and filtered using a sterilized 200 μm nylon mesh. Viability was greater than 92%. The harvested endoderm-like cells were then seeded onto fibronectin-coated collagen gels at a concentration of 5 x 10⁴ cells/well (1.3 x 10⁴ cells/cm²) in C+H medium, consisting of high glucose DMEM supplemented with 10% heat-inactivated fetal bovine serum, penicillin/streptomycin (100 U/ml), hydrocortisone (7.5 g/ml), epidermal growth factor (20 ng/ml), glucagon (14 ng/ml), and insulin (0.5 U/ml). C+H medium was augmented with Bone Morphogenic Protein-2 (BMP2) (10 ng/ml), Hepatocyte Growth Factor (HGF) (20 ng/ml) and Oncostatin M (OSM) (20 ng/ml).

5.3.7 Late stage in vivo syngeneic mouse transplantation

Female 129 mice, (Charles River Laboratories, Boston, MA), syngeneic to D3 ES cells, and weighing between 25 and 35 g were used for this study. The animals were cared for in accordance with the guidelines set forth by the Committee on Laboratory Resources, National Institutes of Health, and Subcommittee on Research Animal Care and Laboratory Animal Resources of Massachusetts General Hospital. Animals had free access to food and water.

ES cells were recovered using dispase digestion from either EB plated culture, standard collagen culture, activin-treated, or follistatin-treated cultures and filtered using a sterilized 200 μm nylon mesh. For support of cellular function, 3x10⁶ mES cells were mixed with 3x10⁴ mouse NIH 3T3 fibroblasts. Mice
were anesthetized with intraperitoneal injections of pentobarbitol (70mg/kg). After shaving and cleaning the site of injection with antiseptic solution, cells were injected subcutaneously such that a subcutaneous wheal could be identified in the paraspinal region between the 6th and 8th rib using a 28-gauge needle. Permanent stitches were placed to mark the injection site. Two weeks after injection, animals were killed by cervical dislocation and the transplantation site was excised and fixed in formalin. Abdominal and thoracic cavities were inspected for tumor formation.

5.3.8 H&E staining
Tissue samples were fixed in buffered formalin (1:10) and placed in tissue cassettes, dehydrated, embedded in paraffin, and sectioned at 4µm. Standard H&E (Hematoxylin and Eosin) staining was performed. Slides were examined using a Nikon Eclipse 800 upright compound microscope and analyzed using Advanced Spot 2 software (Molecular Dynamics).

5.3.9 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)
For each experimental condition, cell lysates were generated using Guanidinium Isothiocyanate-based solution (Clonetech) and stored at -80°C for later use. RNA isolation was conducted using the manufacturer’s instructions for the Nucleospin II RNA Kit (Clonetech). RNA gels were run using 2% Agarose (RNAase free) to ensure that RNA was intact prior to RT-PCR. One-step gene-specific RT-PCR (Qiagen) was performed for gene expression analysis and resolved on a 2% agarose gel. Each reaction represents 10 micrograms of total RNA per condition. Cycle number was 30 for all genes unless otherwise specified. Cycling conditions were 55°C, 30 sec, 94°C, 30 sec, and 72°C 30 sec, with a ten minute extension at 72°C. Thermal cycling was done using the Mastercycler Epigradient X (Eppendorf) with 96 well plates. Gels were imaged using fluorescent gel scanner, the Fluor-S Multi-Imager (BioRad) and captured using Multi-analyst software. Primers were as follows:

- **B-Actin** (F) 5'-GAGGGAAATCGTGCGTGTA-3'; **B-Actin** (R) 5’CCAAGAAGGAAGGCTGGAA-3’;
- **Oct4** (F) 5’-GGAAGCCGACAACAATGA-3’; **Oct4** (R) 5’-CAAGCTGATTGGCGAATGT-3’;
- **Fgf5** (F) 5’-GTTCAGCACTCGAGCGAACAATGA-3’; **Fgf5** (R) 5’T-AGGCACAGACAGAGGGATG-3’;
- **Otx2** (F) 5’-GGAAGGAGGGAAGGTCAT-3’; **Otx2** (F) 5’-CAG-TCGCACAATCCACACA-3’;
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5.3.10 Quantitative Real-Time RT-PCR

Reverse transcription and qPCR was performed using the Superscript III Two-Step qRT-PCR kit.
(In Vitrogen, Catalog No 11735-032). The RT reaction was run using primer containing a mixture of random-hexamers and dT primers, 500ng of total RNA template, and master mix containing a bioengineered MMLV-RT enzyme, nucleotides, and other components. The reaction mix was incubated at 25°C for 10 minutes, and 42°C for 50 minutes, followed by termination at 85°C for 5 min and RNAaseH incubation at 37°C for 20 minutes. Real-time quantitative PCR was performed using the Stratagene MX5000P QPCR machine. Triplicates of 10 μL reactions containing 2 μL of primer mixture (0.2 μM), 10 ng of cDNA template, Rox dye, and SYBR Green master mix containing the Platinum Taq Polymerase were used for all reactions. The cycling temperatures were 94°C for 30s, 57°C for 30s, 72°C for 30s.

5.3.11 Relative Quantitation of Real-Time PCR data

Real time data was analyzed using the Stratagene MX-Pro QPCR software using settings with an amplification-based threshold and adaptive baseline. Melting curves for each reaction were obtained and any reactions without a unique PCR product were not analyzed. Threshold cycle (CT) were determined and used to quantify gene expression using the \(2^{-\Delta\Delta Ct}\) method .(Livak and Schmittgen 2001) Gene expression was measured relative to a normalizer, β-Actin, and calibrated using the day 0 condition for the genes of interest. This data, which was then expressed as relative difference of gene expression on day 24 compared to day 0, was normalized to the untreated condition, termed control. Thus the activin-treated and the follistatin treated conditions were normalized to the control condition.

5.3.12 Immunofluorescence

Samples were washed three times with PBS, and fixed in 4% EM-grade paraformaldehyde for 10 min at room temperature. Samples were then washed with PBS and were permeabilized for 15 min with 0.1% Triton X-100, blocked for 30 min with 1% bovine serum albumin, 5% donkey serum at room temperature followed by staining with primary antibodies at overnight at 4°C. The dilutions of primary antibodies used were Foxa2 (1:100), Sox 17 (1:100) and albumin (1:500). After additional washes with PBS, samples were stained with fluorescently tagged secondary antibodies at a dilution of 1:500, for 45 min at room temperature and washed three times with PBS. Cells were then imaged using phase contrast and fluorescent microscopy (Ziess) and captured using AxioVert software.
5.3. 13 Flow cytometry

Samples were washed three times with cold PBS, kept on ice, and fixed in 1% EM-grade paraformaldehyde for 15 min at 4°C. Samples were then washed with ice cold PBS and were permeabilized for 15 min with 0.1% Triton X-100 while cells were vortexing, and then washed twice with cold PBS. Cells were blocked for 30 min with 1% bovine serum albumin, 5% donkey serum at room temperature for 30 min. Primary antibodies diluted in blocking buffer (above) were then added for 20 min at room temperature, at the same dilution as for immunofluorescence. After additional washes with ice cold PBS, samples were stained with fluorescently tagged secondary antibodies, at the same dilution as immunofluorescence, for 45 min at room temperature, and then washed twice again. Flow cytometry was conducted on a Coulter Epics Altra Flow Cytometer (Beckman Coulter) and raw data were captured using Expo 32 Multicomponent software.

5.4 RESULTS

5.4.1 Activin decreases endoderm and increases epiblast-specific gene expression

Our previous studies had demonstrated that the culture of ES cells on collagen gels selectively induces endoderm gene and protein expression (see Chapter 3, Figure 3.10). Activin/Nodal is a member of the TGFβ superfamily, which has been previously shown to be critical in regulating endoderm formation in vitro (Kubo, Shinozaki et al. 2004; Tada, Era et al. 2005) and in vivo (Ariizumi and Asashima 2001). To test whether endoderm induction in gel cultures responded to stimulation of the activin/nodal axis, we added activin or follistatin (a soluble inhibitor of activin) to the serum-containing culture medium from day 0 to day 10. The addition of 20, and then 50 ng/ml activin induced the appearance of elongated, spindle-like cells, by day 4, and maintained by day 10 of culture in a dose-dependent manner(Figure 5.1). In contrast, follistatin-treated cells appeared to have epithelial like morphology similar to control (Figure 5.2).

We then analyzed these day 10 populations by RT-PCR for epiblast (Oct4, Fgf5) (Pelton, Sharma et al. 2002), endoderm (Foxa2, Sox 17), mesoderm (GATA1, Runx2, Foxf1) (Tang, Liu et al. 2001; Schroeder, Jensen et al. 2005), early mesodermal patterning (Paraxis, Lefty2) (Burgess, Cserjesi et al. 1995; Merrill, Pasolli et al. 2004), and neurectoderm (Pax6, Ascl1(Mash1), Ngn2) markers (Gotz, Stoykova et al. 1998; Britz, Mattar et al. 2006) (Figure 5.3). Surprisingly, the addition of activin (20 ng/ml and 50 ng/ml)
decreased endoderm gene expression in a dose dependent manner (Figure 5.3), while the addition of follistatin (100 ng/ml) did not. Interestingly, this decrease in Foxa2 and Sox17 expression in the activin condition correlated with an increase in expression of the primitive epiblast markers Oct4 and Fgf5. As expected, follistatin had the opposite effect, decreasing Fgf5 expression compared to controls. Neurectodermal and mesodermal markers were absent in all conditions (data not shown). Mesodermal patterning markers, which are activated prior to mesodermal specification in vivo, showed a dose-dependent increase in gene expression with activin, while follistatin induced a downregulation of these genes (Figure 5.3).

Immunofluorescence staining for Foxa2 and Sox17 indicated that the activin-treated spindle-like cells did not express Foxa2 or Sox 17 on the protein level, while follistatin-treated epithelial cells had strong Foxa2 and Sox 17 expression (Figures 5.4). To quantify the percentage of endodermal cells, we performed flow cytometry for Foxa2 on day 10 of culture. Activin-treated cells (50 ng/ml) had approximately 10% Foxa2 positive cells while follistatin-treated cells were approximately 78% Foxa2 positive cells (Figure 5.5). Thus, activin decreased the endodermal fraction by 80% while follistatin caused a 47% increase. These results suggest that activin might delay endoderm induction by maintaining upregulation of early genes for epiblast or mesodermal patterning.

5.4.2 Activin alters the gene expression kinetics of epiblast, mesendoderm, and endoderm

Previous studies have indicated that the and Oct4 positive, Fgf5 positive epiblast population is a transient population which emerges on embryonic day 2 (E2) and is down-regulated by d5 as the cells differentiate into the three major germ layers (ectoderm, mesoderm, endoderm) (Pelton, Bettess et al. 1998). Our previous results (Figure 5.3) demonstrated that while activin downregulated endoderm genes by day 10 of culture, it also upregulated the early epiblast gene Fgf5. To determine if the kinetics of the epiblast precursor population is altered in the presence of activin, we analyzed epiblast (Oct4, Fgf5, Otx2 ) (Ang, Jin et al. 1996) mesendoderm (Brachyury, Goosecoid, and Lim1) (Kubo, Shinozaki et al. 2004; Tada, Era et al. 2005) and endoderm (Foxa2, Foxa1, Sox17, HNF1β) specific gene expression at days 4, 6, and 10 of culture, on collagen gel in the presence of activin (20 and 50 ng/ml) or follistatin (100 ng/ml). Oct4 is a specific marker for the inner cell mass as well as the epiblast, Fgf5 is specific for epiblast, while Otx2 is
expressed in epiblast in addition to other tissues during development (Fossat, Chatelain et al. 2006). While control cells have a decreasing expression of Oct4, Fgf5 and Otx2, activin-treated cells appear to have a constant (20 ng/ml) or increasing (50 ng/ml) expression of the same genes, suggesting an earlier phenotype or delayed differentiation (Figure 5.6.) Similarly to the changes in epiblast expression, markers of mesendoderm differentiation (Brachyury, Goosecoid and Lim1) (Kubo, Shinozaki et al. 2004; Tada, Era et al. 2005) were downregulated by day 10 of culture in control and follistatin-treated condition following transient expression, but remained upregulated by day 10 of culture in activin-treated cells (Figure 5.7).

The expression kinetics of the endoderm-specific transcription factors Foxa2 and Sox17 appear to be similar in all conditions, increasing over time (Figure 5.8). However, activin-treated cells appear to express lower levels of the genes in a dose-dependent manner while follistatin-treated cells express relatively higher levels of endodermal expression. Furthermore, increasing the dose of activin from 20 ng/ml to 50 ng/ml delayed the expression of Foxa1, and diminished expression of HNF1β by day 10 of culture (Figure 5.8.) Taken together, the data suggests that increasing activin concentration may shift the kinetics of epiblast, and mesendoderm differentiation, from a rapid downregulation to a persistent expression of the same markers under our culture conditions.

### 5.4.3 Long term endoderm differentiation following activin stimulation

The patterning of endoderm into its downstream lineages, such as liver, pancreas, and gut, is subject to complex mechanisms in vivo and as a result, is poorly understood (Wells and Melton 1999). To test the differentiation potential of activin and follistatin-treated cells, we reseeded day 10 cells in hepatic differentiation medium containing BMP2, HGF and Oncostatin (Zaret 2000) but without follistatin or activin. Analysis of cell morphology on days 14, 18, and 22 demonstrated that the cells originating from all three early-differentiation conditions, control, activin (50 ng/ml) and follistatin (100 ng/ml) acquired an epithelial morphology (Figure 5.9). However, activin-treated cells initially formed small clusters reminiscent of EBs formed by primitive mES cells (Figure 5.9).

To determine the extent of endoderm induction and patterning, we analyzed gene expression for endoderm patterning markers following 14 days of late-stage differentiation (day 24 of total culture) by RT-PCR. We tested Foxa2 and Sox17, as well the foregut marker alpha fetoprotein (AFP), Albumin, Pdx1,
and Insulin (Zaret 2002), the midgut marker Cdx2 (Wells and Melton 2000) and the hindgut marker Intestinal Fatty Acid Binding Protein, (IFABP) (Wells and Melton 2000). Day 10 mouse embryonic RNA was used for a positive control for all primers. Expression of Foxa2, Sox17 and AFP was similar in all three conditions (Figure 5.10). However, the expression foregut marker albumin as well as the hindgut marker IFABP were inhibited by activin but stimulated by follistatin (Figure 5.10), suggesting that activin might delay hepatic differentiation. To quantify these phenomenon, we performed qRT-PCR on two genes known to be markers for hepatic differentiation, cytokeratin 8 (CK8) and cytokeratin 18(CK18) (Figure 5.12). When compared to control, activin-treated cells displayed decreased relative expression of CK 8 (0.705 ± 0.106) and CK18 (0.79± 0.14). On the other hand follistatin-treated cells, displayed increased relative expression of both CK8 (1.43±0.32) and CK 18 (1.71± 0.84). The ratio of follistatin to activin expression at day 24, was calculated to be 2.02 ± 0.153 for CK8 and 2.09 ± 0.68 for CK18. We extended the analysis of day 24 samples to examine concomitant differences in protein expression. Immunofluorescence staining for intracellular albumin on day 24 showed higher expression in control and follistatin-treated cells than in activin-treated cells (Figure 5.11). To quantify the changes in albumin, we performed flow cytometric analysis of control, activin-treated, and follistatin-treated cells (Figure 5.13). In control and follistatin conditions, 21.8% and 19.3% of cells were positive for albumin, whereas only 5.9% of the activin-treated cells were albumin positive. The mean intensity values of the albumin-positive population were 25.1% for control, 30.2% for follistatin, and 15.8 % for activin. Taken together with the endpoint PCR data, this data strongly suggests that hepatic differentiation was diminished following activin-treatment.

5.4.4 Long term in vivo differentiation of activin and follistatin-treated cells in a syngeneic mouse transplant model

The in vitro differentiation data on days 10 and 24 of culture suggests that while follistatin induces an endoderm-specific differentiation, activin delays differentiation through the induction of primitive lineages. To assess the differences in potency and commitment between these conditions, we transplanted day 10 cultured ES cells subcutaneously in a syngeneic mouse model. The four conditions transplanted were embryoid body (EB), control (no treatment), activin (50 ng/ml), and follistatin (100 ng/ml). Each cell population was mixed with a low ratio of NIH 3T3 fibroblasts (1:100), for mesenchymal support upon
transplantation (Van Vranken, Romanska et al. 2005). All implants gave rise to an observable mass (Figure 5a) by day 14 of transplant (day 24 total culture). The cell mass was significantly larger in the case of activin-treated cells (Figure 5.14).

The excised tissue was analyzed by histological examination and was reviewed by a blinded pathologist. When cells were recovered from an embryoid body (EB) and implanted, they generated a teratoma-like cell mass with extensive extracellular matrix suggesting immature bone or cartilage, evidence of skin, glandular tube-like structures and immature cells (Figure 5.15). On the other hand, the endoderm-like cell population differentiated on fibronectin-coated collagen (control) generated an encapsulated tissue consisting of immature and mature epithelial cells, reminiscent of intestine, with intervening mesenchymal-like septae (Figure 5.15). Importantly, activin-treated cells gave rise to a heterogeneous teratoma-like cell mass with both mesodermal components as well as tubular structures of neural or epithelial origin (Figure 5.16). Furthermore, follistatin-treated cells generated an encapsulated tissue with epithelial-like morphology, similar to control, and demonstrated cords of cells with intervening mesenchymal septae (Figure 5.16). Both embryoid body and activin cases were not encapsulated and invaded into underlying tissue (4x, Figures 5.15 and 5.16).

5.5 DISCUSSION

Previously, we had shown that an endoderm-like cell population can be induced by culture on fibronectin-coated collagen gel, without the use of activin, complex serum free medium, or serial cell sorting, which were previously thought to be essential for endoderm induction (Kubo, Shinozaki et al. 2004; Yasunaga, Tada et al. 2005). Here, we demonstrate several important findings. We found that primitive epiblast and mesendoderm markers were transiently expressed in both control and follistatin cases (Figures 5.6, 5.7). Since these cell populations are the direct precursors to endoderm, this suggests that definitive endoderm was induced by day 10 of culture on collagen gel. Activation of albumin (foregut), IFABP (midgut) and Cdx2 (hindgut) in subsequent late-stage differentiation (day 24) supports this assertion. In vivo differentiation of the day 10 endoderm-like cells with or without follistatin generated a homogenous population of epithelial cells, with intervening fibrous septae, suggesting that our endoderm-like cells remain committed. To our knowledge, this has yet to be reported in literature. Previously, renal capsule
injection of endoderm-enriched populations resulted in heterogeneous groups of endoderm and mesoderm derivatives in mouse and human ES cell models of endoderm induction (Kubo, Shinozaki et al. 2004; D'Amour, Agulnick et al. 2005). One of the surprising results in our studies is that activin, a known endoderm inducer, caused a decrease in endoderm specific gene and an 80% decrease in protein expression by day 10 of culture. (Figures 5.3, 5.5) Previous work demonstrated that activin-nodal-TGFβ signaling through Smad2/3 is essential for endoderm induction both in vivo and in vitro (Chang, Brown et al. 2002; Okabayashi and Asashima 2003; Kubo, Shinozaki et al. 2004; Yasunaga, Tada et al. 2005). However, significant evidence suggests activin-nodal signaling might inhibit the early stages of ES cell differentiation in vitro (Beattie, Lopez et al. 2005; Vallier, Alexander et al. 2005; Ogawa, Saito et al. 2007). Our results demonstrate that in vivo transplantation of activin-treated cells generate a heterogeneous teratoma-like mass with mesodermal, neural, and epithelial components (Figure 5.16) suggesting that the activin-treated cells were still pluripotent. Gene expression kinetics data (Figures 5.6, 5.7) shows that day 10 activin-treated cells remain positive to major epiblast (Oct4, Fgf5 and Otx2) and mesendoderm markers (Brachyury, Goosecoid, Lhx 1) further strengthening the in vivo results. The activin-treated cells also demonstrated delayed hepatic gene and protein expression as shown in (Figures 5.10, 5.11, 5.12, 5.13). However, activin-treated cells were still competent to generate endoderm in vitro (Figure 5.10) and showed a remarkable proliferative potential in vivo (Figure 5.14). Interestingly, the follistatin-treated population showed a 47% increase in the Foxa2-positive ES cell fraction by day 10, suggesting a role for endogenously secreted activin in ES cell self-renewal (Beattie, Lopez et al. 2005). Alternatively, follistatin might interact with other pathways such as Wnt (Willert, Epping et al. 2002) and BMP (Iemura, Yamamoto et al. 1998) to induce differentiation. Further studies will be needed to determine the role of activin in ES cell differentiation using lineage tracing and proliferation studies.

To summarize, in this work we established a novel approach to induce a homogenous endoderm-like cell population that demonstrates lineage-appropriate gene and protein expression without resorting to cell sorting. Activin, normally an endoderm inducer, caused a dose-dependent decrease in endoderm induction, associated with an increase in its precursor epiblast population. One the other hand follistatin, a known activin inhibitor, increased the Foxa2 positive endoderm fraction to 78.4%. These studies demonstrate a
novel technique to induce the direct differentiation of endoderm from ES cells in vitro without resorting to cell sorting, and a new ability to induce critical, transient precursors which will assist in the development of ES-cell based cellular therapies.

5.6 REFERENCES


Figure 5.1. Effects of activin concentration on morphology of embryonic stem cells in gel culture. Phase contrast images (10x) of day 4 and day 10 ES cells cultured on fibronectin coated collagen gel. Medium was DMEM + 10% FBS.
Figure 5.2. Effects of activin versus follistatin concentration on morphology of embryonic stem cells in gel culture. Phase contrast images (10x) of day 6 ES cells cultured on fibronectin coated collagen gel. Medium was DMEM + 10% FBS. Bar equals 100μm.
Figure 5.3. Effects of activin and follistatin (activin inhibitor) on germ layer gene expression of embryonic stem cells cultured in gel culture. Germ layer gene expression measured by RT-PCR (30 cycles, 5ng RNA) of embryonic stem cells cultured on day 10 in gel culture. Primitive (Oct4, Fgf5), Endoderm (Foa2 and Sox 17), Mesoderm (Foxf1, Runx2, GATA1), Early Mesoderm Patterning (Lefty2, Paraxis) and Neuroectoderm (Ascl1, Pax6, Ngn2) markers were used.
Figure 5.4. Effects of activin and follistatin (activin inhibitor) on endoderm protein expression of embryonic stem cells cultured in gel culture. Immunofluorescence images (10x) of nuclear staining (DAPI) and either Foxa2 (red), or Sox17 (green) staining of day 10 embryonic stem cells cultured in gel culture, treated with Activin or Follistatin.
Figure 5.5. Effects of activin and follistatin (activin inhibitor) on endoderm protein fraction. Flow cytometric analysis of Foxa2 positive cells of gel cultured embryonic stem cells on day 10. Positive fraction determined by threshold as shown.
**Figure 5.6. Kinetics of primitive gene expression in control, activin and follistatin-treated cells.** Embryonic stem cells cultured in gel culture were treated with Activin (20 ng/ml, 50ng/ml) and Follistatin (100ng/ml), and RNA was isolated on days 4, 6, and 10. Gene expression was measured by RT-PCR (30 cycles, 10ng RNA) on days 4, 6, and 10. Markers for inner cell mass/epiblast (Oct4, Fgf5, Otx2) were measured.

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<td>Activin 50 ng/ml</td>
<td>Oct4</td>
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<td>Follistatin 100 ng/ml</td>
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Figure 5.7. Kinetics of mesendoderm gene expression in control, activin, and follistatin-treated cells. Embryonic stem cells cultured in gel culture were treated with Activin (20 ng/ml, 50 ng/ml) and Follistatin (100 ng/ml), and RNA was isolated on days 4, 6, and 10. Gene expression was measured by RT-PCR (30 cycles, 10 ng RNA) on days 4, 6, and 10. Markers for mesendoderm (Brachyury, Goosecoid, Lim 1 Homeobox) were measured.
Figure 5.8. Kinetics of endoderm gene expression in control, activin, and follistatin-treated cells. Embryonic stem cells cultured in gel culture were treated with Activin (20 ng/ml, 50ng/ml) and Follistatin (100ng/ml), and RNA was isolated on days 4, 6, and 10. Gene expression was measured by RT-PCR (30 cycles, 10ng RNA) on days 4, 6, and 10. Markers for endoderm (Foa2, Foa1, Sox17, HNF1β) were measured.
Figure 5.9. Morphologic comparison of late stage differentiation in control, activin, and follistatin-treated conditions. Morphologic comparison of day 10 control, activin or follistatin-treated embryonic stem cells re-cultured on fibronectin-coated collagen gels (Gel culture) in the presence of hepatic medium (C+H, see methods) with BMP2 (10 ng/ml), HGF (20 ng/ml) and Oncostatin 20 ng/ml). Bar = 100µm.
Figure 5.10. Gene expression analysis of late stage differentiation in control, activin, and follistatin-treated conditions. Gene expression was measured by RT-PCR (35 cycles, 10ng RNA) for endoderm (Foxa2 and Sox17, foregut (AFP, Alb, Pdx1, Ins), midgut (Cdx2), and hindgut (IFABP) markers.
Figure 5.11. Immunofluorescence of late stage differentiation in control, activin, and follistatin-treated conditions. Immunofluorescence images (10x) of nuclear staining (DAPI) and either Foxa2 (red), or Albumin (green) staining of day 24 control, activin, or follistatin-treated cells re-cultured in gel culture, in the presence of hepatic differentiation medium. Bar = 100µm.
Figure 5.12. Quantitative RT-PCR gene expression in late stage differentiation in control, activin, and follistatin-treated conditions. Two hepatic differentiation genes, CK 8 and CK 18 genes, were tested. Data for activin and follistatin treated cells was normalized to β-actin, and then normalized to control condition and is expressed as relative gene expression compared to control.
Figure 5.13. Flow cytometric analysis of day 24 differentiated cells. Albumin-positive cells differentiated analyzed by flow cytometry in control, activin, and follistatin conditions. Positive fraction determined by threshold as shown.
Figure 5.14. In vivo differentiation of control, activin, and follistatin-treated cells. Day 10 gel cultured embryonic stem cells were mixed with a low ratio of NIH3T3 fibroblasts and subcutaneously implanted in syngeneic mice, for another 14 days. Conditions were, control (gel culture), activin (50ng/ml), and follistatin (100 ng/ml), and embryoid body control. (a) Mouse with implanted mass for 14 days. (b,c) Encapsulated superficial mass demonstrated after incision. (d) Day 24 tissue masses after resection were embryoid body (1), gel culture (2), follistatin (100 ng/ml) (3), and activin (50 ng/ml) (4). Note that activin-treated cells resulted in the largest mass. Bar = 1 cm.
Figure 5.15. In vivo differentiation of embryoid body and gel culture (control)
H&E (Hematoxlin and Eosin) staining of implanted tissue masses of EB cells (left) and gel culture (right) Bar = 100 μm.
Figure 5.16. In vivo differentiation of activin treated and follistatin treated cells
H&E (Hematoxlin and Eosin) staining of implanted tissue masses of activin (50ng/ml) (left) and follistatin (100ng/ml) (right) Bar = 100 µm.
CHAPTER 6:
COCULTURE APPROACHES FOR ENDODERM INDUCTION AND DIFFERENTIATION

6.1 ABSTRACT

One of the major hurdles of cellular therapies for the treatment of liver failure is the low availability of functional human hepatocytes. While embryonic stem (ES) cells represent a potential cell source for therapy, current methods for differentiation result in mixed cell populations or low yields of the cells of interest. Here we describe a rapid, direct differentiation method that yields a homogeneous population of endoderm-like cells with 95% purity. Mouse ES cells cultured on top of collagen-sandwiched Hepatocytes differentiated and proliferated into a uniform and homogeneous cell population of endoderm-like cells. The endoderm-like cell population was positive for Foxa2, Sox17, and AFP and could be further differentiated into hepatocyte-like cells, demonstrating hepatic morphology, functionality, and gene and protein expression. Incorporating the hepatocyte-like cells into a bioartificial liver device to treat fulminant hepatic failure improved animal survival, thereby underscoring the therapeutic potential of these cells.

6.2 INTRODUCTION

Loss of liver function causes 25,000 deaths/year and is one of the leading causes of death in the United States (Popovic and Kozak 2000). Potential treatments for liver failure include hepatocyte transplantation (Strom, Fisher et al. 1997; Fox, Chowdhury et al. 1998), transplanted tissue engineered liver (Demetriou, Levenson et al. 1986; Fontaine, Schloo et al. 1995), and extracorporeal bioartificial liver (BAL) device (Chan, Berthiaume et al. 2004; Demetriou, Brown et al. 2004). However, the low availability of functional human hepatocytes severely limits these potential therapies. Therefore, the generation of a hepatic progenitor cell, with the ability to proliferate in vitro while retaining liver-specific function, is a major goal of the field. Embryonic stem (ES) cells are considered as a potential reliable source of cells for gene, cell, or tissue engineering therapies because of their capacity for unlimited self-renewal, proliferative potential, and differentiation of major cell lineages (Wobus, 2005, Odorico, 2001).
Several groups have already reported the differentiation of ES cells into hepatocyte-like cells. Chinzei et al. and others have shown that ES cells cultured as embryoid bodies (EBs) differentiate spontaneously into hepatocyte-like cells (Chinzei, Tanaka et al. 2002; Jones, Tosh et al. 2002; Miyashita, Suzuki et al. 2002; Asahina, Fujimori et al. 2004; Novik, Maguire et al. 2006). Efforts to induce a higher rate of differentiation toward the hepatic phenotype have seen limited success. These included various media and matrix combinations (Shirahashi, Wu et al. 2004), essential growth factors (Hamazaki, Iiboshi et al. 2001), compounds such as sodium butyrate (Rambhatla, Chiu et al. 2003), or encapsulation-based systems (Maguire, Novik et al. 2006). To date, the differentiation of ES cells toward the hepatic phenotype has resulted in mixed cell populations and low yields in the range of 10-50%. Adult liver or hematopoietic cells are one potential source for hepatic progenitor cells. Several groups have discovered potential hepatic progenitors. The liver resident oval cells, small binucleated progenitors, have been shown to restore liver functions in vivo (Wang, Foster et al. 2003). Human bone marrow-derived stem cells have been shown to differentiate to hepatocytes in vitro (Jiang, Jahagirdar et al. 2002; Schwartz, Reyes et al. 2002) and reverse liver failure in vivo (Jang, Collector et al. 2004). However, both cell types are present in minute fractions and thus are tedious to isolate and difficult to expand.

One problem with current ES cell based differentiation protocols is that they do not take advantage of cell-cell interactions and they do not enrich the endoderm progenitor, which gives rise to the liver, prior to differentiation. During endoderm induction, the endoderm is directly influenced by visceral endoderm, mesendoderm, and neurectoderm (Wells and Melton 2000). After endoderm is formed, these surrounding germ layers, most notably the mesoderm, continue to secrete soluble signals and are in high proximity to the endoderm (Wells and Melton 2000). Current in vitro studies have began to use coculture as a differentiation scheme; they have demonstrated that endodermal derivatives, like Liver, Pancreas, and Lung can be induced form ES cells by coculture with their mesodermal counterparts, such as Cardiac Mesenchyme (Fair, Cairns et al. 2005). Together these studies show that both mesodermal tissue as well as primary tissue can induce a liver developmental program.

Here we describe a rapid differentiation method to obtain a homogeneous endoderm-like cell population with 95% purity. The direct differentiation was achieved by culturing mouse ES cell on top of a collagen sandwich of primary rat hepatocytes. We show that the presence of adult hepatocytes, but not liver
endothelial cells or fibroblasts, promotes the differentiation and the proliferation of ES cells into a strikingly uniform population of endoderm-like cells, expressing the major endodermal markers Forkhead box protein A2 (Foa2, formerly HNF3β), SRY-box containing gene 17 (Sox17), and alpha-fetoprotein (AFP). When these ES cell (ESC)-derived endoderm-like cells were replated on a feeder layer of 3T3-J2 fibroblasts, further proliferation and differentiation along the hepatocyte lineage was observed, demonstrating hepatic morphology, functionality, and gene and protein expression. Furthermore, by seeding a bioartificial liver (BAL) device with these ES-derived hepatocyte-like cells we demonstrate an increased survival of rats following D-galactosamine (GalN)-induced fulminant hepatic failure (FHF). This culture system will be useful as in vitro model to induce and direct ES cells into homogeneous early endodermal cell populations of high yield and high purity for application in cellular therapies.

6.3 METHODS

6.3.1 Cell cultures

Hepatocytes were isolated from adult female Lewis rats (Charles River Laboratories, Boston, MA) weighing 150-200g, using a modified two-step collagenase perfusion procedure (Seglen 1976) as described previously (Dunn, Yarmush et al. 1989). Primary rat hepatocytes were sandwiched between two layers of collagen to maintain their stable liver specific functions (Dunn, Yarmush et al. 1989; Dunn, Tompkins et al. 1992; Berthiaume, Moghe et al. 1996). Tissue culture dishes (35-mm) were coated with 0.5 mL of a mixed solution of nine parts of type I rat tail collagen (1.1 mg/mL in 1 mM HCl) and 1 part 10 x DMEM, and incubated for 1 h at 37°C to form a collagen gel. After gelation, one million hepatocytes (12.5 × 10^3 cells/cm^2) in 1 mL hepatocyte culture medium were seeded, and incubated in 90% air/10% CO₂ at 37°C. To achieve uniform densities, the substrates were shaken every 15 min for the first hour after cell seeding. The following day, the culture medium was removed and a second collagen gel layer was overlaid on the hepatocytes and incubated for 1 h at 37°C. After gelation, 1 mL of hepatocyte culture medium were seeded, and incubated in 90% air/10% CO₂ at 37°C. Culture medium was changed daily. Hepatocyte culture medium consisted of DMEM supplemented with 10% fetal bovine serum (Gibco, Gaithersburgh, MD), 7 ng/mL glucagon (Bedford Laboratories, Bedford, OH), 7.5 μg/mL hydrocortisone (Pharmacia Corporation, Kalamazoo, MI), 0.5 U/mL insulin (Eli Lilly,
Indianapolis, IN), 20 ng/mL epidermal growth factor (Sigma Chemical Co., St. Louis, MO), 200 U/mL penicillin, and 200 μg/mL streptomycin (Gibco).

The murine ES cell line D3 (ATCC, Manassas, VA) was used to assess the differentiation of ES cells into endodermal and hepatic lineage cells. The murine Oct4-GFP ES cell line R1 (provided by Dr. A. Nagy, Mount Sinai Hospital, Toronto, Ontario, Canada) was used to monitor the early differentiation of ES cells. Undifferentiated ES cells were cultured in Knockout DMEM (Gibco), supplemented with 15% replacement serum, 4 mM L-glutamine (Cambrex, Walkersville, MD), 100 U/mL penicillin (Gibco), 100 U/mL streptomycin (Gibco), 10 μg/mL gentamycin (Gibco), 1000 units/mL Leukemia Inhibitory Factor (LIF; Chemicon International, Temecula, CA), and 0.1mM 2-mercaptoethanol (Gibco) on gelatin-coated T75 tissue culture flasks. Culture medium was replaced every day and cells were passaged at dilutions ranging from 1:5 to 1:20 at least once a week. Cells from passage numbers 18-25 for ES-D3 and 26-29 for Oct4-GFP ES cells were used in the experiments. Cells were cultured at 37°C and in a 95% air/5% CO₂ atmosphere. Unless otherwise noted, ES cells were seeded at a density of 6.25 x 10³ cells/cm² and cultured as monolayers in hepatocyte culture medium which was changed daily. ES cells were seeded either on top of collagen sandwiched hepatocytes, liver sinusoidal endothelial cells, or murine embryonic fibroblasts. Controls were established by seeding ES cells on top of a single collagen gel.

Murine 3T3-J2 fibroblasts (purchased from Howard Green, Harvard Medical School, Boston, MA) and embryonic fibroblasts (ATCC) were maintained in T175 tissue culture flasks in DMEM (Gibco) plus 10% FBS and 2% penicillin and streptomycin. For coculture with ESC-derived endodermal cells, 3T3-J2 fibroblasts were growth arrested by a 12 μg/mL Mitomycin-C treatment (Sigma Chemical Co.) for 2.5 hours prior to seeding. Liver sinusoidal endothelial cells were isolated from other nonparenchymal cells using a two-step Percoll gradient separation, following the procedure of Zhang et al (Zhang, Borderie et al. 1997). The cells were cultured in hepatocyte culture medium containing 10 ng/mL VEGF (R & D Systems, Minneapolis, MN).

### 6.3.2 Embryonic Stem Cell Culture/Fibroblast Culture

The murine ES cell line D3 (ATCC, Manassas, VA, USA) was used to assess the differentiation of ES cells into endodermal and hepatic lineage cells. The murine Oct4-GFP ES cell line R1 (provided by Dr.
Andras Nagy, Mount Sinai Hospital, Toronto, ON, Canada) was used to monitor the early differentiation of ES cells. Undifferentiated ES cells were cultured in Knockout DMEM (Life Technologies, Inc.), supplemented with 15% replacement serum, 4 mM L-glutamine (Cambrex, Walkersville, MD, USA), 100 U/mL penicillin (Life Technologies, Inc.), 100 U/mL streptomycin (Life Technologies, Inc.), 10 μg/mL gentamicin (Life Technologies, Inc.), 1000 U/mL Leukemia Inhibitory Factor (LIF; Chemicon International, Temecula, CA, USA), and 0.1 mM 2-mercaptoethanol (Life Technologies, Inc.) on gelatin-coated T75 tissue culture flasks. Culture medium was replaced every day, and cells were passaged at dilutions that ranged from 1:5 to 1:20 at least once a week. Cells from passage numbers 18–25 for ES-D3 and 26–29 for Oct4-GFP ES cells were used in the experiments. Cells were cultured at 37°C and in a 95% air/5% CO₂ atmosphere. Unless otherwise noted, ES cells were seeded at a density of 6.25 x 10³ cells/cm² and cultured as monolayers in hepatocyte culture medium, which was changed daily. ES cells were seeded either on top of collagen-sandwiched hepatocytes, liver sinusoidal endothelial cells, or murine embryonic fibroblasts. Controls were established by seeding ES cells on top of a single collagen gel. Murine 3T3-J2 fibroblasts (purchased from Howard Green, Harvard Medical School, Boston, MA, USA) and embryonic fibroblasts (ATCC) were maintained in T175 tissue culture flasks in DMEM (Life Technologies, Inc.) plus 10% FBS and 2% penicillin and streptomycin. For coculture with ES-derived endodermal cells, 3T3-J2 fibroblasts were growth arrested by a 12 μg/mL mitomycin-C treatment (Sigma) for 2.5 h prior to seeding. Liver sinusoidal endothelial cells were isolated from other nonparenchymal cells using a two-step Percoll gradient separation, following the procedure of Zhang et al. The cells were cultured in hepatocyte culture medium containing 10 ng/mL VEGF (R & D Systems, Minneapolis, MN, USA).

6.3.3 Isolation and culture of differentiated Embryonic Stem cells

To separate the ES cell-derived cells from the hepatocytes in coculture, the cells were treated on day 10 with 1 mg/mL dispase (Life Technologies, Inc.) for 60 min at 37°C. After the dispase treatment, the ES cell-derived cells could be detached by gentle pipetting without disturbing the collagen gel. The collagen-sandwiched hepatocytes remained at the bottom of the dish, thus allowing separation of the ES cell-derived cells from the primary hepatocytes. The ES cell-derived cells were then treated with trypsin to achieve single-cell suspension. The purified ES cell-derived cells were either analyzed for gene and protein
expression or were replated in 35-mm culture dishes at a density of 50,000 cells per dish (6.25x10^3 ES cells/cm^2). For further differentiation, isolated ES cell-derived cells were plated either on collagen gels or growth-arrested 3T3-J2 fibroblast feeder layers and were cultured in hepatocyte culture medium supplemented with 100 ng/mL oncostatin-M (Sigma), 10^{-7} M dexamethasone (Sigma) and insulin/transferrin/selenious acid (5 ug/mL, 5 ug/mL, 5 ng/mL, respectively) (BD Biosciences, San Jose, CA, USA). To rule out the possibility of cell fusion occurring during the Coculture process, we performed a simple genotype analysis of the cocultured ES cells on day 10. Since the rat hepatocytes were of female origin and the ES cells were male, we used a polymerase chain reaction (PCR)-based gender identification approach published previously to analyze the DNA of the cocultured population. This technique uses primers for conserved zinc finger regions of the X and Y chromosomes of the DNA of interest. We reasoned that if appreciable cell fusion had occurred, the XY chromosome analysis of cocultured ES cells would appear similar to the male XY genotype for undifferentiated mouse ES cells rather than the XX profile for female rat hepatocytes.

6.3.4 Experimental animals

Male Sprague-Dawley rats (Charles River Laboratories, Boston, MA, USA), weighing 250–350 g, were used for this study. All animals were acclimated to the animal research laboratory for 5 days prior to experiments and were maintained in a light-controlled room (12-h light–dark cycle) at an ambient temperature of 25°C with chow diet and water ad libitum. These rats were maintained in accordance with National Research Council guidelines, and the experimental protocols were approved by the Subcommittee on Animal Care, Committee on Research, MA General Hospital.

6.3.5 Surgical Procedures and Induction of Fulminant Hepatic Failure (FGF)

Surgical procedures and induction of FHF is described in detail elsewhere (Shito, Tilles et al. 2003). Briefly, male Sprague-Dawley rats (250-350 g) were anesthetized with an intraperitoneal (i.p.) injection of ketamine (Abbott Laboratories, N. Chicago, IL) and xylazine (Phoenix Pharmaceuticals, St. Joseph, MO) at 110 and 0.4 mg/kg, respectively. The carotid artery and jugular vein were cannulated with 40 cm lengths of PE 50 polyethylene tubing (Becton Dickinson, Sparks, MD) through a dorsal incision. The wound was
sutured and the animal was transferred into a cage, and was fasted until the first D-galactosamine (GalN; Sigma, St Louis, MO) injection. To prevent blood clotting, heparinized (20 U/mL) saline solution was continuously infused at a rate of 0.2 mL/h through the arterial line by a syringe infusion pump (Fisher Scientific, Pittsburg, PA) until the extracorporeal perfusion experiments were initiated. GalN was freshly dissolved in 0.5 mL of physiological saline and adjusted to pH 6.8 with 1N NaOH. The first dose of GalN (1.2 g/kg i.p.) was administered 24 hours after cannulation, and a second dose was given 12 hours later. After the first injection, the rats had free access to food and water until sacrifice.

6.3.6 Bioartificial liver (BAL) Device and Cell Seeding

The flat-plate BAL (bioartificial liver) device consisted of two plates fabricated of polycarbonate as described previously (Shito, Tilles et al. 2003). The glass surface comprising the lower plate of the BAL was coated with 0.2 mg/mL rat tail collagen and incubated at 37°C for 1 hour. ESC-derived hepatocyte-like cells (d25-d28) or fibroblasts (3T3-J2) were seeded onto the glass surface at an average density of 20 million cells per seeding. A second seeding of the hepatocyte-like cells was performed after a 1-hour incubation period. The cultures were maintained in hepatocyte culture medium supplemented with oncostatin-M, dexamethasone, and insulin/transferrin/selenious acid as noted above for two days. On day 3 after seeding, the medium was aspirated from the lower plate and the BAL was assembled. The BAL device and perfusion circuit were primed with 6 mL of sterile, heparinized Sprague-Dawley rat plasma (Rockland, Gilbertsville, PA).

6.3.7 Extracorporeal Perfusion System

Arterial blood was pumped at 0.55-0.85 mL/min through #13 Masterflex silicone tubing (Cole-Parmer, Vernon Hills, IL) using a digital peristaltic pump (Cole-Parmer). A plasma separator (mixed cellulose esters MicroKros, 0.2 µm pore size, 16 cm² surface area; Spectrum Labs, Laguna Hills, CA) was placed after the pump as an interface between the animal blood line and the BAL device line. Separated plasma was pumped through the BAL by means of two peristaltic pumps set at a flow rate of 0.1 mL/min. Separated plasma and blood were reunited before entering a bubble trap, and the reconstituted blood returned to the animal through the venous cannula. During perfusion, heparin (41.5 U/mL) with 5%
Dextrose solution was administered continuously through the venous line at a rate of 0.2 mL/hour via a syringe infusion pump. The dead volume of the entire perfusion system was 12 mL, of which 6 mL was accounted for by the BAL device. Oxygenated gas (21% O₂, 5% CO₂, 74% N₂) flow was established through a chamber above the internal gas permeable membrane of the BAL device.

6.3.8 Biochemical analysis of liver damage

Blood metabolites were measured using the Piccolo-Portable Blood Analyzer (Abaxis, Union City, CA). A blood sample of 100 µL volume was collected from the BAL perfusate following the 10 hour extracorporeal treatment and used to measure liver enzymes in the Piccolo cartridge.

6.3.9 Immunofluorescence/ Flow Cytometry analysis

For immunostaining, cultures were washed twice with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde in PBS at room temperature for 30 min. The fixed sample was then washed twice in PBS, permeabilized using 0.2% Triton X-100 in PBS for 10 min at room temperature. The permeabilized cells were then incubated in blocking buffer (PBS/3% BSA/5% donkey serum) for 60 min at room temperature to block nonspecific antibody binding. Following incubation, the cells were stained for 2 h at room temperature with the following primary antibodies: rabbit anti-Foxa2 (R&D Systems, Minneapolis, MN, USA), goat anti-AFP (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit or goat antialbumin (ICN Pharmaceuticals, Aurora, OH, USA), mouse anti-CK-18 (Sigma), or isotype-matched antibodies as controls (Santa Cruz Biotechnology). After washing twice in blocking solution, the cells were incubated with the following secondary antibodies: FITC or Cy3-conjugated rabbit IgG, goat IgG, or mouse IgG (ICN Pharmaceuticals), for 60 min at room temperature. For bromodeoxyuridine (BrdU) staining, the cells were incubated with 10 uM BrdU (Sigma) in the culture medium for 24 h at 37°C and fixed in 70% ethanol for 45 min at room temperature, then treated with 4N HCl for 20 min at room temperature to denature DNA. After incubation in blocking buffer for 30 min, the cells were stained for 60 min at 37°C with anti-BrdUAlex594 (Invitrogen, Carlsbad, CA, USA). In some cases, cells were counterstained with 4,6-diamidino-2-phenylindole (DAPI; Invitrogen) for nuclear staining. Cells were visualized...
by fluorescence microscopy (Zeiss, Thornwood, NY, USA). FACS analysis Cocultured ES-D3 cells were isolated with dispase and trypsin treatment on day 10. Collected cell suspensions were fixed, permeabilized, and stained with antibodies as described above. The primary and secondary antibody concentrations used were the following: rabbit anti-Foxa2, 1:1000 (R&D Systems), goat anti-AFP, 1:1000 (Santa Cruz), donkey antirabbit IgG-FITC, 1:1000 (ICN Pharmaceuticals), and donkey anti-goat IgG-FITC, 1:1000 (ICN Pharmaceuticals). Cell suspensions were analyzed by flow cytometry (Becton Dickinson). For each analysis, 10,000 events were recorded. FACScan data were analyzed using CellQuest software (Becton Dickinson). For flow cytometric analysis of Oct4-GFP ES-R1 cells, cultured ES cells were isolated with dispase and trypsin treatment on day 5 of differentiation. Collected cell suspensions were analyzed by flow cytometry for Oct4-GFP expression. Undifferentiated Oct4-GFP ES-R1 cells were used as a positive control.

6.3.10 Functional analysis
The culture medium samples were collected and stored at -20°C for analysis for urea content. Urea content was determined with diacetylmonoxime with a commercially available kit (StanBio Laboratory, Boerne, TX). The absorbance was measured with a Thermomax microplate reader (Molecular Devices, Sunnyvale, CA).

6.3.11 Quantitative image analysis
To quantify the growth of differentiating ES cells, the surface area of cultured ES cells was measured and quantified by Sigmascan Pro image software. Measured surface areas were normalized to the control condition. The average fluorescence intensity of Oct4-GFP expression in each condition was also quantified by image analysis and compared to the control. Three to four random fields of image per sample were acquired and quantified by image analysis. Two independent experiments in duplicate were performed and the data were represented as an average with the standard deviation.

6.3.12 RNA isolation and RT-PCR analysis
RNA isolation was carried out using the Nucleospin RNAII protocol (Clontech, Mountain View, CA). Following the removal of genomic DNA with DNase, the column was washed and RNA eluted with distilled water. The RNA purity was quantified using the absorbance ratio at 260 nm (Nucleic Acids) and 280 nm (Protein), and was greater than 1.9. One-Step RT-PCR kit (Qiagen, Valencia, CA) was used to analyze gene expression. Reactions were initiated using 10 ng RNA, and 0.6 μM primer. RT reaction was conducted at 50 °C and 95 °C for 15 min. Three-step cycling was performed: Denaturation at 94 °C for 30 seconds, Annealing at 55 °C for 30 seconds, and Extension at 72 °C for 1 min, with a final extension time of 10 minutes at 72°C. The number of cycles varied between 25 and 35. Following PCR, samples were run on a 2% agarose gel and imaged using the Fluor-X Multiimager (Biorad, Hercules, CA). PCR amplification of markers was performed using the following oligonucleotide primers:

- Foxa2 (HNF3β) (5' ACACGCCAAACCTCCCTAC 3': 5' GGCACCTTGAGAAAGCA 3'),
- AFP (5' AACTCTGGCGATGGGTGTT 3': 5' AAACTGGAAGGGTGAGAC 3'),
- Sox 17 (5' ATCCACCAGCCCACTGA 3': 5' TCGGCAACGTCAAATG 3'),
- Albumin (5' CCCTGTGCTGACTGAGTACG 3': 5' TGGAGGTACTTTCTGGTGT 3'),
- GATA1 (5' CACCATCAGGTCCACAGG 3': 5' TTGAGGCAGGATGAGTGC 3'),
- Runx2 (5' TTCCAGACCAGGCACTGAC 3': 5' GCCGCCAACAGACTCAT 3'),
- Foxf1 (5' CGTGTGTGATGAGTGTGG 3': 5' CTCCGTGGCTTTCA 3'),
- Nkx2.5 (5' CGTCCAAACCACACAAAC 3': 5' TTGCTCTCCGCTGAGTCC 3'),
- Tal1 (5' TTGATCCATCCAGCTTG 3': 5' CACAGCCAACAAAGCAT 3'),
- Pax 6 (5' TGCCCTTCCATCTTGT 3': 5' CCAAGGAAGGCTGGAA 3'),
- β-actin (5' GAGGGAAATCTGCTGTA 3': 5' CCAAGGAAGGCTGGAA 3')

### 6.3.12 Statistical analysis

Data are expressed as the mean ± standard deviation (SD). Statistical significance of quantitative image analysis was determined by a two-tailed Student’s t-test (* P < 0.001). Animal survival data was evaluated using the generalized Wilcoxon’s test and a P value of less than 0.05 was considered statistically
significant. Statistical differences of the biochemical analysis were determined by a two-tailed Student’s t-test (* P < 0.05).

**6.4 RESULTS**

**6.4.1 Adult hepatocytes stimulate ES cell differentiation and proliferation in cocultures**

Primary rat hepatocytes cultured in a collagen-sandwich configuration (Dunn, Yarmush et al. 1989; Dunn, Tompkins et al. 1992) have been shown to maintain a high level of liver specific function and to secrete numerous proteins. To determine the ability of collagen-sandwiched rat hepatocytes to induce ES cell differentiation, the cells were seeded on top of the collagen sandwich. The 400 µm thick collagen layer did not permit direct hepatocyte-ES cell contact.

Octamer-4 (Oct-4) is an important marker of pluripotency, expressed by primitive embryonic cells both in vivo and in vitro (Niwa, Miyazaki et al. 2000). Oct-4 is downregulated during early differentiation. To monitor early differentiation in our system, we studied the ES-R1 mouse ES cell line in which green fluorescent protein (GFP) was knocked in to monitor Oct-4 gene expression. To assess the effect of coculture on ES cell differentiation, we monitored Oct-4 gene expression following 5 days of Coculture with either rat liver parenchymal cells (adult rat hepatocytes), rat liver sinusoidal endothelial cells (LSEC), or mouse embryonic fibroblasts (MEF). Using flow cytometry analysis, we observed that Oct4-GFP expression of ES cells cultured on top of collagen sandwiched hepatocytes was decreased significantly compared with the other three culture conditions on day 5 of culture (Figure 6.1). ES cells cultured on top of collagen-sandwiched embryonic fibroblasts or liver endothelial cells (data not shown) maintained strong Oct4-GFP expression, forming Oct4-GFP positive aggregates. Similar results were observed in direct cocultures of Oct4-GFP ES cells on feeder layers of fibroblasts, endothelial cells, and hepatocytes in the absence of collagen gel layer (data not shown). These results indicate that primary rat hepatocytes, but not endothelial cells or fibroblasts, stimulate the differentiation of ES cells in collagen culture.

One possible route by which coculture can induce ES cell differentiation is by altering the growth kinetics of the ES cells. When cocultured with hepatocytes the ES cells underwent rapid proliferation without forming Oct4-GFP positive aggregates (Figures 6.1 and 6.2). One possibility is that a hepatocyte-secreted soluble factor is responsible for the increased proliferation. To evaluate the effect of hepatocytes
on the proliferation of ES cells (ES-D3), we seeded a constant number of ES cells (6.25x10^3 cells/cm^2) on collagen-sandwiched hepatocytes seeded at low (12.5x10^3), medium (62.5x10^3), and high (125x10^3 hepatocytes/cm^2) seeding densities. A hepatocyte “dose-dependent” increase in the area covered by ES cell-derived cells was observed on day 6 of culture (Figure 6.3). We verified that the area per individual ES cell-derived cell was constant (165 ± 28 µm^2 in area), confirming that the increasing coverage correlates directly with an increase in cell number. Similar to the Oct4-GFP ES cells (derived from ES-R1 cells), ES-D3 cells cultured on top of collagen-sandwiched hepatocytes showed a significantly higher proliferation rate when cultured with hepatocytes at medium and high densities compared to those in single culture (P<0.00001). No significant differences were observed between ES cells in single culture and ES cells cocultured with hepatocytes at low density (P=0.519). The data show that ES cell proliferation increased as a function of hepatocyte density. These results indicate that primary rat hepatocytes stimulate the proliferation of ES cells in collagen culture.

6.4.2 ES cells cocultured with adult hepatocytes differentiate into a homogeneous population of endoderm-like cells

To study the differentiation of ES cells in our coculture system we analyzed gene and protein expression on days 6 and 10 of coculture. Figure 6.4 shows the morphology of differentiating ES cells on culture day 6 when cultured alone (control) or when cocultured with hepatocytes. Using RT-PCR we observed that the ES cells cocultured with hepatocytes clearly expressed higher levels of the endoderm markers Foxa2, Sox17, and AFP on day 6 than the ES cells in single culture (Figure 6.5). The mesendodermal markers Brachyury and Goosecoid (Kubo et al. 2004) were upregulated on day 4 of differentiation and downregulated rapidly on days 6 and 10, indicating a transient mesendodermal population (Figure 6.5). Further characterization of the ES cell-derived cells on day 10 by immunostaining demonstrated high level of Foxa2 and AFP protein expression (Figure 6.6). In addition, flow cytometry analysis of the ES cell-derived population demonstrated that 94.2 ± 0.3% of the cells were Foxa2-positive, and 95.9 ± 0.8% of the cells were AFP-positive, demonstrating the formation of a homogenous endoderm-like population (Figure 6.7). Since ectoderm, endoderm, and mesoderm may all be present in early ES cell differentiation schemes, we determined whether key markers for these cell types were expressed in our culture conditions. We chose
a combination of early transient markers and markers for ectoderm, endoderm, and mesoderm. Using RT-PCR, we examined the expression of the liver transcription factors Foxa2 (required for endoderm specification (Ang, Wierda et al. 1993; Nagy, Bisgaard et al. 1994)) and Sox 17 (an endodermal transcription factor), alpha alphafetoprotein (AFP, an endoderm and early Hepatocyte marker), albumin (a hepatocyte marker), GATA1 (hematopoietic cells), Runx2 (mesenchymal cells), Foxf1 (mesenchymal cells), Nkx2.5 (cardiac cells), Tal1 (endothelial cells), and Pax6 (an ectoderm marker) of the ES cells cultured on top of collagen-sandwiched adult hepatocytes for 6 and 10 days. We found that the differentiating ES cells cultured on top of collagen-sandwiched adult hepatocytes expressed Foxa2, Sox17, and AFP on days 6 and 10, but not albumin, suggesting that the cells differentiated into early endodermal cells (Figure 6.8). The mesodermal and ectodermal markers were not expressed on culture days 6 and 10, suggesting that the cells in this culture condition were endoderm progenitors or derived from endoderm.

To rule out the possibility of cell fusion between the differentiating mouse ES cells and the female rat hepatocytes, we performed a simple genotype analysis using gender determination of the cells. Our analysis demonstrated that the undifferentiated ES cells (day 0) and the cocultured ES cells after 10 days of differentiation had double bands (XY genotype) with identical magnitude indicating that they were from male animals (data not shown). Rat hepatocytes used for ES cell coculture displayed a single band indicating that the animal was female. These results show that the differentiating ES cells in coculture were not fusing with rat hepatocytes.

6.4.3 ESC-derived endoderm-like cells can differentiate into hepatic lineage cells

During development, endoderm-derived hepatic precursors associate with mesenchymal cells of the septum transversum before fully maturing into functional hepatocytes. Therefore, in order to differentiate the ES cell-derived endoderm-like cells, we reseeded the cells on top of growth arrested fibroblast (3T3-J2) feeder layers at a density of $6.25 \times 10^3$ ES cells/cm$^2$ (Fig. 1, Step 2). Figure 6.9 shows a series of phase contrast images of the proliferating and differentiating ES cell-derived endoderm-like cells. Following 28 days in culture, the cells formed epithelial-type clusters and appear as a uniform population with morphology that is very similar to that of adult hepatocytes. Some binucleated cells were observed in the colony clusters at late stage of culture (see inset). Although similar morphology was seen when the cells were cultured alone
on collagen gel, cellular proliferation was significantly retarded (data not shown). To further characterize the phenotype of these ES cell-derived hepatocyte-like cells, we studied gene and protein expression using immunofluorescence and RTPCR. The ES cell-derived hepatocyte-like cells were positive for the mature hepatic markers, albumin and CK-18, on the protein level (day 28) (Figure 6.10). Hepatocyte gene expression of the ES cell-derived hepatocyte-like cells was upregulated on days 18 and 28 (Figure 6.10).

The ES cell-derived hepatocyte-like cells were positive for Hepatocyte markers, including albumin, alpha-1-antitrypsin (AAT), CK-8, CK-18, transthyretin (TTR), and cytochrome P450 3A13 (CYP3A13). Foxa2 was weakly expressed on days 18 and 28, similar to gene expression of mature hepatocytes. However, AFP, an endodermal and early hepatic marker, was upregulated on day 28, suggesting that some of the cells may still be in early stages of hepatic lineage. In addition, the high level of AFP expression on day 28 might be due to the proliferation of hepatic progenitors. No expression of mesodermal or ectodermal markers was detected in any of these cultures (Figure 6.10). Immunofluorescence of the cocultured ES cells demonstrated positive staining for albumin and CK 18 (6.11) We also investigated urea synthetis of differentiating ES cell-derived cells to assess hepatocyte-specific function. The activity of urea synthesis on day 18 was 6.1 µg/48 h/35 mm dish (Figure 6.12). The level of urea production increased to 17.9 µg/48 h/35 mm dish in late stage of culture (day 28) (Figure 6.12). These results indicate that the ES cell-derived endoderm-like cell population could be further differentiated to hepatocyte-like cells.

6.4.4 Biochemical analysis and animal survival from liver failure

To evaluate the therapeutic efficacy of the ES cell-derived hepatocyte-like cells, we attempted to rescue rats undergoing GalN-induced FHF by incorporating the hepatocyte-like cells in a BAL device and treating for 10 h. We measured changes in liver enzymes [aspartate transaminase (AST), alanine transaminase (ALT)], total bilirubin (TBIL), and blood urea nitrogen (BUN)] immediately following the 10 h extracorporeal perfusion. Significant reductions were found in the plasma levels of TBIL and BUN ($P<0.025$, $P<0.009$, respectively), and trends were noted for decreased AST and ALT levels ($P<0.149$, $P<0.057$, respectively) in the animals treated with the BAL device containing ES cell derived hepatocytes compared to the control group (Figure 6.13). Animal survival was evaluated up to 120 h (5 days) after the first GalN injection (Figure 6.14). In the animal group treated with the BAL device seeded with the
hepatocyte-like cells, 5 out of 6 animals (83.3%) survived at 48 h compared to 3 out of 8 (37.5%) rats that were treated with a BAL device seeded with fibroblasts (control group). On day 5, 3 out of 6 (50.0%) rats survived in the ES cell-derived hepatocyte seeded BAL group compared to 1 out of 8 (12.5%) rats in the control group. The differences between the two groups were significant on day 5 (P<0.034) using the generalized Wilcoxon’s test. Animal survival from the ES cell derived cell seeded BAL group was also compared to our previously published results in which rats with GalN-induced FHF receiving treatment with a BAL device containing primary porcine hepatocytes had 58.3% survival on day 5 (Shito, Tilles et al. 2003). Similar results in animal survival were observed in both groups through day 5.

6.5 DISCUSSION

The results of our studies indicate that the culture of mouse ES cell on top of collagen-sandwiched primary rat hepatocytes stimulates their differentiation and proliferation into a homogeneous population of endoderm-like cells. The endoderm-like cell population which emerged within the first week of culture had cubical cell morphology, bright cell borders, and maintained a high level of gene and protein expression for the endoderm specific genes Foxa2, Sox17, and AFP (Zaret 1999; Zaret 2002). This cell population was also negative for major mesoderm and ectoderm markers suggesting our system induced an endoderm-specific differentiation. This endoderm-like cell population could be further differentiated to hepatocyte-like cells, by subculturing the cells on a feeder layer of 3T3-J2 fibroblasts. The hepatocyte-like cell population that emerged had a hepatic morphology, expressed albumin on both the gene and protein level, stained positive for CK-18 filaments, and secreted urea. To our knowledge, this is the first demonstration of ES cell differentiation into functional hepatocyte-like cells with a yield approaching 95%. In addition, these hepatocyte-like cells were shown to be efficacious in treating a rat model of GalN-induced FHF when seeded in an extracorporeal BAL device.

During development, the first sign of liver morphogenesis is a thickening of the ventral endoderm, which occurs around embryonic day 8 in the mouse (Zaret 2001). Little is known about the signals involved in initial endoderm formation and subsequent endoderm specification, but recent studies suggest a role for FGF, BMP and activin. FGF, both acidic and basic, produced by the cardiac mesoderm was shown to induce the foregut endoderm to the hepatic lineage (Zaret 2001), while BMP produced by the
transversum mesenchyme was shown to increase levels of GATA4 (Wells and Melton 1999). Activin was also shown to participate in the early induction of endoderm through smad2 signaling (Ball and Risbridger 2001). By embryonic day 8.5 in the mouse, definitive endoderm has formed the gut tube and expresses Foxa2. As liver morphogenesis progresses, Foxa2 positive cells proliferate to form the hepatic diverticulum while expressing AFP. Final maturation of these hepatic progenitors occurs when hepatic cords associate with the mesenchymal cells of the septum transversum, forming the liver sinusoids while expressing albumin and urea (Zaret 2001).

A similar path of differentiation occurs during culture of embryoid bodies (EB), which are the most common method of ES cell differentiation (Hamazaki, Iiboshi et al. 2001). Normally the early cell populations that arise during initial ES cell differentiation consist of neuroectoderm, mesoderm, definitive endoderm, and extraembryonic endoderm. The biopotential mesendoderm may also be present (Kubo, Shinozaki et al. 2004; Tada, Era et al. 2005). Although this mixed cell population does give rise to hepatocyte-like cells, usually in close proximity to cardiac-like tissue, the yield ranges from 5 to 20% make it essential to purify the cell population to further explore their potential. In an effort to create a homogenous cell population, several groups have studied endoderm differentiation in monolayer culture thereby exposing the ES cells to uniform cues from their microenvironment. Tada et al. has shown the presence of mesendodermal precursors in these cultures as well as the importance of collagen type IV to induce endoderm with a yield of up to 50%. Both this group and Hisatomi et al. have shown a characteristic epithelial morphology of Foxa2 positive endodermal cells in culture (Hisatomi, Okumura et al. 2004; Tada, Era et al. 2005). In addition to in vitro culture of ES cells, several groups have shown that liver cells and regenerating livers can induce hepatocyte-like gene expression in stem cells. Lange et al. and Jang et al. have shown that liver cells can induce hepatic gene expression in cultures of mesenchymal and hematopoietic stem cells (Jang, Collector et al. 2004; Lange, Bassler et al. 2005). Similarly, Imamura et al. has shown that the regenerative liver can drive ES cell differentiation into a hepatic phenotype (Imamura, Cui et al. 2004).

Several groups have shown the importance of collagen in the differentiation of ES cells toward the hepatic phenotype. However, the differentiation of ES cells on three-dimensional collagen gels has not been previously investigated. The ability of three-dimensional collagen gels to induce and maintain
epithelial morphology and function is well established suggesting a similar enhancement of epithelial differentiation might occur during ES culture. Therefore, to create an environment conducive for hepatic differentiation we cultured ES cells on top of collagen-sandwiched hepatocytes. The uniform microenvironment, three-dimensional collagen gel, and cues from isolated hepatocyte contributed for the formation of a homogenous cell population which was 94.2% positive for Foxa2 and 95.9% positive for AFP.

Our group has previously shown that primary hepatocyte trapped between two layers of collagen gel (sandwich) maintain a high level of liver specific function (Dunn, Yarmush et al. 1989; Dunn, Tompkins et al. 1992). One explanation for the uniform differentiation observed in our coculture is activin secretion. Hepatocytes have been shown to secrete activin and other TGF-β family mitogens (Yasuda, Mine et al. 1993), which has been shown in numerous studies to promote endoderm induction (Kubo, Shinozaki et al. 2004; D'Amour, Agulnick et al. 2005). Our results demonstrate that ES cells cocultured with hepatocytes rapidly lose the Oct-4 gene which is important for the maintenance of pluripotency. On the other hand, coculture with isolated liver endothelial cells has failed to induce ES cell differentiation. This result is in agreement with in vivo studies suggesting that endothelial cells are important for the later stage of differentiation but not for the initial induction. Similarly, embryonic fibroblasts failed to cause a decrease in Oct-4 expression in ES cells. Mouse embryonic fibroblast feeders have been used to support the maintenance of undifferentiated ES cells. Conditioned media from the fibroblasts was also known to support the ES cell growth in undifferentiated state (Xu, Inokuma et al. 2001). Similar results were observed in this study.

In addition to differentiation, hepatocytes have also been shown to enhance the proliferation of various cell types (Gressner, Brenzel et al. 1993; Skrtic, Wallenius et al. 2000; Endo, Kogure et al. 2004). In a similar fashion, our results demonstrate that hepatocytes stimulate ES proliferation in a dose-dependent fashion due to secreted factors. Similar increase in ES cell proliferation was also seen when the cells were separated by transwells although the proliferation rate was decreased (data not shown). In both cases of differentiation or proliferation in coculture the ES cells were separated more than 400 µm from the hepatocytes and were never in contact, therefore cell fusion or cell-cell contacts could be ruled out.
The endoderm-like population that emerged in our studies had the characteristic epithelial morphology of Foxa2 positive endodermal cells which was previously demonstrated (Hisatomi, Okumura et al. 2004; Tada, Era et al. 2005). Spindle-like, elongated, or elliptical cells were not observed, along with the gene expression data demonstrating endodermal but not mesodermal (Gata-1, Runx2, Foxf1, Nkx2.5, Tal1) or neuroectodermal (Pax6) marker, suggests that the enhanced proliferation and differentiation observed in our system was lineage-restricted to endoderm. The gene expression panel and cellular morphology were similar on day 6 and day 10 of coculture, suggesting that the proliferation was symmetric, and not asymmetric. This, however, needs to be carefully studied using single cell lineage tracing and mapping techniques.

Previous studies by Hamazaki et al. demonstrated the importance of FGF, HGF, oncostatin-M, and cortisone on the differentiation of ES cells into hepatocyte-like cells (Hamazaki, Iiboshi et al. 2001). These factors are commonly secreted by the cardiac mesoderm and the mesenchymal cells. In order to induce the late stage hepatic differentiation we chose to culture the endoderm-like cells on growth arrested 3T3-J2 fibroblasts. Fibroblasts are known to produce a variety of soluble growth factors and ECM components, including FGF, HGF, and proteoglycans (Story 1989; Schmidtchen, Carlstedt et al. 1990; Matsumoto, Okazaki et al. 1992). We found that this system provided the best survival and proliferation of the hepatocyte-like cells, when compared to cultures of these cells on single gel, in double gel, and matrigel (data not shown). The ESC-derived endoderm-like cells continued to proliferate and differentiate into a uniform population of hepatocyte-like cells forming epithelium-like clusters. Some binucleated cells were observed in the colony clusters. Previous reports have shown that endodermal cells alone fail to differentiate and mature (Ishii, Yasuchika et al. 2005; Tada, Era et al. 2005), suggesting that significant mesenchymal/mesodermal cues are necessary for the maturation of the early endodermal cells (Wells and Melton 2000). Taken together, these studies suggest that direct cell-cell contact with endoderm cells provides a suitable environment for endoderm proliferation and differentiation, consistent with our studies.

We have previously shown that 3T3-J2 fibroblasts promote a high level of hepatic gene expression, protein expression and function in isolated hepatocytes (Bhatia, Balis et al. 1999). Similarly, our results demonstrate a strong activation of the albumin gene by day 28 of culture, stained positive for albumin and CK-18, and secreted urea. Morphologically the cells appear hepatocyte-like including many binucleated
cells, dense cytoplasm, and bile canaliculi. Optimization of the culture conditions for terminally differentiating the hepatocyte progenitor cells into mature hepatocytes resulted in albumin and urea secretion levels similar to those seen with rat primary hepatocytes (unpublished data). Although urea production and albumin expression are characteristic of hepatocyte activity, kidney tubular epithelium also produces urea (Dunn, Tompkins et al. 1991), while extraembryonic cells express albumin, to our knowledge only hepatocyte do both suggesting the cells attain a hepatic phenotype. Further studies are needed to characterize these cells at a molecular level.

A means of further evaluating the functional capacity of the ESC-derived hepatocyte-like cells is to determine their therapeutic efficacy in treating FHF. Rats with GalN-induced FHF received a 10 hour perfusion with a BAL device seeded with the ESC-derived hepatocyte-like cells. Plasma levels of liver enzymes were reduced in the animals treated with the device containing the hepatocyte-like cells compared to the animals treated with the device containing fibroblasts. Animal survival on day 5 was significantly increased compared to the animals receiving treatment with a BAL device seeded with fibroblasts. Comparing these results to those from our prior study using primary porcine hepatocytes in the BAL device to treat FHF in the same rat model revealed similar survival trends. This suggests that the ESC-derived hepatocyte-like cells were providing biochemical support to the animals that was similar to primary hepatocytes, and resulted in increased animal survival. Also, it may be possible to increase the liver specific functions of the ESC-derived hepatocyte-like cells in the BAL device by coculturing them with non-parenchymal cells, such as fibroblasts or endothelial cells, as seen with primary hepatocytes. Future studies are ongoing to describe the in vivo therapeutic efficacy of the ESC-derived hepatocyte-like cells.

The production of a hepatic progenitor cell population could lead to a variety of new products. In theory, a committed progenitor, such as a hepatic progenitor cell could be generated from the endoderm and used for cell therapies to treat acute or chronic liver failure as well as for further maturation and confinement within a tissue-engineered extracorporeal BAL device. Similarly, new generations of other cellular products and tissue engineered products could be designed using an ESC-derived endodermal cell. We speculate that a reproducible culture system described in this study could be useful to obtain a unique and homogeneous population of endoderm-like cells that can be further differentiated into hepatic lineages.
without losing their characteristics. These populations derived from ES cells have the potential to become a reliable source of cells for cell transplantation, toxicology screens, and the development of BAL devices.

6.6 REFERENCES


6.7 FIGURES

Figure 6.1. Fraction of Oct4 GFP cells in coculture after early differentiation
Flow cytometric analysis of Oct4-GFP expression in ES-R1 cells in various conditions on day 5 of culture. ES cells were cocultured on top of collagen-sandwiched hepatocytes, mouse embryonic fibroblasts (MEF), or liver sinusoidal endothelial cells (LSEC). As a control, ES cells were cultured on collagen gels with no hepatocytes. Results shown are representative of two independent experiments in duplicate.
Figure 6.2. Phase contrast and fluorescence images of differentiation of Oct4 GFP cells in coculture
Expression of Oct-4-GFP fluorescence (upper panel) and phase-contrast images (lower panel) in ES cell-hepatocyte and ES-cell-fibroblast (MEF) cocultures on day 5 of culture. Scale bar 100 µm.
Figure 6.3. Dose-response of hepatocytes on the proliferation of isolated ES-D3 cells on day 6 of culture. ES cells were cultured on collagen gel with no hepatocytes (control) or cocultured on top of collagen-sandwiched hepatocytes at low (12.5x10^3 hepatocytes/cm^2), medium (62.5x10^3 hepatocytes/cm^2), and high (125x10^3 hepatocytes/cm^2) seeding densities. Relative cell growth of cultured ES-D3 cells was quantified by image analysis. Data shown are mean/ sd of two independent experiments in duplicate. *P < 0.0001. Similar results were reproduced in more than ten independent experiments for control and coculture with high hepatocyte density.
Figure 6.4. Morphology of differentiating ES cell in hepatic coculture
ES cells cultured in control (ES cell only) and coculture (125x10^3 hepatocytes/cm^2). Medium on control was IMDM + 20% FBS and medium in coculture was C+H (hepatic) medium.
Figure 6.5. Analysis of endoderm and mesendoderm gene expression in ES cell–hepatic coculture. Endoderm and mesendoderm gene expression measured by RT-PCR (30 cycles) of embryonic stem cells cultured on day 6 (endoderm) and days 4-10 (mesendoderm) on collagen gels and cocultures. Foxa2, Sox17, and AFP were used to assess endoderm. Brachyury and Goosecoid were used to assess mesendoderm.
Figure 6.6. Immunofluorescence of ES cell-hepatic cocultures on day 10
Expression of Foxa2 and alpha-fetoprotein (AFP) by ES-derived cells after dispase isolation on day 10 and replating into tissue culture dishes. ES cells cultured in control coculture (125x10^3 hepatocytes/cm^2). Medium in coculture was C+H (hepatic) medium. Scale bar = 100 µm.
Figure 6.7. Determination of endoderm fraction in cocultures
Flow cytometric analysis of isolated ESC-derived cells on day 10. AFP and Foxa2 were used for assessment of endoderm fraction.
Figure 6.8. Analysis of germ layer gene expression in ES cell –hepatic coculture. Germ layer expression measured by RT-PCR (30 cycles) of cocultured-ES cells on days 6 and 10 on collagen gels and cocultures. Foxa2, Sox17, and AFP were used to assess endoderm. GATA1, Runx2, Foxf1, Nkx2.5, and Tal1 were used to assess mesoderm. Pax 6 was used to assess ectoderm.
Figure 6.9. Morphology of ES cell-derived cells replated onto growth-arrested 3T3-J2 fibroblasts feeder layers. Cocultured ES-D3 cells on top of collagen-sandwiched hepatocytes were isolated by dispase and trypsin treatment on day 10, replated in 35 mm culture dishes at a density of 50,000 cells per dish (6.25x10^3 ES cells/cm^2) onto growth-arrested 3T3-J2 fibroblast feeder layers, and cultured for up to 20 days (days 10-30 of culture). Primary adult rat hepatocytes cultured in the collagen gel sandwich configuration 7 days after seeding, shown for comparison (bottom right panel). Scale bar = 100 µm.
Figure 6.10. Analysis of gene expression on day 18 and day 30 in ES cell-fibroblast cocultures. Germ layer expression measured by RT-PCR (30 cycles) of ES cells-fibroblast coculture on days 18 and 28. Cocultured ES-D3 cells on top of collagen-sandwiched hepatocytes were isolated by dispase and trypsin treatment on day 10, replated in 35 mm culture dishes at a density of 50,000 cells per dish (6.25x10^3 ES cells/cm^2) onto growth-arrested 3T3-J2 fibroblast feeder layers, and cultured for up to 20 days (days 10–30 of culture). Albumin, Alanine Amino transferase (AAT), Cytokeratin 8 (CK8), Cytokeratin 18 (CK18), Tranthyretin(TTR), Cytochrome P450 3a13 (Cyp 3a13), Foxa2, and AFP were used to assess hepatic differentiation. GATA1, Nkx2.5, and Tal 1 and Pax6 were used to assess mesoderm and ectoderm, respectively.
Figure 6.11. Immunofluorescence for Albumin and CK18
Albumin and CK-18 immunofluorescence staining on day 28 of ES cell/fibroblast coculture. Cocultured ES-D3 cells on top of collagen-sandwiched hepatocytes were isolated by dispase and trypsin treatment on day 10, replated in 35 mm culture dishes at a density of 50,000 cells per dish (6.25x10^3 ES cells/cm^2) onto growth-arrested 3T3-J2 fibroblast feeder layers, and cultured for up to 20 days (days 10–30 of culture). Scale bar =100 µm.
Figure 6.12. Urea synthesis in ES cell cell-fibroblast cocultures
Cocultured ES-D3 cells on top of collagen-sandwiched hepatocytes were isolated by dispase and trypsin treatment on day 10, replated in 35 mm culture dishes at a density of 50,000 cells per dish (6.25x10^3 ES cells/cm²) onto growth-arrested 3T3-J2 fibroblast feeder layers, and cultured for up to 20 days (days 10–30 of culture). Data shown is mean and standard deviation (n=3).
Figure 6.13. Biochemical analysis after treatment for liver failure with ES cell-derived hepatocyte-like cells. Biochemical analysis of GalN-induced Fulminant Hepatic Failure in rats connected to a bioartificial liver device (BAL) for 10 h either with fibroblasts (3T3-J2) as a control or with ESC-derived hepatocyte-like cells. The first dose of GalN (1.2 g/kg i.p.) was administered 24 h after cannulation, and the second dose was given 12 h later. The BAL perfusion started 24 h after the first GalN injection. Total bilirubin (TBIL), blood urea nitrogen (BUN), aspartate transaminase (AST), and alanine transaminase (ALT). *P - 0.05
Figure 6.14. Animal survival curve after treatment ES cell derived hepatocyte-like cells for liver failure. Animal survival curve (B) of GaIN-induced Fulminant Hepatic Failure in rats connected to the bioartificial liver device (BAL) device for 10 h either with fibroblasts (3T3-J2) as a control or with ESC-derived hepatocyte-like cells. The first dose of GaIN (1.2 g/kg i.p.) was administered 24 h after cannulation, and the second dose was given 12 h later. The BAL perfusion started 24 h after the first GaIN injection. Animal survival data using BAL device with primary porcine hepatocytes are from our previously published study (39).
CHAPTER 7:
SUMMARY AND FUTURE DIRECTIONS

7.1 Summary of research objectives met

Overall, Chapters 3-6 each meet portions of more than one objective listed at the end of Chapter 2. Here we will review the extent to which various objectives were met. Below, a summary of the overall objectives of this thesis are now presented, followed by future work for each objective.

7.2 A framework for analyzing early differentiation

A framework was established and used in Chapters 3-6 for measuring differentiation. This allows the ability to measure differentiation accurately, which can be quite challenging. Transcripts for lineage-specific transcription factors were measured for early differentiation, including the endoderm, mesoderm and ectoderm. In Chapter 5 the precursors to endoderm, including epiblast and mesendoderm were also measured. Late stage differentiation was also measured using RT-PCR in Chapters 4, 5, and 6. Transcription factors for different domains of endoderm patterning were measured in Chapter 5. In Chapters 4 and 5, and especially in Chapter 6, the focus was on extensive measurements of hepatic induction. The assay was robust and reproducible.

The future work for this assay is to continue to add more targets, to optimize the cycle number for those targets. For example, the list of gene targets now consists of only differentiation genes associated with differentiation. However, if a soluble factor, or growth factor is added to the system, it can activate specific genes associated with it. These can then be measured using RT-PCR. For example, genes associated with the activin pathway, that are known direct targets of activin, can be added to the analysis to act as a positive control for particular condition. This would strengthen the data overall and increase understanding of how particular factors affect differentiation.

7.3 New culture configurations that promote endoderm induction and endoderm-enriched populations

The next major objective was to establish or develop new culture systems that enhance endoderm induction. Here, three major culture systems are presented for endoderm induction, and highlighted in
Figure 7.1. At the top is the culture system that was initially used. This system consisted of the embryoid body culture with both serum containing (20% FBS) and serum free medium. In this system, there was minimal endoderm induction (Figure 3.2). To solve this problem, three approaches were developed. In each case, multiple changes were made in the culture system initially. For example, to the left in Figure 7.1 is the monolayer culture system on collagen gels. The monolayer system was developed because of the difficulties of the EB based system. However, at late stages, the rigid tissue culture surfaces, whether treated or not, were found to not maintain epithelial phenotypes (Figure 3.10). Thus the collagen gel culture system was retained. To improve the EB system, microfabrication approaches were used in Chapter 4, as shown in Figure 7.1, center, top. This allowed precise control over the diameter of the EB, which had not previously been achieved. The rationale behind this approach was that at particular EB sizes, particular germ layers would be favored. Lastly, a hepatic coculture system was also developed (Figure 7.1, right top) The rationale for this was that a controlled microenvironment could be obtained in hepatic coculture because geometrically the cells are below and the ES cells are plated above. All three culture systems support endoderm induction can have different advantages or disadvantages.

The future work for this objective would be to further develop these culture systems. This can include optimization protocols regarding the medium conditions, the serum content, the extracellular matrix, and soluble factors. This could be accomplished using the current data. For example, medium secreted from the EB, could be used to enhance endoderm induction in monolayer systems. Overall, the monolayer system is the most well characterized, the simplest to set up, and the most reproducible. The microfabricated system for ES cell culture was important because it provided scaleable and uniform aggregates that remain attached. While this system was not evaluated extensively, it could be useful for future studies. For example, a purified progenitor, such as an endoderm progenitor cell or a hepatic progenitor cell could be placed in the microfabricated stencil and homotypic cell-cell interactions could be studied. Furthermore, mixed populations of endoderm with mesodermal cell types could be mixed in various ratios and placed in microfabricated stencils. This type of study would lead to enhanced understanding of heterotypic interactions and how they effect differentiation.
7.4 Microenvironmental factors that effect endoderm induction

Objective three will be to clearly identify factors that effect endoderm induction and the pathway to definitive endoderm induction. An important mechanisms of endoderm induction appear to be the collagen gel, which enhanced endoderm induction in both monolayers (Left, Figure 7.1), and in coculture (Right, Figure 7.1). Alteration of the thickness of the gel appeared to have effects on overall morphology/aggregation (data not shown). This suggests that biophysical, substrate based mechanisms may be at play in enhancing endoderm induction. The mechanisms by which the collagen gel is signaling through cell surface receptors, potentially via integrin receptors, is not known, but would be an important area of future study.

In the monolayer system, decreasing serum did not change endoderm induction, but did reduce the amount of epiblast cells that differentiated towards endoderm (Figure 3.7 and 3.8). This suggests that endoderm induction occurs independently of serum and thus may be intrinsic to ES cells secreting autocrine factors. Serum-free medium seemed to eliminate cells from gels by day 10, probably due to decreased attachment. This suggests that attachment factors added to a serum-free formulation could be used for endoderm induction. The factors that ES cells secrete, and how they activate pathways in an autocrine fashion is an important area for future study.

The second mechanism that may be worthwhile exploring in the future is the hepatic coculture system. It would be interesting to determine if follistatin, which was found to enhance endoderm induction, is a player in the coculture system. Other soluble factors important during development, such as wnt and BMP2, may result in enhanced endoderm induction, or activation of pathways which may not be measurable using the current framework. The interplay of ECM, soluble factors, and medium conditions that affect endoderm induction is only at its infancy. The action of activin, in which it represses endoderm induction is a novel finding in this study. This highlights that specific effects of specific soluble factors or other factors may be dependent on culture configuration. This further implies that universal characterization of the state of the cell, in terms of capacity to differentiate or proliferate, is critical to further studies which characterize the effects of perturbations on differentiation.
The pathway by which endoderm is induced, that is epiblast to mesendoderm to definitive endoderm, was also established in these studies. A clear transition was seen in gene expression when examining the kinetics in collagen gel system (Figure 5.6), as well as the coculture system (Figure 6.5). Thus, in two culture systems, the appropriate pathway was established implying that definitive endoderm was present. Future studies would take advantage of cell labeling techniques and better understand the mechanisms by which transcription factors are sequentially activated and then deactivated during progression to definitive endoderm. The fact that activin delays differentiation may suggest a starting point for mechanisms by which these developmental transcription factor networks push forward or progress.

7.5 Approaches for endoderm patterning/differentiation

One of the ultimate objectives from endoderm induction was to differentiate these cells into an enriched or pure population. Differentiation protocols for ES cells from all three culture configurations, the monolayer gel culture system, the ES system, and the coculture system were developed (Figure 7.2). When differentiating in the gel culture cell system, cells were replated back on to collagen gels. In this case the only variable that was changed was the medium, and the result was evidence of foregut, midgut, and hindgut genes by day 24. In coculture with fibroblasts, cells were replated from the gel condition (in Hepatocyte coculture) onto fibroblasts plated on tissue culture plastic. The result was increased liver gene and protein expression for albumin by day 28. In this case, differentiation was induced with multiple changes at once (change to direct fibroblast coculture and tissue culture plastic). In the case of microfabricated EB’s, a staged protocol was used, called the Hamazaki protocol (Hamazaki, 2000). He stages were divided on days 11-14, 14-17, and 17-21, and different soluble factors were added at different times, including FGF, HGF, Oncostatin, Dexamethasone, and ITS. Overall, the system that produced the greatest differentiation was unclear, because slightly different analytical tools were used for all three. However, it was shown that albumin positive cells could be generated in all three types of culture systems.

There is significant future work for the differentiation of endoderm, since this is where the clinical utility will be. In vivo, endoderm is influenced by its neighboring germ layers (Wells and Melton 2000) as well as by local mesodermal conditions which might specify cell fate. This implies that understanding how
endoderm interacts with mesodermally-derived cocultures might be an important approach. While staged protocols have shown some promise, an important aspect would be to understand the mechanisms by which a staged protocol works. One question to ask for hepatic differentiation would be how does the staged protocol induce critical cell surface receptors or the hepatic nuclear network changes to allow differentiation to progress. A further understanding of differentiation, of the ability to boost differentiation, and the ability to make pure populations of a particular cell of interest would be critical to further understanding the differentiation process.

7.6 In vivo differentiation and clinical applications

The fifth objective was to understand if these endoderm progenitor cells or their derivatives are applicable in these settings. To address this question, two approaches were used, as shown in Figure 7.3. One approach was to place differentiated cells into the extracorporeal bioartificial liver (Shito, Tilles, and Yarmush 2003). In this approach, cells were first differentiated and then cultured in a flat plate bioreactor overnight and then placed in a circuit with the mouse in a clinical model of liver failure. Interestingly, 50% of the animals were surviving by day 5, which was much higher than controls. Importantly, these cells could interact with the plasma, after anticoagulation, survive, and actually support liver functions. This interesting result has important implications and raises key questions for future work. For example, do the cells differentiate further when exposed to plasma? Do the cells secrete substances which improves liver failure or enhance liver generation? What is the long term survival in the animal reactor, and how long can the cells be placed in circuit? These types of questions need to be asked in future studies.

The second system for in vivo application was implanted endoderm progenitor cells combined with a small amount of fibroblasts subcutaneously into syngeneic mice. One reason to differentiate cells in vivo is that it is not known whether early progenitor cells or late committed cells will be the cell of choice for therapies. Another aspect is that it is not known whether in vitro differentiation can ever result in terminally differentiated cells, and thus in vivo differentiation may provide an alternative to in vitro differentiation. In this scenario, cells would be implanted, differentiated, and then recovered in vivo. Lastly, in vivo differentiation may reveal novel insights how morphogenesis occurs, and how tissue architecture is established from progenitor cells, without the need for scaffolding.
The homogenous masses recovered in control and follistatin cases were epithelial in nature, and not previously reported. While this study only established commitment, it also established that these cells can form histologic structures in vivo by interacting with mesenchymal elements. These masses also demonstrate can undergo angiogenesis, and demonstrated evidence of immature blood vessels. This in vivo differentiation assay can act as a tool to study stage of differentiation, the homogeneity in a population, and the ability of the cells to survive, recruit blood vessels, and form 3D structures in vivo.

Since these studies involved studying only histology, future studies can be used to analyze the implanted populations even further. For example, the masses can be recovered not only for H&E staining but also for gene expression analysis. Furthermore, the cells can be genetically modified so that differentiation can be monitored in vivo. Lastly, these cells can be recovered further and isolated and cultured in vitro, and in this case in vivo differentiation could be a tool for obtaining differentiated populations of cells. The ability to form 3D structures in vivo may serve as an important approach to tissue engineering of artificial organs. Lastly, these approaches can be combined with real disease models and orthotopic transplantation or ectopic transplantation.
7.7 FIGURES

**Figure 7.1. Schematic of endoderm induction schemes.** The overall endoderm induction protocols developed are shown above. Arrows are shown to depict changes to the culture system, with labels above the arrows indicating the changes. At each stage, the culture configuration (monolayer, embryoid body(aggregate) or coculture) is described, as is the presence of extracellular matrix (ie. collagen gel), particular type of medium (ie. 20% FBS), or growth factors(ie. Activin). Figures which demonstrate the analysis of that particular condition are referenced each stage in bold type( Fig 3.4). When necessary, expression of Foxa2 is described as a general gauge for level of endoderm induction.
Figure 7.2. Schematic of endoderm differentiation schemes. The starting population for differentiation is shown above, and an abbreviated description of the differentiation protocol is shown below. The three main starting populations are ES cells on collagen gel only, microfabricated aggregates, and hepatocyte cocultured ES cells, listed at top from left to right. A brief description of how each population was differentiated is shown below. C+H is a hepatic differentiation medium containing EGF, Insulin, glucagon, and hydrocortisone. GF represents growth factors, in this case maturating growth factors.
Figure 7.3. Schematic of in vivo applications of ES cell derived cells. Two types of applications are described here. To the left is endoderm progenitor cells that are implanted in a subcutaneous implant model, in order to understand more fundamental questions about how cells behave in vivo. The second, to the right is late stage hepatocyte like cells that are used in an extracorporeal artificial liver with continuous perfusion in a rat liver failure model.
CURRICULUM VITAE  
NATESH PARASHURAMA  

EDUCATION  
1994  
Bachelor’s in Science (BS)  
Chemical Engineering with Biology, The Massachusetts Institute of Technology (MIT),  
with Honors Thesis  

1999  
Doctor of Medicine (MD)  
State University of New York @Buffalo School of Medicine and Biomedical Science  

1999-2001  
Residency in General Surgery  
Boston Medical Center/Boston University  

2001-2007  
Doctor of Philosophy (Ph.D.)  
Department of Chemical Engineering, Rutgers University, The State University of New Jersey  

Title- Stem Cell Engineering of the Endoderm: Approaches to Controlling Endoderm Induction and Differentiation from Embryonic Stem Cells  

Thesis Advisor: Martin L. Yarmush MD, PhD. Professor of Biomedical Engineering Rutgers University and Professor of Bioengineering, (Harvard)  

2004-2007  
Graduate Research Fellow  
Harvard Medical School, Massachusetts General Hospital, Dept of Surgery,  
Center for Engineering in Medicine  
Thesis Advisor: Martin L. Yarmush MD, PhD  

Jan 1, 2008-  
Postdoctoral Fellowship- Molecular Imaging  
Stanford University/Stanford University Medical Center  
Departments of Radiology/Nuclear Medicine  
Mentor: Sanjiv Sam Gambhir MD, PhD  
Director, Molecular Imaging Program at Stanford (MIPS)  
Head, Nuclear Medicine  
Professor, Departments of Radiology and Bioengineering  
Bio-X Program  

HONORS AND AWARDS  
1990  
Class of ‘90 Valedictorian, Smithtown High School West, Smithtown, NY  
1989  
Recipient: NYS Regents Scholarship  
1991-1993  
MIT Undergraduate Research Opportunities Program  
1994  
MIT Undergraduate Honors Thesis  
1993  
MIT’s William H. Stewart Award, for Community Service  
1996  
Roswell Park Cancer Institute, Buffalo, NY Summer Fellowship  
1997  
SUNY@Buffalo Research Foundation Fellowship  
1997  
Accepted for National Research Presentation-Eastern Student Research Forum  
2001-2002  
NIH Biotechnology Fellowship  
2002  
Best Teaching Assistant Award, Department of Chemical and Biochemical Engineering, Rutgers University, NJ  
2004  
NRSA(NIH T32) Research Fellowship, Department Surgery, Division of Burns and Trauma  
2008  
Invited Speaker and Travel Award, Society for Biological Engineering, AIChE, Stem Cell Bioengineering Conference  
2008  
Dean’s Postdoctoral Fellowship, Stanford University
APPOINTMENTS

2004-2007  Scientific Staff, Shriners Hospital, Boston MA
2004-2007  Research Fellow, Massachusetts General Hospital, Harvard Medical School

PUBLICATIONS

Parashurama N, Y Nahmias, C Cho, Berthiaume F, A Tilles, Yarmush ML. “Activin alters kinetics of endoderm induction in collagen gel cultures of embryonic stem cells” In Press, Stem Cells

Parashurama, N, Saygili B., Berthiaume F, A Tilles, Yarmush ML. “Exploring embryonic stem cell derived endoderm differentiation in cocultures in ” In Preparation

Parashurama, N, Banerjee, I, Berthiaume F, A Tilles, Yarmush ML. “Kinetic modeling of activin effects on a developmental gene network in embryonic stem cells ” In Preparation

Cho, C Parashurama N, A Tilles, Yarmush ML Homogenous Differentiation of Hepatocyte-like Cells from Embryonic Stem Cells: Applications for the Treatment of Liver Failure” Accepted FASEB


PRESENTATIONS/POSTERS

“Tracheal Lumen Responds to Chemical Injury with Localized and Time-Dependent Expression of KGF and bFGF in an Organ Explant Culture System” (1997)
SUNY@Buffalo School of Medicine
Accepted for presentation to Eastern Student Research Forum, Miami, Fl.

SUNY@Buffalo School of Medicine - Student Research Forum, Poster Presentation


Tony & Shelly Malkin Stem Cell Symposium, Harvard Stem Cell Institute, Boston MA

AICHE Annual Meeting Engineering Fundamentals in Life Science: Stem Cell Engineering (15D07)

AICHE Annual Meeting 2007-Presentation Sesssion Cell Culture for Cell Therapies

BMES Annual Meeting 2007 -Poster

Accepted for Presentation
Stem Cell Bioengineering International Conference, Society for Biological Engineering, AICHE Annual Meeting 2008 -Presentation

PROFESSIONAL ASSOCIATIONS

1999-current AMA American Medical Association
2003-current ACS American College of Surgeons
2003-current BMES Biomedical Engineering Society
1997-current AICHE American Institute of Chemical Engineering
2005-current HHSCI Harvard Stem Cell Institute
2004-current CIMIT- Center for Integration of Medicine and Innovative Technology
<table>
<thead>
<tr>
<th>Year</th>
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<tbody>
<tr>
<td>2001</td>
<td>Graduate Teaching Assistant, Department of Chemical Engineering</td>
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<tr>
<td>2002</td>
<td>Graduate Teaching Department of Biomedical Engineering, Tissue Engineering: Fundamentals and Tools, Assisted in syllabus and design, homework design and solutions</td>
</tr>
<tr>
<td>2003</td>
<td>Graduate Teaching Department of Biomedical Engineering, Tissue Engineering: Fundamentals and Tools, Assisted in syllabus and design, homework design and solutions</td>
</tr>
<tr>
<td>1998</td>
<td>Graduate Teaching Assistant, Rutgers, The State University of New Jersey, Chemical Engineering Thermodynamics</td>
</tr>
<tr>
<td>1998</td>
<td>Graduate Teaching Assistant, Rutgers, The State University of New Jersey, Tissue Engineering: Fundamentals and Tools, Assisted in syllabus and design, homework design and solutions</td>
</tr>
<tr>
<td>2001</td>
<td>Graduate Teaching Assistant, SUNY@Buffalo School of Medicine, Surgical Management of Peptic Ulcer Disease, Surgical Management of Crohn’s Disease</td>
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<tr>
<td>2002</td>
<td>Graduate Teaching Assistant, SUNY@Buffalo School of Medicine, Surgical Management of Adrenal Pheochromocytoma</td>
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<td>2003</td>
<td>Graduate Teaching Assistant, SUNY@Buffalo School of Medicine, Development of Strategies for Identification and Profiling Progenitor Cells</td>
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<tr>
<td>2004</td>
<td>Graduate Teaching Assistant, Rutgers University, Mini-Stem Cell Conference, Development of Strategies for Identification and Profiling Progenitor Cells</td>
</tr>
<tr>
<td>2005-6</td>
<td>Graduate Teaching Assistant, Rutgers University, Mini-Stem Cell Conference, Development of Strategies for Identification and Profiling Progenitor Cells</td>
</tr>
</tbody>
</table>
PREVIOUS RESEARCH EXPERIENCE

1992-1994  Massachusetts General Hospital (MGH) and Harvard Medical School  
Mehmet Toner, Ph.D., Professor of Surgery/Bioengineering  
**Hepatic Tissue Engineering**  
The goal of our work was to create a packed-bed bioreactor containing encapsulated hepatocytes, to serve as an extracorporeal bioartificial liver. We found that hepatocyte morphology and function could be maintained long term when sandwiched by mixtures of polysaccharides when compared with collagen sandwich cultures.

1994  
**Hepatic Ischemia-Reperfusion Injury**  
I used an in-vitro quantitative assay to estimate the number of cells injured with time of ischemia and developed a simple, reproducible method of inducing ischemia and reperfusion. Cell death was found to be 50% at 6 hours. In addition, reperfusion with high levels of oxygen caused further injury to ischemic cells but not control cells, at a rate faster than ischemic injury.

1994-1995  
**Cellular Biomechanics and Mechanisms of Cell Mechanotransduction**  
Robert M. Ezzell, Ph.D., Asst. Professor of Cell Biology  
Mammalian cells transducer environmental cues into mechanical stresses by interactions between the focal adhesion complex (FAC) and the ECM, and the cytoskeleton. We studied the FAC localization of key cytoskeleton proteins in an F9 embryonal carcinoma cell line (5.51) which lacked Vinculin. We found that transfection of Vinculin promoted stress fiber formation, cell spreading, and cytoskeleton stiffness, as measured with RGD–coated ferromagnetic 5 micron beads.

1994-96  
**Quantitative, Co focal Microscopy and 3D Image Reconstruction**  
Prabhas V. Moshe, PhD  
Critical cellular function of hepatocytes is improved in collagen double gel but the mechanism is unknown. The main findings of the study were that a rapid redistribution of Integrins occurred with overlay of collagen, and that blocking the integrin response with polyclonal antibody resulted in rapid loss of cytoskeleton organization.

1997  
**Roswell Park Cancer Institute(RPCI), Buffalo, NY, Surgical Oncology**  
Wesley L. Hicks, Jr., MD, DDS  
**Regeneration of Tracheal Epithelium**  
An initial step in the development of a treatment strategy for tracheal injury is to identify cells which are involved in repair of the epithelium. I purified a heterogeneous populations of human upper epithelial cells, and obtained a purified basal cell population stained with the lection GSI-B4. The sorted population was studied with electron microscopy, and immunohistochemistry and was maintained in culture for 7 days.

1997  
**Regeneration of Tracheal Epithelium**  
The response to tracheal injury involves migration, proliferation, and differentiation into a columnar epithelium. We developed a method for injuring trachea in a controlled manner, by using tracheal organ explants. Upon epithelial denudation, our results suggested that KGF expression may cause an increase in cell migration followed by bFGF which may cause an increase in cell proliferation.
CLINICAL TRAINING AND EXPERIENCE

1995-1999  SUNY@ Buffalo School of Medicine

1999-2001  Boston University Medical Center
           General Surgery Residency
           • Preoperative, Operative, and Postoperative Care of Surgical Patients
             Over 1000 patients managed, over 200 operations
           • Rotations: Gastrointestinal, Breast, Cancer, Transplant, Vascular, Trauma,
             Cardiothoracic, Neurosurgery, Plastics, Colorectal, Endocrine, Complex Wound Care,
             Surgical Intensive Care, Cardiac Intensive Care, Outpatient
           • Management of Medical Devices/Procedures: Ventilator, Intubation,
             Tracheostomy, Cardiopulmonary Bypass, Hemodialysis, Bronchoscopry, Upper and
             Lower Endoscopy, Laparoscopic Surgery, Intracranial Pressure Monitoring, Intraortic
             Balloon Pump, Swan-Ganz (Pulmonary Artery) Catheterization, Coagulation
           • Two-Year Surgical Topics Curriculum

INDUSTRIAL EXPERIENCE

2002       Summer Internship
           Novartis Institute for Biomedical Research, Novartis Pharmaceuticals
           NIH Biotechnology Fellowship Program
           Supervisor: Larry Wennogle, Ph.D., Cardiovascular/Metabolic Division
           • Elucidated the effects of transgenic expression of protein tyrosine phosphatases
             on platelet activation with the goal of developing cardiovascular therapies.

           Industrial Topics Course, Rutgers University
           “Bioengineering in the Pharmaceutical and Biotechnology Industries: Fundamental and
           Real World Perspectives”
           Course Organizer: Marin L. Yarmush MD, PhD and Greg Rusotti, PhD, Senior
           Bioprocess Engineer, Merck