ABSTRACT

SARKAR, PRASENJIT. Trophoblast Differentiation of Human Embryonic Stem Cells. (Under the direction of Balaji M. Rao).

Human embryonic stem cells (hESCs) are cell lines isolated from the inner cell mass of the human blastocyst and have the ability to form all the tissues of the human body. However, there is considerable debate as to whether they can form the ‘trophoblast’ tissues, which are found in the human placenta during pregnancy. Mouse embryonic stem cells are analogous to hESCs, and do not have the ability to form trophoblast cells. Moreover, currently used approaches for deriving ‘trophoblast-like’ cells from hESCs suffer from significant limitations. These ‘trophoblast-like’ cells do not have the important properties that are otherwise found in the trophoblast cells of the human placenta. There is also ambiguity as to whether these ‘trophoblast-like’ cells belong to the trophoblast lineage or the mesoderm lineage.

We have used an alternate approach to derive trophoblast cells from hESCs and have shown that these cells have the key properties that are found in trophoblast cells of the human placenta. These properties include demethylation of the ELF5 promoter region, the downregulation of HLA Class I type A, B and C antigens and the expression of CDX2 and ELF5 proteins. Our approach involves suppression of the BMP and Activin/Nodal signaling pathways, while current reports suggest that BMP signaling triggers trophoblast gene expression in hESCs. Therefore, we show that trophoblast cells can be obtained even when BMP signaling is inhibited.

Since signaling pathways have an important role in controlling the behavior of hESCs, we have also developed a new method for studying the endogenous signaling factors secreted by
hESCs. We reasoned that secreted proteins transit through the classical secretory pathway, and an analysis of the secretory pathway organelles should allow us to identify secreted proteins as well. Therefore, we isolated organelles that are involved the classical secretory pathway and analyzed them using mass spectrometry. Currently used methods probe the cell culture medium where the secreted proteins are allowed to collect. Since cell culture media contain serum proteins in a far excess quantity, most secreted proteins remain undetected with these methods. Using our alternative approach, we have obtained a deeper coverage of the hESC secretome, and have shown a proof of principle for our method.
Trophoblast Differentiation of Human Embryonic Stem Cells

by
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DEDICATION

To my parents, for all their love and support

“Imagination is more important than knowledge”- Albert Einstein
BIOGRAPHY

Prasenjit Sarkar was born on January 7\textsuperscript{th} 1978 in Shillong, India. He was brought up in New Delhi, and received his Bachelor in Technology degree in 2006 from the Indian Institute of Technology at Guwahati. He worked on an internship at the Jacobs University Bremen, Germany after completing his B.Tech. He started his graduate studies at North Carolina State University, Raleigh in Fall 2007 under the guidance of Dr. Balaji M. Rao.
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# TABLE OF CONTENTS

LIST OF FIGURES .................................................................................................................. viii

CHAPTER 1: HUMAN EMBRYONIC STEM CELLS AS A MODEL SYSTEM FOR EARLY DEVELOPMENT .................................................................................................................. 1

1.1 Introduction ...................................................................................................................... 2
  1.1.1 Formation of embryonic tissues ................................................................................. 3
  1.1.2 Observations from animal models ............................................................................. 3
  1.1.3 Observations from hESC cultures ............................................................................. 4
1.2 Derivation of trophoblasts from hESCs ................................................................. 6
  1.2.1 Trophoblast markers ................................................................................................. 7
  1.2.2 Current approaches ................................................................................................. 8
  1.2.3 Role of signaling pathways ..................................................................................... 10
1.3 Challenges in experimental design .......................................................................... 11
  1.3.1 Crosstalk among signaling pathways ....................................................................... 11
  1.3.2 Heterogeneity in cell culture .................................................................................... 12
  1.3.3 Diverse culture conditions ....................................................................................... 13
1.4 Signaling pathways involved in determining hESC fate ........................................ 14
  1.4.1 Activin/Nodal pathway ............................................................................................ 14
  1.4.2 BMP pathway ........................................................................................................ 15
  1.4.3 FGF pathway .......................................................................................................... 15
  1.4.4 Wnt pathway ........................................................................................................ 16
  1.4.5 Akt pathway ........................................................................................................... 16
  1.4.6 Hippo pathway ....................................................................................................... 17
1.5 Thesis overview ............................................................................................................. 17
  1.5.1 Hypotheses ............................................................................................................. 17
  1.5.2 Thesis layout .......................................................................................................... 18
1.6 References ...................................................................................................................... 19

CHAPTER 2: TROPHOBLAST DIFFERENTIATION FROM HUMAN EMBRYONIC STEM CELLS ........................................................................................................... 49

2.1 Introduction ...................................................................................................................... 50
2.2 Results ........................................................................................................................... 50
  2.2.1 Derivation of cells with DNA hypomethylation at ELF5
2.2.2 Expression of CYTOKERATIN7 and HLA class I type A, B and C antigens ........................................... 52
2.2.3 Derivation of syncytiotrophoblasts (STBs) .................................................. 53
2.2.4 Derivation of iCTBs .................................................................................... 53
2.2.5 Derivation of trophoblast cells that co-express CDX2 and ELF5 ............. 54
2.2.6 The switch between trophoblast and neural fate of hESCs .................. 56
2.3 Discussion and future work ......................................................................... 58
2.3.1 Derivation of cells with properties of placental trophoblast cells ......... 58
2.3.2 The role of BMP pathway in trophoblast differentiation ......................... 59
2.3.3 The role of Activin/Nodal pathway in trophoblast differentiation .......... 60
2.3.4 The role of serum factors in trophoblast differentiation ......................... 60
2.4 Materials and methods .............................................................................. 62
2.4.1 Cell culture and differentiation ................................................................. 62
2.4.2 Isolation of iCTB and STB populations .................................................... 63
2.4.3 RNA isolation, cDNA synthesis and quantitative PCR ......................... 63
2.4.4 Immunofluorescence .............................................................................. 64
2.4.5 Immunohistochemistry and histology analysis ....................................... 65
2.4.6 Flow cytometry ....................................................................................... 65
2.4.7 Subcellular fractionation ......................................................................... 66
2.4.8 Protein fractionation and in-gel digestion ................................................. 66
2.4.9 LC-MS/MS and data analysis ................................................................. 67
2.5 Author contributions .................................................................................... 69
2.6 References ................................................................................................... 70

CHAPTER 3: CHARACTERIZATION OF SUBCELLULAR HESC PROTEOME AND SECRETOME .......................................................... 87

3.1 Introduction .................................................................................................. 88
3.2 Results ......................................................................................................... 88
  3.2.1 Enrichment of secretory pathway organelles from mouse embryonic fibroblasts and hESCs ................................................................. 88
  3.2.2 The secretome of MEFs and hESCs .......................................................... 90
  3.2.3 The secretome of hESCs changes upon exposure to unconditioned medium ......................................................................................... 91
  3.2.4 Subcellular fractionation of plasma membrane, cytoplasm and nuclei
LIST OF FIGURES

Figure 1.1  Schematic of a blastocyst stage embryo .................................................. 37

Figure 1.2  Schematic of a peri-implantation embryo .............................................. 38

Figure 1.3  Schematic showing origin of the three germ layer tissues, viz. ectoderm, mesoderm and definitive endoderm ................................................. 39

Figure 1.4  Schematic showing role of signaling pathways in formation of mesoderm and definitive endoderm from hESCs .................................. 40

Figure 1.5  Schematic showing the role of signaling pathways in the formation of neural and neural crest cells from hESCs .............................. 41

Figure 1.6  Schematic showing the role of signaling pathways in self-renewal of hESCs and in differentiation to germ cells and parietal endoderm cells ........................................ 42

Figure 1.7  A schematic of the Activin/Nodal pathway ............................................. 43

Figure 1.8  A schematic of the BMP pathway ............................................................ 44

Figure 1.9  A schematic of the FGF pathway ................................................................. 45

Figure 1.10 A schematic of the Wnt pathway ............................................................... 46

Figure 1.11 A schematic of the Akt pathway initiated by Insulin-like growth Factors (IGFs) ........................................................................................................ 47

Figure 1.12 A schematic of the Hippo signaling pathway ......................................... 48
Figure 2.1  Methylation levels at the ELF5 promoter region decrease when hESCs are treated with SB431542 for longer durations .......................... 78

Figure 2.2  Expression of ELF5 protein................................................................. 79

Figure 2.3  Downregulation of HLA antigens ...................................................... 81

Figure 2.4  Formation of STBs from hESCs............................................................. 82

Figure 2.5  Formation of mesenchymal cells upon prolonged treatment of H9 cells with SB431542 ................................................................. 84

Figure 2.6  Co-expression of CDX2 and ELF5 is sporadically seen.......................... 85

Figure 3.1  Schematic showing endogenous signaling in hESC cultures .................. 107

Figure 3.2  Schematic showing the protocol for enrichment of secretory pathway organelles.................................................................. 108

Figure 3.3  Secretory pathway organelles were enriched during subcellular fractionation ................................................................. 109

Figure 3.4  Secretory proteins identified in the subcellular fraction from MEF cells ................................................................. 110

Figure 3.5  Secretory proteins identified in the subcellular fraction from H1 and H9 hESCs ................................................................. 111

Figure 3.6  Proteins associated with various signaling pathways identified in the secretome of hESCs and MEFs .................................................. 112

Figure 3.7  Changing the overlying medium changes the secretome of hESCs........ 113
Figure 3.8  Schematic showing the protocol for isolation of plasma membrane, cytoplasm and nuclear fractions from hESCs
CHAPTER 1

HUMAN EMBRYONIC STEM CELLS AS A MODEL SYSTEM FOR EARLY DEVELOPMENT

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1.1 Introduction

The human embryo at its blastocyst stage consists of two tissues – the inner cell mass (ICM) and the trophectoderm (TE) (Figure 1.1). ICM cells can be isolated and maintained in cell culture as a stem cell line, termed as human embryonic stem cells (hESCs) (1). ICM cells in the embryo subsequently give rise to all the tissues of the human body. Similarly, hESCs can form all of these tissues in cell culture, through a process known as differentiation. Therefore, hESCs are a valuable tool for understanding early developmental processes that lead to the formation of these tissues. HESCs can also be used as a source for deriving various tissues of the body, which can be used for drug evaluation and for regenerative medicine applications. This thesis focuses on the use of hESCs for deriving trophoblast tissues that occur in the human placenta, and on understanding the role of various signaling pathways involved in this differentiation process.

Studies with other model systems such as fruit flies, chick embryos, frog embryos and mice embryos have demonstrated that the formation of tissues during embryonic development is controlled by various signaling pathways (2-5). These pathways include the Bone Morphogenetic Protein (BMP) pathway, Activin/Nodal pathway, Fibroblast Growth Factor (FGF) pathway, Wnt pathway, Akt pathway, Notch pathway, Hippo pathway, as well as various others. A brief description of these pathways is provided in section 1.5. These pathways have been identified in hESCs and can be manipulated to obtain various human tissues in cell culture conditions. A review of current approaches used for differentiation of
hESCs to various cell-types is provided below, focusing in particular on the trophoblast tissues.

1.1.1 Formation of embryonic tissues

Upon implantation of the embryo, the ICM cells give rise to the hypoblast and the epiblast (Figure 1.2). The epiblast then goes through the process of gastrulation to form the primitive streak. The primitive streak cells form the mesoderm and the definitive endoderm, while the remaining epiblast cells form the ectoderm. The ectoderm, mesoderm and the definitive endoderm are together known as the three germ layers (Figure 1.3). At later stages, the ectoderm forms specialized cells such as neurons, skin cells, etc. while the mesoderm forms myocytes, osteocytes, erythrocytes, etc, and the definitive endoderm forms hepatocytes, alveolar cells, pancreatic cells, etc. HESCs can also be induced to form these cell-types in vitro through the concerted action of Activin/Nodal, BMP, Wnt and various other signaling pathways.

1.1.2 Observations from animal models

During the development of vertebrate embryos, activation of the Activin/Nodal pathway is required for the formation of mesoderm and endoderm (6-7). High levels of Activin/Nodal signaling lead to endoderm formation, and low levels lead to mesoderm formation (8). FGF signaling is also involved in mesoderm formation during gastrulation (9-10). Knockouts in Erk2, a protein involved in the FGF signaling pathway, abolish the formation of mesoderm in
mice embryos (11). Wnt signaling is activated during gastrulation and is also required for the formation of mesoderm and definitive endoderm (12-15).

Inhibition of Activin/Nodal signaling causes ectoderm formation (7, 16). Within the ectoderm, high levels of BMP signaling lead to the formation of surface ectoderm, while low levels of BMP signaling lead to neural plate formation (17). Intermediate levels of BMP signaling are found in ectoderm cells that form the neural crest (18). Yes associated protein (YAP), a downstream target of Hippo signaling, is involved in neural crest formation (19). FGF signaling is also involved in the specification of different domains of the neural plate (20).

Hippo signaling is suppressed in the outer cells of the mouse embryo, leading to trophectoderm formation in the blastocyst (21). The hypoblast, which is formed from ICM cells, is also known as visceral endoderm in rodents (22). FGF4 signaling is required for the development of visceral endoderm in mice embryos (23-25). However, the formation of hypoblast in human embryos is not dependent on FGF signaling (26).

1.1.3 Observations in hESC cultures

In hESCs, BMP, Activin and Wnt signaling can all induce primitive streak gene expression (27). Inhibition of Notch signaling pathway prevents primitive streak gene expression in hESCs (28). Active Notch signaling is required for differentiation of hESCs to the three germ layers (29). FGF signaling is required for BMP to induce primitive streak gene expression
The Activin/Nodal signaling pathway is involved in definitive endoderm differentiation (31-35), while BMP signaling is involved in mesoderm differentiation (36-39). FGF signaling improves definitive endoderm formation from hESCs (40). Wnt signaling is required for mesoderm differentiation (41-43). Inhibition of Akt signaling in the presence of Activin signaling also leads to definitive endoderm differentiation through a primitive streak-like intermediate (31-32). These observations are summarized in Figure 1.4.

Inhibition of both Activin/Nodal and BMP signaling pathways leads to neural differentiation (44-46). Inhibition of Activin/Nodal signaling pathway alone can also lead to neural differentiation (47-51). Some reports have shown that FGF signaling inhibits neural differentiation (52), while others suggest that FGF signaling has a positive role in neural differentiation from hESCs (53-54). It has also been shown that neural differentiation of hESCs can occur independently of FGF signaling (54). Notch signaling promotes neural differentiation of hESCs, and FGF signaling is required for this process (55). Suppression of TAZ, which is downstream of the Hippo signaling pathway, leads to neural differentiation (51). Activation of the Wnt signaling pathway along-with inhibition of both Activin/Nodal and BMP pathways leads to neural crest formation (56). These observations are summarized in Figure 1.5.

Activin and FGF signaling pathways are required to maintain hESCs in a self-renewing state (57-61). However, certain reports have proposed that inhibition of FGF promotes hESC self-renewal when exogenous Activin A levels are increased (62). Notch pathway components are
present in hESCs, but the pathway is inactive in hESC cultures (63). MEF feeders and FGF signaling cause germ cell differentiation if hESCs are not passaged for long durations (64). BMP signaling is also involved in germ cell differentiation (65). Activation of Protein Kinase C (PKC) causes hESC differentiation to parietal endoderm (66). These observations are summarized in Figure 1.6.

1.2 Derivation of Trophoblast from hESCs

At the preimplantation stage, the human blastocyst embryo consists of the ICM and TE tissues. After implantation, the TE differentiates further to form the trophoblast tissues. These tissues are extraembryonic and are present only in the placenta during pregnancy. These tissues include the villous cytotrophoblast (vCTB), the syncytiotrophoblast (STB), the column cytotrophoblast (cCTB), the invasive cytotrophoblast (iCTB) and the derivatives of iCTBs (67-68). These tissues play an important role in fetal growth during pregnancy by regulating the supply of nutrients from the maternal blood to the fetus. In particular, iCTBs invade through the endometrial tissue of the uterus within the first few weeks of gestation and enter the maternal arteries, where they are responsible for remodeling the artery and allowing for sufficient blood flow towards the placenta (69). The STB acts as a barrier between the fetal side and the maternal side, allowing nutrients to diffuse through and enter the fetal blood flow. VCTBs act as progenitors to STBs and iCTBs, and have been proposed to harbor a stem cell population during 6-8 weeks of gestational age (70-74). The study of these early trophoblast tissues, however, is largely impeded due to restrictions on the
availability of these tissues from patients. Therefore, hESCs are seen as an alternative for derivation and study of these tissues.

The ability of hESCs to form trophoblast tissues in cell culture, however, has been a subject of intense debate (75-81). Studies with mouse embryonic stem cells (mESCs) have failed to derive trophoblasts in cell culture conditions (82-83), except through genetic manipulations (83-86). MESCs, when injected into mice blastocyst embryos, become a part of the fetus but do not incorporate into the placenta (82, 87). Therefore, it has been proposed that mESCs are already committed towards forming tissues of the fetus, and cannot form trophoblast tissues. It has been further proposed that the ICM and TE tissues of the embryo are segregated in their ability to form various tissues, and an “epigenetic barrier” mechanism maintains this segregation (73). Since hESCs are derived from ICM cells of the human embryo, such segregation has been proposed to exist in hESCs as well (75), leading to the hypothesis that hESCs do not have the ability to form trophoblast tissues. On the other hand, however, each of the trophoblast tissues is characterized by specific properties (70-74), and some of these properties have indeed been observed in differentiated cells obtained from hESCs. Therefore, it has been proposed that hESCs may retain the ability to form trophoblast tissues, unlike their mESC counterparts (77).

1.2.1 Trophoblast markers

While attempting to derive specific types of cells from hESCs, it is important to characterize the resultant type of cell appropriately. This is accomplished by probing for specific proteins,
known as markers, which are expressed only in specific cells of the human body. However, the early human trophoblast tissues remain poorly characterized in terms of marker proteins, due to the restricted availability of these tissues. Nevertheless, certain key features of the human trophoblast are known (73-74). It is known that vCTBs and STB do not express HLA Class I type A, B and C antigens (74). It has been proposed that this feature allows these tissues to evade the maternal immune system, thereby allowing the fetus to develop. ICTBs express HLA Class I type C and G antigens and can enter the maternal arteries without evoking an immune response. ICTBs also express matrix metalloproteinases MMP 1, 2, 3, 7 and 9, allowing them to invade the endometrium. The STB secretes human placental lactogen (HPL) and human chorionic gonadotropin (HCG) hormones during pregnancy, and is also multinucleate. VCTBs during 6-8 weeks of gestation also harbor a pool of cells that expresses CDX2 and ELF5, similar to their mouse counterparts (73). All trophoblast tissues also express CYTOKERATIN7 (74). Taken together, these properties can serve as a reference for validating trophoblast differentiation from hESCs.

1.2.2 Current approaches

Current attempts at deriving trophoblast cells from hESCs have led to the creation of cells that have a few properties of in vivo trophoblast cells (76). However, many of the key marker properties of in vivo trophoblast cells are not found in these hESC-derived cells (75), thereby leading to a debate about the identity of these hESC-derived “trophoblast-like” cells (88). Current approaches for obtaining these cells have largely focused on activating the BMP
signaling pathway in hESCs (89). The inhibition of Activin/Nodal pathway and inhibition of the FGF pathway in conjunction with the activation of BMP pathway (90-91) has also been carried out. Inhibition of Activin/Nodal pathway has been shown to upregulate the endogenous BMP pathway, and the BMP pathway has been proposed to be responsible for trophoblast formation (90). On the other hand, inhibition of the FGF and Activin/Nodal pathways along-with the activation of the BMP pathway causes accelerated differentiation to “trophoblast-like” cells (91). All of these treatments lead to the formation of cells that express some trophoblast markers such as CYTOKERATIN7 and HCG (29, 81, 90-102). These cells progress to form multinucleate STB-like cells (80-81, 91-93, 95-96, 103). However, these epithelial cells also express HLA-G (80), which is not present in vCTBs or STBs of the placenta (74). These cells do not express ELF5 (75, 80) and continue to express HLA Class I type A, B and C antigens (75). These cells continue to have DNA hypermethylation at the ELF5-2b promoter locus, while vCTBs of the placenta have hypomethylated DNA at the ELF5-2b promoter locus (73, 75). While these cells express CDX2, which is a marker of placental vCTBs (73), the expression of CDX2 has been shown to depend on BRACHYURY, a marker of primitive streak cells (75). Therefore, it has also been proposed that these BMP4-treated cells belong to the mesoderm germ layer and are not trophoblasts.

Other approaches have focused on generating trophoblast cells through ‘embryoid body’ formation (97, 103-108). Embryoid bodies are aggregates of hESCs that are maintained in suspension culture and induced to differentiate. Cells in different regions of the embryoid
body are influenced by different levels of exogenous and endogenous signaling, leading to heterogeneous differentiation and the formation of numerous cell-types. These aggregates are subsequently dissociated into single cells and the required type of cell is selected out. These approaches have led to the isolation of invasive iCTB-like cells which express HLA-G, VE-CADHERIN and CYTOKERATIN7 (80, 109), though it is not known whether these iCTB-like cells downregulate the expression of HLA Class I type A and B antigens. Also, vCTB-like cell lines have been isolated which express CYTOKERATIN7, HLA-G, CD9, MMP2, MMP9 and HSD3B1 (105). Since placental vCTBs are known to not express HLA-G, CD9 and HSD3B1 (74, 110-111), the identity of these cells remains unknown.

1.2.3 Role of Signaling pathways

Currently used methods for deriving trophoblast-like cells have relied on activating the BMP signaling pathway in hESCs, as well as using embryoid body methods. Activation of the BMP pathway is achieved by adding BMP4 protein to hESCs grown in a monolayer (81). Therefore, this method allows a homogeneous treatment of hESCs with BMP4, and for the mechanistic study of the effects of BMP4 signaling on hESCs. However, activation of the BMP4 signaling pathway in hESCs has also resulted in the formation of mesoderm, extraembryonic mesoderm and parietal endoderm cells (29-30, 38, 75, 80, 97, 102, 112). Also, it is not known whether BMP signaling is essential for the formation of trophoblast-like cells. Inhibition of Activin/Nodal and FGF signaling pathways along-with activating the BMP pathway has been described to increase the efficiency of trophoblast-like differentiation.
and it is not known whether these pathways have an independent role in trophoblast differentiation. Nevertheless, experiments with monolayer cultures of hESCs permit mechanistic studies to be performed, for probing the effect of these signaling pathways on hESCs.

1.3 Challenges in experimental design

1.3.1 Crosstalk among signaling pathways

To understand the effect of various signaling pathways on hESCs, experiments on monolayer cultures of hESCs are preferred (37). However, significant challenges remain with regards to understanding the role of signaling pathways in causing differentiation of hESCs. One such challenge is posed by crosstalk that exists among various signaling pathways in hESCs. Crosstalk occurs when a signaling pathway activates or inhibits another signaling pathway. This may happen due to intracellular interaction of proteins. For example, crosstalk between Akt, Wnt and MAPK pathways has been identified in hESCs (113-114), wherein Akt inhibits the MAPK signaling pathway as well as the Wnt signaling pathway. YAP, which is controlled by the Hippo signaling pathway, can regulate the Activin/Nodal pathway (51).

Crosstalk can also occur due to secretion of extracellular proteins, which then activate another signaling pathway. For example, activation of Wnt pathway in hESCs causes transcriptional upregulation of BMP2, BMP4 and NODAL (43). Treatment of hESCs with Activin A and BMP4, which activate the Activin/Nodal and BMP signaling pathways, respectively, causes upregulation of WNT1, WNT3A and WNT8A, all of which activate the
Wnt signaling pathway (41). Activin A upregulates the expression of FGF8, which activates the FGF signaling pathway (60). Activin A also upregulates WNT3 and downregulates SFRP1, which can inhibit the Wnt signaling pathway. Inhibition of Activin/Nodal signaling causes downregulation of WNT3, FGF2, FGF4 and FGF8, while upregulating BMP2, BMP4 and BMP7 (90). The expression of CRIPTO, which is required for Nodal signaling, is upregulated by the FGF signaling pathway (61). Treatment of hESCs with rapamycin, an inhibitor of mTOR, causes upregulation of BMP2 (39). Treatment of hESCs with LY294002, an inhibitor of phosphatidylinositol 3-kinase (PI3K) does not have the same effect. Both mTOR and PI3K are involved in the Akt signaling pathway. Together, these studies show that a perturbation in one signaling pathway may affect various other signaling pathways through crosstalk mechanisms in hESCs.

1.3.2 Heterogeneity in cell culture

Additional complexities in monolayer experiments arise due to the fact that hESCs in cell culture exist in heterogeneous populations. For example, hESC cultures have been shown to comprise of two populations of cells with differential states of Wnt signaling (115). These distinct populations are biased towards differentiating to different lineages. HESCs respond heterogeneously to Wnt signaling agonists (42). Other reports have identified heterogeneous populations of hESCs in cell culture with respect to the expression of various marker proteins. For example REX1, which is a marker of ICM in mice embryos and is downregulated in the epiblast, is heterogeneously expressed (116). SSEA-3, an hESC
marker, is also heterogeneously expressed (117-118). However, the implications of such heterogeneity are not fully understood.

### 1.3.3 Diverse culture conditions

Yet another challenge in understanding the behavior of hESCs exists due to the fact that they can be cultured under varied media conditions, which target different signaling pathways. HESCs were originally derived in co-culture with mouse embryonic fibroblast (MEF) cells (1). MEF cells secrete proteins that activate specific pathways in hESCs allowing them to proliferate and remain as hESCs, thereby arresting differentiation. MEF-free conditions for culturing hESCs were subsequently devised (119). Briefly, MEF cells are grown in media, allowing secreted proteins to accumulate. This medium, known as MEF-conditioned medium (MEF-CM), is then used for culturing hESCs. However, the composition of MEF-CM remains unknown, although recent reports have sought to identify secreted proteins present in MEF-CM (120-121). Recently, defined media formulations for culture of hESCs have also been derived (122). These media formulations include mTeSR1 (122), XFT (123), HESCO (124), NBF (125), N2/B27-CDM (36), FTDA (126), ‘52300+bFGF’ (127), “BMP11+Myostatin” (128) and StemPro (129). Defined media conditions are a valuable tool for understanding hESC behavior since the exogenous signaling factors present in the media are fully known. However, these media formulations vary in terms of signaling pathway activators/inhibitors used. For example, mTeSR1, HESCO and ‘52300+bFGF’ contain activators of Wnt signaling pathway while the rest do not. ‘52300+bFGF’ does not contain an
activator of the Activin/Nodal pathway, unlike the rest of the media conditions. MEF-CM contains serum-associated lipid factors such as sphingosine 1-phosphate (S1P) and lysophosphatidic acid (LPA), while defined media formulations do not. S1P can activate YAP which is downstream of the Hippo signaling pathway (130). Although the implications of such differences are largely unknown, a recent report on deriving trophoblast-like cells from hESCs has reported lower efficiencies upon using CDM, as compared to MEF-CM (80). Moreover, most studies for obtaining trophoblast-like cells have used MEF-CM (80-81, 91-93, 95-96, 103), while the usage of serum-free media has resulted in the formation of mesoderm, extraembryonic mesoderm and parietal endoderm (29-30, 38, 75, 80, 97, 102, 112). The reason for these differences remains largely unexplored.

1.4 Signaling pathways involved in determining hESC fate

We provide a brief description of the various pathways that have been shown to affect the fate of hESCs. These pathways can be manipulated in concert to allow for the formation of specific cell-types from hESC cultures.

1.4.1 Activin/Nodal pathway

The Activin/Nodal pathway is a branch of the transforming growth factor-β (TGF-β) superfamily of signaling pathways. The TGF-β superfamily of signaling pathways has been reviewed in (131-134). The other branch of this superfamily comprises of the BMP signaling pathway. The Activin/Nodal pathway is initiated by the Activin, TGFβ and Nodal ligands,
which bind to their specific Type I receptors, Type II receptors and co-receptors. This leads to phosphorylation of the Type I receptor by the Type II receptor, and recruitment of SMAD2/3 proteins to the Type I receptor. SMAD2/3 proteins are then phosphorylated by the Type I receptor. Phosphorylated SMAD2/3 proteins can now enter the nucleus and cause the expression of target genes. A schematic of the pathway is shown in Figure 1.7.

1.4.2 BMP pathway

The BMP pathway forms the other branch of the TGF-β superfamily of signaling pathways. The BMP pathway is activated by BMP ligands as well as the Growth and Differentiation Factor (GDF) ligands. These ligands bind to their corresponding Type I receptors, Type II receptors and co-receptors, leading to the phosphorylation of the Type I receptor by the Type II receptor. The Type I receptor then recruits SMAD1/5/8 proteins leading to their phosphorylation. These phosphorylated proteins can now enter the nucleus and regulate their target genes. A schematic of the BMP pathway is shown in Figure 1.8.

1.4.3 FGF pathway

The FGF pathway is activated when FGF ligands bind to heparan sulfate proteoglycans (HSPGs) and FGF receptors (4, 135-138). This causes FGF receptors to dimerize and transphosphorylate each other. The phosphorylated receptors recruit the GRB2/SOS complex, leading to the activation of SOS, which then phosphorylates and activates RAS. This triggers a cascade of phosphorylations, ultimately resulting in the phosphorylation of
ERK1/2. Once phosphorylated, ERK1/2 can either phosphorylate other target proteins, or act as transcription factors. A schematic of the FGF pathway is shown in Figure 1.9.

1.4.4 Wnt pathway

The Wnt signaling is activated by the Wnt ligands, and is divided into the canonical and non-canonical branches (5, 139). In the absence of Wnt signaling, the transcription factor β-catenin forms a complex with Axin, GSK3β, APC and CK1, leading to phosphorylation and subsequent degradation of β-catenin. When Wnt ligands are present, they are recruited by Frizzled and LRP receptors, leading to deactivation of Axin. Deactivated Axin can no longer facilitate the phosphorylation of β-catenin by GSK3β, and β-catenin can not enter the nucleus to regulate gene expression. The noncanonical Wnt pathway, however, acts independently of β-catenin. A schematic of the Wnt pathway is shown in Figure 1.10.

1.4.5 Akt pathway

The Akt pathway can be activated by various growth factors such as Insulin, Insulin-like growth factor (IGF), FGF, TGFβ and others (140-142). Binding of these growth factors to their receptors leads to the activation of phosphatidylinositol 3-kinase (PI3K), which phosphorylates phosphatidylinositol (4, 5)-biphosphate to phosphatidylinositol (3, 4, 5)-triphosphate, thereby allowing Akt to bind. The binding of Akt allows it get phosphorylated by PDK2 and PDK1, leading to its activation. Akt then acts as a kinase for its substrates. A schematic of the Akt pathway initiated by IGFs is shown in Figure 1.11.
1.4.6 Hippo pathway

The hippo pathway is activated by cell-cell contact, which leads to the phosphorylation of MST1/2 kinases (143-144). These kinases then associate with SAV1 and phosphorylate LATS1/2. Upon phosphorylation, LATS1/2 associate with YAP and TAZ and phosphorylate them, causing to become retained in the cytoplasm. YAP and TAZ are otherwise localized to the nucleus, where they are co-factors for other transcription factors (145). A schematic of the Hippo signaling pathway is shown in Figure 1.12.

1.5 Thesis overview

We propose that trophoblast cells which have the key properties of human placental vCTBs, such as hypomethylation at the ELF5 promoter region, the expression of ELF5 and CYTOKERATIN7 proteins, the downregulation of HLA class I Type A, B and C antigens, and the ability to form multinucleate STBs and mesenchymal iCTBs, can be obtained from hESCs in cell culture through the manipulation of various signaling pathways.

1.5.1 Hypotheses

We study the role of various signaling pathways in the formation of trophoblast cells from hESCs. We find that trophoblast cells can be derived when BMP signaling is suppressed using inhibitors of BMP signaling. This is counter to current reports which propose that BMP signaling induces trophoblast gene expression. We also find that inhibition of the Activin/Nodal signaling has a role in trophoblast differentiation, and that its role is not
dependent on activation of BMP signaling. Our current preliminary studies show that Wnt signaling has a negative role in trophoblast differentiation, and that activation of Wnt signaling, when Activin/Nodal and BMP signaling are inhibited, leads to neural crest formation.

1.5.2 Thesis layout

Chapter 2 describes our results pertaining to trophoblast differentiation from hESCs. We describe the derivation of trophoblast cells with the marker properties that are present in placental vCTBs. We describe the involvement of Activin/Nodal and BMP signaling pathways in this process. We also describe our preliminary experiments which study the role of serum factors in this process. A significant impediment in our experiments has been the fact that different hESC cell lines have responded differently to the same signaling pathways because of varying levels of endogenous signaling.

Chapter 3 describes our efforts to characterize the endogenous signaling factors present in hESC cultures by studying their secretome. We describe a new approach to study the secretome of cells grown in cell culture. Using this approach, we explore the secretome of hESCs and MEFs. We also describe the characterization of hESC the hESC proteome at the subcellular level by fractionation of the cytoplasm, nucleus and plasma membrane from hESCs.
1.6 References


27


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Figure 1.1: Schematic of a blastocyst stage embryo. The embryo consists of two tissues, viz.
the trophectoderm and the inner cell mass.
Figure 1.2: Schematic of a peri-implantation embryo. The ICM segregates into the hypoblast and the epiblast tissues.
Figure 1.3: Schematic showing origin of the three germ layer tissues, viz. ectoderm, mesoderm and definitive endoderm.
Figure 1.4: Schematic showing role of signaling pathways in formation of mesoderm and definitive endoderm from hESCs.
Figure 1.5: Schematic showing the role of signaling pathways in the formation of neural and neural crest cells from hESCs.
Figure 1.6: Schematic showing the role of signaling pathways in self-renewal of hESCs and in differentiation to germ cells and parietal endoderm cells.
Figure 1.7: A schematic of the Activin/Nodal pathway.
Figure 1.8: A schematic of the BMP pathway.
Figure 1.9: A schematic of the FGF pathway.
Figure 1.10: A schematic of the Wnt pathway.
Figure 1.11: A schematic of the Akt pathway initiated by Insulin-like growth factors (IGFs).
Figure 1.12: A schematic of the Hippo signaling pathway.
CHAPTER 2

TROPHOBLAST DIFFERENTIATION FROM HUMAN EMBRYONIC STEM CELLS

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2.1 Introduction

Derivation of trophoblast cells from human embryonic stem cells (hESCs) in cell culture is desirable since the availability of trophoblast tissues from very early gestation placentas is scarce, and in certain cases impossible (1). Derivation of trophoblast cells from hESCs also provides a convenient model system for studying the events of very early embryonic development, such as implantation of the trophectoderm and its interaction with endometrial cells of the uterus. However, it is not known if hESCs have the ability to form trophoblast cells or whether they are committed to form only the fetal tissues of the embryo (2). Such a restriction has been observed in mice embryonic stem cells (mESCs) due to their epigenetic properties (3). Moreover, trophoblast-like cells derived from hESCs have been under criticism since they do not possess the properties that trophoblast cells of the placenta have (2). These properties include DNA hypomethylation in the \( ELF5 \) promoter region, the expression of ELF5 protein, the downregulation of HLA Class I type A, B and C antigens, as well as the co-expression of CDX2 and ELF5. Therefore, it is currently not known whether \textit{bona fide} trophoblast cells can be derived from hESCs (2).

2.2 Results

2.2.1 Derivation of cells with DNA hypomethylation at \( ELF5 \) promoter region

We hypothesized that it was possible for hESCs to differentiate into trophoblasts that possess the required properties of placental trophoblast cells. In mESCs, it has been hypothesized that
trophoblast differentiation is prevented due to an “epigenetic barrier” that causes mESCs to suppress ELF5 expression (3). This epigenetic barrier causes the DNA at the ELF5 promoter region to be hypermethylated, causing transcriptional repression of ELF5. This barrier is lifted in mESCs with a homozygous knockout in the DNA methyltransferase gene Dnmt1. We investigated whether DNA hypermethylation persists during hESCs differentiation as well. Two hESC cell lines, H1 and H9 were used in our experiments. HESCs treated with BMP4 have been shown to retain DNA hypermethylation in the ELF5 promoter region. We therefore looked at inhibition of the Activin/Nodal pathway using SB431542 (4) in the presence of mouse embryonic fibroblast-conditioned medium (MEF-CM), which has also been reported to lead to the formation of trophoblast-like cells (5). We observed that, while treatment of hESCs with SB431542 for 6 days caused minimal demethylation of the promoter region, passaging of cells and continued treatment till 12 days led to a significant demethylation (Figure 2.1). The levels of demethylation were similar to those seen in vCTBs of the human placenta (6). Treatment of hESCs with SB431542 and BMP4 together also led to similar results, causing demethylation of the ELF5 promoter after 12 days of treatment.

Methylation of the ELF5 promoter region has been proposed to control the expression of ELF5 (3). Therefore, it is important to confirm the expression of ELF5 protein directly. The expression of ELF5 in differentiated hESCs was confirmed in various experimental conditions using immunofluorescence assays (Figure 2.2). We therefore show that the proposed “epigenetic barrier” on the expression of ELF5 in mESCs, is not present in hESCs.
2.2.2 Expression of CYTOKERATIN7 and HLA Class I type A, B and C antigens

All human trophoblast cells express CYTOKERATIN7 (7-8). Villous cytotrophoblast cells (vCTBs) and syncytiotrophoblast (STB) do not express HLA Class I type A, B and C antigens, while invasive cytotrophoblasts (iCTB) express HLA Class I type C and G antigens. For probing the expression of these membrane proteins during hESC differentiation, we isolated the membrane fraction of hESCs and of hESCs treated with SB435142 for 6 and 12 days. HESCs were grown in media containing isotope labeled amino acids, viz. $^{13}\text{C}_6$ L-arginine and $^{13}\text{C}_6\ ^{15}\text{N}_2$ L-lysine for 5 passages. Greater than 98% incorporation of isotope labeled amino acids was observed (9). Subcellular fractionation was carried out to obtain the plasma membrane of hESC samples. Undifferentiated hESCs, which contained the isotope labels, were then compared with differentiated hESCs, which did not contain the isotope labels, using mass spectrometry. While the expression of HLA Class I type A and B antigens were vastly downregulated at day 12 (Figure 2.3). The expression of HLA Class I type C antigens also decreased at day 12. We observed upregulation of various cytokeratins, including CYTOKERATIN7 at day 6. We concluded that the expression of HLA Class I type A, B and C antigens were downregulated by SB431542 treatment, while that of CYTOKERATIN7 was upregulated, and that the cells obtained by SB431542 treatment were similar to placental vCTBs in these characteristics.
2.2.3 Derivation of syncytiotrophoblasts (STBs)

The formation of multinucleate STBs coupled with the secretion of hormones such as hCG, has been the most widely used marker for assessing trophoblast formation (10-17). While these markers are not sufficient to demonstrate trophoblast differentiation (2), they are, nevertheless, necessary. Studies with human placental vCTBs have shown that STB formation occurs from vCTBs spontaneously in cell culture (18). We observed that treatment of hESCs with SB431542 for 6 days and subsequent passage into MEF-CM lacking SB431542 caused the formation of multinucleate cells (Figure 2.4). These cells upregulated the expression of hCG and of HSD3B1, which is involved in the synthesis of progesterone and is also a marker of STB (19). We therefore concluded that hESCs treated with SB431542 were capable of forming STBs.

2.2.4 Derivation of iCTBs

In the human placenta, vCTBs also give rise to iCTBs through an epithelial to mesenchymal transition (EMT). First trimester placental explants retain this behavior and undergo an EMT when grown in cell culture (18). We observed that continued treatment of hESCs with SB431542 in the presence of MEF-CM induced an EMT in H9 hESCs (Figure 2.5). These mesenchymal cells expressed VE-CADHERIN and CD9, which are markers of iCTBs (20-21). To test for invasiveness, SB431542-treated H9 cells were plated on a solidified block of matrigel, which is an extracellular matrix comprising of collagen, fibronectin and laminin.
Mesenchymal cells that stained for VE-CADHERIN and CD9 were formed and were seen to invade into the matrigel block. This proved the invasive ability of these cells.

However, H1 hESCs did not undergo an EMT when treated with SB431542. Nevertheless, they expressed CD9 (data not shown). We concluded that differentiation might have started in H1 cells, but that EMT had been suppressed. Also, upon passage of H9 hESCs as large clumps, they failed to undergo an EMT. Therefore, we reasoned that an endogenously secreted cytokine might be responsible for this behavior. Human placental vCTBs are known to express BMP7 (22). We hypothesized that endogenous BMP signaling may be responsible for this discrepancy between H1 and H9 cells, and sought to inhibit BMP signaling. For this, we added Noggin and DMH1 to hESCs along-with SB431542. Noggin inhibits BMP signaling through its ability to bind BMP2 and BMP4 with a very high affinity (23). DMH1 is a small molecule inhibitor of ALK2, ALK3 and ALK6 receptors that are involved in BMP signaling (24). Treatment of hESCs with SB431542, Noggin and DMH1 resulted in the onset of an EMT in both H1 and H9 cells, thereby abolishing the discrepancy in their behavior. Further characterization of these mesenchymal cells will reveal if they retain the marker expression characteristics of iCTBs.

2.2.5 Derivation of trophoblast cells that co-express CDX2 and ELF5

A recent report has identified a pool of vCTBs in the human placenta which co-expresses CDX2 and ELF5 (6). These cells occur only during early pregnancy and are almost entirely lost after 8 week of gestation. These cells are analogous to the extraembryonic ectoderm in
mice embryos which co-expresses Cdx2 and Elf5 (25). Cdx2 and Elf5 are also the markers of mouse trophoblast stem cells (mTSCs), which can be maintained in cell culture as a cell line, and are capable of forming all of the placental tissues in vitro (26). Therefore, it has been hypothesized that the pool of vCTBs which co-expresses CDX2 and ELF5 in the first trimester human placenta may also harbor a stem cell population (6). CDX2 is also a marker of the trophectoderm tissue in blastocyst-stage embryos (27). It is currently not known whether CDX2+ ELF5+ cells can be derived from hESCs. Therefore, we studied the expression of CDX2 in our differentiated cultures.

We did not detect the expression of CDX2 in hESCs treated with SB431542 for 12 days (data not shown). Moreover, in the case of H9 cells, these cells had already started to form iCTBs. Upon further passage of these cells at day 12, all epithelial cells were lost. We therefore reasoned that the absence of CDX2 might be a result of triggering iCTB differentiation. Addition of exogenous BMP4 along-with SB431542 to hESCs abolished EMT, but we were not able to detect CDX2 expression. We then probed the effect of the Activin/Nodal pathway on these cells. The Activin/Nodal pathway is activated by the Transforming and growth factor-β (TGFβ), Nodal and Activin proteins (28-30). We blocked each of these ligands separately to study their effect on EMT. Briefly, hESCs were treated with SB431542 in MEF-CM for 6 days and then passaged into MEF-CM containing various inhibitors of the Activin/Nodal pathway. Blocking of TGFβ using soluble Betaglycan (31) led to the formation of flattened multinucleate cells that resembled STBs. Blocking of Activin using Follistatin-288 (32) also led to the formation of flattened multinucleate cells. Inhibition of
both TGFβ and Activin abolished the formation of multinucleate cells and significantly reduced the formation of mesenchymal cells, but did not abolish it. We studied the pool epithelial cells that were seen to remain in these conditions, for the presence of CDX2 and ELF5. We also added Noggin and DMH1 to abolish variability across H1 and H9 cells lines, as shown previously. Therefore, hESCs were treated with SB431542, Noggin and DMH1 in MEF-CM for 6 days and then passaged into MEF-CM with Follistatin, Betaglycan, Noggin and DMH1. Expression of ELF5 was seen in some cells, and faint expression of CDX2 could be observed in few cells (Figure 2.6), suggesting a microenvironment-based effect on the expression of CDX2. We concluded that it may be possible to derive CDX2⁺ELF5⁺ cells and that further studies are required. The temporal pattern of CDX2 expression also needs to be investigated further.

2.2.6 The switch between trophoblast and neural fate of hESCs

A significant caveat of our results is that we have observed trophoblast formation through the inhibition of Activin/Nodal and BMP signaling pathways. SB431542, an inhibitor of Activin/Nodal signaling, is reported to cause neural differentiation in hESCs (33-37). The addition of Noggin and DMH1 in the presence of SB431542 does not change the fate of hESCs and also leads to neural differentiation (38-40). These reports have argued that hESCs have low levels of endogenous BMP signaling, and that exogenous Noggin and DMH1 are not required in many cases for neural differentiation to occur (33-37). Contrary to these reports, we have obtained trophoblast cells with the use of SB431542, Noggin and DMH1.
To resolve this contradiction, we looked at other possible signaling pathways that may differ between our experiments and previously reported experiments. While our experiments have been carried out in MEF-CM, neural differentiation has been carried out in defined media conditions lacking serum and serum-replacement (33-41). Previous reports have shown that serum factors suppress neural differentiation in mouse embryonic stem cells (mESCs) (42-43). Therefore, we tested our hypotheses using mTeSR1, a defined medium that lacks serum and serum-replacement. We observed that the addition of SB431542, Noggin and DMH1 to hESCs in mTeSR1 led to the formation of ‘neural rosettes’ (data not shown). Neural rosettes are radial arrangements of columnar neural precursor cells that form during neural differentiation in vitro, and resemble the radially arranged columnar neural precursor cells of the neural tube which occur during embryogenesis (44). Multinucleate STB-like cells could not be derived from these cells. We therefore inferred that trophoblast differentiation had not occurred in this condition.

We then tested the effect of serum factors on hESC differentiation. MEF-CM contains 20% Knockout® serum replacement (KOSR), which contains lipid-rich bovine serum albumin known as AlbuMAX®. The complete composition of AlbuMAX® is not known. However, it is known to contain serum-associated factors such as sphingosine 1-phosphate (S1P) and lysophosphatidic acid (LPA). It was reported that the lipid component, and not the protein component of AlbuMAX®, is required for maintenance of hESCs (45). Of these factors, S1P is involved in the maintenance of hESCs and can be used in place of serum (46-47). Since lipid factors are not present in mTeSR1, we probed whether these lipid factors are
responsible for causing trophoblast differentiation in MEF-CM conditions. We therefore added AlbuMAX to mTeSR1 along-with SB431542, Noggin and DMH1. However, this also led to the formation of neural rosettes (data not shown). Multinucleate cells could not be derived, implying that trophoblast differentiation had not occurred. We also observed that the addition of AlbuMAX® or KOSR to mTeSR1 causes the cells to change into a more spread-out morphology, indicating an adaptation to the new conditions. Since cells in MEF-CM are already adapted to AlbuMAX®, and form trophoblast cells, we tested whether this ‘adaptation’ step was needed for trophoblast differentiation to occur in mTeSR1. Therefore, we treated hESCs in mTeSR1 with AlbuMAX® for 4 days and then added SB431542, Noggin and DMH1 along-with AlbuMAX® for 6 more days. We observed that neural rosettes had not formed and that some multinucleate cells were formed, suggesting that trophoblast differentiation may have occurred in some cells (data not shown). However, we also observed some astrocyte-like cells, which demonstrated heterogeneity and the possible formation of neural crest cells. Therefore, further investigation is required to study the signaling factors responsible for the formation of trophoblast cells from hESCs.

2.3 Discussion and future work

2.3.1 Derivation of cells with properties of placental trophoblast cells

We have shown that hESCs can be made to differentiate into trophoblast cells that possess the key properties of human placental trophoblast cells. These include DNA hypomethylation at the ELF5 promoter region, the downregulation of HLA Class I type A, B and C antigens,
and the expression of ELF5 protein. These properties have not been demonstrated in previous reports. We also show that STB and iCTB derivatives can be obtained from hESCs, and that these cells express the markers of placental STB and iCTB, respectively. Trophoblast cells downregulate HLA Class I type A, B and C antigens were obtained through inhibition of Activin/Nodal pathway alone, while the rest of the properties were seen in cells where both Activin/Nodal and BMP pathways were inhibited. Therefore, the expression of HLA Class I type A, B and C antigens need to be further studied in hESCs where both Activin/Nodal and BMP pathways have been inhibited.

2.3.2 The role of BMP pathway in trophoblast differentiation

More importantly, we uncover the role of various signaling pathways in the differentiation of hESCs to trophoblast cells. Our results show that trophoblast cells can be obtained in the presence of exogenous BMP signaling (Figures 2.1, 2.2A, 2.4I, 2.4N) and also when BMP signaling is inhibited through Noggin and DMH1. This is contrary to the current paradigm, which suggests that exogenous BMP signaling induces trophoblast gene expression (1, 17, 48-49). However, BMP-induced trophoblast gene expression is reported as being mediated through BRACHYURY (2). Sh-RNA induced knockdown of BRACHYURY abolishes the expression of CDX2. BRACHYURY is a marker for mesoderm cells, so it is not known whether BMP-treated hESCs belong to the trophoblast lineage or the mesoderm lineage. However, the study of BMP-treated hESCs is beyond the scope of this work. Nevertheless, we show that trophoblast formation can also occur when BMP signaling is inhibited.
2.3.3 The role of Activin/Nodal pathway in trophoblast differentiation

The role of Activin/Nodal pathway towards trophoblast differentiation has not received much attention. Inhibition of Activin/Nodal pathway enhances endogenous BMP signaling, and is reported to complement BMP in its ability to induce trophoblast gene expression. We, however, show that inhibition of Activin/Nodal pathway in MEF-CM leads to trophoblast formation even when BMP signaling is inhibited using Noggin and DMH1, suggesting an independent role of Activin/Nodal signaling in inducing trophoblast differentiation.

2.3.4 The role of serum factors in trophoblast differentiation

An important caveat of our approach is the inhibition of Activin/Nodal and BMP signaling pathways with SB431542, Noggin and DMH1. While we show that this treatment leads to the formation of trophoblast cells in MEF-CM, our preliminary data also suggests that the same treatment leads to the formation of neural progenitors in mTeSR1. Since mTeSR1 lacks serum factors, we tested the effects of adding AlbuMAX® to mTeSR1 cultures. Our preliminary data suggests that hESCs grown in mTeSR1 can be adapted to serum factors by adding AlbuMAX®, and these cells do not undergo neural differentiation, but form STB-like cells and neural crest cells. Previous reports have shown that serum factors suppress neural differentiation in mouse embryonic stem cells (mESCs) (42-43). Therefore, a possible explanation for our results may be through the action of serum factors. We then probed for the signaling pathway that was being activated by serum factors. To begin, we probed whether these differences were being caused by the Akt signaling pathway. However, the
inhibition of Akt pathway in MEF-CM by the addition of LY294002 did not abolish the formation of multinucleate STB-like cells (data not shown). This showed that the effect of serum factors present in MEF-CM was not mediated through the Akt signaling pathway.

We then studied if the Erk signaling may be responsible for the discrepancy observed between MEF-CM and mTeSR1 cultures. However, the addition of the MEK inhibitor PD0325901 also did not abolish the formation of multinucleate STB-like cells (data not shown), showing that the Erk signaling was also not responsible for the discrepancy. Alternatively, S1P present in AlbuMAX® can activate YAP in hESCs (50). Overexpression of YAP reduces neural differentiation in mESCs (51). Also, YAP is involved in patterning the mouse embryo at its 8-cell stage, and leads to the commitment of outer cells to the trophectoderm lineage (52). YAP acts as a coactivator for TEAD4 (53-55), which is required for the expression of CDX2 in mouse embryos (52, 56-57). Therefore, it can be hypothesized that the formation of trophoblast cells in MEF-CM is mediated through activation of YAP by serum factors. Since these factors are absent in mTeSR1, trophoblast differentiation was not observed in mTeSR1.

Our preliminary data also suggests that astrocyte-like cells are obtained in MEF-CM cultures when Wnt signaling is activated using BIO, which is a Wnt signaling agonist, even in the presence of SB431542, Noggin and DMH1. This is consistent with previous reports of neural crest formation from hESCs (58). We failed to derive multinucleate STB-like cells in these experiments, showing that trophoblast differentiation had not occurred. This suggests a
negative role for Wnt signaling in trophoblast formation. Another Wnt signaling agonist, namely lithium chloride (LiCl), is present in the formulation of mTeSR1 medium. As an alternative hypothesis, LiCl may also be responsible for our observations wherein trophoblast cells are not formed in mTeSR1 cultures. MEF-CM, on the other hand, is known to contain Wnt signaling inhibitors (59). Therefore, further work is required to study the role of Wnt signaling in trophoblast differentiation of hESCs.

2.4 Materials and methods

2.4.1 Cell culture and differentiation

H1 and H9 hESCs were cultured on feeder layers of mouse embryonic fibroblasts (MEFs) isolated from E13.5 pregnant CD-1 mice embryos (Charles River, Wilmington, MA). For feeder free culture, hESCs were grown on Growth Factor Reduced Matrigel™ (BD Biosciences, Bedford, MA), in MEF CM; CM was prepared using previously published protocols (60). Differentiation was carried out by adding SB431542 (25μM), SB525334 (10μM) or FOLLISTATIN (1nM) (Sigma-Aldrich, St. Louis, MO) to H1 or H9 cultures, as specified, in the presence of CM. The CM containing the inhibitor or FOLLISTATIN was refreshed every day. Enzymatic passaging was carried out by scoring confluent colonies into equally spaced grids of cells using a Pasteur pipet, and lifting off the cells using collagenase IV (Invitrogen). For SILAC labeling, H9 cells were grown in CM without L-lysine and L-arginine, but containing the stable isotopes $^{13}$C$_6$, $^{15}$N$_2$ L-lysine and $^{13}$C$_6$ L-arginine (Pierce, Rockford, IL) (SILAC-CM), as previously described (61). Stable isotope labeled arginine
and lysine incorporation of 98.5% and 98.0%, respectively, was achieved. Arginine-to-proline conversion was determined to be approximately 5% in our system (9).

2.4.2 Isolation of iCTB and STB populations

ICTBs were isolated using a very short Trypsin/EDTA (Sigma-Aldrich) treatment of the cells with gentle shaking to dislodge only the mesenchymal cells. STBs were found to be in co-culture with small epithelial cell colonies, which were removed carefully under a stereomicroscope to obtain STBs.

2.4.3 RNA isolation, cDNA synthesis and quantitative PCR

RNA was isolated using Trizol™ reagent (Invitrogen) using the manufacturer’s protocol. For cDNA synthesis, the RNA pellet was dissolved in diethyl pyrocarbonate (DEPC, Sigma)-treated water and 15 µg RNA was heated at 70°C for 5min with oligo-dT 15-mer primers (Integrated DNA Technologies (IDT), Coralville, IA). M-MLV reverse transcriptase (Invitrogen) and dNTP mix (Invitrogen) were added and the reaction was carried out for 50min at 42°C. The reaction mixture was heated to 70°C for 10min and further incubated with 0.5M sodium hydroxide for 30min at 65°C. The solution was neutralized with 1M hydrochloric acid and stored at -20°C until further use.

Quantitative PCR (qPCR) reactions were carried out using SYBR Green Supermix® (Bio-Rad, Hercules, CA) in a Mastercycler® ep Realplex system (Eppendorf, Hauppage, NY). The primers used for qPCR analysis are listed in Supplementary Table 1. ANOVA
analysis of the data was carried out using the SAS software. QPCR analysis was carried out using three biological replicates for H9 hESCs and one biological replicate for H1 hESCs.

2.4.4 Immunofluorescence

For immunofluorescence analysis, cells were passaged on to glass-bottom culture dishes (Greiner Bio-one, Monroe, NC) coated with Matrigel and cultured in CM or CM supplemented with SB431542. Cells were fixed using 4% paraformaldehyde (Fisher Scientific, Houston, TX) and permeabilized with 0.5% Triton X-100 (Acros Organics, Geel, Belgium). Subsequently, cells were blocked in 1X PBS with 5% BSA and 0.3% Triton X-100, and incubated overnight with the primary antibody diluted in the same blocking buffer. Rabbit anti-human antibodies for OCT4, NANOG, β-CATENIN, VE-CADHERIN, β-ACTIN (Cell Signaling, Danvers, MA), CDX2 (Epitomics, Burlingame, CA), and mouse anti-human antibodies for SSEA4, CD9 (Millipore, Billerica, MA), and ELF5 (Santa Cruz Biotechnology, Santa Cruz, CA) were used. Corresponding rabbit and mouse isotype antibodies for controls were purchased from Cell Signaling, Millipore, Epitomics and Santa Cruz Biotechnology. CellMask™ Deep Red plasma membrane stain was purchased from Invitrogen. Cells were labeled with one or more of Alexa 633-conjugated goat-anti-rabbit IgG, Alexa 488-conjugated goat-anti-rabbit IgG (Invitrogen) and DAPI (Invitrogen), as appropriate, and imaged using a Zeiss LSM 710 confocal microscope.
2.4.5 Immunohistochemistry and histology analysis

Growth factor reduced Matrigel diluted in ice-cold coating buffer (0.01M Tris, 0.7% sodium chloride, pH 8.0) was plated at 5 mg/ml on 24 well plate inserts (12μm, Millipore) and allowed to solidify. H9-vCTBs were passaged on solidified Matrigel surface and cultured in CM. Samples were obtained at specified time points and fixed overnight using 10% neutral buffered formalin (Thermo Scientific, Rockford, IL). Immunohistochemistry (IHC) and H&E staining were carried out at the Histology facility at NCSU, using standard paraffinization protocols. Antibodies used for CD9, VE-CADHERIN, β-CATENIN and E-CADHERIN were the same as previously mentioned.

2.4.6 Flow cytometry

Cells were dissociated using Trypsin/EDTA, fixed in 4% paraformaldehyde and permeabilized in Saponin Buffer containing 1mg/ml Saponin (Sigma-Aldrich) and 1% BSA in PBS. Cells were incubated with the specified primary antibodies for 1 hour at room temperature and subsequently with corresponding secondary antibodies for 1 hour. 3β-HSD1 antibody was purchased from Abcam (Cambridge, MA) and propidium iodide was purchased from Sigma-Aldrich. Cells were analyzed using a BD FacsAria flow cytometer. Propidium iodide analysis of multinuclear cells was carried out using previously published protocols (62).
2.4.7 Subcellular fractionation

Membrane, cytoplasmic and nuclear fractions were isolated from hESCs, hESC-derived vCTBs and hESC-derived eCTBs using previously published protocols (61). The membrane and nuclear pellets were homogenized in 8 M Urea and 50 mM ammonium bicarbonate and used for mass spectrometric analysis.

2.4.8 Protein fractionation and in-gel digestion

The cytosolic, membrane, and nuclear fractions were prepared separately for LC-MS/MS analysis. 25 µg of SILAC labeled protein sample (0 Day, undifferentiated hESCs) was combined with 25 µg of the unlabeled protein sample (either 6 Day hESC-vCTBs or 12 Day hESC-eCTBs) and loaded onto a Criterion 10-20 % Tris-HCl gel (Bio-Rad); additionally, 50 µg of protein sample each from hESCs, hESC-vCTBs and hESC-eCTBs was used for quantification of cytoplasmic protein samples by spectral counting. Proteins were separated by constantly applying 200 V for approximately 1 hour and visualized with Coomassie Stain (Bio-Rad). Each gel lane was dissected into 12 fractions, which were then subjected to reduction with dithiothreitol (DTT), alkylation with iodoacetamide, and digestion with trypsin per a protocol adapted from Shevchenko et al.(63) Extracted peptides were dried in vacuo and reconstituted in mobile phase A (98 % water, 2 % acetonitrile, and 0.2 % formic acid) prior to analysis.
2.4.9 LC-MS/MS and data analysis

An Eksigent 1D+ nano-LC system (Eksigent, Dublin, CA) utilizing a vented column configuration (64) was used for reversed-phase separation of peptides. Magic C18AQ stationary phase (5 µm particle size, 200 Å pore size; Microm BioResources, Auburn, CA, USA) was packed to 15 cm in a 75 i.d. PicoFrit capillary (New Objective, Woburn, MA, USA) for the analytical column and to 5 cm in a 75 i.d. IntegraFrit capillary (New Objective) for the trapping column. LC solvents were purchased from Burdick and Jackson (Muskegon, MI,). Mobile phase A contained 98 % water, 2 % acetonitrile, and 0.2 % formic acid, and mobile phase B consisted of 2 % water, 98 % acetonitrile, and 0.2 % formic acid. Flow rate during the gradient was set to 350 nL/min. The gradient was held at 2 % B for 5 min, adjusted to 10 % B at 7 min and gradually increased to 50 % B over the next 120 min. The gradient was then increased to 95 % B and held for 5 min before re-equilibrating at 2 % B for 10 min.

All measurements were made using a LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). A precursor scan with 60,000 resolving power at 400 m/z was performed in the Orbitrap mass analyzer followed by eight data-dependent MS/MS scan events in the ion trap. Collision-induced dissociation was employed with an isolation width of 2 m/z and normalized collision energy of 35 % for 30 ms. Unassigned, 1+, and ≥ 4+ charge states were rejected from MS/MS analysis. Dynamic exclusion was employed and set to 3 min with a repeat count of 1, a repeat duration of 0 s, and an exclusion list size as
large as 500. Automatic gain control settings were $8 \times 10^3$ and $1 \times 10^6$ ions in the ion trap and Orbitrap, respectively.

Peak lists were created from LC-MS/MS .RAW files using MASCOT Distiller (Matrix Science, Boston, MA) and subsequently searched using the MASCOT server (Matrix Science) (65). Search parameters included asparagine and glutamine deamidation, methionine oxidation, $^{13}$C$_6$-arginine, and $^{13}$C$_6$, $^{15}$N$_2$-lysine as variable modifications as well as cysteine carbamidomethylation as a fixed modification. Precursor search tolerance was set to $\pm 5$ ppm and product ions were searched with a tolerance of $\pm 0.6$ Da. SwissProt human database was concatenated with reverse sequences and used for searching. ProteoIQ (NuSep, Athens, GA, USA) was used to create protein lists filtered using a 1% false discovery rate and to perform SILAC quantification. The Normalized Spectral Abundance Factor (NSAF) for each protein was manually calculated using the corresponding (non-normalized) spectral count value obtained from ProteoIQ as follows:

$$\text{NSAF}_{x} = \frac{\sum_{i,1}^{N} \text{SpC}_{i} / \text{L}_{i}}{\sum_{i,1}^{N} \text{SpC}_{i} / \text{L}_{i}}.$$ 

where $\text{L}=\text{number of amino acids}$

$\text{SpC}=\text{total number of MS/MS spectra that identify protein x}$
Only those proteins which showed a statistically significant change in abundance (p-value < 0.05) and greater 2-fold change (hESC vs. hESC-vCTB or hESC-vCTB vs. hESC-eCTB) have been reported here.

2.5 Author contributions

P.S and B.R designed research; P.S, T.C, S.R, A.N and T.R performed research; D.M contributed analytic tools; P.S analyzed data; and P.S wrote the manuscripts.
2.6 References


Figures

Figure 2.1: Methylation levels at the ELF5 promoter region decrease when hESCs are treated with SB431542 for longer durations.
Figure 2.2: Expression of ELF5 protein. (A) Expression of ELF5 in H1 cells treated with SB431542, BMP4, PD0325901 and LY249002 for 6 days and with Follistatin, Betaglycan, Noggin and DMH1 for the next 6 days. (B) Staining for DAPI. (C) Expression of ELF5 in H1 cells treated with SB431542, Noggin and DMH1 for 6 days and with Follistatin, Betaglycan, Noggin and DMH1 for the next 6 days. (D) Staining for DAPI. (E) Expression of ELF5 in H1 cells treated with SB431542, Noggin, DMH1 and PD0325901 for 6 days and with Follistatin, Betaglycan, Noggin and DMH1 for the next 6 days. (F) Staining for DAPI. (G) Expression of ELF5 in H9 cells treated with SB431542, Noggin, DMH1 and PD0325901 for 6 days and with Follistatin, Betaglycan, Noggin and DMH1 for the next 6 days. (H) Staining for DAPI.
Figure 2.3: Downregulation of HLA antigens. Mass spectrometric analysis of the membrane fraction of SB431542-treated hESCs shows downregulation of (A) HLA Class I type A, (B) type B and (C) type C antigens at day 12, and upregulation of (D) CYTOKERATIN7 (K2C7) at day 6.
Figure 2.4: Formation of STBs from hESCs. H1 and H9 cells were treated with SB431542 for 6 days and then passaged into MEF-CM and grown for 6 more days. STBs were isolated and the expression of (A) CSH1, (B) β-HCG, (C) HLA-G, (D) 3β-HSD1 and (E) PSG9 were studied. (F) Staining with DAPI (blue) and a membrane dye (red) shows multinucleate cells in SB431542-treated H9 cultures. (G) Flow cytometry results for the expression of 3β-HSD1 in SB431542-treated H9 cultures. (H) H1 cells were treated with SB431542, Noggin and DMH1 for 6 days and passaged into MEF-CM with BMP4 and TGFβ3 for another 6 days. Staining with DAPI (blue) and a membrane dye (red) shows multinucleate cells. H1 cells were treated with SB431542, BMP4, PD0325901 and LY294002 for 6 days and passaged into MEF-CM with BMP4 and TGFβ3 for another 6 days. (I) Shows SYNCYTIN staining and (J) shows DAPI along-with SYNCYTIN. H1 cells were treated with SB431542, Noggin and DMH1 for 6 days and then passaged into MEF-CM containing BMP4 and TGFβ3 for 6 days. (K) Shows SYNCYTIN staining and (L) shows DAPI along-with SYNCYTIN. (M) H1 cells were treated with SB431542, Noggin, DMH1 and PD0325901 for 6 days and then passaged into MEF-CM containing BMP4 and TGFβ3 for 6 days. Staining with DAPI (blue) and a membrane dye (red) shows multinucleate cells. (N) H9 cells were treated with SB431542 and BMP4 for 6 days and then passaged into MEF-CM containing BMP4 and TGFβ3 for 6 days. Staining with DAPI (blue) and a membrane dye (red) shows multinucleate cells.
**Figure 2.5**: Formation of mesenchymal cells upon prolonged treatment of H9 cells with SB431542. (A) DIC image of mesenchymal cells. Immunoflourescence staining is shown for (B) DAPI, (C) CD9 and (D) VE-CADHERIN. (E) To test for invasiveness, SB431542-treated H9 cells were plated on solidified matrigel and grown for 15 days. The matrigel sample was sectioned and stained for CD9 and VE-CADHERIN. Invasive cells that express these markers were seen invading into matrigel.
**Figure 2.6:** Co-expression of CDX2 and ELF5 is sporadically seen. H1 cells were treated with SB431542, Noggin and DMH1 for 6 days and then passaged into MEF-CM containing Follistatin, Betaglycan, Noggin and DMH1 for another 6 days. (A) Shows staining for CDX2 and (B) shows composite of CDX2 and DAPI (blue). H1 cells were treated with SB431542, Noggin, DMH1 and PD0325901 for 6 days and then passaged into MEF-CM containing Follistatin, Betaglycan, Noggin and DMH1 for another 6 days. (C) Shows staining for ELF5, (D) shows staining for CDX2, and (E) shows composite of CDX2 (green), ELF5 (red) and DAPI (blue). H9 cells were treated with SB431542, Noggin, DMH1 and PD0325901 for 6 days and then passaged into MEF-CM containing Follistatin, Betaglycan, Noggin and DMH1 for another 6 days. (F) Shows staining for ELF5, (G) shows staining for CDX2, and (H) shows composite of CDX2 (green), ELF5 (red) and DAPI (blue).
CHAPTER 3

CHARACTERIZATION OF SUBCELLULAR HESC PROTEOME AND SECRETOME

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3.1 Introduction

A better understanding of the behavior of human embryonic stem cells (hESCs) can be obtained through the identification of signaling pathways and signaling proteins present in the hESC microenvironment (1-2). The hESC microenvironment is constituted by the exogenous cytokines present in the growth medium, the extracellular matrix (ECM) used for growing hESCs, the endogenous cytokines secreted by hESCs themselves and the endogenously secreted ECM. It is also important to characterize the expression of signaling receptors and proteins within hESCs that confer responsiveness towards the signaling environment. Together, these factors control the behavior of hESCs in cell culture. To characterize the signaling microenvironment of hESCs, we describe an approach to study the secretome of hESCs. This involved subcellular fractionation and enrichment of the secretory pathway organelles from hESCs, followed by analysis through mass spectrometry (MS). To characterize hESCs themselves, we describe the subcellular fractionation of the plasma membrane, cytosol and nuclei from hESCs followed by MS analysis.

3.2 Results

3.2.1 Enrichment of secretory pathway organelles from mouse embryonic fibroblasts and hESCs

HESCs are routinely grown in co-culture with mouse embryonic fibroblasts (MEFs) (3). Under these conditions, signaling factors secreted by MEF cells maintain hESCs in an
undifferentiated state. Alternatively, the factors secreted by MEF cells can be collected in the overlying medium (known as MEF-conditioned medium, or MEF-CM), and this medium can be used for the growth of hESCs (4). A schematic depicting these processes is shown in Figure 3.1. However, the signaling factors present in MEF-CM remain poorly characterized.

Traditional methods for identifying secreted proteins have relied on MS analysis of the overlying medium (5). However, serum and serum-replacement are routinely used components of cell culture media and are present at far exceeding concentrations as compared to endogenously secreted proteins. This causes secreted proteins to go largely undetected, and necessitates the use of serum-free media for collecting secreted proteins (5). In the case of hESCs however, this also leads to significant cell death, and can alter the behavior of hESCs themselves. We therefore used an alternative approach wherein we isolated secretory pathway organelles from inside hESCs. Secretory pathway organelles are organelles that are involved in the classical secretory pathway. These include the endoplasmic reticulum (ER), where secretory proteins are translated, the Golgi apparatus, where these proteins are post-translationally modified, and the secretory vesicles where these proteins are stored and finally secreted outside the cell. Since these organelles are involved in the production and trafficking of the secretory cargo, we reasoned that an MS analysis of these organelles should also lead to the identification of the secretory cargo. Since it was important to maintain the integrity of these organelles, we avoided ultracentrifugation steps during our fractionation procedures.
To enrich for the ER and Golgi organelles, we homogenized cells in an isotonic medium and pelleted the nucleus. The post-nuclear supernatant was depleted of mitochondria using affinity capture of mitochondria through TOM-22 magnetic beads. This supernatant was then centrifuged at moderate speeds to obtain a pellet that was enriched in the ER and Golgi organelles, as has been previously reported (6). The procedure is outlined as a schematic in Figure 3.2. Enrichment of these organelles was confirmed using the DAVID software, and also using a previously reported method of Gilchrist et al. (7) (Figure 3.3). In this method, peptides identified from the MS analysis are counted if they are associated with the proteins of a specific organelle. The percentage of such peptides in the sample is used as a measure of the representation of that organelle in the sample. Using this approach, we compared the representation of secretory organelles in the total lysate of hESCs with that in the fractionated sample, and observed enrichment in ER and Golgi organelles. We also observed enrichment in the secretory cargo proteins.

3.2.2 The secretome of MEFs and hESCs

We isolated fractions that were enriched in secretory pathway organelles, from MEFs and hESCs. The MS analysis of these fractions revealed the secretory cargo present in these cells. The secretome of MEF cells gets accumulated in MEF-CM, which is then used for growing hESC cultures, as illustrated in Figure 3.2. Therefore, the MEF secretome gives rise to exogenous signaling in the hESC microenvironment. On the other hand, the secretome of hESCs gives rise to endogenous signaling in the hESC microenvironment.
In the MEF fraction, we identified 129 proteins that are known to be present in the extracellular space (Figure 3.4), 76 of which had not been identified in previous MS analysis of MEF cells (5). In the hESC fraction, we identified 99 such extracellular proteins, 37 of which had not been identified in previous MS analysis (Figure 3.5). Of interest, we identified regulators of various signaling pathways in both the fractions. These included the Activin/Nodal, bone morphogenetic protein (BMP), fibroblast growth factor (FGF), Wnt and Insulin-like growth factor (IGF) signaling pathways (Figure 3.6).

3.2.3 The secretome of hESCs changes upon exposure to unconditioned medium

Previous reports for probing the secretome of hESCs had used an altered medium that lacked serum proteins, to overlay on hESCs and collect secreted proteins from hESCs (5). In our experiments, we found that this led to massive cell death. Alternatively, we exposed hESCs to unconditioned medium for 24 hours. We then isolated fractions that were enriched for secretory pathway organelles and compared them to normally grown hESC fractions. Our MS analysis revealed that the secretome of hESCs had changed upon exposure to unconditioned medium (Figure 3.7). We concluded that altering media conditions can change the secretome of hESCs. Therefore, we highlight a key problem associated with the approach of using altered media for probing the secretome of cells. On the other hand, our method probes the organelles within the cells. Therefore, our protocol does not require changing of media conditions for studying the secretome of cells.
3.2.4 Subcellular fractionation of plasma membrane, cytoplasm and nuclei from hESCs

Subcellular fractionation was carried out according to previously known protocols (6). A schematic for the protocol is shown in Figure 3.8. Briefly, hESCs were homogenized and nuclei were pelleted. This fraction is also known to contain heavy plasma membrane sheets. Therefore, this pellet was subjected to discontinuous sucrose gradient centrifugation, resulting in separation of heavy nuclei from the light plasma membrane sheets (6). The supernatant from the lysate was subjected to ultracentrifugation to pellet all the other organelles and microsomes, leaving the cleared cytoplasmic fraction. These fractions were subjected to MS analysis using stable isotope labeling of amino acids in cell culture (SILAC). The cytoplasmic fraction was also subjected to spectral counting. Our SILAC analysis identified 893 proteins in the nuclear sample, 1397 proteins in the cytosolic fraction and 1185 proteins in the membrane sample. Spectral counting analysis on the cytoplasmic sample identified 2439 proteins. Combining the SILAC and spectral counting analysis, we identified 2475 proteins in the cytoplasmic fraction. A total of 3359 proteins were identified, of which 2491 (74%) were unique to one single subcellular fraction and only 26% were shared among the subcellular fractions. Only 9.7% of the proteins were shared among all the three fractions. We concluded that proteins in hESCs have distinct subcellular localization. We also concluded that subcellular fractionation reduces sample complexity and provides a deeper coverage of the hESC proteome.
3.2.5 Identification of proteins involved in various signaling pathways

Our MS analysis of the cytoplasm led to the identification of various proteins that are involved in the Activin/Nodal pathway, canonical and noncanonical Wnt pathways, BMP pathway, FGF pathway, Akt pathway, IGF pathway, and the histone deacetylase (HDAC) Classes I, II, and III pathways (data not shown). Our analysis of the membrane fraction led to the identification of many growth factor receptors, integrins, G-proteins and cell–cell junction proteins associated with the adherens junctions, tight junctions, gap junctions, and desmosomes. Our analysis of the nuclear fraction led us to identify many epigenetic factors, including chromatin-remodeling enzymes, histone acetyltransferases, histone deacetylases, histone methyltransferases, DNA methyltransferases and transcription factors. A comprehensive characterization of the epigenetic factors present in the nucleus of hESCs had not been carried out previously. We were also able to identify serine/threonine/tyrosine kinases, phosphatases, and cell-cycle regulators in the subcellular fractions.

3.3 Discussion

We report the development of an alternate approach for studying the secretome of cells. Our method does not require any perturbation to the medium that may affect the cells under investigation. Our approach is also applicable to complex systems such as co-cultures, where the analysis of the overlaying medium is not sufficient to know the secretome of specific cells. Our approach can also be applied to differentiating cultures where the proteome changes with time, since our approach gives a temporal snapshot of the secretome of cells,
whereas conventional approaches allow for identification of secreted proteins collected over a certain period of time. We also show that subcellular fractionation coupled with MS analysis is a powerful tool to probe the proteome of hESCs, since subcellular fractionation decreases sample complexity and allows for the study of specific organelles of interest.

3.4 Materials and methods

3.4.1 Cell culture

MEFs were manually dissected from E13.5 pregnant CD-1 mice embryos (Charles River, Wilmington, MA). MEFs were dissociated using Trypsin-EDTA (Sigma-Aldrich, St. Louis, MO) and cultured in MEF medium, consisting of DMEM-High Glucose (Invitrogen, Carlsbad, CA), 1% penicillin-streptomycin (Invitrogen), 10% fetal bovine serum (Sigma-Aldrich) and 1% l-glutamine (Invitrogen). H9 and H1 hESCs (WiCell, Madison, WI) were co-cultured with MEF feeders, as described elsewhere (8). HESCs were passaged every 5 to 7 days, at confluence. For feeder free culture of hESCs, they were grown on Growth Factor Reduced Matrigel™ (BD Biosciences, Bedford, MA) in MEF-CM. Passaging was carried out by scoring confluent colonies and treating with Collagenase IV (Invitrogen) to lift off the subcolonies. MEF-CM was prepared from passage-3 MEFs using a previously described protocol (4). For isotope labeling of hESCs, cells were grown in MEF-CM which lacked L-lysine and L-arginine, but contained $^{13}$C$_6$, $^{15}$N$_2$ L-lysine and $^{13}$C$_6$ L-arginine (Pierce, Rockford, IL, USA) (SILAC-CM) as described previously (9). 98.5% incorporation of
isotope-labeled arginine and 98.0% incorporation of isotope-labeled lysine were achieved. An arginine-to-proline conversion rate of approximately 5% was observed in our cultures (8).

### 3.4.2 Enrichment of secretory pathway organelles

MEFs were harvested after making MEF-CM, as described previously (4). MEFs were washed with Dulbecco's PBS lacking Ca$^{2+}$ and Mg$^{2+}$ (DPBS) (Sigma-Aldrich) and exposed to hypotonic PBS (25% DPBS in water) for 20 min at 4 °C. MEF cells were collected and vigorously vortexed to dislodge individual cells from the deposited ECM. The solution was centrifuged at 300g for 5 min at 4 °C, and the MEF pellet was retained. The MEF pellet was resuspended in sucrose buffer containing 250 mm sucrose (Fisher), 5 mm magnesium chloride (Fisher), 25 mm potassium chloride (Fisher), 10 mm acetic acid (Fisher), 10 mm triethanolamine (Sigma-Aldrich), and complete mini® protease inhibitor mixture tablets (Roche, Indianapolis, IN) at a pH of 7.6. Cells were homogenized by being passed through a 25G needle twice. The homogenate was centrifuged at 3000g for 10 min at 4 °C to pellet nuclei (6). 50 μl of anti-Tom22 magnetic microbeads (Miltenyi Biotec, Auburn, CA) were added to the supernatant. Beads were incubated with the supernatant at 4 °C for 1 h, and subsequently cleared from the solution using a DynaMag-15 magnet (Invitrogen) by incubating at 4 °C for 5 h. The lysate was now centrifuged at 15,000g for 30 min at 4 °C to pellet the ER and Golgi organelles (6). The pellet was solubilized in Urea buffer, consisting of 8 m urea (Sigma) and 50 mm ammonium bicarbonate (VWR, West Chester, PA), and stored at −80 °C.
Confluent cultures of H1 and H9 cells were taken for subcellular fractionation. When stated, H1 and H9 cells were exposed to unconditioned hESC medium for 1 day, prior to subcellular fractionation. Cultures were washed with DPBS to remove dead cells and were incubated in DPBS with 1 mm EDTA (ACROS, Geel, Belgium) to dissociate the culture into single cells which floated off. Cells were isolated from the suspension by centrifuging at 300g for 5 min at 4 °C. Sucrose buffer was used to resuspend the cell pellet. The rest of the procedure was similar to that used for MEF cells, as described above. Anti-human TOM22 magnetic microbeads (Miltenyi Biotec) were used for depletion of mitochondria. The whole cell lysate was prepared by solubilizing hESCs in Urea buffer, and stored at −80 °C.

3.4.3 Subcellular fractionation of cytoplasm, plasma membrane and nuclei

Membrane, cytoplasmic, and nuclear fractions were isolated using protocols described previously (9). HESCs were homogenized in sucrose buffer and centrifuged at 300g for 5 min at 4°C to pellet the nuclei. This pellet also contained heavy plasma membrane sheets (6). Therefore, this pellet was resuspended in sucrose buffer comprising of 1.4M sucrose, subjected to a discontinuous sucrose gradient centrifugation. Briefly, the 1.4M sucrose solution containing the resuspended pellet was layered over a 2.0M sucrose cushion, and 0.25M sucrose was layered at the top. The gradient was centrifuged at 180,000g for 80 min at 4°C. Nuclei were collected from the pellet below the 2.0M sucrose cushion. Plasma membrane was collected from the 0.25-1.4M sucrose interface. The nuclear fraction was solubilized in Urea buffer and stored at −80 °C. The plasma membrane sample was diluted in
0.25M sucrose buffer and pelleted at 3000g for 10 min at 4°C. The pellet was solubilized in Urea buffer and stored at −80 °C. For isolating the cytoplasm, the supernatant from the hESC homogenate was used. The supernatant was centrifuged at 180,000g for 80 min at 4°C to pellet all remaining organelles and microsomes. The supernatant was reserved as the cytoplasmic fraction and stored at −80 °C.

3.4.4 LC-MS/MS and data analysis for secretory pathway organelle-enriched fractions

Approximately 100 μg of sample protein was taken and digested using filtered-aided sample preparation, as previously described (10). Vivacon filters with a 30k molecular weight cutoff (Sartorius Stedim Biotech, Goettingen, Germany) were used for carrying out the reaction. Briefly, samples were reduced using dithiothreitol, alkylated using iodoacetamide and then digested overnight using trypsin. The reaction was quenched by adding 1% formic acid and peptides were collected by centrifugation. Peptides were then fractionated using an anion exchange protocol, as described previously (11). StageTips were created from 200 μl pipette tips by filling them with a solid phase anion exchange resin (3M, St. Paul, MN). These were used for peptide fractionation (12). Britton & Robinson buffer at pH 5.0 (Ricca Chemical Company, Arlington, TX) was titrated using HCl or NaOH to create six different buffers at pH readings of 3.0, 4.0, 5.0, 6.0, 8.0 and 11.0. StageTips were conditioned by treating with methanol, followed by 1 m NaOH and finally with pH 11 buffer. Peptides were loaded into the StageTips using the pH 11 buffer. Peptides were then eluted using each of the
aforementioned buffers, in descending order of their pH, by centrifugation. Peptide fractions were collected, vacuum-dried, and reconstituted in mobile phase A.

The Eksigent 1D+ nano-LC system (Eksigent, Dublin, CA), equipped with a cHiPLC-Nanoflex system for reversed-phase separation of peptides, was used in a trap and elute configuration. The nano cHiPLC analytical column was 75 μm in inner diameter and 15 cm in length. The trapping column was 200 μm in inner diameter and 0.5 mm in length. ChromXP C18-CL (3 μm, 120 Å) was used for packing both the columns, by the manufacturer (Eksigent). Mobile phase A consisted of water (98%), acetonitrile (2%), and formic acid (0.2%). Mobile phase B consisted of water (2%), acetonitrile (98%), and formic acid (0.2%). Peptides were loaded at 2 μl/min and then switched in-line with the analytical column at a flow rate of 350 nL/min. The gradient was initiated at 2% B. Over the first 5 min, it was adjusted to 7% B, and over the next 211 min, the gradient was ramped to 40% B. It was finally adjusted to 95% B over a period of 2 min. The gradient was kept at 95% B for 8 min and then adjusted to 2% B over the duration of 2 min. The gradient was held at 2% B for another 10 min for re-equilibration.

An LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) was used for carrying out mass measurements, as described previously (13). A broadband scan using 60,000 resolving power at 400 m/z was collected by the Orbitrap mass analyzer in profile mode. This was followed by eight data-dependent MS/MS scan events collected using collision-induced dissociation in centroid mode in the LTQ. Charge state screening was
enabled. ≥4+, 1+ and unassigned charge states were rejected. Dynamic exclusion was set to 180 s with a repeat count of 1, repeat duration of 0 s. The exclusion list size was set to 500 ions. Automatic gain control was set at $8 \times 10^3$ ions for the LTQ and at $1 \times 10^6$ ions for the Orbitrap.

For analyzing the whole cell lysate, 50 μg of the protein was loaded on a polyacrylamide gel and run at a constant 200 V before staining with BioSafe Coomassie stain (BioRad, Hercules, CA). The gel lane was cut into a total of 10 sections, which were then treated with dithiothreitol, alkylated using iodoacetamide, and digested using trypsin. Digested peptides from each sample were separated using the Eksigent 1D+nano-LC system (Eksigent, Dublin, CA) utilizing a vented column configuration (14). A 15 cm PicoFrit column (New Objective, Woburn, MA) as well as a 5 cm IntegraFrit trap column (New Objective) were packed with Magic C18AQ stationary phase (5 μm particles, 200 Å pore size) (Microm Biosources, Auburn, CA). A 7T LTQ-FT-ICR Ultra (Thermo Fisher Scientific, San Jose, CA) mass spectrometer was used for acquiring data, using a 100,000 FWHM resolving power at 400 $m/z$. For every precursor scan up to eight data dependent MS/MS scan events were triggered.

MASCOT Distiller version 2.4.2.0 (Matrix Science, Boston, MA) was used for analyzing RAW data files generated from LC-MS/MS analyses. Peak lists were created and subsequently searched with the MASCOT search engine version 2.3.2 (Matrix Science) (15). Variable modifications, asparagine and glutamine deamidation, and methionine oxidation
were included as parameters for searching. Cysteine carbamidomethylation was set as a fixed
modification. A maximum of two missed trypsin cleavages were allowed. Search tolerances
were ±5 ppm and ±0.6 Da for precursor ions and product ions, respectively. The SwissProt
human database (downloaded January 28, 2012) was appended with reverse sequences for
use as target/decoy database. Search results were imported into ProteoIQ version 2.3.05
(NuSep, Athens, GA) and protein lists were filtered using a 1% false discovery rate.
Normalized spectral abundance factor (NSAF) values were calculated manually using the
raw spectral count data, using the following formula:

\[
(\text{NSAF})_x = \frac{(SpC/L)_x}{\sum_{i=1}^{N} (SpC/L)_i} \times (10^4) \quad \text{(Eq. 1)}
\]

where:

- \( L \) = number of amino acids.
- \( SpC \) = total number of MS/MS spectra that identify protein \( x \).

Search results were imported into ProteoIQ version 2.3.05. Protein lists were filtered using a
1% false discovery rate for spectral counting normalization and for relative quantification
across hESC samples. All data reported in the manuscript originate from three technical
replicates for a single biological replicate for MEFs, H1 and H9 hESCs cultured in MEF-CM,
and H1 and H9 hESCs exposed to unconditioned hESC medium for 24 h.
3.4.5 LC-MS/MS and data analysis for cytoplasmic, plasma membrane and nuclear fractions

This study was conducted in parallel with a study that compared the quantification of protein expression using SILAC and spectral counting methods (9). H9 cells were grown on CM and differentiated by adding 25 μM SB431542 (Sigma-Aldrich) to CM. The medium was changed every day. Protein samples were obtained through subcellular fractionation from both undifferentiated cells grown in SILAC-CM, and from differentiating cells grown in normal CM, at 6 days and 12 days of SB431542 treatment. Corresponding subcellular fractions from undifferentiated and differentiated samples were mixed and analyzed using MS. Heavy peptide identifications were used for the analysis of undifferentiated hESCs, and this has been presented in this article. The cytoplasmic fraction of hESCs cultured in SILAC-CM was used for spectral counting analysis.

For SILAC analysis, 25 μg of protein from differentiated hESCs grown in normal CM was mixed with 25 μg of protein from undifferentiated hESCs grown in SILAC-CM. For spectral counting analysis, 50 μg of cytoplasmic protein sample each was used. Protein samples were run on a 10–20% Tris-HCl Gel (Bio-Rad, Hercules, CA, USA). The gel lane was sectioned into 10 pieces and the subsequent MS analysis and data analysis were carried out as described above. Gene Ontology (GO) annotation analysis for identifying cytoplasmic, nuclear and plasma membrane proteins, was carried out using Blast2Go (16) and DAVID (17-18).
3.4.6 Gene Ontology annotation analysis and estimation of secretory pathway organelle enrichment

Gene Ontology (GO) enrichment analysis was carried out using DAVID (17-18). The list of proteins that were annotated to the secretory pathway organelles—namely, the endoplasmic reticulum (GO:0005783), endoplasmic reticulum–Golgi intermediate compartment (GO:0005793), ER to Golgi transport vesicle (GO:0030134), Golgi apparatus (GO:0005794), secretory granule (GO:0030141), transport vesicle (GO:0030133), and ribosome (GO:0005840)—were obtained using the Princeton GO Term Mapper (go.princeton.edu/cgi-bin/GOTermMapper). Repeated proteins were eliminated. The lists of proteins that were annotated as extracellular—namely, ECM (GO:0031012), extracellular region (GO:0005576), extracellular region part (GO:0044421), extracellular space (GO:0005615), and proteinaceous ECM (GO:0005578), were obtained using the Princeton GO Term Mapper, and repeats were eliminated. These lists were manually curated via comparison with the UniProt database. Proteins that were annotated as “Secreted” in the UniProt database were retained and referred to as “Secretory Cargo.”

To quantify the fractional abundance each organelle in the fractionated sample and in the whole cell lysate sample, a previously described method was used (7). For a given sample, a list of nonredundant peptides that mapped to each organelle $i$ was obtained and denoted as $S_i$. The fractional abundance of peptides associated with the organelle $i$ ($F_i$) was estimated as
\[ F_i = \frac{\text{Spectral Counts due to peptides in } S_i}{\text{Total Spectral Counts in MS analysis of the sample}} \]  
(Eq. 2)

\[ E_i = \frac{F_i \text{ in subcellular fraction}}{F_i \text{ in whole cell lysate}} \]  
(Eq. 3)

\( F_i \) was used as a metric for fractional abundance of organelle \( i \) in a given sample. The fold-enrichment of organelle \( i \) in the fractionated sample was calculated as:

The two-tailed Fisher's exact test was used to calculate the \( p \) value of enrichment \( E_i \).

3.5 Author contributions

P.S, D.M and B.R designed research; P.S, T.C and S.R performed research; D.M contributed analytic tools; P.S, T.C and S.R analyzed data; and P.S wrote the manuscripts.
3.5 References


Figure 3.1: Schematic showing endogenous signaling in hESC cultures. (A) HESCs grown in co-culture with MEFs can affect each other in a paracrine fashion due to endogenous signaling. (B) MEF-CM contains endogenously secreted MEF proteins. (C) HESCs grown in MEF-CM are exposed to proteins secreted by MEF cells. These cultures also contain endogenously produced hESC proteins.
Figure 3.2: Schematic showing the protocol for enrichment of secretory pathway organelles.
**Figure 3.3:** Secretory pathway organelles were enriched during subcellular fractionation. DAVID analysis results are shown for subcellular fractions obtained from (A) H1 cells, (B) H9 cells and (C) MEF cells. (D) Enrichment was confirmed using a method of Gilchrist et al. (7) for H9 cells.
Figure 3.4: Secretory proteins identified in the subcellular fraction from MEF cells. The NSAF values, which correspond to the abundance of these proteins in the sample, are plotted.
Figure 3.5: Secretory proteins identified in the subcellular fraction from H1 and H9 hESCs. The NSAF values, which correspond to the abundance of these proteins in the sample, are plotted.
**Figure 3.6:** Proteins associated with various signaling pathways identified in the secretome of hESCs and MEFs.
Figure 3.7: Changing the overlying medium changes the secretome of hESCs. HESCS were exposed to unconditioned medium for 24 hours. Subcellular fractions were collected and analyzed. DAVID analysis of shows enrichment of secretory pathway organelles in fractions obtained from (A) H1 cells and (B) H9 cells. This dataset was compared with the data obtained from normal H1 and H9 cells, and the fold-change in secreted protein levels that were statistically significant were identified for (C) H9 cells and (D) H1 cells.
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### C

- **IGF-binding protein 10**
- **Glucose-6-phosphate isomerase**
- **Laminin subunit beta-1**
- **Phospholipase B-like 1**
- **Gamma-glutamyl hydrolase**

### D

- **Laminin subunit beta-2**
- **Epididymal secretory protein E1**
- **Discodin receptor tyrosine kinase**
- **Polypeptide N-acetylgalactosaminyltransferase-2**
- **Disintegrin and metalloproteinase domain-containing protein 9**
- **Cysteine-rich with EGF-like domain protein 2**
- **Sulfhydryl oxidase 2**
- **Peroxidase homolog**
- **Retinoid-inducible serine carboxypeptidase**

Value (Log2)
Figure 3.8: Schematic showing the protocol for isolation of plasma membrane, cytoplasm and nuclear fractions from hESCs.
CONCLUSIONS AND FUTURE WORK

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Chapter 1 provides a general introduction to signaling pathways and their control over the behavior of human embryonic stem cells (hESCs). It focuses on the currently known pathways involved in trophoblast differentiation of hESCs, and discusses the limitations of currently used approaches to derive ‘trophoblast-like’ cells.

Chapter 2 discusses our approaches for obtaining trophoblast cells which have the marker properties of placental trophoblast cells. Specifically, we focus on the expression of CDX2 and ELF5 proteins, which are critical markers of mouse trophoblast stem cells. We also focus on the lack of HLA antigens, which is a distinctive property of placental trophoblasts. Our work can be used for various applications as listed below:

1. Regenerative medicine: We describe the derivation of invasive cytotrophoblast-like cells that lack the expression of HLA A, B and C antigens. The lack of these antigens is thought to provide immune privileges to trophoblast cells, whereby they do not evoke an immune response in the maternal bloodstream. The lack of an immune response is critical for the success of regenerative medicine, so that hESC-derived tissues can be grafted into the patient’s body. Current efforts for deriving HLA A, B and C negative cells have only succeeded through genetic knockdown of these genes. However, genetically altered cells may have undesirable properties, and are not preferred for regenerative medicine applications. We have shown that cells that downregulate HLA A, B and C antigens can be derived from hESCs without genetic
alterations. The protocol for deriving these cells can be of potential use in regenerative medicine applications.

2. In vitro model: Invasive cytotrophoblasts (iCTBs) remain embedded into the endometrial tissue and cannot be isolated considerable quantities from the human placenta. HESC-derived iCTBs can therefore be used as an alternative source of these cells. These cells can be used to study intravasation and remodeling of endometrial arteries which occurs during the first trimester of pregnancy. The lack of sufficient intravasation and remodeling leads to pre-eclampsia. Therefore, these cells may be useful candidates as in vitro models for studying the remodeling of uterine arteries.

3. Formulation of defined media conditions: We describe preliminary work involving the derivation of trophoblast cells in mTeSR1 medium. Since this medium is completely defined, this work can potentially lead to the development of formulated media that can be used for deriving early gestation trophoblast cells. Derivation of formulated media will also allow for mechanistic studies on the role of various growth factors and cytokines involved in the maintenance and differentiation of these cells in vitro.

4. Derivation of human trophoblast stem cells: We describe the development of methods that lead to the formation of some CDX2⁺ELF5⁺ cells. Future work can be devoted to identify culture conditions for maintaining these cells, and to test whether these cells can be clonally expanded and if they retain the ability to form syncytiotrophoblasts and invasive cytotrophoblasts. If these conditions are met, human trophoblast stem
cells (hTSCs) can be derived from these cells. Currently, mouse TSCs have been derived but their human counterparts remain to be derived.

Chapter 3 describes our efforts to characterize the secretome of human embryonic stem cells, as well as the proteome of its plasma membrane, cytoplasm and nucleus. Future work can be focused towards the following applications:

1. While characterizing the secretome, we observed that only 8% of the proteome of the secretory pathway organelles corresponded to the secretory cargo. Future work can be devoted to increase the coverage of the secretory cargo. We are currently developing affinity ligands for capturing secretory vesicles. Secretory vesicles are vesicles that are packaged with the secretory cargo. Therefore, an affinity-based purification of the secretory vesicles can potentially lead to a very high representation of the secretory cargo in the proteomic sample, leading to increased proteomic coverage of the secretome. This may lead to a better understanding of the endogenous signaling factors that are present in cell co-culture systems, specifically in hESCs grown in MEF co-cultures.

2. Our efforts to characterize the plasma membrane of hESCs led to the identification of various HLA antigens that are present in hESCs and are downregulated in differentiated hESCs. Currently, there are no independent methods for studying the expression of specific HLA antigens in cells. Currently available antibodies against HLA antigens have poor specificity and do not discriminate among different HLA
antigens of the same class. However, our MS analyses have shown that an MS-based approach can discriminate among different antigens of the same HLA class. Therefore, MS approaches provide a very unique opportunity in this regard, and the viability of this approach should be further investigated.