Controlling Cell Density by Micropatterning Regulates Smad Signalling and Mesendoderm Differentiation of Human Embryonic Stem Cells

by

Lawrence Lee

A thesis submitted in conformity with the requirements for the degree of Master of Applied Science

Graduate Department of the Institute of Biomaterials and Biomedical Engineering
University of Toronto

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2008

Abstract

Human embryonic stem cells (hESC) present a potentially unlimited supply of hematopoietic progenitors for cell-based therapies. However, current protocols for generating these progenitors typically also generate undesired cell types due to imprecise control of the hESC microenvironment and poor understanding of the signalling networks regulating mesoderm differentiation (the germ layer from which hematopoietic cells emerge). This report demonstrates that activation of the downstream effectors of Activin/Nodal and bone morphogenetic protein (BMP) signalling (Smad2 (composite of Sma (smaller) and Mad (mothers against decapentaplegic) and Smad1, respectively) are both required for mesoderm differentiation. It is further shown that microcontact printing-mediated control of hESC colony size creates local microenvironments that guide differentiation, via a Smad1-dependent mechanism, preferentially towards the mesoderm lineage. These findings demonstrate the need for precise control of the microenvironment in order to effectively guide hESC differentiation to produce specific cell types for potential therapeutic applications.
Acknowledgements

I would like to acknowledge Dr. Chirag Joshi for his invaluable advice and initial training on generating hematopoietic cells and quantitative gene expression analysis. I would also like to thank Dr. Mark Ungrin for his help in generating microwells and spinEBs. Special thanks to Manuel Alvarez and Ting Yin for providing passage after passage of perfectly cultured hESCs. I also want to acknowledge Weijia Wang for her assistance with the flow cytometry analysis. I would like to express my gratitude to Simon Smuckler and Margot Arntfield for their advice and providing key reagents with the endoderm differentiation. I would like to express my gratitude to Raheem Peerani, but if I did this section would be far too long. To put it simply, he has taught me everything I know. Finally, I would like to thank my supervisor, Dr. Peter Zandstra, for his guidance and support, and providing such a great environment to learn – also for making my thesis defense happen on such short notice.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ActRIIB</td>
<td>Activin receptor type II B</td>
</tr>
<tr>
<td>AGM</td>
<td>Aorta-gonad-mesonephros</td>
</tr>
<tr>
<td>ALK</td>
<td>Activin receptor-like kinase</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>BIO</td>
<td>Bromoindirubin oxime</td>
</tr>
<tr>
<td>BME</td>
<td>β-mercaptoethanol</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>BMPRII</td>
<td>BMP receptor type II</td>
</tr>
<tr>
<td>Bry</td>
<td>Brachyury</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CDX2</td>
<td>Caudal type homeobox 2</td>
</tr>
<tr>
<td>Cer1</td>
<td>Cerberus 1</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>CFU-GEMM</td>
<td>CFU-granulocyte, erythroid, macrophage, megakaryocyte</td>
</tr>
<tr>
<td>CFU-GM</td>
<td>CFU-granulocyte, macrophage</td>
</tr>
<tr>
<td>CFU-M</td>
<td>CFU-macrophage</td>
</tr>
<tr>
<td>Co-Smad</td>
<td>Common Smad</td>
</tr>
<tr>
<td>CXCR4</td>
<td>Chemokine (C-X-C motif) receptor 4</td>
</tr>
<tr>
<td>DKK1</td>
<td>Dickkopf 1</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagles’s medium</td>
</tr>
<tr>
<td>E</td>
<td>Embryonic day</td>
</tr>
<tr>
<td>EB</td>
<td>Embryoid body</td>
</tr>
<tr>
<td>ECM</td>
<td>Extra-cellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra-acetic acid</td>
</tr>
<tr>
<td>ESC</td>
<td>Embryonic stem cell</td>
</tr>
<tr>
<td>ExE</td>
<td>Extraembryonic Endoderm</td>
</tr>
<tr>
<td>FAST-1</td>
<td>Forkhead Activin signal transducer-1</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescent Isothiocyanate</td>
</tr>
<tr>
<td>Flk1</td>
<td>Fetal liver kinase 1</td>
</tr>
<tr>
<td>FoxA2</td>
<td>Forkhead box A2</td>
</tr>
<tr>
<td>FoxH1</td>
<td>Forkhead box H1</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocytic colony stimulating factor</td>
</tr>
<tr>
<td>GDF</td>
<td>Growth and differentiation factor</td>
</tr>
<tr>
<td>GFR</td>
<td>Growth factor reduced</td>
</tr>
<tr>
<td>GSC</td>
<td>Goosecoid</td>
</tr>
<tr>
<td>GSK3</td>
<td>Glycogen synthase kinase 3</td>
</tr>
<tr>
<td>GUSB</td>
<td>Glucuronidase beta</td>
</tr>
<tr>
<td>hESC</td>
<td>Human embryonic stem cell</td>
</tr>
<tr>
<td>HNF1β</td>
<td>Hepatocyte nuclear factor 1β</td>
</tr>
<tr>
<td>HNF3β</td>
<td>Hepatocyte nuclear factor 3β</td>
</tr>
<tr>
<td>HNF4α</td>
<td>Hepatocyte nuclear factor 4α</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
</tr>
<tr>
<td>ICM</td>
<td>Inner cell mass</td>
</tr>
</tbody>
</table>
IL-3 Interleukin-3
IL-6 Interleukin-6
IPF1 Insulin promoter factor-1
KDR Kinase insert domain receptor
KGF Keratinocyte growth factor
KO Knockout
LEF1 Lymphoid enhancer binding factor 1
Lefty1 Left-right determination factor 1
LTC-IC Long-term culture-initiating cell
MAP Mitogen-activated protein
mEF Mouse embryonic feeders
mESC Mouse embryonic stem cell
Mesp1 Mesoderm posterior 1
MIXL1 Mix 1 homeobox-like 1
MNX1 Motor neuron and pancreas homeobox-1
NEAA Nonessential amino acids
NOD/SCID Nonobese diabetic/sever combined immunodeficiency
OAZ Olf-1/EBF associated zing finger
Oct4 Octamer-4
Pax6 Paired box 6
PBS Phosphate buffered solution
PDGFRα1 Platelet derived growth factor receptor alpha 1
PDMS Poly(dimethylsiloxane)
PDX1 Pancreas/duodenum homeobox-1
PE Phycoerythrin
PECAM1 Platelet endothelial cell adhesion molecule 1
pSmad Phosphorylated Smad
RA Retinoic Acid
rh Recombinant human
RNA Ribonucleic Acid
ROCK Rho kinase
R-Smad Receptor smad
SCF Stem cell factor
Smad Composite of Sma (smaller, Caenorhabditis Elegans) and Mad (mothers against decapentaplegic, Drosophila Melanogaster)
Sox17 SRY-box containing gene 17
Sox2 SRY-box containing gene 2
Sox7 SRY-box containing gene 7
SR Serum replacement
TCF T-cell factor
TGFβ Transforming growth factor β
TβRI TGFβ receptor type I
TβRII TGFβ receptor type II
UV Ultraviolet
VEGF Vascular endothelial growth factor
VEGFR2 Vascular endothelial growth factor receptor 2
Wnt Wingless-related mouse mammary tumour virus integration site
XTC-MIF Xenopus cell line XTC mesoderm inducing factor
Mathematical Model Parameters

\( y \)  Percent of cells expressing Brachyury
\( c_1 \)  Intercept term
\( c_2 \)  BMP2 main effect coefficient
\( c_3 \)  Activin A main effect coefficient
\( c_4 \)  BMP2 quadratic coefficient
\( c_5 \)  Activin A quadratic coefficient
\( c_6 \)  Second order interaction coefficient
1 Introduction

1.1 Motivation

Currently, a variety of blood and immune disorders can be treated through transplantation of hematopoietic stem cells (HSC) from a healthy donor. Such HSC can be extracted from the bone marrow (Thomas et al. 1970), peripheral blood (Korbling et al. 1981), or umbilical cord blood (Gluckman et al. 1989; Goldberg et al. 2007) obtained immediately following childbirth. These treatments, however, are restricted by the limited supply of donors and the requirement of human leukocyte antigen matching. Alternatively, human embryonic stem cells (hESC) have the potential to form all cell types present in an adult body, and therefore present a potentially unlimited supply of HSC that can be used for transplantation. Additionally, recent studies have shown the ability to induce pluripotency from human somatic cells (Takahashi et al. 2007; Yu et al. 2007). These induced pluripotent stem (iPS) cells offer a patient specific cell source for cell based therapies and would obviate concerns of immune rejection. However, much remains to be studied in the embryonic stem cell system before further advances can be made. Current protocols for generating hESC derived HSC include attached coculture of hESC with various hemogenic stromal cell lines or three-dimensional embryoid body differentiation in the presence of serum or a cocktail of cytokines (Kaufman et al. 2001; Chadwick et al. 2003; Vodyanik et al. 2005; Zambidis et al. 2005). These approaches are limited by their undefined culture medium or underlying mechanism. Other challenges include the purity of cell types that are produced from hESC and the sheer number of HSC that would be required for a therapeutic application in humans.
In order to overcome these challenges, a greater understanding of the mechanisms underlying hESC fate decisions is required. A key player in these mechanisms is the interaction of hESC and their microenvironment, or ‘niche.’ This stem cell niche includes cell-cell contact, secreted and/or exogenously added cytokines and extracellular protein interactions, all of which play pivotal roles in determining hESC cell fate. In order to precisely control hESC differentiation so that the desired cell type with sufficiently high purity can be obtained, the hESC niche must also be controlled during the early stages of differentiation. To achieve this goal, a combination of exogenous cytokines will be added to hESC cultures while simultaneously controlling hESC colony size. This will allow for the robust control of cell-cell contact and signalling in order to guide hESC differentiation towards the hematopoietic lineage.

1.2 Background

1.2.1 Key Developmental Signalling Pathways

In order to develop an full understanding of embryonic stem cells and how to best make use of their potential, it is often useful to first discuss the biology of a developing embryo and fetus. During the first several days of an implanting embryo, roughly 4 and 7 days following fertilization in mice and humans, respectively, a handful of developmental signalling pathways are responsible for its proper development and tissue patterning. The most important pathways include transforming growth factor-β (TGFβ) signalling, Wnt signalling, and fibroblast growth factor (FGF) signalling, the basic elements of which will be briefly discussed here prior to discussing embryogenesis and embryonic stem cells.
1.2.1.1 Transforming Growth Factor-β Signalling

Transforming growth factor-β (TGFβ) signalling has been shown to be important in early mammalian embryogenesis and patterning, as well as key regulators of embryonic stem cell (ESC) self-renewal and differentiation (Chu et al. 2004; Peerani et al. 2007; Smith et al. 2008). The TGFβ superfamily can be divided into two subclasses of ligands. The first includes the TGFβs, Activins and Nodals while the second class includes the BMP (bone morphogenetic protein) and GDF (growth and differentiation factor) ligands. The variety of TGFβ ligands (the human genome encodes for at least 29 different ligands (Feng et al. 2005)) is accommodated by various permutations of the TGFβ signalling complex, which consists of two type II and two type I receptors. In the absence of ligands, type I and type II receptors are found as homodimers on the cell surface. However, when ligand is present homodimers of each type I and type II receptors form a complex with the ligand which allows for phosphorylation of a conserved Glycine/Serine-rich sequence in the type II receptor and leads to autophosphorylation of the type I receptor (Derynck et al. 2003; Shi et al. 2003). TGFβ1 and Activins/Nodals bind to TβRII (TGFβ receptor type II) and ActRIIB (Activin receptor type II B) type II receptors, respectively, and type I receptors TβRI (also known as ALK5, Activin receptor-like kinase 5) and ALK4/7, respectively. This leads to phosphorylation of the receptor Smads (R-Smads, composite of Sma (smaller, Caenorhabditis Elegans) and Mad (mothers against decapentaplegic, Drosophila Melanogaster)) 2 and 3 by the type I receptor. BMPs, on the other hand, such as BMP2, 4 and 7 bind to BMPRII (BMP receptor type II) type II receptor and ALK3/6 type I receptor that leads to phosphorylation of R-Smads 1, 5 or 8. In either case, the phosphorylated R-Smads form a complex with a common Smad (co-Smad), Smad4 in vertebrates, which translocates to the nucleus where it interacts with transcription factors to initiate or suppress transcription. Reviewed in Feng et al. (2005).
### 1.2.1.2 Wnt Signalling

Wnt (wingless-related mouse mammary tumour virus integration site) signalling also plays a crucial role in early embryo axis formation and mice with *Wnt3* null mutations fail to form early germ layers (Liu et al. 1999). As many as 19 Wnt mammalian homologues have been identified and play key developmental roles in gastrulation and further tissue patterning. *Wnt* genes encode secreted cysteine-rich signalling proteins that regulate cell processes such as proliferation, differentiation and movement. The most studied Wnt signalling pathway is the canonical pathway that acts primarily through the intracellular signalling molecule β-catenin. In the absence of Wnt ligand, β-catenin is complexed with Axin and is phosphorylated by casein kinase 1α (CK1α) and glycogen synthase kinase 3β (GSK3β) destining β-catenin for ubiquitination and degradation. However, when Wnt ligand is present the Wnt protein forms a signalling complex with the Frizzled (Fz) family of receptors and LRP (low density lipoprotein receptor-related protein) 5 and LRP6 coreceptors. This leads to the association of Dishevelled (Dvl) with the Frizzled receptor and ultimately inhibits the phosphorylation of β-catenin by GSK3β. This allows for the nuclear accumulation of β-catenin where it associates with the TCF/LEF (T-cell factor/lymphoid enhancer factor) family of transcription factors leading to transcriptional activation. Reviewed in Katoh (2007) and Nusse (2008).

The Wnt pathway also interacts with TGFβ through GSK3β. It has been shown that the receptor Smads contain a region, termed the linker region, that is susceptible to phosphorylation by activated GSK3β. This prevents its nuclear accumulation and targets it for degradation (Fuentealba et al. 2007). Through activation of the Wnt receptors and inhibition of GSK3β, Wnt pathway activation is able to prolong BMP signalling and Smad1 activation.
1.2.1.3 Fibroblast Growth Factor Signalling

Fibroblast growth factor (FGF) signalling has also been shown to play a central role in regulating embryogenesis. The cells of a pre- and post-implantation embryo express FGF4 (Niswander et al. 1992) and FGF2 is commonly used to maintain human embryonic stem cells in an undifferentiated state (Thomson et al. 1998; Xu et al. 2005). Fibroblast growth factor are soluble proteins that signal through transmembrane receptor tyrosine kinases (RTK). Most RTKs are monomeric but binding of the ligand to the extracellular domain induces dimerization of the receptor-ligand complex. In the unbound, unstimulated state, the intrinsic kinase activity of the RTK is very low, however, once dimerized the RTK is activated causing phosphorylation of the cytosolic tyrosine residues. The phosphorylation of these residues leads to the activation of a membrane-bound protein called Ras. Activated Ras then leads to a cascade of protein kinases causing the activation of the kinase MAP (mitogen activated protein). Activated MAP kinases then form dimers which translocate to the nucleus to activate various transcription factors (Lodish et al. 2004). Additionally, it has been shown the FGF signalling pathway interacts with the TGFβ pathway through MAP kinase in a very similar fashion as GSK3β mentioned above. MAP kinases are able to phosphorylate receptor Smads in the linker region which prevents their nuclear accumulation and transcriptional activity (Kretzschmar et al. 1997). In this way, FGF signalling is able to regulate the TGFβ pathway to control its activity.

1.2.2 Gastrulation, Hematopoietic and Pancreatic Development in vivo

With an introduction to the signalling pathways responsible embryogenesis and proper tissue patterning, the early stages of development of the mammalian embryo will now be discussed with a particular emphasis on the tissues that give rise to the hematopoietic system and pancreatic organs.
1.2.2.1 Gastrulation

In the post-implantation embryo, gastrulation marks one of the earliest morphological changes when tissue formation and germ layer development begins. Starting at roughly embryonic day 6 (E6) of the developing mouse embryo the three primary germ layers (ectoderm, endoderm and mesoderm), from which all somatic cell types are derived, begin to emerge. The ectoderm gives rise to neural tissues such as the central nervous system as well as the outer epithelium of the body. The endoderm gives rise to the gut such as the liver, lungs and pancreas, while the mesoderm gives rise to skeletal muscle, bones, and the hematopoietic system. This tissue patterning is the result of morphogenetic signals secreted by the epiblast and the surrounding extraembryonic tissue. The epiblast secretes Nodal, while the proximal extraembryonic endoderm (ExE) secretes bone morphogenetic proteins (BMP), and Wnt ligands. In contrast, the distal ExE, and later the anterior ExE, secretes antagonists to these morphogens including Cerberus1, Lefty1 (left-right determination factor 1), and DKK1 (dickkopf 1) (Pfister et al. 2007). This creates a signalling gradient along the proximal/distal axis that leads to the development and distal expansion of the primitive streak starting at the proximal-posterior region of the embryonic cup (Brennan et al. 2001; Lowe et al. 2001; Vincent et al. 2003). It is in the primitive streak where both the definitive endoderm and mesoderm emerge and this region is marked by the expression of T-Brachyury (T-Bry), Goosecoid (GSC) and Mixl1 (Mix 1 like homeobox 1). Reviewed in Pfister et al. (2007). At roughly E7.5 when the primitive streak reaches the distal tip of the egg cylinder, a distinct morphological object, known as the node, becomes apparent. This structure has a crucial role in organizing and patterning the midline axis of the embryo (Nagy 2003).
1.2.2 Hematopoiesis

During mouse development hematopoiesis is initiated independently at two distinct sites: 1) the extraembryonic yolk sac; and 2) the aorta-gonad-mesonephros (AGM) region in the embryo proper. Extraembryonic, also known as primitive, hematopoiesis first emerges from clusters of primitive mesoderm in the extraembryonic area at E8. These clusters, known as yolk sac blood islands, give rise to both endothelial and hematopoietic cells leading researchers to postulate the existence of a bipotential ‘hemangioblast’ giving rise to both endothelial and hematopoietic lineages (Sabin 1920; Murray 1932). This has since been confirmed using both mouse and human ESC models (Choi et al. 1998; Nishikawa et al. 1998; Kennedy et al. 2007; Zambidis et al. 2007). This extraembryonic hematopoiesis, however, is characterized by predominantly nucleated erythroblasts expressing strictly embryonic and fetal hemoglobins (Barker 1968; Brotherton et al. 1979), the lack of lymphoid progenitors, and the inability to repopulate adult mice. Reviewed in Tavian et al. (2005) and Zambidis et al. (2006). This transient wave of
hematopoiesis is thought to provide a temporary hematopoietic system for the developing embryo.

The appearance of HSC with the ability to generate complex hematopoietic progenitors (myeloid, lymphoid, erythroid) and to repopulate adult mice first appears in the AGM region of the E9 mouse embryo proper (Medvinsky et al. 1993; Tavian et al. 2001). During gastrulation, mesoderm cells in the posterior region of the primitive streak migrate laterally out of the primitive streak to form the splanchnopleura and dorsal aorta. It is in this region, the AGM, where embryonic hematopoietic cells first emerge. It has been shown that this embryonic source of hematopoiesis, known as definitive hematopoiesis, also has its origins in a bipotential hemogenic endothelium lining the wall of the dorsal aorta (Dieterlen-Lievre et al. 1994; Jaffredo et al. 1998; Nishikawa et al. 1998).

Signalling gradients and morphogens have also been described in the *Xenopus* embryo. A *Xenopus* homologue of Activin A, XTC-MIF (Xenopus cell line XTC mesoderm inducing factor) (Savage et al. 1989; Green et al. 1990; Green et al. 1990; Smith et al. 1990), has been shown to induce ventral mesoderm, dorsal mesoderm, and neural tissue at low, moderate and high concentrations, respectively suggesting an instructive action of XTC-MIF. Similar *Xenopus* studies have also shown a role for BMP4 to induce hemogenic mesoderm from early mesodermal precursors during gastrulation (Dale et al. 1992; Maeno et al. 1996; Huber et al. 1998). These studies show that BMP4 acts following gastrulation to preferentially induce ventral hemogenic mesoderm rather than axial tissue. The hemogenic role of BMP4 appears to be conserved in humans where it has been found that *BMP4* is expressed at high levels in the AGM, specifically in regions of densely packed cells underlying intra-aortic hematopoietic clusters suggesting its direct role in specifying hematopoietic cells from mesodermal precursors (Marshall et al. 2000).
Other signalling proteins have also been identified as inducers of hemogenic mesoderm. Mouse embryos with homozygous mutations for Flk1 (fetal liver kinase 1), which encodes for a receptor of vascular endothelial growth factor (VEGF) (Millauer et al. 1993), undergo gastrulation but die at E9 due to defects in primitive hematopoietic and endothelial differentiation. Moreover, when Flk1−/− mESC are made into chimeric embryos, they are unable to contribute to definitive hematopoiesis demonstrating that the gene is required for both types of hematopoiesis (Shalaby et al. 1995), suggesting its importance in not only vascularization but also hematopoiesis.

A variety of other hemogenic cytokines have also been identified as maintaining or expanding HSC in culture including Flt3-ligand, stem cell factor (SCF), granulocytic colony stimulating factor (G-CSF), interleukin-3 (IL-3) and IL-6 (Bhatia et al. 1997; Conneally et al. 1997; Brugger et al. 2000; Zheng et al. 2003). These factors expand the number of long-term culture-initiating cells (LTC-IC) and improve their engraftment ability in subletheally irradiated nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice models. Due to their positive effects on somatic HSC expansion, they have also been applied to deriving hematopoietic lineages from hESC (Chadwick et al. 2003) but a later report has shown that the addition of G-SCF, IL-6, and IL-3 are not required for hemogenic induction from hESC (Pick et al. 2007).

1.2.2.3 Pancreatic Development

During gastrulation, the definitive (or embryonic) endoderm originates from the anterior region of the primitive streak between E6 and E6.5 (Lawson et al. 1986). The endoderm precursors migrate through the primitive streak and intercalate with the overlying extraembryonic endoderm. Following gastrulation, gut endoderm appears as a sheet of cells stretching from the primitive streak to the anterior region of the embryonic cup. This flat sheet then elongates and
forms a primitive gut tube along which the different endoderm lineages are derived. The liver emerges from the most anterior portion of the primitive gut tube while the most posterior region forms the hindgut from which with the large intestine and colon are formed. In between the anterior and posterior regions lies the primitive foregut and midgut, which give rise to the stomach, duodenum, and pancreas. Reviewed in Wells et al. (1999). The entire primitive gut tube is marked by the expression of hepatocyte nuclear factor-1β (HNF1β) (Coffinier et al. 1999), FoxA2 (forkhead box A2, or HNF3β) (Sasaki et al. 1993), and HNF4α (Duncan et al. 1994). At this time pancreatic development requires retinoic acid signalling as well as the inhibition of hedgehog signalling (Kim et al. 1998; Hebrok et al. 2000; Stafford et al. 2004). The developing pancreatic epithelium expresses hepatocyte nuclear factor-6 (HNF6) (Rausa et al. 1997), insulin promoter factor-1 (IPF1, or pancreas/duodenum homeobox-1, PDX1) (Wilson et al. 2003), and motor neuron and pancreas homeobox 1 (MNX1) (Wilson et al. 2003), which are important regulators of pancreatic development.

1.2.3 Embryonic Stem Cells

Embryonic stem cells (ESC) are the in vitro equivalent of the inner cell mass (ICM) of a blastocyst stage embryo and were first derived from mice (Evans et al. 1981; Martin 1981) and researchers have since isolated ESC from other vertebrates including humans (Thomson et al. 1998) and other primates. Although ESC are removed from their native embryonic environment, they nonetheless retain the capacity to develop into all cell types found in an adult, as demonstrated by chimera (Bradley et al. 1984) and tetraploid embryo complementation (Nagy et al. 1993) experiments where ESC are reintroduced into a developing embryo. This unique ability coupled with their capacity to expand and self-renew indefinitely gives ESC great potential for cell-based regenerative medicine applications. The commitment of embryonic stem cells from a
pluripotent state to more restricted cell types, a process known as differentiation, will now be discussed as it relates to the in vivo model discussed above.

1.2.3.1 Primitive Streak-like Development from Embryonic Stem Cells

1.2.3.1.1 Mesendoderm- and Primitive Streak-like Precursors

A bipotential mesendoderm precursor capable of giving rise to both endoderm and mesoderm lineages has been well characterized in the nematode worm, Caenorhabditis Elegans (Sulston et al. 1983), as well as the frog, Xenopus Laevis (Rodaway et al. 2001), but its existence in mammalian species is more contentious. Several attempts have been made using mouse embryonic stem cells but have fallen short of definitively proving the existence of a developmentally restricted single cell capable of giving rise to both endoderm and mesoderm lineages. Tada and colleagues inserted a green fluorescent protein (GFP) reporter into the Goosecoid (GSC) gene locus, an early marker of mesoderm and endoderm differentiation (Norris et al. 2002), to monitor mesendoderm differentiation (Tada et al. 2005). Using Activin A, a protein closely related to Nodal that signals through the same set of receptors and downstream effectors, they were able to induce GSC expression and show that a population expressing GFP-goosecoid, epithelial-cadherin, and platelet-derived growth factor receptor (PDGFR) α1 is capable of giving rise to mesoderm and endoderm lineages. Clonogenic single cell assays, however, were limited to the measurement of early transcription factor and cell surface marker expression and no functional output. Another report also attempted to generate developmentally restricted mesendodermal clonal cell lines through sustained activation of the Wnt pathway (Bakre et al. 2007). The functional assays shown in the study, however, are restricted to the mesoderm lineage and do not show production of definitive endoderm. These results
demonstrate that although a bipotential mesendoderm precursor has been shown to exist in lower order species there is no conclusive evidence thus far to prove its existence in mammals.

**1.2.3.2 Mechanisms Governing Mesoderm and Endoderm Differentiation**

Regardless of the existence of a mesendoderm precursor, the molecular mechanism that govern whether a pluripotent, or restricted multipotent, cell will become endoderm or mesoderm fated remains poorly understood. However, what is known in several *in vivo* and *in vitro* embryonic stem cell models will be discussed here.

**1.2.3.2.1 Foundations in the Xenopus Model**

It has been shown that the direct action of Activin/Nodal signalling on mesoderm and endoderm differentiation is the result of an Activin responsive element contained in the promoter region of the Mix family of homeobox genes in the *Xenopus* embryo (Huang et al. 1995; Chen et al. 1996). It was determined that the downstream effector of Activin signalling, XSmad2 (*Xenopus* homologue of Smad2), forms a complex with a transcriptional partner named forkhead Activin signal transducer-1 (FAST-1), and later renamed FoxH1 (forkhead box H1). Its homologue was subsequently identified in mammalian species and further shown that in mice that Smad2, along with Smad4, forms a complex with FoxH1 that binds directly to the *MIXL1* (Mix1 homeobox-like 1) promoter region and induces its transcription (Hart et al. 2005). However, FoxH1 can also act as a negative regulator of *MIXL1* expression by recruiting Goosecoid to the FoxH1/Smad2/Smad4 complex which suppresses the expression of *MIXL1* to act as a negative feedback loop (Izzi et al. 2007). Other genes required for mesoderm and endoderm differentiation such as Goosecoid and Lim1 also contain Activin responsive elements further demonstrating the role of Activin/Nodal signalling (Labbe et al. 1998; Watanabe et al. 2002).
Although Smad1 and Smad2 are structurally very similar, the region within Smad2 responsible for binding with FoxH1 is different in Smad1 such that it does not interact with FoxH1 nor does it induce \textit{MIXL1} expression (Attisano et al. 2001; Hart et al. 2005). Despite this, Smad1 activation through BMP signalling is required for primitive streak development and mesoderm differentiation (Winnier et al. 1995; Fujiwara et al. 2002; Rivera-Perez et al. 2005). This can be explained by reports describing an analogous BMP responsive element contained in the promoter regions of genes required for ventral patterning and mesoderm formation in the \textit{Xenopus} embryo. Moreover, BMP signalling involves a transcriptional coactivator termed Olf-1/EBF associated zing finger (OAZ) that interacts with XSmad1, but not XSmad2, to initiate transcription (Hata et al. 2000). Direct targets of Smad1 signalling include \textit{Xvent-2b}, a homeobox gene involved in ventral patterning of the \textit{Xenopus} embryo (Henningfeld et al. 2000) as well as the \textit{Xenopus} Brachyury homologue, \textit{Xbra}. These results demonstrate the role of Smad1 activation through BMP signalling in ventral patterning and mesoderm formation in the \textit{Xenopus} embryo.

\textit{1.2.3.2.2 Extending into the Mouse Embryonic Stem Cell}

TGFβ and Smad signalling have also been shown to induce endoderm and mesoderm differentiation in mammalian species. Using mouse embryonic stem cells is found that high levels of Activin/Nodal signalling specifies definitive endoderm differentiation while low levels of Activin/Nodal or BMP signalling specifies mesoderm differentiation (Johansson et al. 1995; Kubo et al. 2004; Ng et al. 2005; Tada et al. 2005; Gadue et al. 2006; Nostro et al. 2008; Shiraki et al. 2008). This is similar to the \textit{Xenopus} model described above where Smad2 activation targets primarily endoderm-related genes while Smad1 activation targets mesoderm genes. This suggests a balance between Smad2 and Smad1 activation where Smad2 induces anterior cell
fates while Smad1 has the effect of posteriorizing primitive streak-like cells. This model is supported by the observation that mESC derived definitive endoderm precursors are characterized by high levels of phosphorylated (p)Smad2 and low levels of pSmad1 while mesoderm precursors are characterized by high levels of pSmad1 (Shiraki et al. 2008).

The involvement of Wnt signalling, as well as Smad1 and Smad2 activation, in endoderm and mesoderm development has also been suggested through studies using mouse embryonic stem cells. Several studies have shown that Wnt signalling is required for the induction of a primitive streak-like phenotype (Gadue et al. 2006; Nostro et al. 2008). The implication of Wnt signalling in primitive streak development is supported by studies showing that the β-catenin-LEF-1/TCF transcription factor complex promotes Nodal expression during gastrulation in the Xenopus embryo and in mESC (McKendry et al. 1997; Gadue et al. 2006). However, inhibition of Wnt signalling in the presence of Activin/Nodal signalling blocks mesoderm and endoderm differentiation suggesting key targets of Wnt signalling in primitive streak development. This is supported by studies using mouse embryos and mESC models showing that T-Bry is a direct target of the Wnt-β-catenin signalling pathway (Yamaguchi et al. 1999; Arnold et al. 2000). Moreover, it has been shown that high concentrations of Wnt3a alone is able to induce mesoderm differentiation, but little to no endoderm (Gadue et al. 2006) – an effect that can be explained by the deactivation of GSK3β, a known antagonist of Smad1 signalling (Fuentealba et al. 2007).

1.2.3.2.3 From Mouse to Human Embryonic Stem Cells

Despite that mesoderm and endoderm differentiation is mouse embryonic stem and other animal models is fairly well described, there is a significant lack of understanding when it comes to human embryonic stem cells. A detailed analysis of Smad signalling in hESC with respect to
mesoderm and endoderm differentiation has yet to be performed. Moreover, despite that many developmental mechanisms are conserved amongst mouse and human, an important distinction has been observed whereby Activin/Nodal signalling alone is insufficient to induce mesoderm differentiation from hESC. Sustained treatment with Activin A, regardless of concentration does not induce mesoderm differentiation and primarily results in endoderm differentiation at higher concentrations (D'Amour et al. 2005; D'Amour et al. 2006; McLean et al. 2007; Kroon et al. 2008). This suggests that a greater activation of Smad1 is required in hESC for mesoderm differentiation, or greater basal activation of Smad1 is present in mESC. Also, although Wnt signalling has been found to play key roles in mesoderm development of mESC, there are no such reports to date implicating its role in hESC. An improved understanding of not only Smad signalling, but also other developmental signalling pathways is required in order to fully exploit the potential of human embryonic stem cells. By understanding these mechanisms, embryonic stem cell differentiation can be more precisely controlled in order to generate specific cell types and avoid the generation of undesired cell types which would otherwise need to be removed through cell sorting techniques such as fluorescent or magnetic activated cell sorting.

1.2.3.3 Other Mechanisms Regulating Embryonic Stem Cell Differentiation

1.2.3.3.1 Cell-cell and Extra-cellular Matrix Mediated Interactions

Factors other than soluble ligands have also been implicated in ESC derived hematopoiesis. It has been shown that hematopoietic development from mESC, without the support of a stromal layer, is completely inhibited when cultured as an attached colony during the first 3 days of differentiation but is not impaired if cultured initially in suspension and then reattached (Dang et al. 2002). This suggests a cell-cell contact or extra-cellular matrix- (ECM) adhesion mediated process required only for early hemogenic mesoderm induction and not for subsequent
hematopoietic specification and expansion. This also suggests that stromal coculture
differentiation acts in part by fulfilling this cell-contact/adhesion step.

1.2.3.3.2 Niche-mediated Control of Embryonic Stem Cell Differentiation

The importance of the stem cell niche on self-renewal and differentiation of both mESC and hESC has been previously reported (Davey et al. 2006; Peerani et al. 2007). These reports describe how the radial organization of ESC coupled with auto/paracrine signalling loops affect cell fate in the absence or withdrawal of growth factors. Cells near the centre of colonies and at a higher local cell density show increased pluripotency due to the secretion of self-renewal factors while cells at lower densities secrete excess differentiating factors leading to loss of pluripotency. It is also shown that the balance of self-renewal and differentiation can be controlled through the use of micropatterning techniques to independently control colony size (Peerani et al. 2007; Peerani In Press). Further data (Peerani, unpublished) also shows that control of colony size can be used to dictate the fate of differentiation hESC where larger colonies assume a neural fate while smaller colonies predominantly differentiate into extraembryonic endoderm but a small proportion show primitive streak-like phenotype. Moreover, it has also been shown that spatial patterns can affect the proliferation of endothelial cells through a process mediated by mechanical stresses (Nelson et al. 2005). These reports highlight the importance of spatial organization and cell density in the stem cell niche as well as the ability to control differentiation by manipulating the niche in the absence of exogenous growth factors.
1.3 Research Objectives

The overall objective of this project is to optimize conditions allowing for hematopoietic differentiation of hESC. However, the differentiation of ES cell to hematopoietic progenitor is a multifaceted process involving complex signalling interactions governing sequential cell lineage specification. Therefore, this project has been subdivided into three specific aims:

- **Specific Aim 1**: Induction of mesendoderm/primitive streak-like development from hESC using a combination of BMP2 and Activin A
- **Specific Aim 2**: Optimization of conditions allowing for mesoderm specification from the mixed mesendoderm precursors
- **Specific Aim 3**: Differentiation of mesoderm precursors towards the hematopoietic lineage

The deconstruction of the complex process of hematopoiesis from hESC allows for identifying signalling process involved during the step-wise differentiation of hESC similar to the ontogeny of hematopoietic stem cells *in vivo*.

1.4 Hypothesis

It is hypothesized that both exogenous and endogenous signalling factors can influence the differentiation of hESC towards mesoderm and hemogenic mesoderm lineages. Specifically, Activin/Nodal and BMP signalling have been shown to be crucial in the development of mesoderm lineages in not only *in vivo* but also *in vitro* ESC models. Due to the observation that signalling gradients are responsible for patterning early mouse embryo, it is anticipated that different concentrations of exogenously added Activin/Nodal and BMP signalling ligands will lead to different cell fates. Moreover, it is also believed that the endogenous secretion of agonists or antagonists to the TGFβ pathway, and possibly others, in response to the added cytokines will also play a central role in fate decision. By controlling the colony size of hESC, one can dictate
the level of not only exogenous but also endogenous signalling to optimize the proportion of mesoderm fated cells. In addition, controlling colony size decreases heterogeneity by maintaining constant local cell density and endogenous signalling effects within a given sample. Furthermore, it is hypothesized that this mesoderm fated population can be differentiated further towards hemogenic mesoderm through the addition of hemogenic cytokines through three-dimensional embryoid body differentiation. It is anticipated that this optimal hemogenic mesoderm population will correlate with a maximum yield of hematopoietic progenitors.
2 Specific Aim #1: Induction of Mesendoderm/Primitive Streak-like Development from hESC

2.1 Overview

Using embryogenesis as a model, this objective takes the first step in developing definitive hematopoiesis from human embryonic stem cells. Embryonic stem cells represent the in vitro analog of the inner cell mass of the blastocyst stage embryo. Prior to mesoderm formation in the mouse embryo, the primitive ectoderm, or epiblast, migrates through the primitive streak under the influence of an Activin/Nodal, BMP and Wnt signalling gradient. With this model as a guide, this objective is to use a combination of BMP2 and Activin A to induce primitive streak-like development from hESC. Although studies from mouse embryos have shown the activity of BMP4 in primitive streak induction, BMP2 and BMP4 have been found to be functionally interchangeable (Massague 1990; Sampath et al. 1993; Lyons et al. 1995; Zhang et al. 1996). The endpoint of this objective is to determine the optimal concentration of both Activin A and BMP2 to maximize primitive streak-like development.

2.2 Materials and Methods

2.2.1 Human Embryonic Stem Cell Culture and Differentiation

Human embryonic stem cell lines H9 (Technion – Israeli Institute of Technology), HES2 (WiCell) and I6 (Technion – Israeli Institute of Technology) were used for all experiments described in this thesis. H9, HES2, and I6 human ES cell lines were maintained on irradiated mouse embryonic feeder (mEF) cells plated at a density of 20,000 cells per cm². H9 and I6 human ES cell lines were maintained in serum-free medium consisting of knockout (KO)-Dubelcco’s modified Eagle’s medium (DMEM, Invitrogen) and 20% KO-serum replacement.
(SR, Invitrogen) supplemented with 4ng/mL recombinant human (rh) basic fibroblast growth factor (bFGF, PeproTech). Cells were dissociated into clumps using 0.1 % collagenase IV (Invitrogen) and passaged at a ratio of 1:6 every 5 to 6 days. The HES2 human embryonic cell line was maintained in serum-free medium consisting of DMEM-F12 (Invitrogen) supplemented with 2 mM L-Glutamine (Invitrogen), 50 u/mL Penicillin/Streptomycin (Invitrogen), 20% KO-SR, 1 × nonessential amino acids (NEAA, Invitrogen), 0.1 µM β-mercaptoethanol (BME, Sigma), 5% mEF conditioned medium, and 20 ng/mL bFGF. HES2 cells were dissociated into single cells using 0.25% Trypsin-EDTA (Ethylene diamine tetra-acetic acide, Cellgro) and passaged at a ratio of 1:6 every 4 to 5 days.

In order to remove mEFs prior to differentiation cells were passaged as clumps onto tissue-culture treated plastic coated with growth factor reduced Matrigel™ (GFR-MG, 1:30, BD Biosciences) in defined serum-free medium (XV-FAT) consisting of X-VIVO10™ medium (Cambrex) supplemented with 2 mM GlutaMAX™ (Invitrogen), 1× nonessential amino acids (NEAA, Invitrogen), 0.1 µM β-mercaptoethanol (BME, Sigma), 40 ng/mL bFGF, 0.1 ng/mL rh-transforming growth factor β-1 (TGFβ1, R&D Systems), and 5 ng/mL rh-Activin A (R&D Systems). Following overnight seeding in XV-FAT maintenance medium, medium was replaced with X-VIVO10™ medium supplemented with 2 mM GlutaMAX™ (Invitrogen), 1× nonessential amino acids (NEAA, Invitrogen), 0.1 µM β-mercaptoethanol (BME, Sigma), and indicated cytokines. Recombinant human bone morphogenetic protein-2 (BMP2, R&D Systems) and rhActivin A (R&D Systems) and rhWnt3a (R&D Systems) were used at 50 ng/mL unless otherwise indicated. TGFβ inhibitor SB431542 (Sigma) was used at a concentration of 10 nM.
2.2.2 Immunofluorescence

Cells were fixed in 3.7% para-formaldehyde in phosphate buffered solution (PBS, Invitrogen) for 15 minutes at room temperature. Following fixation, cells were blocked and permeabilized for 45 minutes at room temperature with PBS containing 0.1% Triton X-100, 10% donkey serum (Jackson ImmunoResearch) and 1% BSA (bovine serum albumin, Sigma). Cells were then rinsed in PBS three times and incubated overnight at 4°C with primary antibody at indicated dilution in PBS containing 10% fetal bovine serum (FBS, Gibco). Following primary antibody incubation, cells were rinsed three times with PBS and incubated at room temperature for 90 minutes with the appropriate secondary antibody and Hoechst 33342 nuclear dye (1 µg/mL, Invitrogen). Goat anti-human Brachyury antibody (R&D Systems) was used at 0.5 µg/mL, rabbit anti-phospho-Smad1/5/8 antibody (Cell Signaling Technologies) was used at 1:200 dilution, mouse anti Smad2/3 antibody (BD Biosciences) was used at 1:200 dilution, mouse anti-human Sox17 (R&D Systems) was used at 10 µg/mL, rabbit anti-FoxA2 (Abcam) was used at 1:4000 dilution, goat anti-HNF1β (Santa Cruz Biotechnology) was used at a dilution of 1:100, goat anti-HNF4α (Santa Cruz Biotechnology) was used at a dilution of 1:100. Primary antibodies were counterstained with Alexa Fluor® 488 donkey anti-goat IgG secondary antibody (Invitrogen, 1:200), AlexaFluor® 647 donkey anti-rabbit IgG secondary antibody (1:200), or AlexaFluor® 555 donkey anti-mouse IgG secondary antibody (1:200). Fluorescent images were taken with an AxioCam MRm (Zeiss) digital fluorescent camera connected to an Axio Observer.D1 (Zeiss) fluorescent microscope.

2.2.3 Quantitative Immunofluorescence and ‘Neighbours Analysis’

Quantitative image analysis was performed using the ArrayScan® VTI imaging platform and the Target Activation assay algorithm. The imaging platform captures successive fluorescent images
in order to image an entire area of a well of a 96-well plate. The Target Activation assay
algorithm uses the Hoechst nuclear stain to identify and draw a mask surrounding the nuclei of
each cell. The average fluorescent intensity of each channel is then calculated for the area
encompassed by the nuclear mask (Figure 2-1). The spatial coordinates \((x, y)\) of the centroid of
each nucleus are also recorded (Figure 2-2). Positive gating for cells defined as expressing a
given protein is established based on negative controls stained with secondary antibody only.

Figure 2-1 Example of Target Activation Assay algorithm. Mask is drawn around the nucleus of
each cell, and the mask is applied to all other channels to calculate average nuclear fluorescent
intensity of desired channel
Figure 2-2 The nuclear mask (Figure 2-1) is used to calculate the \((x, y)\) coordinates of the centroid of each cell. For every cell in a well, the Neighbours Analysis algorithm iteratively calculates the number of cells within a 200 µm radius for that cell.

An algorithm, termed ‘Neighbours Analysis’ was performed using the spatial information and fluorescent intensities gathered from quantitative immunofluorescence and is originally described by Peerani and colleagues (Peerani et al. 2007). The algorithm iteratively calculates for each cell within a given well the total number of neighbouring cells within a 200 µm radius (Figure 2-2). A radius of 200 µm was chosen because it is the largest radius for which a significant trend in Smad signalling or Brachyury expression is observed.

2.2.4 Mathematical Modeling

Fitting the empirical data of Bry expression frequency as a function of BMP2 and Activin A concentration was performed using JMP IN software (version 3.2.6, http://www.jmp.com). The
data was initially fitted using a quadratic model, which includes all main-effect coefficients, quadratic coefficients, and second-order interaction coefficients. All terms that were found to be insignificant (p>0.05) were removed from the model, the data was fitted again to the revised model, and the process was repeated until only significant terms remained.

### 2.2.5 Quantitative Gene Expression Analysis

Total ribonucleic acid (RNA) was extracted from cells using TRIzol® reagent (Invitrogen) and purified using the RNeasy® Mini RNA isolation kit (Qiagen). Complementary deoxyribonucleic acid (cDNA) was synthesized from purified RNA using the SuperScript™ III Reverse Transcriptase kit. cDNA synthesized without reverse transcriptase was used as a negative control. Real time quantification of transcripts using polymerase chain reaction was performed using *Power SYBR® Green PCR Master Mix* (Applied Biosystems) and a 7900HT Fast Real-Time PCR System (Applied Biosystems). Both β-actin and GUSB (glucuronidase beta) were used as endogenous controls and yielded very similar results, but the transcript levels presented here were calculated as $2^{\Delta(\Delta^{Ct})}$ normalized to β-actin. The list of primer sequences used is listed in Table 2-1. Total RNA isolated from human fetal lung (Biochain), human fetal brain (Biochain), and Caco-2 human colorectal adenocarcinoma cell line (American Type Culture Collection) were used as positive controls.

<table>
<thead>
<tr>
<th>Transcript Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Actin – forward</td>
<td>5’-CATGTACGTTGCTATCCAGGC</td>
</tr>
<tr>
<td>β-Actin – reverse</td>
<td>5’-CTCCTTAATGTCACGCACGAT</td>
</tr>
<tr>
<td>GUSB – forward</td>
<td>5’-GTCTGCGGCATTTTGTCCGG</td>
</tr>
<tr>
<td>GUSB – reverse</td>
<td>5’-ATGGCCGATAGTGATTCCGGAGC</td>
</tr>
<tr>
<td>Oct4 – forward</td>
<td>5’-TGGGGCTCGAGAAGGATGTG</td>
</tr>
<tr>
<td>Oct4 – reverse</td>
<td>5’-GCATAGTCGCTGCTTTGATCG</td>
</tr>
<tr>
<td>T-Bry – forward</td>
<td>5’-TGCTTCCCTGAGACCCAGTT</td>
</tr>
<tr>
<td>T-Bry – reverse</td>
<td>5’-GATCACTTCTTTTCCATCAAAG</td>
</tr>
<tr>
<td>MIXL1 – forward</td>
<td>5’-CCGAGTCCAGGATCCAGGTA</td>
</tr>
<tr>
<td>Primer</td>
<td>Sequence</td>
</tr>
<tr>
<td>--------</td>
<td>----------</td>
</tr>
<tr>
<td>MIXL1 – reverse</td>
<td>5’-CTCTGACGCGAGACTTGG</td>
</tr>
<tr>
<td>GSC – forward</td>
<td>5’-GAGGAGAAAGTGGAGGCTGTT</td>
</tr>
<tr>
<td>GSC – reverse</td>
<td>5’-CTCTGATGAGGCCAGCGCTT</td>
</tr>
<tr>
<td>KDR – forward</td>
<td>5’-ACTTTGGAACAGAACAAATATC</td>
</tr>
<tr>
<td>KDR – reverse</td>
<td>5’-TGAGGCACTTTCAGCAGAAA</td>
</tr>
<tr>
<td>Nodal – forward</td>
<td>5’-CCGGAGCGACACATCATCC</td>
</tr>
<tr>
<td>Nodal – reverse</td>
<td>5’-CCATCCACTGCCACATCTTCT</td>
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<tr>
<td>Wnt3 – forward</td>
<td>5’-CCACAACACGGAGCGAGAAC</td>
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<tr>
<td>Wnt3 – reverse</td>
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<tr>
<td>Cer1 – forward</td>
<td>5’-ACAGTGCCCTTCAGCCAGACT</td>
</tr>
<tr>
<td>Cer1 – reverse</td>
<td>5’-ACAACCTACTTTTTCAGCCAGCTT</td>
</tr>
<tr>
<td>Sox17 – forward</td>
<td>5’-GGCGCAGCAGAACATCCAGA</td>
</tr>
<tr>
<td>Sox17 – reverse</td>
<td>5’-CCAGCAGCTGGCCAGCAT</td>
</tr>
<tr>
<td>CDX2 – forward</td>
<td>5’-GGCTCTTCTCTAGAGAGCGAGT</td>
</tr>
<tr>
<td>CDX2 – reverse</td>
<td>5’-CCTTTTGTCTGCGGTTCT</td>
</tr>
<tr>
<td>FoxA2 – forward</td>
<td>5’-GGAGGCGGTGAAGATGGA</td>
</tr>
<tr>
<td>FoxA2 – reverse</td>
<td>5’-TCATGTTGCTCACGGAGAGTA</td>
</tr>
<tr>
<td>PDGFRα1 – forward</td>
<td>5’-AAATTGTGTCCACCGTACG</td>
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<tr>
<td>PDGFRα1 – reverse</td>
<td>5’-CGACACATAGTTCCAGAATCATG</td>
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<tr>
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<tr>
<td>Lefty1 – reverse</td>
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<tr>
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<tr>
<td>Mesp1 – reverse</td>
<td>5’-GTCTGCCAAGGACCCACTTC</td>
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<tr>
<td>Pax6 – forward</td>
<td>5’-ATGTGTGAGTTAAATTTGCCC</td>
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<tr>
<td>Pax6 – reverse</td>
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<td>Sox7 – forward</td>
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<td>Sox7 – reverse</td>
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<td>HNF1β – forward</td>
<td>5’-CAAGGGCACCCTATGAAGA</td>
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<td>HNF4α – forward</td>
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<td>MNX1 – forward</td>
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<td>MNX1 – reverse</td>
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</tr>
<tr>
<td>HNF6 – forward</td>
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</tr>
<tr>
<td>HNF6 – reverse</td>
<td>5’-TCCACATCTCCGGAAGGT</td>
</tr>
</tbody>
</table>

**Table 2-1** List of primer sequences used for quantitative gene expression analysis.
2.3 Results

2.3.1 Brachyury Expressing Mesoderm Cells Have High Smad2 Signalling

As BMP4 is well known to induce mesoderm differentiation of mouse and human ESC, the effect of BMP2 was investigated to examine if the mesoderm inducing effect is specific to BMP4. It was found that 48 hours of treatment with 50 ng/mL of BMP2 is effective at inducing the expression of Bry (Figure 2-3a), an established marker of early mesoderm differentiation (Rivera-Perez et al. 2005), suggesting that their common effector, Smad1, is responsible for mesoderm differentiation. Interestingly it is found that Bry expressing cells are also characterized by high levels of Smad2 nuclear localization indicating active Smad2 signalling (Figure 2-3a and Figure 2-4).

![Figure 2-3](image_url)

**Figure 2-3** Immunofluorescent images showing Bry expression and Smad2 nuclear localization. Panel a) BMP2 induces the expression of Bry but Bry expressing cells also show nuclear localization of Smad2. Panel b) Inhibition of Smad2 signalling using SB431532 (TGFβi) blocks nuclear localization of Smad2 and Bry expression. Panel c) Activin A alone induces nuclear localization of Smad2 but no Bry expression. Original magnification is 40×, scale bar represents 100 µm.

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Figure 2-4 Quantitative immunofluorescence shows that Bry expressing (+ve) cells are characterized by greater levels of nuclear Smad2.

Although BMP2 is not believed to directly activate Smad2 signalling, treatment with BMP2 induces the expression of *Nodal*, an endogenously secreted ligand that is an activating ligand of Smad2 (Figure 2-5). This suggests that Smad2 is activated indirectly through the endogenous expression of *Nodal* which in turn activates ALK4/7 and triggers nuclear translocation of Smad2. To investigate if the mesoderm inducing effect of BMPs is dependent on Smad2 signalling, hESC were treated with 50 ng/mL of BMP2 and SB431532 (TGFβi), a specific inhibitor of ALK4, ALK5, and ALK7, but has no effect on the BMP receptors ALK1, ALK2, ALK3, ALK6 (Inman et al. 2002; Laping et al. 2002). Treatment with TGFβi completely blocks nuclear accumulation of Smad2 as well as Bry expression (Figure 2-3b). This demonstrates that Smad2 signalling is required for mesoderm differentiation. To examine if Smad2 signalling alone is sufficient to induce mesoderm differentiation, hESC were treated with 0, 5, 25 or 50 ng/mL Activin A, a Smad2 activator, for 48 hours. A range of concentrations was tested due to previous observations that moderate concentrations are required for mesoderm differentiation of mESC. Despite the fact that Activin A alone effectively induces nuclear accumulation of Smad2, it is not
sufficient to promote Bry expression (Figure 2-3c). Together, these findings show that both Smad1 and Smad2 activation are required for mesoderm differentiation.

![Bar chart showing fold increase expression](image)

**Figure 2-5** Quantitative gene expression analysis reveals that treatment with BMP2 induces the expression of *Nodal*.

### 2.3.2 Activin A and BMP2 Synergize to Maximize Mesoderm Differentiation

Due to the finding that both Smad1 and Smad2 activation are required for mesoderm differentiation, combinations of different concentrations of BMP2 and Activin A were tested to determine if supplementing endogenously expressed Nodal with Activin A would further increase mesoderm differentiation. An initial dose-response using BMP2 and Activin A on the effect of Brachyury expression was determined using a high-throughput 96-well platform. The concentration of Activin A and BMP2 were independently varied from 0 to 50 ng/mL in X-VIVO 10™ serum-free medium. Brachyury protein expression was measured through quantitative immunofluorescence using the Cellomics™ ArrayScanV platform and Target Activation algorithm. This method allows for automated high-throughput analysis of different
samples using standard culture-ware. An alternative to quantitative immunofluorescence would be flow-cytometry, but would typically require 10 times the number of cells for a reliable measurement. An additional advantage of the ArrayScanV platform is that the spatial coordinates of each cell are also measured. This allows for the computation of geometric relationships between various cell types and the effects of local cell density (Davey et al. 2006; Peerani et al. 2007), which would be otherwise lost in a flow-cytometric analysis.

It was found that the frequency of Brachyury expressing cells increases with Activin A and BMP2 concentration in a dose dependent manner following 48 hours of treatment. The highest concentrations of Activin A and BMP2 tested (both 50 ng/mL) yielded the highest percentage of Brachyury expressing cells, 25.6 ± 0.9 % (Figure 2-6). As described above, it is also found that treatment with Activin A alone, regardless of concentration, resulted in minimal Brachyury expression, confirming previous reports (D'Amour et al. 2005; McLean et al. 2007) and demonstrating that Activin/Nodal signalling alone is not sufficient for mesoderm differentiation of hESC. Instead it is found that BMP signalling is also required and that it synergizes with Activin/Nodal to induce mesoderm differentiation. This is in agreement with the mouse embryo model where the primitive streak emerges from the proximal-posterior region of the embryonic cup, which also corresponds to the highest level of Nodal and BMP4 expression.
Further analysis was also performed to determine if the 48-hour time point chosen is the optimal time to measure mesoderm differentiation. Quantitative immunofluorescence and quantitative gene expression analysis was used to measure Bry expression following 12, 24, 48, 72 and 120 hours of treatment with Activin A and BMP2. These experiments showed that Bry expression is first detected at 12 hours, peaks at 48 hours, and declines thereafter (Figure 2-7) in agreement with previous studies (Gadue et al. 2006; McLean et al. 2007). This confirms that, under the conditions tested here, the 48 hours is an appropriate time point for analyzing early mesoderm differentiation.
Figure 2-7 Analysis of Bry protein (a) and gene expression (b) at various time points following treatment with BMP2 and Activin A showing that Bry expression peaks at 48 hours.

In order to quantitatively confirm if BMP2 and Activin A synergize, the numerical data were fitted to a quadratic model (Audet et al. 2002). The initial model is as follows:

\[
y = c_1 + c_2 \times [\text{BMP2}] + c_3 \times [\text{Activin A}] + c_4 \times [\text{BMP2}]^2 + c_5 \times [\text{Activin A}]^2 + c_6 \times [\text{BMP2}] \times [\text{Activin A}] \]

Eq. 2-1

where \( y \) represents the percent of cells expressing Brachyury, \( c_1 \) is the intercept term, \( c_2 \) and \( c_3 \) are the main effect coefficients of BMP2 and Activin A concentrations, respectively, \( c_4 \) and \( c_5 \) are the quadratic coefficients of BMP2 and Activin A concentrations, respectively, and \( c_6 \) is the second-order interaction coefficient of BMP2 with Activin A. An initial fit of the expression data with Eq. 2-1 shows that only the intercept term, BMP2 main effect coefficient, and second-order interaction coefficient are significant (\( p<0.05 \), Table 2-2)
Table 2-2 Table of parameter estimates and associated errors and statistical significance for quadratic model (Eq. 2-1)

<table>
<thead>
<tr>
<th>Term</th>
<th>Estimate</th>
<th>Error</th>
<th>pValue</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_1$</td>
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<td>0.91</td>
<td>0.0049</td>
</tr>
<tr>
<td>$C_2$</td>
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<td>0.07</td>
<td>0.0488</td>
</tr>
<tr>
<td>$C_3$</td>
<td>0.062</td>
<td>0.075</td>
<td>0.4266</td>
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<tr>
<td>$C_4$</td>
<td>0.00070</td>
<td>0.0013</td>
<td>0.6114</td>
</tr>
<tr>
<td>$C_5$</td>
<td>-0.0018</td>
<td>0.0014</td>
<td>0.236</td>
</tr>
<tr>
<td>$C_6$</td>
<td>0.0054</td>
<td>0.0010</td>
<td>0.0002</td>
</tr>
</tbody>
</table>

**R² Adjusted** 0.955447

The result that the quadratic terms are not significant suggests that the concentrations of BMP2 and Activin A have not reached saturation and that greater concentrations will induce even greater expression of Bry. However, the cost of high concentrations of cytokines limits the regular use of even greater concentrations. Once the insignificant terms were removed the data was fitted again to Eq. 2-1, but with the terms $c_3$, $c_4$ and $c_5$ removed:

$$y = c_1 + c_2 \times [BMP2] + c_6 \times [BMP2] \times [Activin A]$$

Eq. 2-2

All the terms of the revised model (Eq. 2-2) are significant and both the BMP2 main effect coefficient and the second-order interaction coefficient are positive demonstrating that Bry expression increases with increasing BMP2 concentration and that BMP2 and Activin A do in fact quantitatively synergize. A negative value would be indicative of an inhibitory relationship. The result that Bry expression increases with BMP2 concentration suggests that increasing levels of Smad1 signalling increases Bry expression. Moreover, the $R^2$ value does not decrease using the second model equation despite the removal of parameters. A plot of the predicted values versus the experimental value shows good agreement between predicted and experimental values (Figure 2-8). Additionally, a plot of the residuals as a function of the predicted value shows that the residuals are evenly distributed and the model does not show significant bias (Figure 2-9).
Table 2-3 Table of parameter estimates and associated errors and significance for simplified model (Eq. 2-2)

<table>
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<th>Estimate</th>
<th>Error</th>
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</tr>
</thead>
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<tr>
<td>$C_2$</td>
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<td>&lt;0.0001</td>
</tr>
<tr>
<td>$C_6$</td>
<td>0.0047</td>
<td>0.0006</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>$R^2$</td>
<td>0.96</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 2-8 Plot of experimental values and predicted values of Bry expression using the simplified quadratic model (Eq. 2-2)
Figure 2-9 Plot of residuals as a function of predicted values for simplified quadratic model (Eq. 2-2)

2.3.3 The Combination of BMP2 and Activin A Induces Both Mesoderm and Endoderm Precursors but Not Other Lineages

Although the combination of BMP2 and Activin A efficiently induces mesoderm differentiation, endoderm precursors also emerge as evidenced by expression of Sox17, a marker of definitive endoderm precursors (Figure 2-10). Quantitative gene expression analysis was performed to confirm the immunofluorescent results and additionally characterize the cell population. It was found that in addition to T-Bry (Brachyury), the expression of other early mesoderm-associated genes were also upregulated including KDR (kinase insert domain receptor, human homologue of Flk1, or vascular endothelial growth factor receptor 2, VEGFR2) (Kennedy et al. 2007), PDGFRα1 (platelet derived growth factor receptor alpha 1) (Davis et al. 2008), and Mesp1 (mesoderm posterior 1) (Saga et al. 1996) (Figure 2-11). However, the expression of endoderm-
associated genes were also upregulated including *MIXL1* (Mix1 homeobox-like 1) (Izumi et al. 2007), *GSC* (Goosecoid) (Perea-Gomez et al. 1999), and *Sox17* (SRY-box containing gene 17) (Kanai-Azuma et al. 2002) (Figure 2-11). The expression of both mesoderm- and endoderm-associated transcripts suggests the emergence of a mixed population including both mesoderm and endoderm precursors, reminiscent of the primitive streak, but there is no evidence of a bipotential mesendoderm precursors. Immunofluorescence and quantitative immunofluorescence shows that neither Bry nor Sox17 are found to be coexpressed following 48 hours of treatment with BMP2 and Activin A (Figure 2-10 and Figure 2-12), suggesting that lineage commitment to either mesoderm or endoderm has taken place. A previous report, however, has shown coexpression of Bry and Sox17 following 48 hours of treatment with Activin A and low concentrations of FBS (D'Amour et al. 2005). In the model proposed by D’Amour and colleagues, Activin is shown to induce Bry expression and the removal of Activin permits mesoderm differentiation. However, this is in the presence of serum, which is known to contain BMPs. In order to definitively prove the existence of a mesendoderm precursor, sorting cell populations and performing clonogenic assays would be required to show that a single cell can give rise to both endoderm and mesoderm but not endoderm lineages.
Figure 2-10 Immunofluorescent images of Sox17 and Bry expression. Original magnification is 40×, scale bar represents 100 µm.

Figure 2-11 Quantitative gene expression analysis showing expression of mesoderm- and endoderm-associated transcripts.
The population was further characterized using flow cytometry to assess Oct4 (Octamer-4, or POU5F1, POU domain class 5 transcription factor 1) and Sox2 (SRY-box containing gene 2) expression, transcription factors associated with pluripotency (Nichols et al. 1998; Avilion et al. 2003). As expected, cultures of undifferentiated hESC mostly contain cells coexpressing Oct4 and Sox2, indicative of pluripotency (Figure 2-13). However, following treatment with BMP2 and Activin A, Sox2 expression is highly downregulated while Oct4 expression remains high (Figure 2-13). This suggests that the cells are no longer pluripotent, due to loss of Sox2 expression, but are slowly undergoing differentiation with retention of Oct4. This accounts for the large proportion of cells that do not express either Bry or Sox17 (Figure 2-12), suggesting that these cells have lost pluripotency but have not yet expressed markers of mesoderm or endoderm differentiation.
Figure 2-13 Oct4 and Sox2 flow cytometry analysis of undifferentiated hESC and cultures treated with BMP2 and Activin A for 48 hours. Cells treated with secondary antibodies only were used as negative control.

Quantitative gene expression analysis was also used to assess the differentiation of other lineages. Minimal differentiation towards neural, extraembryonic endoderm, or trophectoderm lineages was found as evidenced by low expression of Sox2 (Ellis et al. 2004), Sox7 (Kanai-Azuma et al. 2002), and CDX2 (caudal type homeobox 2) (Beck et al. 1995), respectively (Figure 2-14), demonstrating that BMP2 and Activin A together preferentially drives mesoderm and endoderm differentiation.
2.3.4 Wnt Signalling Does Not Significantly Affect Mesoderm Differentiation in the Presence of Activin/Nodal and BMP Signalling

In addition to Nodal the expression of Wnt3 also increased following treatment with BMP2 (Figure 2-11) as previously reported (Nostro et al. 2008; Zhang et al. 2008). However, unlike Activin A, the addition of exogenous Wnt ligand (Wnt3a, 50 ng/mL) does not significantly increase Bry expression, nor does the addition of BIO (bromoindirubin oxime, 500 nM) a soluble GSK3β (glycogen synthase kinase 3β) inhibitor in the presence of Activin A and BMP2 (Figure 2-15). A previous report has also demonstrated that blocking the Wnt pathway using high concentrations of Dkk1, an inhibitor of Wnt signalling, in the presence of BMP4 does not affect mesoderm differentiation suggesting that Wnt signalling is dispensable for mesoderm induction from hESC in the presence of active BMP signalling, contrary to evidence found for mESC (Nostro et al. 2008).
2.3.5 Local Cell Density Correlates with Smad1 Signalling and Mesendoderm Differentiation

In addition to Bry, Sox17 (a transcription factor marking definitive endoderm precursors (Kanai-Azuma et al. 2002)) expressing cells are also detected following 48 hours of treatment with Activin A and BMP2. This demonstrates that both mesoderm and endoderm precursors are arising in this condition indicative of mesendoderm/primitive streak-like development. From the analysis of immunofluorescent images, it was observed that cells expressing Bry are typically found in areas of high cell density while cells expressing Sox17 are typically found in areas of low cell density. To quantify this observation a previously developed algorithm, termed ‘Neighbours Analysis’ (Peerani et al. 2007), was used in conjunction with the ArrayScan® VTI (Cellomics) imaging platform to measure the frequency of Bry expressing cells as a function of
the number of cells within a 200 µm radius. The number of neighbouring cells is used a measure of local cell density. Each cell within a well of a 96-well microplate, was binned according to the number of cells within a 200 µm radius of that cell (Figure 2-2). The frequency of Bry and Sox17 expressing cells was calculated for each bin and plotted. This algorithm quantitatively shows that Bry expressing cells are enriched in areas of high local cell density while Sox17 expressing cells are enriched in areas of low cell density (Figure 2-16). This relationship is visually confirmed through chloropleth maps with dots representing spatial location of cells and colour representing Bry or Sox17 protein level. It can be seen that expression of Sox17 is predominant at lower cell densities at the periphery of colonies while Bry expressing cells are enriched in areas of higher cell density towards the centre of colonies (Figure 2-17).

**Figure 2-16** Plot of Bry and Sox17 expression frequency as a function of local cell density. (*) indicates significance (p<0.05) over second adjacent bin.
To investigate a possible mechanism governing this effect a similar algorithm was employed to measure Smad1 signalling, measured by levels of nuclear phosphorylated Smad1 (pSmad1), as function of local cell density. Following a 90 minutes pulse with Activin A and BMP2 it was found that pSmad1 increases with local cell density (Figure 2-18) and is visually confirmed through a chloropleth map showing higher nuclear pSmad1 levels in areas of high local cell density (Figure 2-19). The 90 minute time point was chosen because Smad1 and Smad2 signalling peaks after 90 minutes of treatment before decaying to near or below baseline after 48 hours of treatment with BMP2 and Activin A (Figure 2-20). This suggests that a high Smad1 signalling environment is required for mesoderm differentiation whereas low Smad1 is required for definitive endoderm differentiation. The effect of Smad signalling on mesendoderm differentiation has been previously described in mESC where Foxa2 expressing definitive endoderm precursors are characterized by low levels of pSmad1 while Bry expressing mesoderm precursors are characterized by high levels of pSmad1 (Shiraki et al. 2008). A similar observation is made here. Although the average nuclear pSmad1 level for all cells has decreased

Figure 2-17 Chloropleth map showing spatial location of Sox17 and Bry expressing cells. Orange/red colour indicates protein expression, blue indicates no protein expression.
to near baseline after 48 hours, it is found that Bry positive cells maintain significantly greater levels of nuclear pSmad1 when compared to the Bry negative population (Figure 2-21) demonstrating a direct correlation between Smad1 signalling and Bry expression.

**Figure 2-18** Plot of nuclear pSmad1 intensity as a function of local cell density. (*) indicates p < 0.05.
Figure 2-19 Chloropleth map of nuclear pSmad1 intensity showing higher intensity in areas of greater local cell density. Orange/red colour indicates high intensity, blue indicates low intensity.

Figure 2-20 Smad1 and Smad2 activation kinetics following treatment with BMP2 and Activin A.
Figure 2-21 Quantitative immunofluorescence shows that Bry expressing cells are characterized by higher levels of nuclear pSmad1. (*) indicates p<0.05.

2.4 Discussion

The results presented here is the first detailed report of the synergistic effects of BMP and Activin/Nodal signalling in a dose-dependent manner on mesoderm differentiation from human embryonic stem cells. Previous reports has shown that Activin A alone is able to induce both mesoderm and endoderm differentiation of mESC, with high concentrations of Activin A promoting endoderm and medium to low concentrations promoting mesoderm differentiation (Smith et al. 1990; Johansson et al. 1995; Robertson et al. 2003; Ang et al. 2004; Gadue et al. 2006; McKiernan et al. 2007). Moreover, the combinatorial effect of Activin A and BMP4 on mesoderm development has also been shown in mESC (Huber et al. 1998; Nostro et al. 2008) and recently in hESC (Laflamme et al. 2007; Yang et al. 2008). However, this is the first detailed analysis of the downstream effectors of Activin/Nodal and BMP signalling on mesoderm and endoderm differentiation in hESC. It is shown here that not only BMP signalling but also Smad2 activation are required for mesoderm differentiation, as evidenced by Bry expression. Moreover,
the addition of an exogenous Activin/Nodal agonist, such as Activin A, increases the mesoderm inductive effects of BMP signalling. It is also shown that Bry expressing cells are characterized by greater levels of pSmad1 consistent with the results of Shiraki and colleagues who found that, in mESC derived populations, Bry expressing cells show high levels of pSmad1 while FoxA2 expressing endoderm precursors show low levels of pSmad1 (Shiraki et al. 2008). It is demonstrated here that this mechanism is conserved in hESC. Despite the fact that the average nuclear pSmad1 level has decayed to near baseline levels after 48 hours of treatment with Activin A and BMP2, Bry expressing cells maintain a relatively high level of Smad1 signalling suggesting that duration of BMP signalling also plays a role in mesoderm specification.

On the other hand, in hESC the role of Smad2 activation has been overlooked in the signalling pathway regulating mesoderm differentiation. Although several groups have identified that TGFβ signalling through ALK4, 5, and 7 is required for mesoderm and endoderm differentiation (Gadue et al. 2006; Nostro et al. 2008; Zhang et al. 2008), this is the first report showing that Bry expressing mesoderm precursors show high levels of Smad2 activation and that the addition of exogenous Smad2 activating ligands, such as Activin A, increases Bry expression. Similar to Smad1, Smad2 signalling remains active in Bry expressing cells despite the decay in Smad2 signalling in the remaining cells after 48 hours. This suggests that, in addition to Smad1, sustained Smad2 signalling is also required for mesoderm differentiation. Despite the previous associations of high Activin A concentrations with endoderm differentiation protocols of mouse and human ESC we find that, in the presence of BMP signalling, Activin A increases mesoderm differentiation. It is believed that Smad1 signalling has the effect of posteriorizing cell fates of hESC, driving differentiation of hESC away from anterior endoderm towards a mesoderm fate, a phenomenon that has been observed in mESC (Nostro et al. 2008). Without sufficiently high levels of Smad1 activation hESC in the presence of Activin A become endoderm fated.
The effect of Wnt signalling was also investigated here. A canonical Wnt ligand, Wnt3a, was added with BMP2 and Activin to determine if it would further increase mesoderm differentiation. It was found, however, that this is not the case. Wnt3a had no significant effect on Bry expression, neither did BIO, a synthetic activator of the Wnt pathway. This suggests that either Wnt signalling is not required for mesoderm induction from hESC or that endogenous secretion of Wnt ligand is sufficient for mesoderm specification in the presence of BMP2 and Activin A.

This relationship between Smad1 signalling and mesoderm differentiation is strengthened by observation that both nuclear pSmad1 intensity and Bry expression increases with local cell density. In these regions of higher local cell density and higher Smad1 signalling, Bry expressing mesoderm precursors emerge whereas in regions of lower local cell density and lower Smad1 signalling, Sox17 expressing endoderm precursors emerge. Smad1 signalling was also investigated but the opposite relationship between Smad1 activation and local cell density is observed when compared to the report by Peerani and colleagues (Peerani et al. 2007). They find that Smad1 signalling decreases with local cell density and show that opposing signals from endogenous secretion of BMP2 and GDF3 (growth and differentiation factor 3), an antagonist of BMP signalling, is responsible for this phenomenon. However, their experiments were conducted in serum-free medium containing no cytokines, whereas the experiments presented here are conducted in the presence of high concentrations of Activin A and BMP2. These two signalling environments are drastically different considering that Activin A and BMP2 elicit the expression of signalling proteins including Wnt3 and Nodal, as demonstrated here, and expression of BMP2 and BMP4 as described previously (Pera et al. 2004; Nostro et al. 2008).
Although Bry expression is used here as a marker of mesoderm differentiation, there is considerable evidence suggesting that Bry is also expressed on endoderm precursors. Using an EGFP-Bry/CD4-FoxA2 reporting mouse embryonic stem cell line it has been shown that coexpression of Bry and FoxA2 delineates endoderm fated cells, while Bry positive FoxA2 negative cells are mesoderm precursors (Gadue et al. 2006; Nostro et al. 2008). Due to the fact GFP has been shown to have a half-life of roughly 24 hours (Fukumura et al. 1998), it is possible that Bry and FoxA2 were not coexpressed but that Bry expression had been downregulated prior to FoxA2 expression. Nonetheless, it does demonstrate that the endoderm fated cells were derived from a Bry expressing precursor. Moreover, reports have shown that Bry and Sox17 are coexpressed on hESC derived definitive endoderm precursors following 48 hours of treatment with Activin A and low concentrations of FBS but that Bry expression is subsequently downregulated while Sox17 expression persists at the 72 hour time point (D'Amour et al. 2005; McLean et al. 2007). However, in the conditions described here using high concentrations of BMP2 and Activin A, it is found that Bry and Sox17 are not coexpressed, but it does not rule out the possibility that the Bry expressing cells observed here are also capable of giving rise to endoderm lineages. Further investigation into the developmental potential of the Bry expressing population through cell sorting techniques as well as temporal analysis of Bry and Sox17 expression are required to confirm the assumption that the Bry expressing cells obtained here are in fact mesoderm and not endoderm precursors.

The use of a quadratic model to fit the experimental data provides the first reported quantitative evidence demonstrating that Activin A synergizes with BMP2 with respect to Bry expression. This is a simple yet powerful tool that allows one to extract quantitative relationships between BMP2 and Activin A concentrations with mesoderm differentiation. The insignificance of the quadratic terms for both Activin A and BMP2 concentrations suggests that the response of these
ligands has not reached saturation. If that were the case, the quadratic terms would have a negative value. Also, the insignificance of the Activin A main effect coefficient demonstrates that Activin A alone has no effect on Bry expression, regardless of concentration, showing that Smad2 signalling alone is insufficient for mesoderm differentiation. However, the second-order interaction coefficient is significant and greater than zero, demonstrating that Activin A synergizes with BMP2. Had the second-order interaction coefficient been negative, this would suggest an inhibitory effect of Activin A.

The role of Smad1 regulating the differentiation axis between endoderm and mesoderm of ESC is supported by the correlation between nuclear pSmad1 levels and local cell density. It is shown that cells in a higher local cell density microenvironment show higher activation of Smad1 and it is in these regions of greater local cell density where Bry expressing mesoderm precursors are most highly enriched. The activity of Smad1 signalling specifically in mesoderm precursors is supported by a previous report showing that Smad1 transcript levels are greater in the Bry expressing population compared to the Bry negative population in differentiating mESC (Zafonte et al. 2007).
3 Specific Aim #2: Maximizing Mesoderm Development from Mixed Mesendoderm Population

3.1 Overview

Considering that BMP2 together with Activin A induces both mesoderm and endoderm precursors, further control of the stem cell microenvironment is required for hESC differentiation such that mesoderm rather than endoderm precursors emerge. As described previously, local cell density and colony size have a significant effect on hESC self-renewal and differentiation mediated by endogenous signalling. This objective is to use techniques developed by Peerani and colleagues (Peerani In Press) to precisely control hESC colony size in order to manipulate local cell density and modulate endogenous signalling effects in the presence of Activin A and BMP2. Activin/Nodal signalling is autoregulated through feedforward promotion of its own signalling cascade as well as the expression of inhibitors, namely Lefty1 (left-right determination factor-1), and Cer1 (Cerberus1) in the overlaying visceral endoderm (Brennan et al. 2001; Ulloa et al. 2001; Norris et al. 2002; Perea-Gomez et al. 2002; Robertson et al. 2003). TGFβ signalling has also been shown to induce the expression of Wnt ligands (Zhou et al. 2004) and that Wnt and TGFβ signalling act cooperatively to activate the transcription of developmentally regulated genes in the Xenopus embryo through physical interactions between LEF-1/TCF and Smad3, a downstream signalling protein in the TGFβ pathway (Labbe et al. 2000). The spatially controlled expression of these ligands that interact with TGFβ signalling is essential for correct tissue patterning and specifying the fate of cells migrating through the primitive streak. It is believed that by controlling the colony size of differentiating hESC in the presence of Activin A and BMP2, one can concurrently control the relative signalling effects of other ligands that are known to interact with the TGFβ superfamily in specifying cell fates. Considering the results of Specific Aim 1, it is anticipated that hESC differentiated as larger colonies, which mimic the
microenvironment of high local cell density, will result in selective specification of the mesoderm lineage, while smaller colonies, which mimic the microenvironment of low local cell density, will result in selective specification of the endoderm lineage. It is believed that this will allow for uniform differentiation of hESC towards a single lineage when compared to random seeding of hESC clumps.

3.2 Materials and Methods

3.2.1 Creation of Micropatterned Substrate

Microfabricated poly(dimethylsiloxane) (PDMS) stamps were created using standard soft lithography techniques (Xia et al. 1998) and as described in (Peerani In Press). Three-inch <100> silicon wafers (Silicon Sense Inc.) were spin-coated with SU-8 25 photoresist (Microchem Corp) and exposed to ultraviolet (UV) radiation through a mask with opaque features. Areas exposed to UV radiation are crosslinked and resistant to dissolution by developing solvent. Areas not exposed were dissolved in SU-8 developer (Microchem Corp) leaving behind an etched layer of photoresist polymer between 40 to 60 µm in thickness. This creates a mould from which Poly(dimethylsiloxane) (PDMS) stamps were cast. PDMS prepolymer was mixed with a crosslinking agent (Sylgard® 184 Silicone Elastomer Kit) and poured onto the microfabricated silicon wafer. The PDMS was cured at 70°C for 4 hours then peeled away from silicon wafer creating a stamp with raised features corresponding to the areas exposed by UV radiation. The stamps were ultrasonically cleaned and sterilized in 70% ethanol for 24 hours. PDMS stamps were inked with growth factor reduced- (GFR) Matrigel™ diluted 1:30 with pH 5 PBS for at least 1 hour at 4°C. Excess GFR-Matrigel™ was rinsed away with deionized water and quickly dried using pressurized nitrogen gas. Dried stamps were then pressed onto 3”×1” tissue-culture treated microscope slides (Nunc) or 60 mm tissue-culture treated dishes (BD) then incubated at
room temperature in a humidity controlled chamber (85% relative humidity) for 10 minutes to allow rehydration of Matrigel™ protein and transfer to culture substrate. The stamps are removed and the surface is passivated using 5% (w/v) Pluronic F-127 (Sigma) for at least 1 hour. Pluronic acid surfactant coats areas not printed with protein to prevent non-specific attachment of cells (Figure 3-1).

3.2.2 Seeding of hESC onto Micropatterned Substrate

Human ESC maintained on mEFs were dissociated into single cells using TrypLE™ Express for 4 minutes. TrypLE™ was inactivated and diluted using culture media saved from hESC culture and then the single cell suspension was strained through a 40 µm cell strainer (BD Falcon) to remove cell clumps and large debris. hESC were resuspended in XV-FAT maintenance medium and seeded onto micropatterned Matrigel™ substrates at a concentration of 1500 cells/mm². Following overnight incubation, non-attached cells were rinsed away three times with X-VIVO10™ medium, then cultured with X-VIVO™ supplemented with specified cytokines.

Figure 3-1 Overview of microcontact printing. A) PDMS stamps are inked with Matrigel™; b) Excess Matrigel™ is rinsed away and dried; c) Matrigel™ coated stamp is pressed onto tissue-culture substrate; d) Stamp and substrate are incubated at 85% humidity then stamp is removed transferring Matrigel™ protein onto substrate; e) Single cell suspension is placed on micropatterned substrate and cultured overnight; f) Non-attached cells are rinsed away.
3.2.3 Flow Cytometry Analysis

All flow cytometry analysis was conducted using a FACSCanto flow cytometer (BD Biosciences). Micropatterned colonies and embryoid bodies were dissociated into single cells using TrypLE™ Express for 4 and 10 minutes, respectively. For intracellular staining of Bry, cells were fixed in 3.7 % formaldehyde for 15 minutes then permeabilized with methanol on ice for 2 minutes. Goat anti-human Bry antibody (R&D Systems) was used at 1 µg/mL, and counterstained with Alexa Fluor® 488 donkey anti-goat IgG secondary antibody (1:100). Cells stained with secondary antibody only were used as a negative control. Allophycocyanin- (APC) conjugated mouse anti-human KDR antibody (R&D Systems), fluorescent isothiocyanate- (FITC) conjugated mouse anti-human CD31 antibody (BD Pharmingen), Phycoerythrin- (PE) conjugated mouse anti-human CD34 antibody (Beckman Coulter) were used at 1:10 dilution. PE-conjugated mouse anti-human CD117 (also known as c-kit, BD Biosciences) and APC-conjugated mouse anti-human CD184 (also known as CXCR4, BD Pharmingen) were used at a 1:50 dilution. FITC-, APC-, and PE-conjugated mouse IgG isotype controls (Beckman Coulter) were used as controls at a dilution of 1:10.

3.3 Results

3.3.1 Higher Cell Densities Cause Increased Brachyury Expression

Although high concentrations of both BMP2 and Activin A maximize mesoderm differentiation, endoderm precursors also emerge in this condition as evidenced by expression of MIXL1, Sox17, and GSC. In light of the observation that the frequency of Bry expressing mesoderm precursors is greatest in areas of high cell density while Sox17 expressing endoderm precursors are enriched in areas of low local cell density, it is hypothesized that directly manipulating cell density can control mesoderm versus endoderm differentiation. To test this hypothesis, microcontact printing
technology was used to create hESC colonies of controlled size and geometry. Circular colonies of 200, 400, 800, and 1200 µm in diameter were successfully produced. The ratio of the diameter to the pitch (spacing between colonies) was kept constant so that the fraction of the total area covered by hESC was also held constant to minimize the effects of macroscopic cell density (i.e. the total number of cells per well) (Figure 3-2). The larger colonies are designed to mimic the microenvironment of areas of high local cell density while smaller colonies represent areas of low cell density. The Neighbours Analysis algorithm was applied to micropatterned cultures to confirm that cells cultured as small 200 µm colonies are all found to have low local cell density while the majority of cells cultured as large 1200 µm colonies are found to have high local cell density (Figure 3-3).

Figure 3-2 Phase contrast image of micropatterned colonies of indicated diameter.
The micropatterned colonies were treated in an identical manner as the hESC that were seeded randomly as clumps described in Specific Aim 1. Following 24 hours of seeding in serum-free maintenance medium, the micropatterned colonies were treated with 50 ng/mL of Activin A and BMP2 for 48 hours and analyzed for mesoderm and endoderm differentiation. Confirming the initial hypothesis, the expression of $T-Bry$ and $KDR$ increases with increasing colony size indicating greater mesoderm differentiation in larger, higher density colonies (Figure 3-4). Conversely, the expression of the endoderm-associated transcripts $MIXL1$, $GSC$, $Sox17$ increases with decreasing colony size indicating greater endoderm differentiation in smaller, lower density colonies (Figure 3-4). Gene expression analysis was confirmed through protein level analysis of Bry and KDR by flow cytometry showing greater frequency of protein expression in larger colonies (Figure 3-5).
Figure 3-4 Quantitative gene expression analysis of micropatterned cultures following 48 hours of treatment with BMP2 and Activin A. (*) indicates significance (p<0.05) compared to 200 µm colonies.

Figure 3-5 Flow cytometry analysis of Bry and KDR expression of micropatterned cultures. (*) indicates significance (p<0.05) compared to 200 µm colonies.

3.3.2 Larger Colonies Have Greater Smad1 Signalling

As described in Specific Aim 1 (Chapter 2), it was shown that differential levels of Smad1 signalling might explain the increased mesoderm differentiation in areas of high local cell
density. Nuclear pSmad1 levels increased as a function of local cell density leading to mesoderm differentiation at high local cell density and endoderm differentiation at low local cell density. This relationship is once again evident in the micropatterned colonies where cells in the large 1200 µm colonies are characterized by higher levels of nuclear pSmad1 when compared to the smaller 200 µm colonies following a 90 minute treatment with BMP2 and Activin A (Figure 3-6). This reinforces the relationship between local cell density, pSmad1 and Bry expression.

Independently controlling colony size and local cell density demonstrates that effect of local cell density on Bry expression and Smad1 signalling is in fact causative and not merely correlative.

![Graph showing relative fluorescent intensity](Figure 3-6)

**Figure 3-6** Quantitative immunofluorescence of nuclear pSmad1 intensity of micropatterned cultures. (*) indicates p<0.05

### 3.3.3 Smaller Colonies Express Greater Levels of BMP Antagonists

To investigate a possible mechanism governing the different levels of Smad1 signalling in large and small colonies, the expression of BMP signalling antagonists was measured. Quantitative gene expression analysis shows that expression of Cer1 (Cerberus 1) and Lefty1 (left-right determination factor 1) are greater in smaller colonies (Figure 3-4). Cer1 encodes a secreted protein that binds to BMP proteins extracellularly preventing its signalling activity (Piccolo et al.
1999). Lefty1, on the other hand, encodes an intracellular protein that inhibits the heterodimerization of Smad5 with Smad4, preventing its nuclear localization (Ulloa et al. 2001). Together, it is believed that both these inhibitors of BMP signalling are responsible for attenuating Smad1 signalling in smaller colonies.

3.4 Discussion

The findings discussed in Specific Aim 1 (Chapter 2) show that increased local cell density positively correlates with mesoderm differentiation and negatively correlates with endoderm differentiation. It is conceivable that mesoderm and endoderm precursors emerge stochastically in culture and migrate leading to the observed relationship with local cell density. However, controlling colony size through microcontact printing shows that this relationship is causative. Independently controlling colony size shows that a higher local cell density microenvironment is conducive to mesoderm differentiation while a lower local cell density microenvironment is conducive to endoderm differentiation. This is not to say, however, that the combination of Activin A and BMP2 is ideal for endoderm differentiation even if colony size is restricted. This condition has been identified as inducing optimal mesoderm differentiation but it is also found to support endoderm differentiation. Typical definitive endoderm differentiation protocols employ high concentrations (100 ng/mL) of Activin A alone (D'Amour et al. 2005; D'Amour et al. 2006). Although the combination of Activin A and BMP2 at 50 ng/mL is permissive to both mesoderm and endoderm differentiation, the control of colony size is able to direct lineage specification.

Furthermore, the relationship between local cell density and Smad1 signalling, as was seen in Specific Aim 1, again holds true in the micropatterned scenario. Independently controlling colony size is able to manipulate Smad1 signalling in the presence of BMP2 and Activin A. The
trend of increasing Smad1 activation with greater local cell density is again demonstrated in the micropatterned colonies whereby larger 1200 µm colonies are characterized by higher levels of pSmad1 compared to the smaller 200 µm colonies demonstrating that local cell density causes differences in Smad1 signalling. As mentioned in Specific Aim 1, this trend is opposite to that described by Peerani and colleagues (Peerani et al. 2007) where they report increasing Smad1 activation in smaller colony sizes. Again, this discrepancy is due to the fact that their assays were conducted in the absence of any exogenous cytokines. In this assay, high levels of BMP and Activin/Nodal ligands are present creating a drastically different signalling environment. The addition of these cytokines induces the expression of a different set of endogenous signalling proteins including Wnt3, Nodal, Cerberus 1, and Lefty 1.

In the presence of BMP2 and Activin A, it is proposed that the Smad1 signalling gradient is due to endogenous expression of Cer1 and Lefty1, inhibitors of BMP signalling. Both Lefty1 and Cer1 are known to be expressed in the overlying visceral endoderm of the early primitive streak-stage mouse embryo where they serve to restrict primitive streak and mesoderm formation to the posterior side of the embryo. In the in vitro model using hESC, it is proposed that Lefty1 and Cer1 are more highly expressed in areas of low cell density and attenuate BMP signalling to limit mesoderm differentiation and allow endoderm formation. To further confirm this mechanism small interfering (si)RNA could be used to block Lefty1 or Cerberus expression. In this proposed model, the addition of siRNA against Lefty1 or Cer1 to small colonies would result in higher nuclear pSmad1 levels, greater mesoderm, and less endoderm differentiation.

These results demonstrate that micropatterning hESC into defined colony sizes allows one to probe different microenvironments by specifying a specific local cell density. By doing so, it has allowed the identification of Lefty1 and Cer1 as potential regulators of mesendoderm
differentiation in hESC. Moreover, it allows for the specification of desired germ layers from hESC allowing for decreased heterogeneity of differentiation that would otherwise occur in traditional, randomly seeded hESC cultures.
4 Specific Aim #3: Differentiation of Mesoderm and Endoderm to Hematopoietic Progenitors and Primitive Gut Tube

4.1 Overview

Once an optimal mesoderm population has been established, the next step is to further differentiate mesoderm precursors into hemogenic mesoderm and hematopoietic progenitors. The mechanisms through which this occurs, however, remains poorly understood. Due to the increased complexity of the mouse and human embryo post-gastrulation, it is increasingly difficult to decipher the signalling network responsible for hematopoietic induction. However, as mentioned earlier, several cytokines have been identified to promote hemogenic mesoderm, but it remains unclear if these cytokines induce hemogenic mesoderm or simply maintain and expand hematopoietic progenitors that arise through an unknown mechanism. Moreover, in an effort to more precisely understand the mechanisms underlying hematopoiesis, a completely defined culture medium is desired to preclude the effects of undefined factors present in serum or secreted by hemogenic stromal cells. Additionally, it has been shown that, in a stromal free system, attached culture inhibits hematopoiesis while promoting endothelial differentiation (Dang et al. 2002).

With these considerations in mind, a previously established differentiation protocol will be employed (Chadwick et al. 2003; Ng et al. 2005). This protocol involves an embryoid body (EB) differentiation system that uses defined culture medium supplemented with several cytokines shown to support hematopoietic differentiation and expansion. The potential to form endoderm lineages will also be assessed using a previously reported protocol for specifying primitive gut tube and pancreatic endoderm (D'Amour et al. 2005; D'Amour et al. 2006; Kroon et al. 2008).
The use of the previously established protocols allows for the unbiased and direct comparison of the mesoderm and endoderm forming potential of the micropatterned colonies.

4.2 Materials and Methods

4.2.1 Fabrication of Microwells

Microwells were fabricated as described by Ungrin and colleagues (Ungrin et al. 2008). Silicon moulds were generated by the Canadian Microsystems Consortium (http://www.cmc.ca). Silicon wafers were coated with a silicon nitride layer followed by a layer of photoresist. The photoresist was selectively removed using an opaque mask, UV radiation, and photoresist developer to produce a protective square grid pattern on top of the silicon nitride layer. Deep reactive ion etching was used to remove the silicon nitride layer in areas not protected by photoresist. The remaining photoresist was then chemically dissolved. Wet anisotropic etching was used to etch square pyramids outlined by the silicon nitride layer producing a silicon master mould consisting of an array of square pyramids 800 µm in width (Figure 4-1). Reviewed in Kovacs et al. (1998). The microfabricated silicon wafer was used to cast a PDMS negative mould. PDMS prepolymer was mixed with a crosslinking agent and poured over the silicon wafer and cured at 70°C for 4 hours then peeled away from silicon wafer creating an array of pyramidal peaks. The negative mould was then used to cast a positive mould using the same process creating a microtextured PDMS sheet containing an array of pyramidal wells of 800 µm in width. The microtextured PDMS sheets were then cut into circular inserts that fit tightly into the bottom of 24-well plates resulting in approximately 300 microwells per insert. The circular inserts were ultrasonically cleaned and sterilized by autoclave. Prior to use, inserts were coated with 5% (w/v) Pluronic-F127 (Sigma) solution for at least 1 hour to prevent cell attachment to PDMS inserts.
4.2.2 Generation of Forced Aggregate Embryoid Bodies

Forced aggregate embryoid bodies (spin EBs) were generated as originally described by Ng and colleagues (Ng et al. 2005) and modified by Ungrin and colleagues (Ungrin et al. 2008). Following 48 hours of treatment with BMP2 and Activin A, micropatterned hESC colonies were dissociated into single cells using TrypLE™ Express for 4 minutes then rinsed thoroughly with X-VIVO10™ medium to remove any residual TrypLE™ Express. Harvested cells were then resuspended in X-VIVO10™ serum-free medium supplemented with 50 ng/mL rhSCF (R&D Systems), 150 ng/mL rhFlt3 Ligand (R&D Systems), 10 ng/mL rhVEGF165 (R&D Systems), and 10 µm Y-27632 Rho kinase (ROCK) inhibitor (Calbiochem). ROCK inhibitor is added in order to improve survival of single cell suspension (Watanabe et al. 2007), though the mechanism through which it acts remains unclear. The single cell suspension (5,000 cells per microwell) was pipetted onto microtextured PDMS inserts within a well of a 24-well plate. The entire plate is centrifuged for 3 minutes at 200g to pellet single cell suspension into the bottom of microwells.

Figure 4-1 Schematic diagram of microwell fabrication.
The cells were then cultured overnight at 37°C to allow embryoid body (EB) formation. EBs were then removed from the microwells by gently pipetting with a 1 mL pipette and rinsed with PBS to remove ROCK inhibitor, resulting in roughly 300 EBs with approximately 5,000 cells per EB. EBs were subsequently cultured in ultralow adhesion 6-well plates (Costar) in the same medium as above without ROCK inhibitor for 9 days (Figure 4-2).

**Figure 4-2** Schematic diagram of microwell spinEB formation. a) Micropatterned cultures are enzymatically dissociated into single cells and pipetted onto microwells; b) Microwells are centrifuged to pellet cells into bottom of microwells and cultured overnight to form embryoid bodies (EB); c) EBs are then removed from the microwells and cultured in suspension.

### 4.2.3 Flow Cytometry

Flow cytometry was performed as described in the Materials and Methods section of Specific Aim 1.

### 4.2.4 Hematopoietic Colony Forming Assay

After 9 days of embryoid body culture, EBs were dissociated into single cells using TrypLE™ Express for 10 minutes and pipetting through a 200 µL micropipette tip. Single cell suspensions
were strained through a 40 µm cell strainer to remove any cell clumps. Single cells were resuspended in MethoCult® GF+ semi-solid medium (H4435, Stem Cell Technologies) at a density of 50,000 cells per mL. Hematopoietic colonies were counted following 14 days of culture. A minimum of 100,000 cells was assayed per condition per replicate.

### 4.2.5 Primitive Gut Endoderm Differentiation

The differentiation protocol implemented here was first described in references (D'Amour et al. 2005; D'Amour et al. 2006; Kroon et al. 2008). Following 48 hours of treatment with BMP2 and Activin A, 200 and 1200 µm diameter micropatterned cultures were reseeded onto Matrigel™ coated 6-well plates by physical scraping with a cell scraper (Sarstedt). Cells from an entire 60 mm culture dish were seeded into 2 wells of a 6-well plate in X-VIVO10™ medium supplemented with 100 ng/mL Activin A. Following 2 days of culture, medium was replaced with RPMI Medium 1640 (Gibco) supplemented with 2% FBS, 2 mM GlutaMAX™ (Invitrogen), 50 U/mL Penicillin/Streptomycin (Invitrogen), and 50 ng/mL fibroblast growth factor 7 (FGF7, or keratinocytes growth factor, KGF, R&D Systems). Following 3 more days of culture, medium was replaced with DMEM supplemented with 1% B27 supplement (Gibco), 2 mM GlutaMAX™ (Invitrogen), 50 U/mL Penicillin/Streptomycin (Invitrogen), 50 ng/mL Noggin (R&D Systems), 250 nM cyclopamine (a kind gift from Dr. Derek van der Kooy, University of Toronto), and 2 µm retinoic acid (RA). Following 3 more days of differentiation, cultures were fixed and stained for immunofluorescence or harvested for quantitative gene expression analysis as described in the Materials and Methods section of Specific Aim 1.
4.3 Results

4.3.1 Larger, Mesoderm Enriched Colonies Demonstrate Greater Hematopoietic Potential

A hematopoietic differentiation assay was used to assess the functional capacity of the mesoderm precursors described in Specific Aim 2. Following the initial 48 hours of mesoderm induction with BMP2 and Activin A, the micropatterned colonies were dissociated into single cells and formed into forced aggregate embryoid bodies as originally described by Ng and colleagues (Ng et al. 2005) and modified by Ungrin and colleagues (Ungrin et al. 2008). The embryoid bodies were cultured in suspension for 9 days in X-VIVO10™ serum-free medium supplemented with hemogenic cytokines VEGF (vascular endothelial growth factor, 10 ng/mL), SCF (stem cell factor, 50 ng/mL) and Flt-3 ligand (150 ng/mL) (Chadwick et al. 2003). Following 9 days of culture, the embryoid bodies were dissociated into single cells and hematopoietic potential was assessed through hematopoietic colony forming assay (Eaves et al. 1999) and flow cytometry analysis. This hematopoietic differentiation protocol was employed because it allows for the tight control of embryoid body size and number so that the differentiation of hESC is uniformly conducted except for the variation of colony size during the initial 48 hours of differentiation. This allows for the isolation of the effect of local cell density on early mesoderm differentiation.

Flow cytometry analysis shows that hESC differentiated in larger colonies give rise to 2.5-fold (p<0.05) greater frequency of CD34, CD31 (PECAM1, platelet endothelial cell adhesion molecule 1) and VEGFR2 expressing cells, markers of hematopoietic progenitors (Tavian et al. 1996; Labastie et al. 1998; Tavian et al. 1999) (Figure 4-3). Hematopoietic colony forming assay further demonstrates that the larger, mesoderm enriched colonies yield significantly greater frequencies of colony forming units (CFU), a 10-fold increase (p<0.05) when compared to the smaller colonies. Such colony forming units include CFU-macrophage (CFU-M), CFU-
granulocytes, macrophage (CFU-GM), and CFU-granulocytes, erythroid, macrophage megakaryocyte (CFU-GEMM) (Figure 4-4 and Figure 4-5). This demonstrates the functional output of the mesoderm precursors described in Specific Aim 2 and shows that the larger, higher local cell density colonies are in fact enriched for mesoderm precursors.

Figure 4-3 Flow cytometry analysis shows that EB cultures derived from 1200 µm colonies express markers of hematopoietic differentiation at a greater frequency compared to 200 µm colonies. (*) indicates significance (p<0.05).
**Figure 4-4** Hematopoietic colony forming assay shows that EB cultures derived from 1200 µm colonies have a greater frequency of colony forming units. (*) indicates significance (p<0.05).
4.3.2 Smaller, Endoderm Enriched Colonies Demonstrate Greater Primitive Gut Endoderm Potential

Although the hematopoietic differentiation assay described above demonstrates that the larger colonies have greater potential for mesoderm differentiation when compared to the smaller colonies, an endoderm differentiation assay is required to support the earlier finding that larger colonies are deficient in endoderm potential while the smaller colonies are enriched in endoderm precursors. To accomplish this the micropatterned hESC colonies were further cultured in conditions supportive of definitive gut tube and pancreatic endoderm differentiation (D'Amour et al. 2005; D'Amour et al. 2006; Kroon et al. 2008). Following 48 hours of treatment with BMP2 and Activin A, 200 and 1200 µm diameter micropatterned colonies were subsequently treated.
with Activin A alone for 2 days, FGF7 for 3 days, followed by RA, cyclopamine and Noggin for 3 more days.

Following 48 hours the treatment with Activin A alone, cultures originating from the 200 µm colonies express CXCR4 (chemokine (C-X-C motif) receptor 4), a marker of definitive endoderm (McGrath et al. 1999; D'Amour et al. 2005; Yasunaga et al. 2005; D'Amour et al. 2006) at a significantly greater frequency when compared to cultures originating from the larger 1200 µm colonies as measured by flow cytometry (Figure 4-6). Moreover, after treatment with FGF7 followed by Noggin, RA, and cyclopamine, cultures originating from 200 µm colonies express hepatocyte nuclear factor-1 β (HNF1β) (Coffinier et al. 1999), FoxA2 (forkhead box A2, or HNF3β) (Sasaki et al. 1993), and HNF4α (Duncan et al. 1994), markers of primitive gut tube differentiation, whereas cultures originating from 1200 µm colonies do not (Figure 4-7). Quantitative gene expression analysis confirms the protein expression showing that HNF1β, FoxA2, and HNF4α gene expression is also greater in cultures derived from the smaller 200 µm colonies (Figure 4-8a). Moreover, it is also found that transcripts associated with pancreatic epithelium such as hepatocyte nuclear factor-6 (HNF6) (Rausa et al. 1997), insulin promoter factor-1 (IPF1, or pancreas/duodenum homeobox-1, PDX1) (Wilson et al. 2003), and motor neuron and pancreas homeobox 1 (MNX1) (Wilson et al. 2003) are also more highly expressed in cultures derived from the smaller colonies, though the overall expression is considerably less than human pancreatic tissue (Figure 4-8b). Together, these results demonstrate that the 200 µm colonies are enriched for definitive endoderm precursors capable of primitive gut tube and pancreatic endoderm differentiation.
Figure 4-6 Flow cytometry analysis of CXCR4 expression of attached cultures derived from 200 and 1200 µm colonies following 2 days of culture in Activin A. Red histogram is of isotype control, green histogram represents sample. (*) indicates p<0.05.
Figure 4-7 Fluorescent images of cultures derived from 200 and 1200 µm colonies following endoderm differentiation assay showing HNF1β, FoxA2, and HNF4alpha staining. Nuclei are shown in blue. Scale bar represents 100 µm; original magnification is 20× and 40×.
Figure 4-8 Quantitative gene expression analysis of primitive gut tube and pancreatic endoderm cultures derived from micropatterned cultures. a) Cultures derived from smaller 200 µm colonies show greater expression of primitive gut tube-associated genes when compared to 1200 µm colonies; b) Expression of pancreatic endoderm-associated genes is also greater in cultures derived from 200 µm colonies, but considerably less when compared to human pancreatic tissue. (*) and (**) indicate significance (p<0.05 and p<0.01, respectively) when comparing 200 and 1200 µm colony derived cultures.

4.4 Discussion

Using markers of early mesoderm and endoderm differentiation such as T-Bry, Sox17, GSC and MIXL1, it was shown that mesoderm precursors are enriched in areas of higher local cell density while endoderm precursors are enriched in areas of lower cell density. However, it is still necessary to demonstrate that these markers of early mesoderm and endoderm differentiation truly reflect their differentiation potential. It is shown here that using differentiation protocols
supportive of either hematopoietic or primitive gut endoderm differentiation, the larger colonies represent precursors of the mesoderm and not endoderm lineage while the opposite is true for small colonies.

A hematopoietic differentiation assay demonstrated the functional output of the mesoderm progenitors. It was shown that there is a 10-fold increase in the number of hematopoietic colony forming units in embryoid bodies derived from the larger 1200 µm colonies when compared to the smaller 200 µm colonies. Although the hematopoietic assay used demonstrates that the larger colonies give rise to greater frequencies of colony forming units, the overall frequency is considerably less than other reports. Using a hematopoietic differentiation protocol that includes a combination of cytokines and serum Chadwick and colleagues report a clonogenic frequency of hematopoietic precursors of roughly 1:400 (Chadwick et al. 2003), while Ng and colleagues report a similar frequency of 1:523 (Ng et al. 2005) using serum-free conditions. The frequency reported here, however, is roughly 1:4300, one tenth of these previous reports. The low frequency of hematopoietic colonies could be explained by the types of mesoderm precursors that arise in the micropatterned colonies. Mesoderm differentiation was assessed by Bry expression, which is a pan-mesodermal marker that is expressed in precursors of all types of mesoderm, including somitic, cardiac as well as hemogenic mesoderm. Treatment with Activin A and BMP2 may have skewed mesodermal differentiation towards one of these other lineages resulting in low hematopoietic frequency. In fact, previous reports have used BMP4 and Activin A to robustly induce cardiac differentiation from hESC suggesting that this condition is supportive of cardiac rather than hematopoietic differentiation (Laflamme et al. 2007; Yang et al. 2008). Previous studies have also shown that Wnt signalling acts during later stages of mesoderm differentiation to specify hematopoietic lineages at the expense of cardiac (Naito et al. 2006; Ueno et al. 2007). In order to more precisely direct hematopoietic differentiation, further
studies could include the addition of exogenous Wnt ligands during embryoid body differentiation.

To demonstrate that the smaller colonies are enriched from endoderm precursors, a differentiation assay supportive of primitive gut tube and pancreatic endoderm was employed. Both protein and gene expression of markers of primitive gut endoderm were shown to be greater in cultures derived from smaller 200 µm colonies. Moreover, expression of genes associated with pancreatic epithelium development were also more highly expressed in cultures derived from smaller colonies, but the overall expression was still considerably less that that of human pancreatic tissue. This suggests that the pancreatic lineage has not fully matured from the primitive gut tube endoderm and that further differentiation is required. Nonetheless, these results demonstrate that the smaller colonies are enriched for endoderm precursors. However, to further confirm the functional capacity of these endoderm precursors, further differentiation and functional assays such as insulin secretion and c-peptide release would be required.

The relationship between colony or EB size and mesendoderm differentiation that is observed here has been previously reported but the mechanism governing this relationship was unknown. Ng and colleagues have reported that a minimum EB size of roughly 500 cells/EB is required for hematopoietic differentiation of hESC (Ng et al. 2005) while another report shows that endoderm differentiation occurs much more efficiently when ESC are seeded at low densities (Cho et al. 2008). It is shown here, however, that the effect of cell density and colony size on mesendoderm differentiation is mediated by Smad1 signalling where greater Smad1 activation is present at higher cell densities while lower Smad1 activation is present at lower cell densities. It is proposed here that this effect of cell density on Smad1 signalling is responsible for controlling mesoderm versus endoderm differentiation. By manipulating cell density it is now possible to
more finely tune the ESC microenvironment allowing for more tight control over ESC differentiation.


5 Conclusions

In order for stem cell researchers to effectively guide embryonic stem cell differentiation and obtain pure population of transplantable cells for therapeutic applications, a greater understanding of the molecular mechanisms controlling ESC differentiation is required. From the results presented here, it is clear that both Smad1 and Smad2 signalling play pivotal roles in mesoderm and endoderm differentiation from hESC. Although previous reports have shown that BMP and Activin/Nodal signalling are required for mesoderm and endoderm differentiation, this is the first report demonstrating that Smad2 signalling directly targets mesoderm precursors. Moreover, the use of a quadratic mathematical model has quantitatively confirmed the synergistic relationship between Activin A and BMP2 in mesoderm differentiation. Activin/Nodal alone is not sufficient for mesoderm differentiation but it does synergize with BMP signalling to maximize the frequency of mesoderm precursors.

This is also the first report to elucidate a possible mechanism governing the relationship between stem cell microenvironment, specifically local cell density, and mesendoderm differentiation whereby expression of BMP antagonists in areas of low local cell density attenuate Smad1 signalling leading to endoderm differentiation while mesoderm differentiation occurs mainly in areas of high local cell density. By controlling colony size one can independently manipulate local cell density, and consequently Smad1 signalling, in order to precisely guide mesendoderm differentiation of human embryonic stem cells (Figure 5-1).

It is finally shown that this method allows for the control of the differentiation of more mature cell types including hematopoietic cells and primitive gut endoderm. Using a hematopoietic differentiation assay, a 10-fold increase in hematopoietic colony forming frequency is observed
in embryoid bodies derived from the larger mesoderm-enriched colonies when compared to the smaller colonies. On the other hand, an endoderm differentiation protocol reveals that cultures derived from smaller endoderm enriched colonies show greater protein and gene expression of markers of primitive gut tube and pancreatic endoderm differentiation. This is a vast improvement over traditional bulk culture systems where the stem cell microenvironment is not controlled leading to heterogeneous differentiation. These results highlight the importance of tightly controlling all aspects of the stem cell niche, including extracellular signalling and cell density, in order to obtain the desired cell types.

**Figure 5-1** Proposed scheme for the regulation of mesendoderm differentiation by local cell density and Smad signalling
6 Future Work

There remain several specific outstanding issues in this thesis that merit further investigation. Firstly, the role of Cer1 and Lefty1 gene expression needs to be validated through protein level analysis and loss of function analysis through RNA interference. Demonstrating the effects of suppressing Cer1 and/or Lefty1 expression through small interfering RNA would definitively demonstrate the direct effect of Smad1 inhibition on mesendoderm differentiation. Moreover, other signalling pathways other than Wnt and the TGFβ warrant investigation, such as cell tension mediated by RhoA activation, or FGF signalling among others. The results of preliminary experiments looking into the role of Rho kinase and FGF signalling are discussed in the appendix and they demonstrate the need for further investigation.

Also, as alluded to earlier, the efficiency of hematopoietic differentiation from the mesoderm precursors is quite low compared to other published reports. This highlights the importance of not only dissecting the mechanisms governing early germ layer differentiation, but also later lineage specification. Several reports have identified Wnt signalling as a factor that influences the differentiation of committed mesoderm precursors to either the cardiac or hematopoietic lineage (Naito et al. 2006; Ueno et al. 2007). Moreover, Sukurai and colleagues (Sakurai et al. 2006) have shown using an in vitro mESC model that two populations representing paraxial and lateral mesoderm have the ability to interconvert between lineages at early stages. The mechanism underlying this interconversion, however, remains unknown. Also, Wnt3a has been implicated in the specification of trunk and tail paraxial mesoderm and that graded concentrations of Wnt3a are required for posterior derivatives of paraxial mesoderm (Takada et al. 1994; Greco et al. 1996). It is clear that complex interactions between various signalling
pathways regulate the specification of mature cell types from early mesoderm precursors and a
greater understanding of these interactions could explain the seemingly low frequency of
hematopoietic progenitors observed, and also improve the purity and number of desired cell
types, be they hematopoietic, osteogenic, or any other mesoderm-derived cell type.

Thinking more broadly, this project has focused heavily on the differentiation of hESC as an
attached culture on a laminin based substrate. This aspect alone brings into question the
difference of two very distinct differentiation paradigms – attached versus non-attached cultures.

_in vivo_, embryogenesis occurs in the uterine wall surrounded by extraembryonic tissue, a very
disparate microenvironment than the stiff substrate of a tissue-culture flask. Future work may
involve completely suspension culture systems, such as embryoid bodies, or even semi-solid
medium that more closely resembles the physical properties a developing embryo would
experience _in vivo_. Similar questions could still be raised, such as the effect of local cell density,
or EB size in this case, on mesendoderm differentiation, or perhaps the role of substrate
mechanical properties on the epithelial-mesenchymal transition (EMT) that characterizes the
development of pluripotent epiblast cells as they migrate through the primitive streak (Moustakas
et al. 2007).

Recently, the ability to generate induced pluripotent stem cells from somatic cells (Takahashi et
al. 2007; Yu et al. 2007) has opened the door to a wide range of possibilities for generating
transplantable hematopoietic stem cells for treating blood disorders. Rather than focusing on
guiding the differentiation of pluripotent cells to hematopoietic progenitors, it is worthwhile to
consider a similar strategy to reprogram somatic cells directly to hematopoietic stem cells
circumventing the need for precise differentiation protocols. This, however, introduces new
challenges such as identifying the minimal combination of factors required for hematopoietic
reprogramming, as well as concerns with tumourogenicity from reintroducing potentially oncogenic genes or proteins. There are clearly a number of challenges that must be overcome before a practical and clinically relevant cell-based therapy for treating blood disorders can be achieved.
References


Zhang, H. and A. Bradley (1996). "Mice deficient for BMP2 are nonviable and have defects in amnion/chorion and cardiac development." Development 122(10): 2977-86.


cord blood-derived hematopoietic stem/progenitor cells with recombinant human stem cell factor can up-regulate levels of homing-essential molecules to increase their transmigratory potential. "Exp Hematol 31(12): 1237-46.

8 Appendix

8.1 Other Signalling Pathways Possibly Regulating Mesendoderm Differentiation

As discussed above, it is possible that other signalling pathways besides the TGFβ pathway are involved in regulating mesendoderm differentiation. During the course of this project, the Rho kinase and FGF signalling pathway were also investigated but the results from these investigations were not included in the main body of this thesis because they did not significantly contribute to the ideas or conclusions presented. However, these results are presented in the appendix for future consideration.

8.1.1 Effect of Rho Kinase Inhibitor Y-27632 on Mesendoderm Differentiation

Although it is proposed here that differential Smad1 activation is responsible for specifying mesoderm differentiation at the expense of endoderm, it is possible that other factors such as cell spreading or cell-cell contact also mediate this effect. It has been previously shown that cell shape and cytoskeletal tension regulate the differentiation of human mesenchymal stem cells towards adipocyte or osteoblast cell types and this process is mediated through RhoA activation (McBeath et al. 2004). Through constitutive activation or inhibition of the RhoA effector, Rho kinase, they were able to induce osteogenesis or adipogenesis, respectively, independent of cell shape. A similar approach can be used here to investigate if cell shape or cytoskeletal tension processes mediated by RhoA activation play a role in mesendoderm differentiation. Preliminary studies were conducted by concomitantly treating micropatterned cultures with BMP2, Activin A and the Rho kinase inhibitor Y-27632 (10 nM). These studies showed that the Rho kinase
inhibitor increases Bry expression in large colonies but has little or no effect on smaller colonies suggesting that the mechanism governing mesendoderm differentiation may be related to RhoA activation (Figure 8-1). However, the Rho kinase inhibitor has also been shown to increase survival (Watanabe et al. 2007), decrease Smad1 activation, and increase self-renewal of hESC (Peerani et al. 2007). The many known interactions of the Rho signalling pathway with various other pathways precludes any conclusions to be drawn here as to the mechanism governing Rho kinase activation and mesoderm differentiation. Further studies are required in order to better understand the effects of Rho signalling on hESC fate.

Figure 8-1 Representative immunofluorescent images of Bry expression of micropatterned colonies following 48 hours of treatment with BMP2 and Activin A with or without Y-27632 ROCK inhibitor. Original magnification is 10×, scale bar indicates 500 µm.

8.1.2 Effect of Fibroblast Growth Factor Inhibition on Mesendoderm Differentiation

Fibroblast growth factor (FGF) signalling has been previously implicated in maintaining pluripotency of hESC (Thomson et al. 1998; Xu et al. 2005) as well as ectoderm differentiation
(Stavridis et al. 2007) but its role in mesendoderm differentiation remains unclear. Therefore, inhibition of FGF signalling through pharmacological inhibition of the FGF receptor (FGFR) 1 and inhibition of MAP kinase was investigated using the small molecules PD173074 (FGFRi, 20 nM) and PD184352 (MAPKi, 500 nM), respectively. It was found that inhibition of FGFR1 or MAP kinase during the initial 48 hours of differentiation in the presence of BMP2 and Activin A was able to significantly increase Bry expression in the larger 1200 µm colonies but not the smaller 200 µm colonies (Figure 8-2). Protein expression levels were further confirmed through quantitative gene expression analysis (Figure 8-3). As previously mentioned, FGF signalling is able to modulate Smad signalling through phosphorylation of receptor Smads in the linker region by MAP kinase which prevents its nuclear accumulation and transcriptional activity.

**Figure 8-2** Representative immunofluorescent images of Bry expression following 48 hours of treatment with BMP2, Activin A, FGFR inhibitor PD173074 (20 nM), or MAP kinase inhibitor PD184352 (500 nM) as indicated. Original magnification is 10×, scale bar indicates 500 µm.
Figure 8-3 Quantitative gene expression analysis of micropatterned colonies following 48 hours of treatment with BMP2, Activin A, and FGFR inhibitor PD173074 (20 nM) as indicated. (*) indicates p<0.05.

To determine if this was the mechanism through which FGF inhibition acts, the levels of pSmad1 were measured following 90 minutes of treatment with BMP2, Activin A and the MAP kinase inhibitor PD184352 (500 nM). Quantitative immunofluorescent analysis demonstrates that pSmad1 activation is greater when MAP kinase is inhibited supporting the role of FGF signalling attenuating Smad1 activation and decreasing mesoderm differentiation (Figure 8-4). However, when the hematopoietic assay was applied to FGF inhibited cultures, there was a striking decrease in hematopoietic colony forming frequency (Figure 8-5). As a result, it is unclear what role FGF signalling plays in mesoderm or hematopoietic specification. It is possible that FGF inhibition does not increase mesoderm differentiation, or FGF inhibition only inhibits hematopoietic development at a very early stage.
**Figure 8-4** Quantitative immunofluorescent analysis of pSmad1 intensity of micropatterned colonies following 90 minute treatment with BMP2, Activin A, and MAP kinase inhibitor PD184352 (500 nM) as indicated. (*) indicates p<0.05.

**Figure 8-5** Frequency of hematopoietic colonies following hematopoietic differentiation assay of 1200 µm cultures with or without FGFR1 inhibition.