Differential modulation of BMP Signaling by Activin/Nodal and FGF pathways in lineage specification of Human Embryonic Stem Cells

Inaugural – Dissertation

to obtain the academic degree

Doctor rerum naturalium (Dr. rer. nat.)

submitted to the

Department of Biology, Chemistry and Pharmacy,

Free University, Berlin

by

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May, 2010

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Date of PhD Defense: 29-11-2010

Acknowledgement

I extend my appreciation to Prof. Hans Lehrach for admitting me as a PhD student in his department.

I would like to thank Dr. James Adjaye for giving me an opportunity to venture into the immensely interesting field of human embryonic stem cell research and also for the helpful comments and corrections. I am grateful to Prof. Heiner Niemann for the fruitful collaborative project on Bovine preimplantation development. I thank Boris for motivating me during my initial days of PhD and whose work has been the basic motivation behind this research work.

I am deeply thankful to Prof. Petra Knaus for being my second supervisor and for her critical comments and constructive discussions. I also thank her for conducting biannual symposiums for all her external PhD students, setting a platform for interactions between each other and also with her.

Special thanks to Ingo and Karol for helping me in learing mouse preimplantation embryo handling. I thank Beatrix and Rudi for the electron microscopy experiments. I thank Aydah for helping me with Illumna microarray experiments and also being very understanding in terms of urgency. I also thank other animal facility staff as well as the library staff for their support.

I extend my gratitude to Aditi, Lukas, Anja, Julia, Thomas, Masoud, Sahar, Marc, Wibke, Anne, Bodo, Sigmar, Pamela, Janett, Sebastian, Anna, Uli, Ingrid and Kathleen for the all the help they have extended, either concerning work or administrative formalities. Thanks to Birgit for taking care of the orders in such an efficient way that sometimes, during the time of urgency, she used to take extra efforts to make sure that the product reaches soon. I thank Frank and Sven for helping me solve computer problems.

Both good and bad times were a part of my stay in this laboratory. I thank my colleagues, Justyna, Marc, Guifre and Thore for all their help, especially duing my initial days and all the times we spent together, sharing our PhD experiences and making the work environment very conducive. I would like to thank all my lab members, also including Mei-Chih, Raed, Katharina, Ying, Bjorn, Mathias and Alessandro for extending a helping hand whenever I needed and also for the enjoyable times. Not knowing german language was never a barrier for me in the lab, just because of all my considerate colleagues and I thank all of them for the same.

Acknowledgement

For a person to perform his best, his happiness in life matters, which is brought about by surroundings, the loved ones, family and friends. I express my deepest gratitude to my beloved parents, brothers and other family members, for their constant support, love and encouragement. I run short of words, in extending my immense gratitude to my life partner and best friend, Raghu Bhushan, whose great support, motivation, love and understanding were the driving forces for me ever since we met, without which accomplishing this goal would have been impossible for me. Also, Raghu has been immensely helpful to me in terms of subject-oriented discussions, critical analysis of the experiments and also in helping me in some experiments in the times of urgency. Special thanks to Antjew, Sandra and Mitko for making the surroundings such a nice place to live, with a beautiful garden as a bonus. I thank all my friends and well-wishers in Berlin and elsewhere for all the moral support and pleasant times together.

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Abbreviations

ACTB: Beta-actin

ALK: Activin Receptor like Kinase

ANOVA: Analysis of variance

ATP: Adenosine TriPhosphate

BMP: Bone Morphogenic Protein

BMPR: Bone Morphogenic Protein Receptor

BSA : Bovine Serum Albumin

CDH1: Cadherin 1 / E-Cadherin

CDK: Cyclin Dependent Kinase

CDKN1A / p21: cyclin-dependent kinase inhibitor 1A

CDKN1C / p57: cyclin-dependent kinase inhibitor 1C

cDNA: Complementary DeoxyriboNucleic Acid

cDNA : Complementary DeoxyriboNucleic Acid

CDX2: Caudal-type homeobox protein 2

CGA: Glycoprotein hormones, alpha polypeptide

CGB/hCG β: chorionic gonadotropin, beta/ human chorionic gonadotropin, beta

CM : Conditioned Medium

Co-SMAD /SMAD4: Co-mediator SMAD

cRNA: Complementary Ribonucleic Acid

CTB: Cytotrophoblast

DAPI: 4',6-diamidino-2-phenylindole

DAVID: DAVID Functional Annotation Bioinformatics Microarray Analysis

DE: Definitive Endoderm

DLX: Distal-Less homeobox

DM: Defined medium

DMEM: Dulbecco's Modified Eagle Medium

DMSO: DiMethyl SulfOxide

dn FRFR : dominant negative FGFR

dNTP : deoxyribonucleotide triphosphate

dpc : days post-coitum

ECL: electrogenerated chemiluminescence

EGF: epidermal growth factor

ELISA : enzyme-linked immunosorbent assay

EMT: Epithelial Mesenchymal transition

EpiSC: Epiblast stem cell

ERK: Extracellular signal-Regulated Kinase

ERVWE1: Endogenous Retroviral family W, env(C7), member 1

EVT: extravillous trophoblast

ExE: Extraembryonic Ectoderm

FGF: Fibroblast Growth Factor

FGFR: Fibroblast Growth Factor receptor

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GATA: GATA binding protein

GC: Giant Cell

GDF: growth differentiation factor

GnRH: gonadotropin-releasing hormone

GPIAP: glycosyl-phosphatidyl-inositol-anchored protein

GSK3β: Glycogen synthase kinase 3 beta

hCG: Human Chorionic Gonadotropin

HERV-FRD: HERV-FRD provirus ancestral Env polyprotein

hESCs: human Embryonic Stem Cells

HLA-G : major histocompatibility complex, class I, G

HOP /HOPX / NECC1 : HOP homeobox / not expressed in choriocarcinoma protein 1

HSPG: Heparan sulfate proteoglycans

ICM: Inner Cell Mass

IgG: Immunoglobulin

IUGR: intrauterine growth restriction

KEGG: Kyoto Encyclopedia of Genes and Genomes

KRT7/CK7: keratin 7 / cytokeratin 7

LEFTY: left-right determination factor 1

LIF: Leukemia inhibitory factor

MAPK: Mitogen-Activated Protein Kinase

MEKK: MAPK/ERK kinase kinase

mEpiSC: Mouse Epiblast Stem Cell

mESC: Mouse Embryonic stem cell

MET: Mesenchymal Epithelial transition

miRNA: micro Ribonucleic Acid

MMP: matrix metallopeptidase

mRNA: messenger Ribonucleic Acid

MS: Microsoft

NANOG: Nanog homeobox

OCT4 / POU5F1: octamer-binding protein 4 / POU class 5 homeobox 1

Oligo dT: oligomeric deoxy-thymine

PAS: Periodic acid-Schiff

PBS : phosphate buffered saline

PE: Primitive Endoderm

PECAM: platelet/endothelial cell adhesion molecule

PFA: Paraformaldehyde

PIG-A : phosphatidyl-inositol-glycan class A

PS: Primitive Streak

RNA: Ribonucleic Acid

RNAi: RNA interference

rRNA: ribosomal Ribonucleic Acid

R-SMAD: Receptor SMAD

RT: room temperature

SDS : sodium dodecyl sulfate

SLUG / SNAI2: snail homolog 2 (Drosophila)

SNAI1: snail homolog 1 (Drosophila)

SOX: SRY (sex determining region Y)-box

SR : serum replacement

STBM: Syncytiotrophoblast membrane

TE: Trophectoderm or trophoblast

TEMED: Tetramethylethylenediamine

TGF: Transforming Growth Factor

TIMP: TIMP metallopeptidase inhibitor

tRNA: transfer Ribonucleic Acid

TS: Trophoblast Stem cell

UM : unconditioned medium

VE: Visceral Endoderm

TREATMENTS

B: BMP4

SB: BMP4+SB431542

F: BMP4+SB431543+FGF2

BSU: BMP4+SU5402

SBSU: SB431543+SU5402

SU: BMP4+SB431543+SU5402

SUO: SU5402

UNITS

°C : degrees Celsius

%: Percentage

g: grams / gravity

hr/hrs : hours

kDa: kilo Daltons

M: mol

mA: milliampere

mg: milligrams

min: minutes

ml: millilitres

mm: millimetres

mM: millimol

ng: nanograms

nm: nanometres

pg: picograms

pmol: picomol

s: seconds

U: units

V: volt

mIU: milli international units

µg : micrograms

µl: microlitres

µM: micromol

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Abstract

Human embryonic stem cells (hESC) can be differentiated into both embryonic and extraembryonic lineages via the modulation of signaling pathways. BMP signalling is known to support differentiation of hESCs into multiple lineages, including trophoblast (TE). It has been shown that that TE formation can be induced in hESCs using BMP4 or BMP2 or SB431542 (a TGFßRI specific inhibitor) or via OCT4 knock down. In our current study we show for the first time that inhibition of FGF signaling in hESCs also supports trophoblast differentiation, inducing hCG secretion. Further, we also show that inhibition of FGF signaling (-FGF), either alone or in conjunction with BMP signaling activation and ACTIVIN/NODAL signaling inhibition (+BMP-TGF-FGF) induces hESC differentiation to non-invasive, epithelial, βhCG hormone secreting multinucleated syncytiotrophoblast. The latter treatment (+BMP-TGF-FGF) induced an accelerated differentiation process. Our results also indicate that BMP signaling activation (+BMP) or an additional inhibition of ACTIVIN/NODAL signaling either with or without exogenous FGF signaling activation (+BMP-TGF or +BMP-TGF+FGF) supports differentiation of hESCs to embryonic mesendoderm and extraembryonic trophoblast lineages, but does not support neurectodermal differentiation. Taken together, the insights from this study can be used to understand early lineage segregation events during embryo development. Further, we can also understand syncytiotrophoblast formation, endocrine functions of placenta, drug metabolism or pathological conditions, which could provide direction for the pre-clinical development of rational therapeutics.

Zusammenfassung

Humane embryonale Stammzellen (hESC) können durch die Modulation von Signaltransduktionskaskaden sowohl in die embryonale als auch in die extraembryonale Linie differenziert werden. Die BMP-Signaltransduktionskaskaden sind bekannt dafür, eine unterstützende Wirkung auf die Differenzierung der hESCs in multiple Linien auszuüben, einschließlich der Differenzierung in Trophoblast (TE). Es konnte gezeigt werden, dass die TE Bildung aus hESCs durch BMP4, BMP2, SB431542 (einem spezifischen TGFßRI Inhibitor) oder mittels eines *OCT4*-Knock-Downs induziert werden kann. In der vorliegenden Studie zeigen wir erstmalig, dass die Inhibition der FGF-Signaltransduktion die Trophoblastdifferenzierung mit anschließender hCG Sekretion fördert. Zudem zeigen wir, dass die Inhibition der FGF-Signaltransduktion (-FGF) allein oder in Kombination mit einer Aktivierung der BMP-Signaltransduktion bei gleichzeitiger ACTIVIN/NODAL Inhibition (+BMP-TGF-FGF) die Differenzierung von hESCs in nicht-invasive, ßhCG-Hormon sezernierende, multinukleäre Synzytiotrophoblasten induziert. Die Wirkung der letztgenannten, kombinierten Behandlung (+BMP-TGF-FGF) von hESC ist dabei besonders effizient. Unsere Ergebnisse deuten darauf hin, dass die Aktivierung der BMPSignaltransduktion (+BMP) oder eine zusätzliche Inhibition der ACTIVIN/NODALSignaltransduktion, entweder mit oder ohne gleichzeitiger Aktivierung der FGFSignaltransduktion (+BMP-TGF oder +BMP-TGF+FGF), die Differenzierung der hESCs in Richtung embryonales Mesoderm und extraembryonalem Trophoblast unterstützt, nicht aber die des Neuroectoderms. Zusammengenommen können die Erkenntnisse dieser Studie helfen die Ereignisse der frühen Keimblatt- oder Liniensegregation der Embryogenese zu verstehen. Außerdem können wir die Bildung der Synzytiotrophoblasten, die endokrine Funktion und den Wirkstoffmetabolismus der Plazenta sowie pathologische Umstände verstehen lernen und somit die Entwicklung prä-klinischer Therapien vorantreiben.

Introduction

The embryo undergoes numerous asymmetric cell divisions before implantation in mother's uterus (pre-implantation), resulting in two distinct cell populations, the inner cell mass (ICM) and trophectoderm (TE), the precursor of the placenta and this marks the first differentiation that ever happens in an embryo (Fig. 1) (Arnold and Robertson, 2009; Johnson and Ziomek, 1981; Rossant and Tam, 2009; Tarkowski and Wroblewska, 1967). Mammalian preimplantation development is dependent on well-orchestrated gene expression patterns and post-fertilization embryos of different vertebrate species show considerable variation in size, form and timing of development (Arnold and Robertson, 2009). For example, large scale synthesis of messenger RNA from the diploid embryonic genome is initiated at species-specific developmental stages in different organisms. In murine embryos, this happens at the end of the first cell cycle, in human embryos at the 4-cell stage and in bovine embryos at the 8-cell stage (Braude et al., 1998; Kues et al., 2008; Misirlioglu et al., 2006; Telford et al., 1990). In spite of the initial differences, the basic signalling pathways that control cell lineage specification and axis patterning, such as the transforming growth factor-β (TGF β), WNT and FGF pathways remain conserved (Arnold and Robertson, 2009). Therefore, after gaining insight into the gene expression changes occurring during bovine preimplantation development (Sudheer and Kues et al., 2008), we used human embryonic stem cells (hESCs) as a model system to understand the influence of ACTIVIN/NODAL and FGF signalling pathways on BMP4-driven differentiation of hESCs. BMP signalling is known to support differentiation of hESCs into multiple lineages (Chadwick et al., 2003; Pera et al., 2004; Zhang et al., 2008), including trophoblast (Xu et al., 2002), which marks the first binary lineage segregation during embryo development and is part of blastocyst formation, the last stage of preimplantation development.

1.1: Human and mouse embryonic stem cells and associated signaling pathways

Embryonic stem cells (hESC) originate in the inner cell mass of blastocyst and are self-renewing and pluripotent (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998), with an innate ability to give rise to all three germ layers - endoderm, mesoderm, and ectoderm, both in vitro (directed differentiation and embryoid body formation) and in vivo (teratomas formed in immunodeficient mice) (Reubinoff et al., 2000; Thomson et al., 1998). They also have the capacity to give rise to germ cells, extra-embryonic tissue and trophoblast cells (Babaie et al., 2007; Hyslop et al., 2005; Xu et al.,

2002). While both human and mouse ES cells (mESC) can grow indefinitely in vitro, they maintain their pluripotent status using different signaling pathways.

mESC self-renewal is supported by LIF signaling (Nichols et al., 1990; Yoshida et al., 1994) through GSK3β inhibition causing activation of WNT signalling (Niwa et al., 2009; Sato et al., 2004) and also by inactivation of FGF/ERK signaling (Guo et al., 2009; Ying et al., 2008). Erk2−/− mESCs lack the ability to differentiate into somatic lineages (Kunath et al., 2007). But LIF signalling is not active in hESCs (Daheron et al., 2004; Humphrey et al., 2004) and under chemically defined conditions WNT/β-catenin signalling induces hESC differentiation (Sumi et al., 2008). The hESC self-renewal can be maintained through the support of TGF β or Activin/Nodal and FGF2 mediated pathways (Amit et al., 2004; Beattie et al., 2005; Greber et al., 2008; James et al., 2005; Liu et al., 2006; Lu et al., 2006; Ludwig et al., 2006b; Vallier et al., 2005; Vallier et al., 2004; Wang et al., 2007; Xiao et al., 2006; Yao et al., 2006). Binding of ActivinA to BMPR I and II receptors causes activation of SMAD6 and 7, which are inhibitory to SMAD1/5/8 signaling (Balemans and Van Hul, 2002).

hESCs resemble mouse epiblast stem cells (mEpiSCs), derived from postimplantation mouse embryos in terms of embryonic identity and pluripotent state (Brons et al., 2007; Tesar et al., 2007). Moreover, in both hESCs (Greber et al., 2008; Vallier et al., 2009; Xu et al., 2008) and mouse epiblast (Camus et al., 2006; Mesnard et al., 2006), activation of ACTIVIN/NODAL signalling, causes induction of NANOG via its effectors, SMAD2/3, blocking neurectoderm differentiation and inhibition of Activin/Nodal signaling induces neurectoderm differentiation (Greber et al., 2010). But in contrast to hESCs, in which FGF2 cooperates with SMAD2/3 in promoting self-renewal through NANOG activation (Greber et al., 2007, 2008; Li et al., 2007; Ludwig et al., 2006a; Ludwig et al., 2006b), in mEpiSCs, it stabilizes the epiblast state by dual inhibition of differentiation to neuroectoderm and of media-induced reversion to a mouse embryonic stem cell-like state (Greber et al., 2010). Hence, both Activin/Nodal and FGF signaling support the maintenance of pluripotency of hESCs, mEpiSCs by blocking neuroectoderm differentiation during early development, though differences occur in the mechanism.

1.2: Blastocyst formation, development and gastrulation

1.2.1: Blastocyst formation

The post-fertilization embryo undergoes numerous asymmetric cell divisions to form a spherical structure called the blastocyst (Fig. 1), having 3 different cell types, trophectoderm (TE), epiblast and the primitive endoderm (PE), also called the hypoblast. The first segregation of cells happens at the morula stage along a basolateral cleavage plane, with the smaller inner cells forming the inner cell mass (ICM) which represent the progenitors of embryo and the larger outer epithelial cells, forming trophectoderm (TE) from which the extraembryonic placenta is formed (Sutherland et al., 1990).

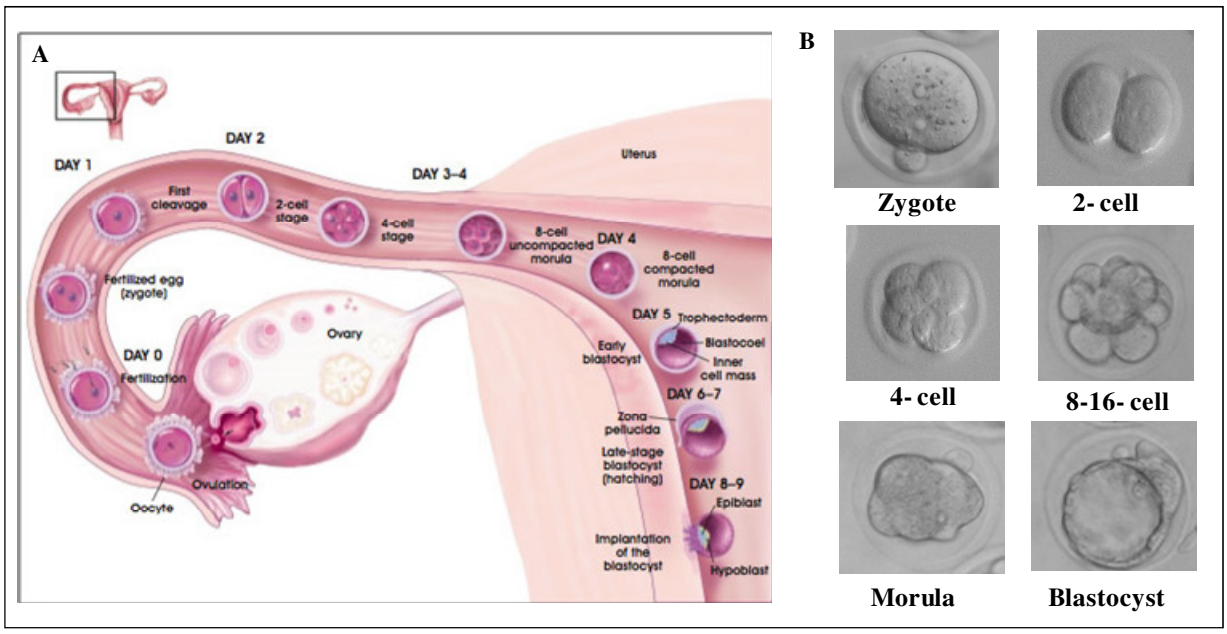


Figure 1.1: A) Human preimplantation development (adapted from http://stemcells.nih.gov/info/scireport/appendixa.asp) B) Developmental stages during preimplantation development (mouse)

This primary cell-type segregation is accompanied by the formation of a fluid filled cavity called the blastocoel. This is followed by the next differentiation event, in which the outermost layer of cells in the ICM, facing the blastocoel form another extraembryonic tissue, the primitive endoderm (PE) that later develops into the yolk sac tissue. The epiblast harbors pluripotent stem cells, being the progenitors, which later on, gives rise to the embryo proper. Embryonic stem cells are self-renewing and pluripotent cells derived from the ICM of blastocysts and can be expanded unlimitedly in vitro (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998). Oct4 (Nichols et al., 1998), Sox2

(Avilion et al., 2003; Nichols et al., 1998) and Nanog (Chambers et al., 2003; Mitsui et al., 2003) are the key transcription factors required for the maintenance of pluripotency in the ICM or epiblast. Any alteration in their expression in both mESCs and hESCs can induce differentiation (Babaie et al., 2007; Fong et al., 2008; Hay et al., 2004; Ivanova et al., 2006; Rodriguez et al., 2007; Zaehres et al., 2005). In cooperation with SALL4 (Zhang et al., 2006), Nanog (Chambers et al., 2003; Mitsui et al., 2003) maintains pluripotency in epiblast progenitors.

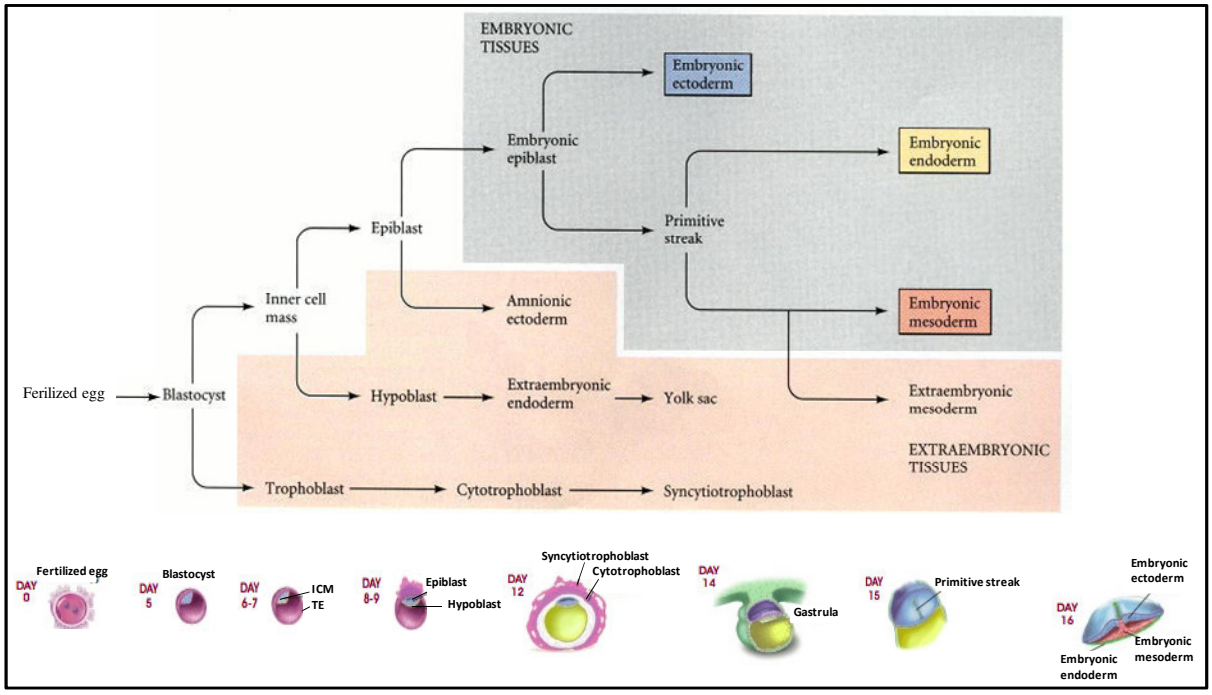


Figure 1.2: Developmental tree depicting lineage allocation of embryonic and extraembryonic lineages (adapted from http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=dbio&part=A2609)

In the mouse preimplantation embryo, the transcription factors, Oct4 and Nanog are expressed in the inner cell mass and Cdx2 is expressed in the trophectoderm (Chambers et al., 2003; Mitsui et al., 2003; Nichols et al., 1998; Tanaka et al., 1998). There exists a transcription factor-driven reciprocal inhibition between the ES cell markers, Oct4 and Nanog and Cdx2 for lineage commitment to ICM or TE, in which both the ES markers antagonize Cdx2 and vice versa (Chen et al., 2009; Niwa et al., 2000; Niwa et al., 2005; Strumpf et al., 2005). Cdx2 is required to repress Oct4 and Nanog during early development, for trophoblast specification (Strumpf et al., 2005). Another transcription factor, GATA3 positively regulates Cdx2 gene expression and is selectively expressed in the trophectoderm of peri-implantation embryo (Home et al., 2009).

Null mutants of the transcription factors, Eomes (Russ et al., 2000) or Cdx2 (Rossant et al., 2003) are trophoblast defective and trophoblast stem cells cannot be derived from them. In cultured mouse trophoblast stem cells, both Cdx2 and Eomes are responsive to FGF signaling (Cross, 2000; Rossant and Cross, 2001; Russ et al., 2000) and differentiation is associated to their down-regulation (Cross, 2000; Hughes et al., 2004; Selesniemi et al., 2005; Tanaka et al., 1998). Trophoblast differentiation studies on mESCs have reemphasized on the importance of Cdx2 in trophoblast lineage specification (He et al., 2008; Schenke-Layland et al., 2007). Both mouse (Tanaka et al., 1998) and human (Baczyk et al., 2006; Ferriani et al., 1994) require FGF signalling for trophoblast stem cell maintenance and proliferation and upon FGF4 withdrawal, they differentiate (Tanaka et al., 1998). In response to Oct4/Sox2 regulation, the ICM acts as an endogenous source of FGF4 (Nichols et al., 1998; Yuan et al., 1995), which can support PE and trophoblast cells in the blastocyst and this could be one of the reasons for no effect on Cdx2 expression upon the inhibition of FGFR/MEK signaling in mouse embryos (Yamanaka et al., 2010). It has been suggested that in spite of the reciprocal inhibition, additional regulatory mechanisms must be involved in Oct4 regulation and restricted expression (Tolkunova et al., 2006) as Oct4 protein was present in all blastomeres even after more than 20 hours of Cdx2 is restriction (Dietrich and Hiiragi, 2007).

GATA6 expression, a marker of PE is dependent on FGF signaling or GRB2 expression, a mediator of FGF signaling and Nanog and GATA6 exhibit mutually exclusive expression in epiblast and PE respectively (Arman et al., 1998; Chazaud et al., 2006; Feldman et al., 1995; Ralston and Rossant, 2005; Wilder et al., 1997; Yamanaka et al., 2010). Recently it has been shown that the fate of Nanog- or Gata6-expressing progenitors of epiblast and PE could be shifted towards either of the fates via modulating FGF signaling by either receptor/MAP kinase pathway inhibition or addition of exogenous FGF respectively, during blastocyst maturation (Yamanaka et al., 2010). Therefore, at the blastocysts stage, the lineage specification into the three initial cell types, epiblast, TE and PE are highly dependent on the fine-tuned-regulation of FGF signalling pathway.

1.2.2: Extraembryonic membranes

There are four extraembryonic membrane associated with fetal development, namely, chorion, allantois (Extraembryonic mesoderm), yolk sac (Extraembryonic endoderm), amnion (Amnionic ectoderm). The major classification of the extraembryonic membranes of birds, reptiles and mammals is the same. The major difference is that in mammals, the embryo is attached to the mother's uterus, where it grows inside the placenta through out the gestation period.

Amnion and allantois arise from the epiblast cells, whereas yolk sac is formed from the PE. Chorion is the outermost protective membrane around the fetus that harbours the trophoblast cells. The major structural and functional components needed to bring the fetal and maternal blood systems into close contact are provided by the trophoblast cells. The chorion provides oxygen and nourishment from the mother, secretes hormones to support the fetal growth in the uterus and produces regulators of immune response, to avoid rejection of the embryo by the mother's body. The allantois gives rise to the fetal vascular compartment that comes in contact with the chorion and during the course of development, blood vessels grow out of the allantois into the chorion. Amnion is the innermost membranous sac enclosing the embryo, which is filled with the amniotic fluid, used for the suspension and protection of the embryo, by serving as a shock absorber for the developing embryo while preventing its desiccation.

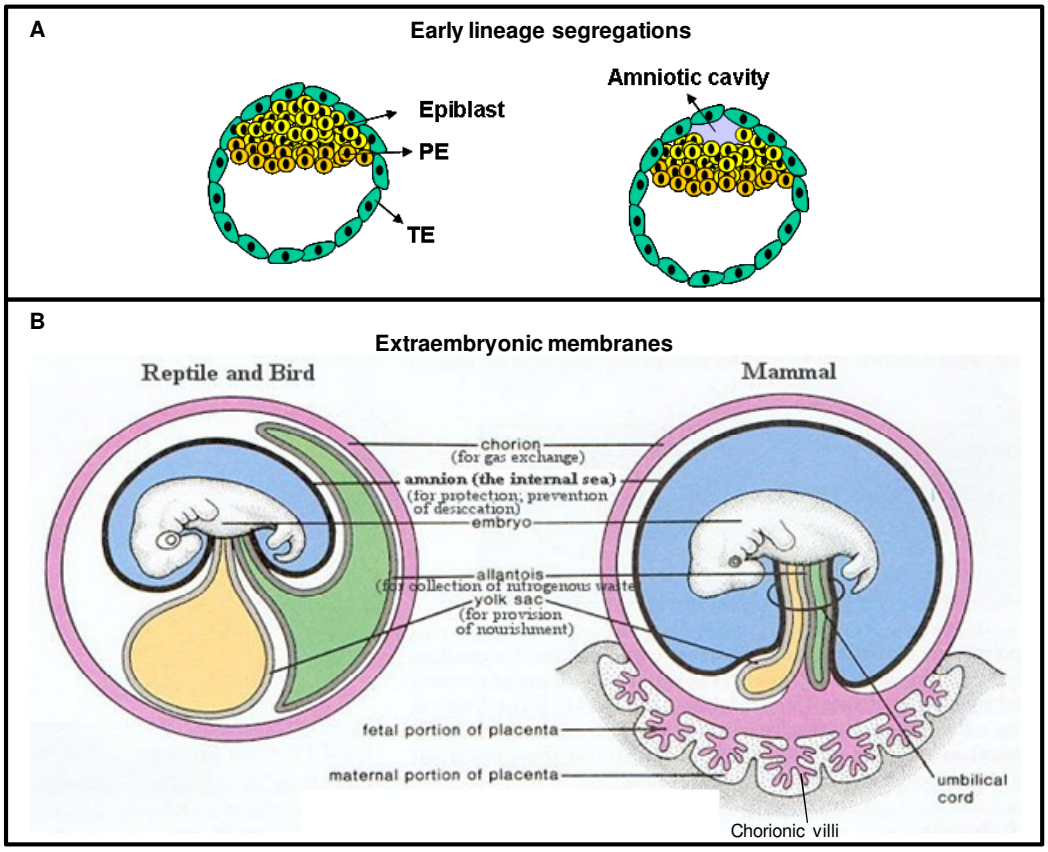


Figure 1.3: A) Early lineage segregation into Epiblast, Hypoblast or Primitive endoderm (PE) and Trophectoderm in a maturing blastocyst, along with the beginning of the amniotic cavity formation B) Extraembryonic membranes in reptiles, birds and mammals (adapted from: http://universe-review.ca/R10-33-anatomy.htm)

1.2.3: Blastocyst development and Gastrulation

Upon the maturation of blastocyst, the three distinct lineage-restricted subpopulations, epiblast, TE and PE undergo further diversifications.

The TE develops to form the progenitors of placenta, namely, extraembryonic ectoderm (ExE) and ectoplacental cone. ExE harbours trophoblast stem cells, expressing markers such as Eomes and Cdx2 (Tanaka et al., 1998). The ectoplacental cone overlying ExE, is an elongated structure that later on forms the connection between the embryo and mother's uterus. The outer regions of ectoplacental cone endoreduplicate to form trophoblast giant cells.

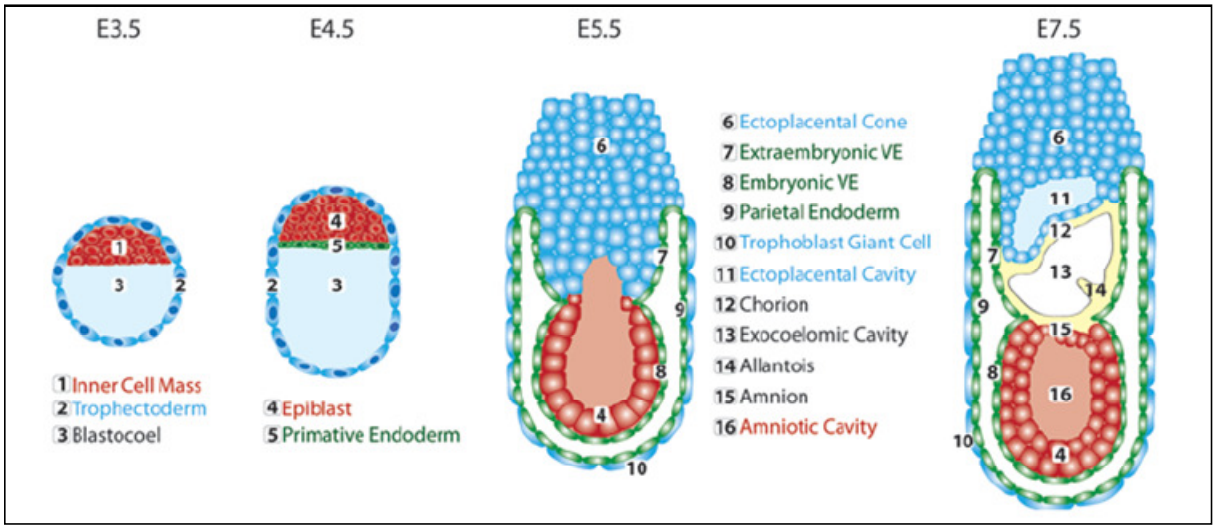


Figure 1.4: Labelled schematic diagrams of mouse embryo development - early blastocyst (E3.5), late blastocyst (E4.5), and post-implantation conceptuses at E5.5 (egg cylinder stage) and E7.5; with red, green and blue representing the embryonic, primitive/extraembryonic endoderm and trophoblast lineages respectively. (adapted from (Roper and Hemberger, 2009)

The ExE contains contains differentiated trophectodermal cells that are thought to form the spongiotrophoblast in mouse (Cross et al., 2003) and is devoid of trophoblast stem cells (Uy et al., 2002). Further development leads to the formation of chorionic epithelium from the extra-embryonic ectoderm (Rossant and Cross, 2001). The event of chorioallantoic fusion takes place when allantois arises from the mesoderm at the posterior end of the embryo and makes contact with the chorion. As development proceeds, feto-placental blood vessels grow into the chorion from the allantois to

generate the fetal components of the placental vascular network. Further, the trophoblast, along with its fetal blood vessels, undergoes extensive villous branching to create a densely packed structure called the labyrith. Along with the beginning of morphogenetic branching, the chorionic trophoblast cells differentiate into various layers of labyrinth trophoblast cells, including the multinucleated syncytiotrophoblast.

The PE, develops into parietal endoderm and visceral endoderm (VE). The parietal endoderm migrates away from the ICM surface and comes in contact with the maternal tissue. The visceral endoderm forms an epithelium on the surface of epiblast and ExE, expanding along the surface of the ExE and epiblast, giving rise to the endoderm lining the extraembryonic yolk sac and a small number of its descendants contributing to the to the endoderm of the embryonic gut (Kwon et al., 2008). Reciprocal signalling between extraembryonic ectoderm (ExE), visceral endoderm (VE) and epiblast, mediated by secreted growth factors of TGF beta superfamily, namely nodal and BMP signalling pathways, WNT and FGF signalling pathways leads to region-specific gene-expression patterns in these tissues (Arnold and Robertson, 2009; Loebel et al., 2003).

The pluripotent epiblast is the origin for the three primary germ layers, ectoderm, mesoderm and definitive endoderm (DE), which constitute the progenitor cells that develop into all fetal tissues during embryo development and the extraembryonic mesoderm, of the yolk sac, the allantois and the amnion (Arnold and Robertson, 2009; Loebel et al., 2003; Tam and Loebel, 2007). The first visible sign of gastrulation is the formation of primitive streak (PS), a structure that forms in the posterior region of the embryo, through which the epiblast cells ingress and are allocated to the mesoderm or definitive endoderm. The epiblast cells forming the progenitors for neurectoderm lineage do not enter the PS. The mesendoderm is formed in the anterior and posterior regions of the PS and extraembryonic mesoderm in the posterior region of the PS. Mesendoderm gives rise to the somitic and paraxial mesoderm and definitive endoderm. Extraembryonic mesoderm gives rise to the amnion and hemangioblasts, the precursors of blood and blood vessels (Tam et al., 2006). The ingression of epiblast cells into PS for the formation of mesoderm and endoderm requires them to undergo epithelial to mesenchymal transition (EMT) (Tam and Loebel, 2007).

1.2.4: Epithelial mesenchymal transition (EMT)

EMT is a process modulated by various signaling pathways, through which polarized epithelial cells are converted to individually motile mesenchymal cells, accompanied by the loss of epithelial

polarities and adherence junctions (Kalluri and Weinberg, 2009; Lee et al., 2006). EMT happens during early embryogenesis and also tumor progression and hallmarks of EMT are the downregulation of a cell-adhesion molecule responsible for intercellular adhesion, E-Cadherin and up-regulation of Vimentin, an intermediate filament family member. Intermediate filamentents, along with microtubules and actin microfilaments, make up the cytoskeleton. E-Cadherin (CDH1) is thought to maintain the epithelial architecture of normal cells, regulating the differentiation of benign, non-invasive cells (MacCalman et al., 1998) and appears to be down-regulated in metastatic malignant cell types (Mori et al., 1998; Siitonen et al., 1996). The zinc-finger transcription factors Snail and Slug repress E-Cadherin in vitro by binding to its promoter (Batlle et al., 2000; Cano et al., 2000) and they have been also shown to promote EMT through β-Catenin–T-Cell Factor-4-dependent expression of TGF β3 (Medici et al., 2008) (Medici et al., 2008). Members of TGF β family, TGF β1, TGF β2, TGF β3 have been implicated as initiators of EMT in both development and cancer (Akhurst and Derynck, 2001; Camenisch et al., 2002; Medici et al., 2008; Zeisberg and Kalluri, 2004).

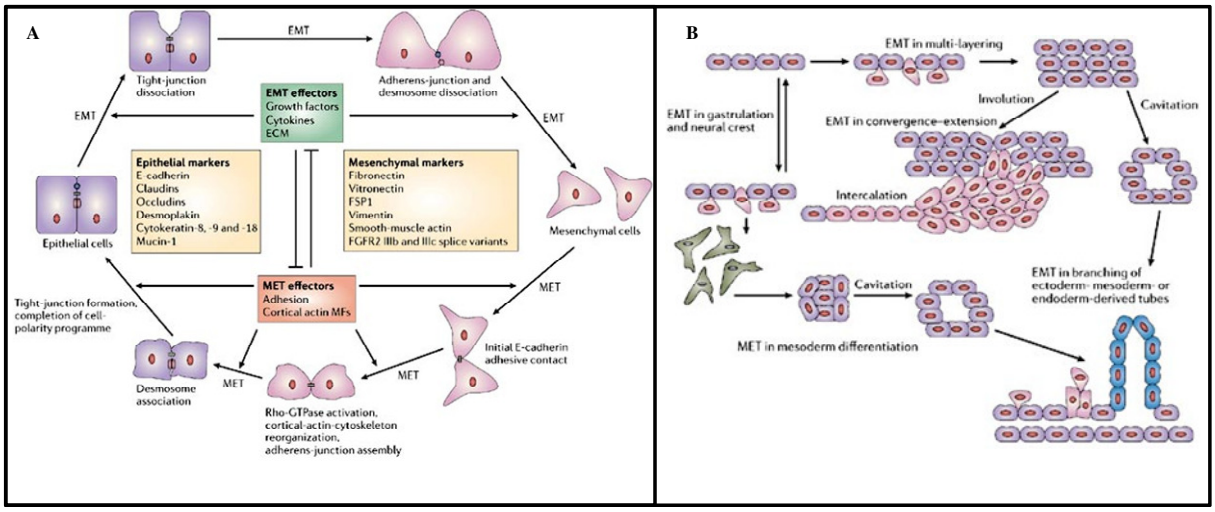


Figure 1.5: A) The cycle of events during which epithelial cells are transformed into mesenchymal cells (EMT) and vice versa (MET). The effectors and markers of the two processes are listed in the boxes B) The involvement of EMT or MET in morphogenesis, tissue formation and development. (adapted from (Thiery and Sleeman, 2006)

There have been evidences that TGFβ and Wnt signaling cooperate in promoting EMT. Wnt signaling has been reported to be essential for stimulating EMT during neural crest formation

(Garcia-Castro and Bronner-Fraser, 1999) and a transcription factor, LEF1, associated with this pathway can repress E-Cadherin (Jamora et al., 2003). LEF-1 can be functionally activated by binding with either β-catenin or Smad proteins (Labbe et al., 2000; Nishita et al., 2000) and TGFβ1 can promote β-catenin–LEF1 signaling and EMT in Madin-Darby canine kidney cells (Medici et al., 2006).

Members of various signalling pathways, including BMP, Wnt, Notch, etc., have the ability to regulate cell fate decisions during embryogenesis (Loebel et al., 2003) and can be used in numerous combinations to influence lineage choices in cultured ES cells. The lineage specification-ability of these pathways can be dependent on the signalling environment or other signalling pathways, which can modulate them by influencing their activity at various levels. Hence, an understanding of the potential influence of a pathway on the other is important for directing more ES cells to a specific lineage. The members of TGF-beta superfamily, including Activins and BMPs are involved in a broad range of cellular events, like differentiation, cell adhesion, apoptosis and repair (Massague, 1998).

1.3: Placenta

1.3.1: Human and mouse (rodent) placenta

Placental mammals originated 100 million years ago (mya) (Springer et al., 2003) and any aberration in the normal development of placenta can cause fetal retardation or death. Morphological differences exist between human and rodent placentas, owing to their unique evolutionary histories (Rawn and Cross, 2008). In spite of the differences in morphology and some cell types, the basic developmental plan is the same and several genes critical for placental development in mice have conserved expression patterns in the human placenta (Cross et al., 2003; Rossant and Cross, 2001).

During the course of evolution, ancient genes, functional in the placenta were co-opted and also duplicated in some cases, to meet the needs of diverse range of pregnancy physiologies (Knox and Baker, 2008). Furthermore, some pathways, like FGF pathway and genes, like Hand1 and Dlx3 are functional both in the placenta and other organ systems, playing similar functional roles (Rawn and Cross, 2008). Fgf signalling is implicated in epithelial branching morphogenesis (Xu et al., 1998), Hand1 is functional in the development of placenta, heart, and blood vessels (Riley et al., 1998) and Dlx3 in the development of the placenta, neural crest epidermis, and limbs (Beanan and Sargent, 2000).

In mouse, implantation and invasion into the uterus is mediated by polyploid trophoblast giant cells (Zybina and Zybina, 1996) that are formed by endoreduplication (MacAuley et al., 1998) and occupy the outer layer of the placenta. The mouse trophoblast giant cells are analogous to the human invasive extravillous cytotrophoblast cells, which also become polyploidy but to a lesser extent (Berezowsky et al., 1995). Unlike in humans, mouse placenta has a middle layer, called the spongiotrophoblast cells that can differentiate into giant cells and arise from the ectoplacental cone. The chorionic villi of mice have two multinucleated syncytiotrophoblast layers, whereas that of humans has a single syncytial layer and an underlying trophoblast stem cell layer. But the labyrinth layer of the mouse placenta is completely analogous to the chorionic villi of the human placenta in terms of function and in both the organisms, the villi are covered by syncytiotrophoblast that comes in direct contact with maternal blood (Rossant and Cross, 2001).

1.3.2: Human Placental development

Trophoblast or trophectoderm formation is the first differentiation and lineage restriction which happens in an embryo. The trophoblast cells are the inhabitants of the extraembryonic membrane called the chorion, which plays a very crucial role in the fetal development and protection. Placental development is primarily dependent on trophoblast stem cell differentiation (Jurisicova et al., 2005).

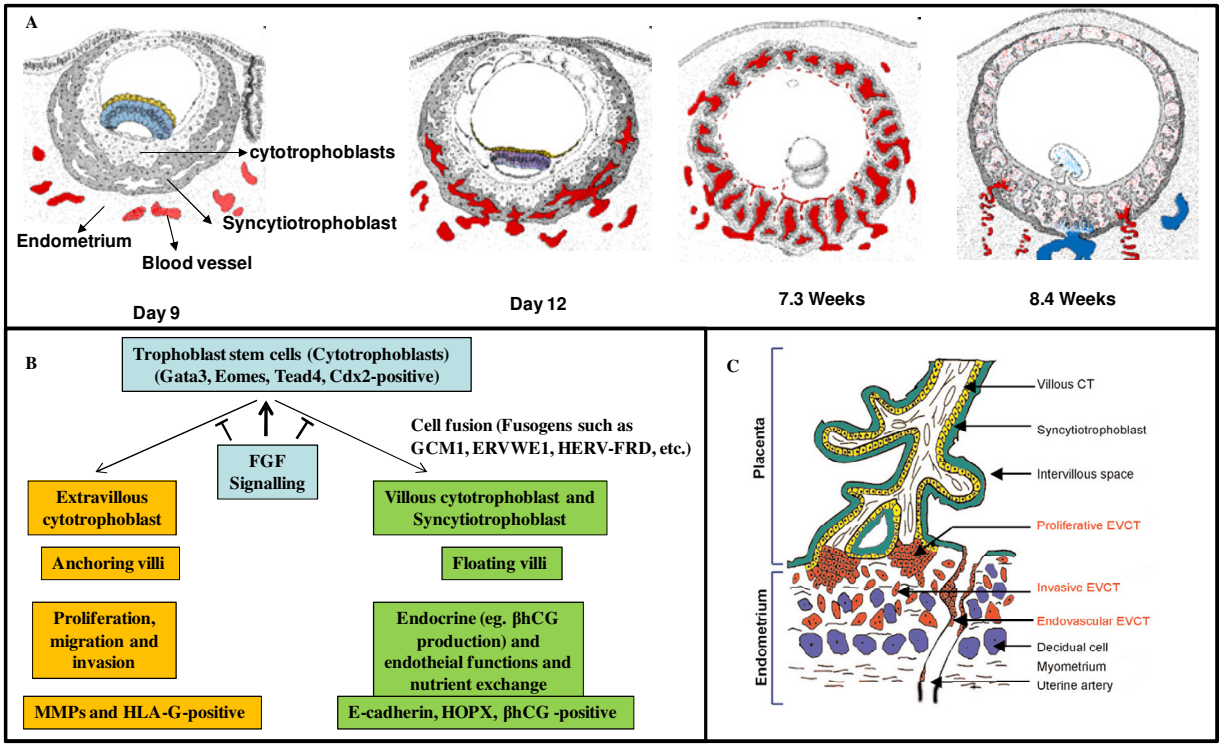


Figure 1.6: A) Implantation of the human embryo in the mother's uterus (endometrium) (adapted from http://www.med.yale.edu/obgyn/kliman/placenta/articles/EOR_Placenta/Trophtoplacenta.html)

B) A schematic diagram representing differentiation of trophoblast stem cells, based on the findings in mouse and human C) Human Chorionic Villi (adapted from (Guibourdenche et al., 2009) CT: Cytotrophoblasts; EVCT: Extravillous cytotrophoblasts

Following the invasion of the endometrium by cytotrophoblast cells, numerous trophoblastic primary villi are formed by the proliferation and differentiation of the cytotrophoblasts (Huppertz and Herrler, 2005). During the primary villi formation, connections are built between the villi and endometrium and the villi are surrounded by maternal blood (Cross et al., 2006). As the villi mature to form secondary villi, a central core is formed within the villus by extra-embryonic mesoderm cell differentiation, responsible for the fetal blood circulation, which allows material-exchange between the fetal blood (inside the villi) and maternal blood (outside the villi) (Cross et al., 2006). These changes take place within three weeks of pregnancy and majority of the villi are arrested at this developmental stage (Aplin et al., 2000). But some villi continue to develop into anchoring villi via further differentiation of cytotrophoblasts, which either invade further into the endometrium to anchor the placenta to the uterus or invade the maternal spiral arteries to remodel these blood vessels (Aplin et al., 2000). Some of the cytotrophoblasts spread out and come in contact with the cells of other villi to ultimately form the cytotrophoblast shell (Lala and Graham, 2001).

The trophoblast stem cells also called cytotrophoblast cells, can adopt two mutually exclusive differentiation paths, either the non-invasive villous syncytiotrophoblast or invasive extravillous cytotrophoblasts (Enders et al., 2001). These two separate pathways lead to the formation of two specialized chorionic villi, the floating villi and the anchoring villi. The floating villi is constituted by an inner layer of mononucleated cytotrophoblast cells, which fuse to form the outer layer, the multinucleated syncytiotrophoblast, characterised by its ability of high levels of βhCG production. The anchoring villi is constituted of invasive extravillous trophoblast cells formed from the cytotrophoblasts streaming out of the trophoblastic shell and adopting the invasive pathway of differentiation. Among the two types of differentiated trophoblast cells, the syncytiotrophoblast cells remain epithelial and the extravillous invasive cytotrophoblast cells undergo epithelial-mesenchymal transition (EMT), which infiltrate into the maternal decidual stroma and blood vessels (Charnock-Jones et al., 2004; Kingdom et al., 2000; Vicovac and Aplin, 1996; Zygmunt et al., 2003).

1.4: Trophoblast formation and differentiation

1.4.1: In vitro differentiation of ESCs into trophoblast

BMP signaling, via BMP4, BMP2, BMP7 or GDF5 support differentiation of hESCs to trophoblast (Chen et al., 2008; Ezashi et al., 2005; Liu et al., 2004; Wu et al., 2008; Xu et al., 2002). Microarray data analysis has revealed that BMP4 and downstream target genes of this pathway, such as human ID2 and HAND1are highly expressed in human TE, when compared to the ICM (Adjaye et al., 2005). hES cell clones lacking the cell surface molecules, glycosyl-phosphatidyl-inositol-anchored proteins (GPI-APs) cannot differentiate to trophoblast, due to the lack of GPI-anchored BMP coreceptors, resulting in the impairment of full BMP4 signaling activation, which reemphasizes the requirement of BMP signaling for trophoblast differentiation (Chen et al., 2008). The lack of GPI-APs is because of the deficiency in the gene expression of PIG-A (phosphatidyl-inositol-glycan class A), which is required for the first step of GPI synthesis. Though BMP signaling plays a central role in driving trophoblast differentiation, it (BMP2, BMP4, BMP7 or BMP2/BMP7 heterodimers) has also been implicated in the differentiation of hESCs to extra-embryonic endoderm lineage, seen from the up-regulation of endoderm specific markers HNF3α, HNF4, GATA4 and GATA6 (Pera et al., 2004). When cultured in 3D-substrate of Matrigel in endothelial cell growth medium-2, BMP4 treatment caused up-regulation in the gene expression of endoderm, mesoderm and endothelial markers (Boyd et al., 2007). This throws light on the possibility of interference of other pathways in BMP-mediated directed differentiation. While BMP signaling can induce trophoblast differentiation in hESCs, in cooperation with LIF/STAT3, it supports mESC self renewal and pluripotency (Daheron et al., 2004; Humphrey et al., 2004). It has been shown that to when mESCs are treated with collagen type IV (Col IV), markers for trophectoderm, hematopoietic, endothelia and smooth muscle cells were induced (Schenke-Layland et al., 2007) and also, Wnt3a, in the absence of LIF in TS cell medium, could trigger trophoblast differentiation by Cdx2 expression (He et al., 2008). The BMP mediated differentiation of hESCs to trophoblast can be influenced by the presence or absence of FGF2 in the culture medium and the former can slow down this differentiation process (Das et al., 2007; Schulz et al., 2008).

Smads can bind to with the NANOG proximal promoter (Greber et al., 2008), the activity of which is enhanced upon TGF b/Activin activation and reduced by BMP signaling in vitro (Xu et al., 2008). Inhibition of Activin/Nodal signaling in hESCs has also been shown to initiate trophoblast differentiation (Wu et al., 2008), though it also supports neurectoderm differentiation (Smith et al.,

2008). The knock down of hESC core transcription factors, OCT4 (Babaie et al., 2007; Hay et al., 2004; Matin et al., 2004), SOX2 (Fong et al., 2008) and NANOG (Hyslop et al., 2005) induce trophoblast differentiation evidenced by the upregulation of specific markers, depending on the media conditions, such as the culture conditions (conditioned media or defined media), or the presence or absence of FGF (Hay et al., 2004), all of which can influence this differentiation. The reduction or silencing of Oct4, Nanog and Sox2 gene expression in mESCs also have shown similar effects (Hay et al., 2004; Hough et al., 2006; Ivanova et al., 2006; Loh et al., 2006; Niwa et al., 2000; Velkey and O'Shea, 2003). Furthermore, overexpression of Oct4 leads to differentiation toward the extraembryonic endoderm lineage (Niwa et al., 2000).

Some studies have enriched trophoblast cells types via embryoid body formation, followed by selection. The differentiation of hESCs to embryoid bodies in suspension leads to spontaneous differentiation of some of the peripheral cells to trophoblast (Gerami-Naini et al., 2004) , which can be sorted out using selection criteria for specific markers, like hCG (Harun et al., 2006), representing villous trophoblast cells or the adhesion molecule PECAM-1, representing extravillous trophoblasts as majority (Peiffer et al., 2007).

1.4.2: Trophoblast stem cells (Cytotrophoblasts)

FGF4 (Tanaka et al., 1998) and TGF β maintain long-term continuous TS cell proliferation (Erlebacher et al., 2004) and Nodal and FGF4 directly act on extraembryonic ectoderm to inhibit differentiation of trophoblast stem cells (Guzman-Ayala et al., 2004). FGF signaling (FGF4) supports in vitro maintenance and proliferation of both mouse (Murohashi et al., ; Tanaka et al., 1998) and human (Baczyk et al., 2006; Ferriani et al., 1994) and its withdrawal causes differentiation, not inducing apoptosis (Simmons and Cross, 2005; Yang et al., 2006). In mouse, the withdrawal causes endoreduplication (Ullah et al., 2008), leading to trophoblast giant cell formation (Tanaka et al., 1998), a cell type analogous to human extravillous trophoblast (Berezowsky et al., 1995). During endoreduplication of mouse TS cells, p57 inhibits CDK1, which is accompanied by suppression of the DNA damage response by p21 (Ullah et al., 2008). Inactivation of MEKK4, which is a signaling hub for FGF4 activation of JNK, in mouse trophoblast stem cells shows a preferential differentiation to spongiotrophoblast and syncytiotrophoblast (Abell et al., 2009).

Null mutants of the trophectoderm-expressed transcription factors, Eomes (Russ et al., 2000) or Cdx2 (Rossant et al., 2003) are trophoblast defective and trophoblast stem cells cannot be derived

from them. Cdx2 is essential for the segregation of inner cell mass and trophectoderm (Beck et al., 1995; Chawengsaksophak et al., 2004; Strumpf et al., 2005) and mutant Cdx2 blastocysts fail to implant due to affected trohoblast formation (Strumpf et al., 2005). In mouse, the transcription factor, Tead4 acts upstream of both Gata3 and Cdx2 as it is required for the expression of both, but Cdx2 is not required for Gata3-induced expression of a subset of trophoblast genes in embryonic stem cells (Ralston et al.). Gata3 is specifically expressed in TE and not in the ICM and RNAi and chromatin immunoprecipitation experiments have shown Gata3 regulates expression of Cdx2, thereby regulating the expression of key genes in TE lineage (Home et al., 2009). As a response to FGF signalling, TS cells express the transcription factors, Cdx2 and Eomes both in vitro and in vivo cells (Rossant and Cross, 2001; Russ et al., 2000; Tanaka et al., 1998). The differentiation of TS cells to more specialised cells types is associated with down-regulation of these genes and up-regulation of transcription factors, specific to the respective cell type (Tanaka et al., 1998) (Hughes et al. 2004; Tanaka et al., 1998; Cross, 2000; Selesniemi et al., 2005).

TGF-beta/activin is crucial for the maintenance and proliferation of trophoblast stem cells (Erlebacher et al., 2004; Natale et al., 2009). Activin but not TGF-beta results in the maintenance of expression of TS cell markers, prolongs the expression of syncytiotrophoblast markers, and significantly delays the expression of spongiotrophoblast and TGC markers and Activin promotes differentiation of cultured mouse trophoblast stem cells towards a labyrinth cell fate (Natale et al., 2009).

Based on known information on transcription factors functional in placenta (Cross et al., 2002) and comparative placentation, a prospective transcription-factor model for human trophoblast differentiation has been suggested (Roberts et al., 2008).

1.4.3: Syncytiotrophoblast

Syncytiotrophoblast layer is a continuous multinucleated cell layer, with no lateral cell borders (Richart, 1961), formed from the fusion of mononucleated mitotically active cytotrophoblast stem cells (Frendo et al., 2003a)

(Boyd and Hamilton, 1970; Benirschke and Kaufmann, 2000). The syncytiotrophoblast layer remains in contact with the maternal blood directly, mediating gas and nutrient exchanges between the developing fetus and the mother and its formation and maintenance is critical for pregnancy. Though this layer is subject to constant renewal throughout pregnancy (Ugur et al., 2006), its nuclei are non-

mitotic (Richart, 1961). Cell cycle disruption, by inhibition of DNA synthesis and replication is a basic trigger for cytotrophoblast differentiation into syncytiotrophoblast (Crocker et al., 2007) and cell cycle arrest is critical for syncytiotrophoblast formation (Simmons and Cross, 2005).

Initially, the syncytiotrophoblast can penetrate or invade into the uterine epithelium to form the primary villus (Frendo et al., 2003a), but later it loses its invasive phenotype, forming the outer surface of the chorionic villus that separates the maternal and fetal blood circulation (Huppertz et al., 2006). Fragments of the syncytiotrophoblast layer, called the syncytiotrophoblast membrane (STBM) are shed into the maternal circulation (Gude et al., 2004), the amount of which is abnormally increased in the circulation of women with preeclampsia (Cockell et al., 1997). The formation and maintenance of syncytiotrophoblast involves a complex cascade of events, with its replenishment via continuous fusion of the underlying proliferating trophoblast stem cells called the villous cytotrophoblasts and elimination of the old nuclei via apoptosis, taking place near the surface in the "syncytial knots" that are a characteristic of human placenta (Burton and Jones, 2009; Caniggia et al., 1997a; Huppertz et al., 1999a; Huppertz et al., 1999b).

Various cytoskeletal components, including actin filaments are present in the developing placenta (Beham et al., 1988; Parast and Otey, 2000) and prominent actin-based microfilamental structures are present in the human syncytiotrophoblast (Smith et al., 1977). The formation of syncytiotrophoblast via cell fusion (Fulton et al., 1981; Ramos et al., 2008) and its functions, such as nutrient transport from the mother's blood, through mechanisms like endocytosis (Fuchs and Ellinger, 2004), the state of ion channels and transport can be tightly regulated by the organization of actin filaments in the human syncytiotrophoblast (Montalbetti et al., 2005).

The mononucleated villous cytotrophoblasts isolated from term placenta, under in vitro conditions, in the presence of fetal calf serum spontaneously differentiated into multinucleated syncytium, that synthesize and secrete a number of cell products as do their counterparts in vivo, including hCG, placental lactogen (Feinman et al., 1986), estrogen and progesterone (Kliman et al., 1986). The culture conditions of the cytotrophoblasts can influence the path of differentiation these cells adopt. hCG treatment can promote the syncytialization of villous cytotrophoblasts derived from term pregnancy placenta (Zhou et al., 2001) and inhibition of its receptor abolishes the fusion-promoting effects of several growth factors, such as EGF, TGFα and LIF (Yang et al., 2003).

Human chorionic gonadotrophin (hCG) is a hormone, secreted by the human placenta, which plays a very important role during early pregnancy, maintaining the corpus leuteum and progesterone production ensuring successful embryo implantation. It can be detected in the mother's serum after 8 days of ovulation (Braunstein et al., 1976). hCG has 2 subunits, α (CGA) and β (CGB). Out of these two, βhCG is essentially restricted to villous syncytiotrophoblast and βhCG hormone secretion is an exclusive characteristic of this cell type (Randeva et al., 2001). The subunit of hCG (βhCG) is encoded by a cluster of genes, CGB, CGB5, CGB7 and CGB8, located on chromosome 19q13.3 (Rull and Laan, 2005), whereas a single gene codes for the α subunit, located on chromosome 6q12-q21. The α subunit (CGA) of hCG is sometimes visible in the cytotrophoblast, but the β subunit is essentially restricted to villous syncytiotrophoblast (Randeva et al., 2001). Pregnancy tests involve the determination of its concentration by an immunoassay (Chard, 1992).

Some of the known genes, responsible for driving cytotrophoblast cell fusion (fusogens) are the retroviral genes, syncytin-1 (ERVWE1) (Frendo et al., 2003b), syncytin-2 (HERV-FRD) (Blaise et al., 2003) and the transcription factor, GCM1 (Baczyk et al., 2004; Nait-Oumesmar et al., 2000). Glial cell missing-1 (GCM1) is a placental-specific transcription factor, localised in a subset of highly differentiated human villous cytotrophoblasts and syncytiotrophoblast (Baczyk et al., 2004; Janatpour et al., 1999; Nait-Oumesmar et al., 2000), regulates the expression of syncytin-1 (Yu et al., 2002) and aromatase (Yamada et al., 1999). GATA 2 and 3 play a very crucial role in trophoblast differentiation (Home et al., 2009; Kim et al., 2009; Ng et al., 1994; Ni et al., 2004) and binding of GATA factors to the enhancer of the human syncytin gene is critical for its cell-specific expression (Cheng and Handwerger, 2005). syncytin-1 (Yu et al., 2002) and aromatase (Yamada et al., 1999) are regulated by the transcription factor, GCM1.

HOPX is a tumor suppressor gene, exclusively expressed in syncytiotrophoblast and not in extravillous trophoblasts (EVT) in humans, whereas in mouse, it is expressed in trophoblast giant (TG) cells which are the orthologues of human EVT, and spongiotrophoblast cells (Asanoma et al., 2007). The diverse localization shows the diverse functions, HOP can have in mouse and human placenta. The exact role of this gene in placenta has not been deciphered yet. But its ability of induction of syncytiotrophoblast formation (Asanoma et al., 2003) and its specific expression in syncytiotrophoblast give clues to its supportive action in human syncytiotrophoblast. Inactivation of Hop in mice by homologous recombination results in heart failure and lethality (Chen et al., 2002b; Shin et al., 2002).

1.4.4: Invasive extravillous cytotrophoblasts (EVTs)

Invasive extravillous cytotrophoblasts (EVTs) are primarily responsible for the invasion of the decidua and myometrium. During the early stages of pregnancy, the cytotrophoblast stem cells at the villous basement membrane form cell columns in the anchoring villi (Caniggia et al., 1997b). EVTs originate from the outer most part of these cell columns, giving rise to the endovascular and interstitial invasive cytotrophoblasts and upon differentiation, stop proliferating, unlike tumor cells (Pollheimer and Knofler, 2005). Both these differentiated cell types are instrumental in invading and anchoring the placenta to the maternal endometrium (Chakraborty et al., 2002). The interstitial invasive cytotrophoblast also differentiate into giant cells (GC) in deeper areas of the placental bed (Huppertz et al., 2006). The endovascular invasive cytotrophoblasts migrate towards the maternal uterine spiral arteries and degrade the muscle cell layer which is necessary to maintain blood vessel integrity, a process called conversion, lowering vascular resistance, and thereby increasing blood flow required to meet the demands of the fetus as pregnancy progresses (Aplin et al., 2000). In patients with preeclampsia, conversion of an insufficient number of maternal arteries is often observed (Aplin et al., 2000).

The EVTs can be distinguished from the non-invasive syncytiotrophoblasts, with the help of markers, specific to this cell type. Human leukocyte antigen (HLA)-G, which is a major histocompatibility gene, expressed in invasive extravillous trophoblast cells all throughout pregnancy (Chumbley et al., 1993; Goldman-Wohl et al., 2000; McMaster et al., 1995; Yelavarthi et al., 1991) and is not found in the non-invasive syncytiotrophoblast of the chorionic villi (McMaster et al., 1995). Mouse glycogen cells, which are a type of cytotrophoblast cells, could be analogous to the human extravillous cytotrophoblasts (Georgiades et al., 2002). Cytokeratin7 (KRT7 or CK7) is a specific marker for villous cytotrophoblasts (Baal et al., 2009; Cervar et al., 1999) and for human invasive trophoblasts (Handschuh et al., 2009; Tarrade et al., 2001). There are evidences that β-hCG secreting trophoblast cells are largely cytokeratin-negative (Maldonado-Estrada et al., 2004; Manoussaka et al., 2005) and the syncytiotrophoblast lining expresses little cytokeratin (Badwaik et al., 1998). E-Cadherin, a marker for epithelial cells has been mainly characterised in the anchoring placental villi and it is either absent or down-regulated in the cells undergoing extravillous differentiation in vitro and in vivo (Babawale et al., 1996; Floridon et al., 2000; Zhou et al., 1997a; Zhou et al., 1997b). In vivo data in human suggests that bFGF is strongly expressed in cytotrophoblast and extravillous trophoblast, but very weakly in syncytiotrophoblast (Ferriani et al.,

1994). Intersitial CTBs, a descendant of EVTs, express transcription factors responsible for EMT, such as Snail and Tcf family members as well as β catenin, signifying Wnt pathway activation, a typical feature of EMT (Knofler, ; Pollheimer et al., 2006).

1.5: Signalling pathways:

1.5.1: The TGF-β super-family: Activin, Nodal TGF-β and BMPs

The members of the TGF-β super-family signal a heteromeric complex of receptor serine kinases which include at least two type I (I and IB) and two type II (II and IIB) receptors. In mammals, five type II and seven type I receptors (also called activin receptor-like kinase or ALK) have been characterized (Schmierer and Hill, 2007). These receptors, which are transmembrane proteins are composed of a ligand-binding extracellular domain with a cysteine-rich region, a transmembrane domain, and a cytoplasmic domain with predicted serine/threonine specificity.

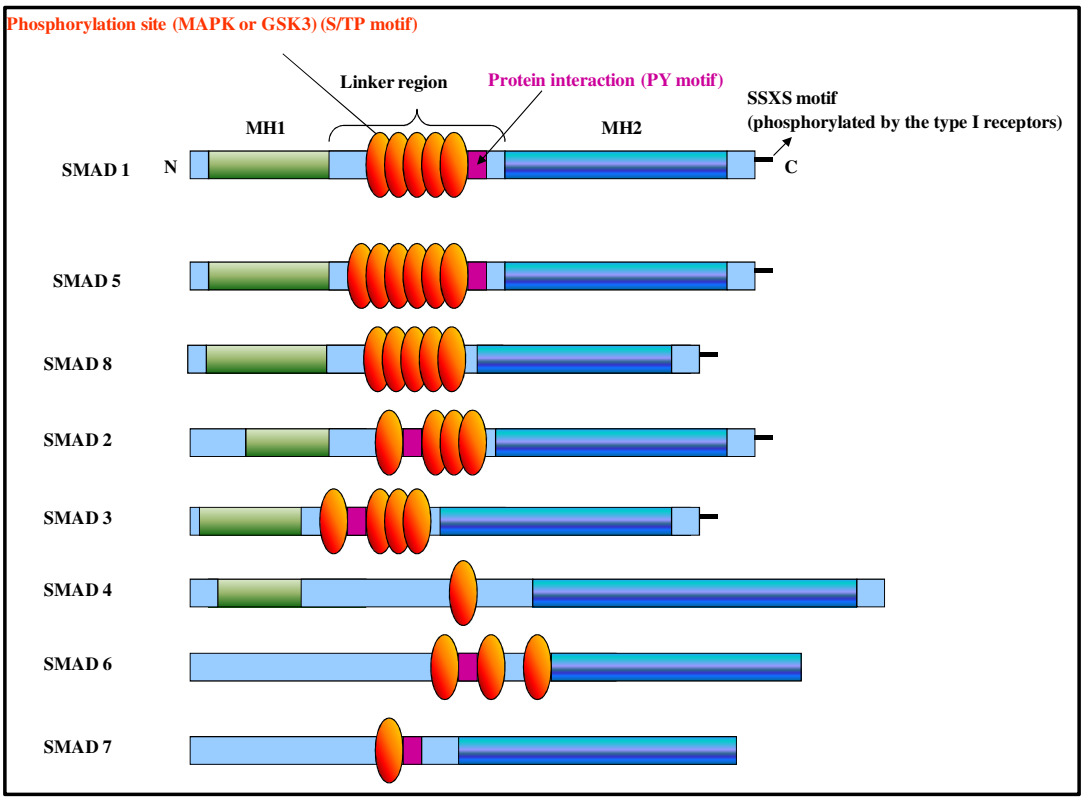


Figure 1.7: Cartoons depicting the structure of SMAD proteins (modified from (Miyazono et al., 2010)

Type I receptors are essential for signaling, and type II receptors are required for binding ligands and for expression of type I receptors. Type I and II receptors form a stable complex after ligand binding, resulting in phosphorylation of type I receptors by type II receptors, thereby activating the Smad proteins. The Smads are a group of intracellular signaling molecules constituting the receptor-regulated Smads (R-Smads) Smad 1, 2, 3, 5, and 8, the co-Smad Smad4, and the inhibitory Smads Smad 6 and 7. The TGF-β superfamily signaling pathway has two major branches, TGF-β/Nodal/Activin branch and the BMP/GDF branch, both of which use distinct receptor the downstream signalling molecules. While Activin/Nodal/TGF β utilize the receptors, ALK4/5/7, activating Smad 2/3, BMP ligands, such as BMP4 use a different set of receptors, ALK 2/3/6, activating Smad1/5/8 (Massague, 2008; Massague et al., 2005). Though Nodal uses the same receptors as that of Activin, in addition it requires Cripto (a GPIAP) as a co-receptor for its signaling though Smad2/3. The type I receptors phosphorylate R-Smads at two serines in an S-M/V-S motif at their extreme C termini (Attisano and Wrana, 2002; Massague, 2008; Massague et al., 2005).

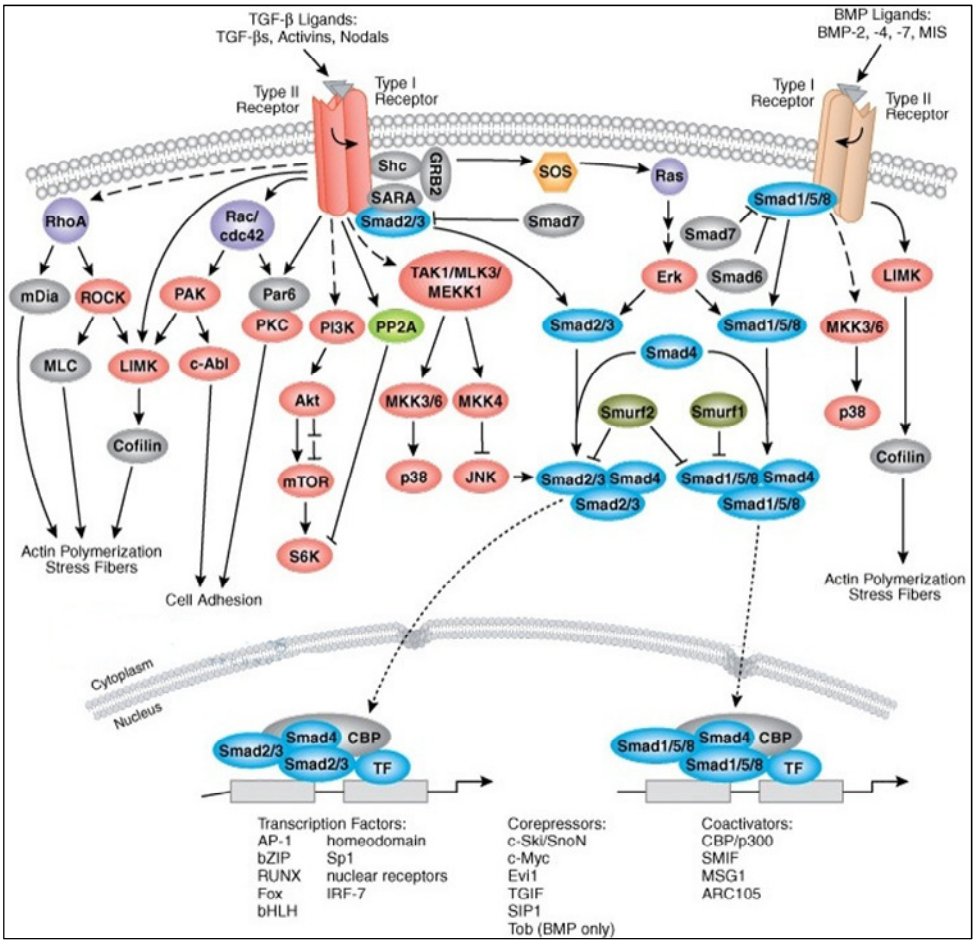


Figure 1.8: TGFβ pathway, representing the two branches, namely the SMAD2/3 (TGFβ) branch and the SMAD1/5/8 branch (BMP) (adapted from Cell signaling Inc.).

The phosphorylated R-Smads, then form both homomeric and heteromeric complexes with Smad4, followed by accumulation in the nucleus, regulating transcription of the target genes. The phosphorylated R-Smads, Smad 1, 5, 8 and Smad 2,3 compete for the co-Smad, Smad4, therefore naturally antagonizing each other.

Recent findings have shown some exceptions in the traditional understanding of the TGF-β superfamily signaling pathway. Some GDFs, such as , 9, and 11, signal through ALKs 4, 5, and 7 (Schmierer and Hill, 2007). In some epithelial, endothelial, fibroblast, and tumor cells, TGF-β activates both Smad2/3 and Smad1/5/8 (Bharathy et al., 2008; Daly et al., 2008; Goumans et al., 2003; Liu et al., 2009). In epithelial cells, this results in the formation of R-Smad complexes of Smad 1 and 2, in addition to the canonical Smad2/3-Smad4 complexes (Daly et al., 2008).

1.5.1.1: BMP signalling pathway

BMP signalling can support differentiation of hESCs, depending on the degree of activation and signalling environment into multiple lineages, namely trophoblast (Greber et al., 2008; Xu et al., 2002), extra-embryonic endoderm (Pera et al., 2004), mesoderm (Zhang et al., 2008) and hematopoietic cells (Chadwick et al., 2003). Inhibition or reduction of BMP signalling is important for neural induction during vertebrate development (Gaulden and Reiter, 2008; Munoz-Sanjuan and Brivanlou, 2002; Vallier et al., 2009). Furthermore, the knockdown of Bmp4 diminishes the inhibitory activity of the extra-embryonic ectoderm in mouse embryo (Soares et al., 2008). The developmental advancement from undifferentiated epiblast stem cells to mesendoderm is marked by the formation of primitive streak, which expresses a conserved T box transcription factor, Brachyury all throughout its structure in diverse organisms, including Xenopus, zebrafish, and mammals (Kispert and Herrmann, 1994; Schulte-Merker et al., 1997; Smith et al., 1991). Experiments using BMP receptor inhibitors on mouse ES cells or homozygous BmprI or II mutant mouse embryos showed that BMP signaling is not necessary for preimplantation or for initial postimplantation development or primitive streak formation or visceral endoderm formation, but is required for epiblast differentiation and mesoderm formation (Beppu et al., 2000; Mishina et al., 1995; Nostro et al., 2008).

BMP signaling inhibits neuronal differentiation in both Xenopus and mouse (Munoz-Sanjuan and Brivanlou, 2002).BMP signaling has been shown to have contrasting roles in mouse and hESCs, in terms of maintenance of pluripotency and differentiation. On one hand, it supports mESC self renewal, on the other, in human and monkey ESCs, it has been shown to induce differentiation to trophectoderm or mesendoderm (Yan-ling et al., 2009).

Gremlin is a homodimer of 28 kDa, which was first isolated from the neural crest of the Xenopus as an antagonist of BMP signaling (Hsu et al., 1998; Topol et al., 2000) and is an important BMP regulator for limb development that acts in a complementary fashion with other BMP antagonists (Khokha et al., 2003). It binds to BMP2, 4 and 7 with high affinity and prevents them from interacting with their receptors (Hsu et al., 1998; McMahon et al., 2000; Shi et al., 2001). During embryogenesis gremlin is required to establish the apical ectodermal ridge and epithelial-mesenchymal feedback signaling and is decisive for early limb outgrowth, patterning and morphogenesis in kidney and lung rudiments (Khokha et al., 2003; Michos et al., 2007; Michos et al., 2004). High levels of gremlin expression were found in non-dividing and terminally differentiated cells such as neurons, alveolar epithelial cells, and goblet cells (Topol et al., 1997). Gremlin transcriptionally activates p21 (Chen et al., 2002a).

1.5.1.2: ACTIVIN/NODAL signaling pathway

ACTIVIN/NODAL signaling is essential for PS formation from hESCs and this pathway, along with β-catenin synergistically induce posterior PS/mesoderm progenitors in the presence of BMP signaling and in its absence, induce anterior PS/endoderm progenitors, in which, the PI3-kinase/Akt, but not MAPK, signaling pathway plays a very crucial role (Sumi et al., 2008).

Other than its role of supporting hESC self-renewal (Amit et al., 2004; Beattie et al., 2005; Greber et al., 2008; James et al., 2005; Liu et al., 2006; Lu et al., 2006; Vallier et al., 2005; Vallier et al., 2004; Wang et al., 2007; Xiao et al., 2006), the members of the TGF-β superfamily, Activin and Nodal is required for primitive streak formation (Conlon et al., 1994; Nostro et al., 2008), supports mesendoderm induction (Schier, 2003) and is crucial during mouse gastrulation in vivo (Arnold and Robertson, 2009). Primtive streak formation is the primary step in gastrulation and marks the formation of mesoderm and endoderm at the posterior side of the embryo. Activin A or Nodal have been used previously for endoderm induction of mESCs and hESCs in vitro (D'Amour et al., 2005; Kubo et al., 2004; Yasunaga et al., 2005). Graded levels of its expression gives rise to of different populations of mesoderm and endoderm cells (Tam and Loebel, 2007). Lefty1, Lefty2 and Cerberus

(Belo et al., 1997; Meno et al., 1999; Perea-Gomez et al., 2002) are the inhibitors of Nodal signaling (Shen, 2007). Lefty2 binds to Cripto, inhibiting nodal signalling by preventing Nodal/Cripto/receptor complex formation (Chen and Shen, 2004; Cheng et al., 2004) and Cerb-S, which is a truncated form of Cerberus blocks this signalling by direct ligand-binding (Piccolo et al., 1999). Lefty plays a role in anterior–posterior patterning in the mouse embryo during peri-implantation stages (Takaoka et al., 2006). Studies in mouse suggest the inhibitory role of Nodal signalling towards neuroectoderm specification as Nodal−/− embryos (Camus et al., 2006) and Cripto−/− mESCs (Liguori et al., 2003; Sonntag et al., 2005) show increased tendency towards neuroectoderm differentiation. In vitro studies show that inhibition of ACTIVIN/NODAL promotes differentiation of hESCs towards neuroectoderm (Smith et al., 2008; Vallier et al., 2004; Vallier et al., 2009) and mesenchymal lineage (Mahmood et al.). LEFTY expression has been characterised during the differentiation of hESCs to early embryoid bodies and has been implicated in ectoderm specification (Dvash et al., 2007). All these independent findings reemphasise on the importance of ACTIVIN/NODAL signalling in supporting self renewal of hESCs, mesendoderm induction and in the inhibition of neurectoderm induction.

1.5.2: FGF signaling pathway

FGFs are members of a large family of multifunctional, heparin-binding proteins (Heparan sulfate proteoglycans: HSPG), showing diverse patterns of interaction with a family of receptors (FGFR-1 to -4), spliceforms of which exist. FGF4 binds and activates FGFR1 to -3 (Kosaka et al., 2009). FGF2 binds to its receptors, principally FGFR1 (Stachowiak et al., 2003), resulting in a complex, which is translocated to the nucleus to regulate target gene expression (Dvorak et al., 2005). FGF signaling is required for hESC self-renewal (Thomson et al., 1998). The most abundant FGF receptor in hESCs is FGFR1, followed by FGFR3, FGFR4, FGFR2, in the order of expression levels. Both FGF (Dvorak et al., 2005; Greber et al., 2007) and FGF4 (Mayshar et al., 2008) are expressed in hESCs through autocrine signaling and support both pluripotency and differentiation, depending on the signaling environment. Inhibition of FGF signaling causes differentiation of hESCs whereas, the expansion of undifferentiated mESCs is not disturbed, but their lineage commitment is restricted, impairing neural and mesodermal induction (Kunath et al., 2007).

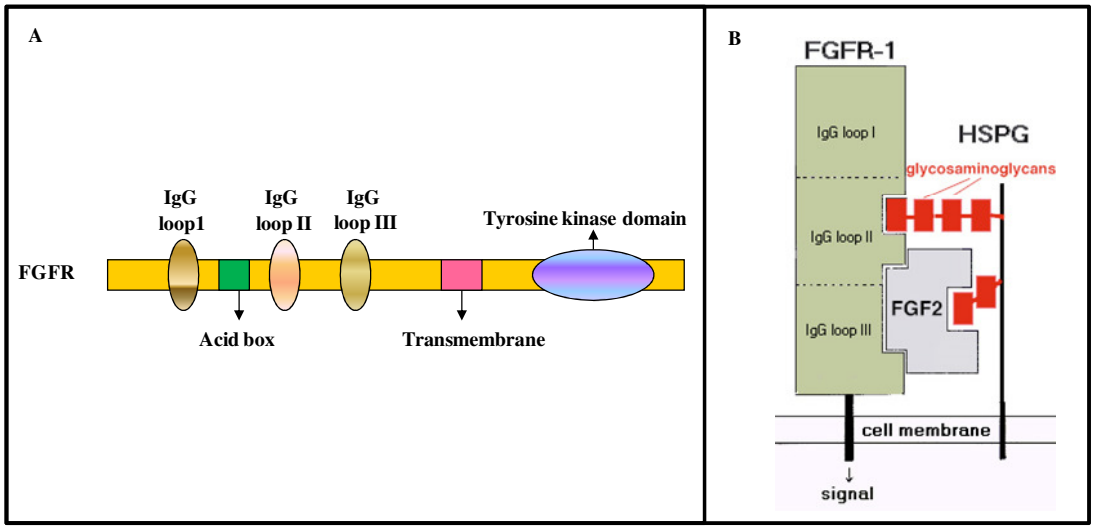


Figure 1.9: Cartoons depicting A: the general structure of FGF receptor (FGFR) (adapted and modified from http://www-personal.umich.edu/~lpt/fgf/fgf.htm) B: the binding of FGF2 with FGFR1 (adapted from Gilbert, SF, Developmental Biology, Eighth Edition)

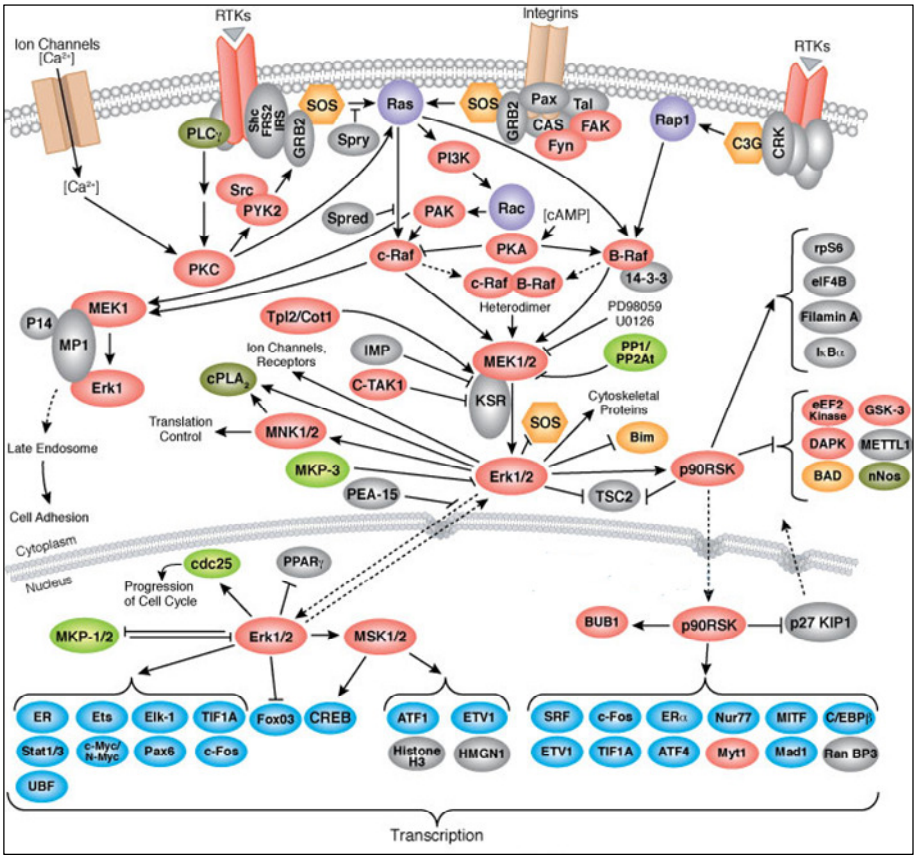


Figure 1.10: FGF signaling pathway (adapted from Cell signaling Inc.)

FGF/MAPK signals are known to oppose BMP/Smad1 in many developing organs (De Robertis and Kuroda, 2004). Smad3 has been shown to also serve as a substrate of the G1 cyclin-dependent kinases Cdk2 and Cdk4. The CDK phosphorylation sites in Smad3 include threonine and serine residues in the linker region (Matsuura et al., 2004) which are also sites for ERK MAP kinases (Kretzschmar et al., 1997; Kretzschmar et al., 1999). SMAD1 has GSK3 phosporylation site at the linker region and the Wnt pathway promotes BMP Smad signaling by repressing GSK3 (Fuentealba et al., 2007; Sapkota et al., 2007). The R-Smad linker region contains conserved MAPK and glycogen synthase kinase 3 (GSK3) phosphorylation sites, which are crucial targets for regulating the stability of activated R-Smads. Sequential Smad1 linker phosphorylation by MAP kinases and GSK3 leads to polyubiquitinylation via Smurf1. Subsequently, Smad1 is targeted for proteasome-dependent degradation (Sieber et al., 2009).

Oct4 and Fgf4 expression patterns are consistent with each other in mouse preimplantation embryos and in postimplantation embryos (Niswander and Martin, 1992; Scholer, 1991) they are expressed in distinct regions as well as in overlapping regions (Niswander and Martin, 1992; Rosner et al., 1990)**.** In stem cells, the FGF4 enhancer contains a consensus octamer binding site, to which the Sox2/Oct-3/4 complex binds and transcriptionally activates FGF4 expression, which is important for maintenance of pluripotency (Yuan et al., 1995).

A lot of studies have shed light of the crucial role played by FGF signaling in early embryonic development and the cell fate decisions taken by the cells based on its presence or absence(Yamanaka et al., 2010; Zernicka-Goetz et al., 2009). There are evidences of the critical role of FGF/MAP kinase signalling pathway in lineage segregation and specification of epiblast, TE and PE in early developmental stages. Experiments sone using transgenic mice with dominant negative FGF receptor (dnFGFR), showed the requirement of FGF signaling in the formation of mitotic trophoblast cells adjacent to the ICM, though formation of postmitotic extraembryonic ectoderm cells at the abembryonic pole (Chai et al., 1998). There is a requirement of FGF4 in the maintenance of trophectoderm and primitive endoderm identity during early mouse development (Goldin and Papaioannou, 2003).

Homozygous mutants of Fgf4, Fgfr2 or Grb2 exhibit periimplantation lethality and lack of PE formation completely in vivo and also in blastocyst outgrowths (Arman et al., 1998; Feldman et al., 1995; Goldin and Papaioannou, 2003; Wen et al., 1998) and overexpression of a dominant-negative FGF receptor in ES cells blocks PE formation in embryoid bodies (EBs) in vitro (Chen et al., 2000).

The lineage segregation of epiblast and PE occurs during maturation of the blastocyst in an FGF/MAP kinase signal-dependent manner, in which the formation of the latter is affected in the absence of this pathway (Yamanaka et al., 2010). Attenuation of FGF/Erk signaling is required for extraembryonic ectoderm differentiation, ectoplacental cone cavity closure, and chorioallantoic attachment (Papadaki et al., 2007).

FGF-2 has been shown to induce development of ectodermal and mesodermal cells from pre-differentiated hESCs (Schuldiner et al., 2000) and to support hESC differentiation into neural lineages (Bendall et al., 2007; Carpenter et al., 2001; Cohen et al., ; Dvorak et al., 2005; Park et al., 2004; Reubinoff et al., 2001; Schuldiner et al., 2000; Schulz et al., 2003; Zhang et al., 2001). Though neural induction of hESCs can occur in the absence of FGF signaling, their neural specification is instructed by this pathway (Cohen et al.). FGF2, together with noggin positively regulate neural specification (Lamb and Harland, 1995) and dominant negative FGF receptor expression inhibited neural tissue formation (Launay et al., 1996) in developing Xenopus embryos. Neural induction in mESCs was blocked when dominant negative FGFRs were overexpressed (Tropepe et al., 2001; Ying et al., 2003). A recent study also throws light on its conserved ability to inhibit neuroectodermal commitment of EpiSCs and hESCs (Greber et al., 2010). FGF signaling has also been shown to support endodermal differentiation of hESCs (Ameri et al., ; Johannesson et al., 2009).

EMT is crucial for mesendoderm formation and Fgf signaling is required for expression of Snail (Zohn et al., 2006). During early stages of gastrulation, Fgf4 expression is restricted to the primitive streak (Drucker and Goldfarb, 1993; Niswander and Martin, 1992) and FGFR1 promotes EMT at the primitive streak by controlling Snail and E-Cadherin expression (Ciruna and Rossant, 2001). Taken together, FGF signaling is functional in diverse processes, of both maintenance of pluripotency and differentiation into the embryonic and extraembryonic lineages.

Methods:

2.1: *In vitro* culture of Human embryonic stem cells (hESCs)

Long-term culture of hESCs can be achieved by either growing on a layer of mitotically inactivated mouse embryonic fibroblasts (MEFs) or under feeder-free conditions in Conditioned Medium (CM) on Matrigel-coated plates to maintain their undifferentiated status. Success of both culture conditions completely depends on the quality of feeders, since they can directly affect the growth of human ES cells.

2.1.1: Mouse Embryonic Fibroblasts Culture

2.1.1.1: Isolation of Mouse Embryonic Fibroblasts (MEFs)

Gelatine (Gelatine from bovine skin, Type B, Sigma) solution (0.2%) was prepared in distilled water, autoclaved and filtered. Tissue culture flasks (T150: TPP) were coated with 0.2% gelatine for 2 hours. Meanwhile, **t**he pregnant female mice (CF-1, Harlan, USA) were sacrificed at 13 or 14 d.p.c. (days post-coitum) by cervical dislocation. Uterine horns were dissected out, briefly rinsed in 70% (v/v) ethanol and placed inside a falcon tube containing PBS without Ca2+ Mg2+ (Gibco, invitrogen). Afterwards, under the laminar flow, they were placed in a petri dish and each embryo was separated from the placenta and its embryonic sac. Next head and red organs were dissected out and the rest of the tissue was washed in PBS and placed in a clean petri dish. Using sterile razor blade the tissues were finely minced until they become pipettable. Then 1ml of 0.05% trypsin/EDTA (Gibco, Invitrogen) and DNase I (USB, 100 Kunitz units per ml of trypsin) was added per embryo. Afterwards the tissue was transferred into a 50 ml falcon tube and incubated for 15 minutes at 37°C. After every 5 minutes of incubation, the cells were dissociated by pipetting up and down several times. After 15 minutes, trypsin was inactivated by adding equal volume of MEF medium (Supplementary protocols) as that of the amount of trypsin. Low-speed short centrifugation facilitated settling down of the undissociated tissue pieces at the bottom, which were removed. The supernatant containing single MEFs were resuspended in warm MEF medium and plated into tissue culture flasks. Approximately 3-4 embryos were plated in each T150 (TPP) flask after aspirating the 0.2% gelatine which was coated on it for the past 2 hours. The advantage of gelatine coating is that it specifically favours the attachment of only fibroblasts (P0, passage 0).

When the cells were 80-90% confluent (usually the next day), a major part of the P0 cells were frozen for future usage. Usually 1 embryo equivalent was frozen per vial. The remaining one T150 flask of P0 was expanded (kept growing) to a maximum of P3 or P4, inactivated and used as feeders to thaw hESCs or to produce conditioned medium (CM).

2.1.1.2: Cryopreservation (freezing) of MEFs

After aspirating the culture media from the T150 flasks in which MEFs were grown, cells were washed with PBS without Ca2+Mg2+ (Gibco, Invitrogen) and trypsinized (0.05% trypsin/EDTA (Gibco, Invitrogen)) for 5 minutes at 37°C. Trypsin was neutralized by adding an equal volume of MEF medium as that of trypsin (Supplementary protocols). Then cells were centrifuged at low speed for 5 minutes. The supernatant was removed and the cells were resuspended in cold freezing media (Supplementary protocols). The cell suspension was transferred into 1 ml cryovials (TPP). Usually 1 embryo equivalent was frozen per vial. Cryovials were placed inside pre-cooled freezing container (Nalgene) and stored at -80°C overnight, which facilitates the cells to freeze very slowly. The next day vials were transfer to the liquid nitrogen tank for long-term storage.

2.1.1.3: Thawing and maintaining of MEFs

MEF medium was taken in a 15 ml falcon tube and placed on water bath at 37°C for warming. The frozen MEFs were removed from liquid nitrogen and quickly thawed in the 37°C water bath till a small bit of ice pellet was left in the vial. After sterilizing the vial with 70% (v/v) ethanol and complete evaporation of ethanol, the thawed cell suspension was transferred to the pre-warmed MEF medium drop-wise, to reduce osmotic shock. To remove the DMSO present in freezing media, the cells were centrifuged at low speed (1000 to 1200 rpm) for 5 minutes, supernatant was discarded and the cell pellet was re-suspended in 20 ml of pre-warmed MEF medium and plated in T150 flasks. The next day, media was replaced with fresh pre-warmed media. In another 2-3 days, when the cells were 90% confluent, they were passaged at a splitting ratio of 1:2 or 1:3 using trypsin.

* Mouse Embryonic Fibroblasts should be only used to a maximum of 5 passages, to support hESC growth as they start attaining a senescence state after that and do not divide any more in a sufficient manner.

2.1.1.4: Inactivation and plating of MEFs (Feeders preparation)

T150 flasks were coated with 0.2% gelatine (Gelatine from bovine skin, Type B, Sigma) and incubated at room temperature for at least 2 hours. During this time mitomycin C (Roche) was diluted in PBS (1mg/ml) and filtered-sterilized and media from MEFs was aspirated and cells were washed with PBS without Ca2+Mg2+ (Gibco, Invitrogen). Then 20ml of MEFs were exposed to MEF media containing 10μg/ml of mitomycin C for 2 hours at 37°C. Then mitomycin C containing media was removed and cells were washed twice with PBS, trypsinized (0.05% trypsin/EDTA), centrifuged and resuspend in warm media. Afterwards the cells were counted and plated in T150 flasks (56,000 cells/cm2) to be used for CM preparation for the next 6 days.

2.1.2: Preparation of FGF2, matrigel and media

2.1.2.1: Preparation of FGF2 (bFGF) stock solution

BSA (Bovine Serum Albumin, Fraction V, 99% purity, Sigma) solution of 0.2% was prepared in PBS and filtered (0.2μM Acrodisc R Syringe Filters, Pall Corporation) under sterile conditions. Then recombinant human basic fibroblast growth factor (bFGF, FGF2, Peprotech) was dissolved in PBS with 0.2% BSA to a final concentration of 10 μg /ml (For example: 50μg FGF2 in 5ml PBS with BSA). Stock aliquots were kept at -20C (for long-term storage).

* FGF2 solution cannot be filtered as it is very sticky.

2.1.2.2: Preparation of Matrigel stock solution and Matrigel-coated plates

Growth Factor-Reduced Matrigel (Becton Dickinson) was thawed at 4°C overnight to avoid gel formation. All the operations, including aliquoting were performed on ice and also the falcon tubes used for aliquoting were cooled down at -20°C before aliquoting. Adequate volume of cold Knockout DMEM (4°C) was added to the thawed matrigel and mixed well and 1 ml each was aliquoted into the pre-cooled 14 ml falcon tubes, followed by storage at -20°C for future use.

For coating the plates, 14 ml of cold Knockout DMEM was added directly to a frozen aliquot and thawed by repeated pipetting in and out. As soon as the matrigel was completely in solution and mixed thoroughly, 1.5 ml and 1 ml of it was added to each well of a 6-well and 12-well plate respectively. Without any delay, the plates were wrapped tightly with parafilm to avoid evaporation

and placed at 4°C at least overnight, for a maximum of 5 days. . Matrigel solution was replaced with the specific cell culture medium and warmed to 37°C just before plating MEFs or hESCs.

2.1.2.3: Conditioned medium preparation

The inactivated MEFs were plated at a density of 56, 000 cells/cm2. The following day, MEF media was replaced with hESC medium (UM, unconditioned medium) (Supplementary protocols) (0.5ml/cm2), supplemented freshly with 4ng/ml of FGF2. CM was collected from feeder flasks after every 24 hours of incubation, followed by addition of fresh hESC medium supplemented with FGF2. The CM was collected for 6 days and stored at -20°C. After 6 days all the collected media was mixed together and filtered (Corning, 0.22μM, PAS). Then 45 ml aliquots were made in 50 ml falcon tubes and stored at -80°C. Before feeding hESCs grown on matrigel coated plates with CM, it was supplemented with 4ng/ml of FGF2.

* As both Knockout DMEM and Knockout Serum Replacement do not tolerate repeated warming and cooling, small aliquots of CM were prepared and stored at -80°C for long-term storage or at -20°C for short-term storage.

* When stored at -80°C, CM swells and therefore, while aliquoting into falcons, they should never be filled till the brim.

2.1.2.4: Defined medium preparation

Though hESCs were maintained on CM, all the experiments (treatments) were carried out in the presence of defined media (N2B27) (Supplementary protocols). The hESCs were passaged and maintained in the presence of CM till they reached a confluency to ~50%. Then they were washed once with pre-warmed PBS and defined medium, followed by various treatments.

2.1.3: Handling and manipulation of hESCs

2.1.3.1: Human embryonic stem cell lines

Human embryonic stem cell (hESC) lines H1 and H9 (WiCell Research Institute, Madison, Wisconsin) were maintained under sterile conditions in a humidified incubator in a 5%CO2-95% air atmosphere at 37 C (INNOVA CO-170 Incubator, New Brunswick Scientific) from passage 36 till 65.

2.1.3.2: Handling and manipulation of hESCs

Manipulation of the colonies, such as the removal of differentiated cells and splitting were done under HERAguard R Clean Bench (Heraeus, Thermo Fischer Scientific Inc.) with Leica MZ 95 Stereo Microscope (Leica, Vashaw Scientific Inc.) placed in it.

2.1.3.3: Thawing human ES cells

Around 3-4 days before thawing the cells, 6-well plate was coated with Matrigel. Two days later 250,000 inactivated MEFs per well (6-well format) were seeded. The next day, when MEFs were attached to the matrigel coating, the MEF media was replaced with UM supplemented with 4ng/ml of FGF2 and placed in an incubator until hESCs were thawed and plated onto them.

The cryovial containing frozen hESCs was removed from the liquid nitrogen storage tank and thawed by gentle swirling in a 37°C water bath until only a small ice pellet was remaining. Afterwards 70% (v/v) ethanol was used to sterilize the cryovial and the vial was allowed to air dry before opening. Cells were added drop-wise, to reduce osmotic shock, into a 15ml falcon containing 10ml of pre-warmed UM. Cells were briefly centrifuged, re-suspended in 2ml of warm UM and added gently to the prepared plates (MEFs plated on Matrigel and UM). Usually cells from one cryovial are plated into one well of 6-well plate. Nevertheless it may take even 2 weeks before cells are ready to be expanded. Human ES cells culture methods and conditions were adapted from Xu et al. (2001). When the hESC colonies were large-enough, they were passaged into matrigel coated plates for maintenance under feeder-free conditions. With continuous passaging, the inactivated MEFs used for thawing are diluted out and lost, retaining pure hESC culture.

2.1.3.4: Daily maintenance of human ES cells in feeder-free culture

Every day human ES cells were fed with fresh CM. Only required amount of CM was placed in a 37°C water bath until warm and supplemented freshly with 4 ng/ml of FGF2, thereby human ES cells were fed by adding 3-4ml of it to each well (6-well format).

* As both Knockout DMEM and Knockout Serum Replacement do not tolerate repeated warming and cooling, small aliquots of CM were prepared and stored at -20°C for short-term storage or at -80°C for long-term storage.

* The final concentration of exogenously added FGF2 in CM will be 8 ng/ml as it is added to a concentration of 4 ng/ml both during the preparation of CM and also just before feeding the hESCs.

2.1.3.5: Passage of human ES cells on Matrigel

The human ES cells were maintained at high density and passaged usually once per week when they reached 70-80% confluency. Matrigel coated plates, stored atleast overnight, for a maximum of 5 days were used for plating hESCs. Matrigel from these plates was removed and replaced with ~ 1.5 ml to 2 ml CM (supplemented with 4ng/ml FGF2) and placed in the CO2 incubator for warming. Meanwhile, differentiated cells were removed manually, by morphological identification and a dark field microscope, using Gilson 20µl tips.

Morphological distinction between hESCs and differentiated cells

Due to high nuclear cytoplasmic ratio, the hESC colonies form ordered, flat, tight colonies with sharp borders, with low light scattering properties while the differentiating colonies are apparently disordered or irregular with uneven edges or transparent centers and exhibit high light-scattering property. And also the differentiated cells look bigger than that of undifferentiated cells due to the reduced nuclear-cytoplasmic ratio.

Then the colonies were cut manually into uniform pieces with a BD MicrolanceTM3 injection needle (Becton Dickinson, Madrid, Spain). At an interval of about 5-10 minutes and also after cutting the colonies, the cells were placed in the incubator, so that the temperature is maintained. Then the media was aspirated and cells were washed with PBS without Ca2+Mg2+ (Gibco, Invitrogen). Into each well of a 6-well plate, 1ml of dispase (2mg/ml, Gibco, Invitrogen) (Supplementary protocols) was added and cells were incubated at 37°C in the incubator for around 2-4 minutes, till it was observed that the edges of the cut colonies started dislodging from the well surface. And then the cells were gently wash thrice with 2ml of pre-warmed UM (Supplementary protocols). After that, 2 ml UM was added to the cells and the cut colonies were lifted out without disrupting the pieces or clumps using a cell spatula (TPP 99010 or Biochrom). Cells clumps were collected and transferred into a 15ml falcon tube for brief centrifugation (1000 to 1200 rpm for 1-2 minutes). Then the supernatant was aspirated out and the cell pellet was resuspended in CM supplemented with FGF2 (4 ng/ml), very gently by pipetting up and down without disrupting the clumps. After that hESCs were seeded into wells of Matrigel-coated plates, in an optimal split ratio of 1:3 or 1:4. The plates were placed in the incubator and gently agitated left to right and back to front to obtain an even

distribution of cells. The next day, undifferentiated cells were visible as small isolated colonies attached to the matrigel coating, which become large and compact with further growth.

2.1.3.6: Freezing human ES cells

Human ES cells freezing medium (Supplementary protocols) was prepared and placed at 4°C for cooling. The procedure for freezing human ES cells is identical to that of passaging these cells till the final step of re-suspending the cell clumps. Shortly, colonies were manually cut, washed with PBS, incubated with dispase, washed thrice with UM and gently lifted out with a cell spatula. Then the clumps were collected, centrifuged for sedimentation and re-suspended gently in cold human ES cells freezing media. Usually cells clumps collected from one well of a 6-well plate were suspended in 1ml of freezing media and accommodated in a 1 ml cryovial, which was placed in a freezing container (Nalgene) and stored at -80°C overnight. Next day, the cryovials were transfer to liquid nitrogen for long-term storage.

* To obtain higher efficiency during thawing, cells clumps for freezing should be slightly larger than those used for splitting.

2.1.4: Human ES cell treatments and experimental set up

2.1.4.1: Recombinant proteins and Inhibitors

The stock solutions for recombinant proteins and inhibitors were prepared and small aliquots were made to avoid repetitive freezing and thawing, followed by storage at -20°C. Both SB431542 and SU5402 were dissolved in DMSO (Sigma D2650). The details for the recombinant proteins and inhibitors used for the experiment are given in Table 2.1.

Treatments	Solvent	Stock concentration	Concentration in media for the treatments	Volume of stock in media (µl/ml)
BMP4*	0.2% BSA in PBS	10 ng/µl	10 ng/ml	1
SB431542**	DMSO	10 mM	20 µM	2
SU5402***	DMSO	10 mM	20 µM	2
FGF2****	0.2% BSA in PBS	10 µg/ml	20 ng/ml	2.5

Table 2.1: Details of preparation, stock and working concentrations of recombinant proteins and inhibitors.

* Recombinant BMP4 (R&D 314-BP-010): Bone morphogenetic protein 4

** SB-431542 (Sigma S4317): a specific inhibitor of TGF β superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7.

*** SU5402 (Calbiochem 572630): A cell-permeable, reversible, and ATP-competitive inhibitor of the tyrosine kinase activity of fibroblast growth factor receptor 1 (FGFR1) and aFGF-induced tyrosine phosphorylation of ERK1 and ERK2.

**** FGF2: Fibroblast growth factor 2

2.1.4.2: Experimental set up

The experiments were mainly carried out on the hESC line, H1, but reproducibility was confirmed in the hESC line, H9 also. All the treatments were carried out at the same time as one single experiment, except for SUO and its respective control. Separate wells were maintained for both total RNA and protein isolations. The details for experimental set-up, including combinations of reagents used for each treatment is given in Table 2.2.

hESCs which were maintained under feeder-free conditions, on matrigel were passaged at 1:4 splitting ratio and grown in the presence of CM till they were ~ 50% confluent. Then they were rinsed gently with pre-warmed PBS, followed by a rinse with defined medium (DM). As the inhibitors were dissolved in DMSO, controls, with hESCs grown in the presence or absence of BMP4 and equivalent volume of DMSO (4 µl: equivalent to the DMSO in the treatment, SU) were also maintained, to rule out the possibility of any effect brought about by its presence. After the required incubation period (time point), the cells were harvested for RNA and protein isolation separately, after rinsing with pre-warmed PBS.

Treatments	Abbreviation used	Replicates	No. of replicates	Time points (hours /days)
Control (Undifferentiated hESCs: with FGF2 (20 ng/ml))	UD	Biological	2	5 D
BMP4	B	Biological	2	3 h, 5 D
BMP4 + SB431542	SB	Biological	2	3 h, 1D, 3D, 5 D
BMP4 + SB431542 + SU5402	SU	Biological	2	3 h, 1D, 3D, 5 D
BMP4 + SB431542 + FGF2	F	Biological	2	3 h, 1D, 3D, 5 D
SU5402	SUO	Biological	2	1D, 3D, 5 D
Placenta	Placenta	Technical	2	
Control (BMP4+DMSO)		Biological	2	5D
Control (DMSO)		Biological	2	5D

Table 2.2: Experimental set-up: Details of treatments, replicates and time points.

2.2: Molecular Biology Techniques:

2.2.1: Gene expression analysis (RNA)

2.2.1.1: RNA extraction

RNA isolation from cell lysates was performed using the RNeasy® Mini Kit (Qiagen) following the manufacturer's instructions, including the optional step of DNase I treatment of the samples on column to get rid of trace amounts of genomic DNA. This technology combines the selective binding properties of a silica-based membrane with centrifugation which enables the capture and purification of up to 100 μg of RNA longer than 200 bases by binding to the silica membrane in a column. The procedure provides an enrichment for mRNA since most RNAs <200 nucleotides (such as 5.8S rRNA, 5S rRNA, and tRNAs, which together comprise 15–20% of total RNA) are selectively excluded.

First, the cells were briefly rinsed with pre-warmed PBS (37°C) and RLT lysis buffer (highly denaturing guanidine-thiocyanate–containing buffer) to which β-mercaptoethanol was added at the

rate of 10 µl per 1 ml RLT Buffer prior to use. 600 µl Then the cells were scraped out using a cell scraper and collected in a 1 ml microcentrifuge tube, followed by thorough vortexing for 1 minute to ensure proper cell lysis. Then the lysates were transferred to RNeasy-columns and further RNA isolation was carried out according to the manufacturer's instructions. RNA was eluted into RNAse-free tubes using 22 µl of RNAse-free sterile water, out of which 2 µl was used for quality check by agarose gel electrophoresis and quantification.

2.2.1.2: RNA and cRNA quantification

The quantity and quality of RNA and cRNA was determined using a spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). This technology combines fibre optics and surface tension to measure small amounts of sample. 1µl of sample was loaded onto the optical pedestal of the NanoDrop and its arm is closed. The sample is held by surface tension as a column during the measurement. If the concentration exceeded saturation limits, the samples were diluted by adding RNAse-free sterile water.

2.2.1.3: Agarose gel electrophoresis

Gels of 1-3% were prepared by mixing agarose (Life Technologies, Paisley, Scotland) in 1x TAE buffer. 1µl of ethidium bromide (10mg/ml; Invitrogen) was added directly to the gel and mixed before solidifying. To assign the length of the

amplicons the GeneRulerTM 1kb DNA ladder (Fermentas, St. Leon-Rot, Germany) was used. Samples were brought to equal volumes using distilled water and mixed with appropriate amount of 6x loading buffer (Fermentas) before loading. Gels were run in an electrophoresis chamber with 50V for 30 to 60min. Nucleotides were visualized with UV light, using the AlphaImagerTM (Alpha Innotech, San Leandro, CA, USA).

2.2.1.4: Microarray experiment: Sample preparation and Illumina bead chip hybridisation

Biotin-labelled cRNA was generated by the means of a linear amplification kit (Ambion, Austin, TX, USA) using 300ng of quality-checked total RNA as input. Chip hybridisations, washing, Cy3-streptavidin staining, and scanning were performed on an Illumina BeadStation 500 platform (Illumina, San Diego, CA, USA) using reagents and following protocols supplied by the manufacturer. cRNA samples were hybridised on human-8 BeadChips (Section 2.1.4.2: Table 2.2).

2.2.1.5: Data analysis and Interpretation

The raw data was normalised using the 'rank invariant' algorithm of the BeadStudio 1.0 (Illumina) software. Data analysis including statistical tests, comparisons and filterations such as significance tests, principal component analysis (PCA), clustering, venn diagram, and data filtering and compilations were performed using the TIGR-MEV software (Saeed et al., 2003), R statistical software (Bioconductor), VENNY interactive tool (Oliveros, 2007), MS Excel and MS Access. R version 2.4.0 was used for implementing the packages of Bioconductor and also for plotting scatter plots. First the entire probe sets, which had a detection p-value, greater than 0.01 (not detected) in all the treatments were removed. One-way ANOVA was performed on the normalized data for finding out the significant of differential regulation of gene expression between the samples. To avoid the inclusion of false positives, p values were calculated based on permutations, with a false discovery rate, setting a cut off of proportion of false significant genes not exceeding 0.05. Out of this set of significant genes, only those genes were taken into consideration for various comparisons, which were at least 2 fold up- or down-regulated. Database for annotation, visualization and integrated discovery (DAVID; http://david.abcc.ncifcrf.gov) was used for pathway analyses. Groups of genes associated with specific pathways, based on the Kyoto Encyclopedia of Genes and Genomes (KEGG), were analyzed together to assess pathway regulation during various treatments of hESCs.

2.2.1.6: Reverse transcription

For reverse transcription using M-MLV reverse transcriptase (Promega, Madison, WI, USA), 2µg of RNA was brought to a final volume of 9.5 µl with distilled water and 0.5µl of Oligo-dT primer (1µg/µl; Invitek, Berlin, Germany) was added to it. The mixture was spun briefly, heated to 70°C for 5 minutes and immediately cooled on ice to prevent secondary structure from reforming. An RT master mix of 15 µl was prepared using the following components for one reaction: 9.4µl of distilled water, 5 µl of 5x reaction buffer (Promega), 0.5 µl of 25mM dNTP and 0.1µl of M-MLV reverse transcriptase (200 units/µl: Promega). After thorough mixing and a short centrifugation, reverse transcription was carried out at 42°C for 1hr and then the reaction was stoped by incubating at 65°C for 10min.

2.2.1.7: Real-time polymerase chain reaction (Real-Time PCR)

cDNA prepared from 2 µg of total RNA was diluted at the rate of 1:8 with distilled water and used for Real-Time PCR, which was performed in 96-Well Optical Reaction Plates (Applied Biosystems,

Foster City, CA, United States). The PCR mix in each well included 5 µl of SYBR®Green PCR Master Mix (Applied Biosystems), 1 µl each of the forward and reverse primers (5pmol/µl; MWG) and 3 µl of the diluted cDNA in a final reaction volume of 10 µl. Triplicate amplifications were carried out per gene with three wells as negative controls without template. GAPDH or ACTB were amplified along with the target genes as endogenous controls for normalization. The PCR reaction was carried out on the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) using the following program,

stage 1: 50°C for 2min,

stage 2: 95°C for 10min,

stage 3: 95°C for 15s and 60°C for 1min, for 40 cycles

stage 4: 95°C for 15s, 60°C for 15s and 95°C for 15s

The last heating step in stage 4 was performed with a ramp rate of 2% in order to enable the generation of a dissociation curve of the product. The output data generated by the SDS 2 software (Applied Biosystems, USA) were transferred to MS Excel (Microsoft, Redmond, WA, USA) for analysis. The differential mRNA expression of each gene was normalized against GAPDH or ACTB mRNA expression in the respective samples and calculated using the comparative Ct (threshold cycle) method and fold over control (undifferentiated) at each of the time point was calculated:

$\delta CT = CT_{gene} - CT_{GAPDH}$

$\delta\delta CT = \delta CT_{treated} - \delta CT_{untreated}$

Fold Change = $2^{-\delta\delta CT}$

2.2.2: Protein expression analysis

2.2.2.1: Measurement of hCG in culture medium

Enzyme-linked immunosorbent assay (ELISA) was carried out using the Human Chorionic Gonadotropin (HCG) ELISA Kit (Bio-Quant, BQ 047F) according to manufacturer's instructions.

This kit makes use of the direct solid phase sandwich ELISA method.

2.2.2.2: Immunocytochemistry

Monolayer culture of cells were washed with PBST (PBS with 0.05% Tween 20) and fixed with 4% paraformaldehyde (PFA) in PBS for 10 minutes at room temperature. Then the cells were washed twice for 5 minutes with PBST with gentle shaking. After washing, cells were permeabilized with 1% Triton X-100 in PBS for 10min. at RT. Afterwards, the cells were blocked in PBST containing 1% BSA (Bovine Serum Albumin, Fraction V, 99% purity, Sigma) and 5% normal chicken serum (Vector Laboratories Inc. Burlingame) for 45 minutes at room temperature with gentle shaking. Thereafter, cells were incubated with primary antibodies for 1 hour (diluted to working concentration in PBST containing 1% BSA and 1% normal chicken serum). Then cells were washed three times for 5 minutes each in PBST with 0.1% BSA and incubated with secondary antibodies (diluted in PBST with 1% BSA) for 1 hour in the dark. Afterwards three washes (PBST with 0.1% BSA) were carried out, with the third wash containing DAPI solution (Molecular Probes, Invitrogen) in it. Fluorescence was examined and images captured under the confocal microscope (LSM510 Meta, Zeiss, Germany). The primary antibodies used were: anti-β hCG (Abcam, ab763), anti-OCT4 (SantaCruz Biotechnology, sc-8629), anti-human smooth muscle actin (Sigma A5316), anti-CDX2 (Chemicon International AB4123), anti-HLA-G (Santa Cruz Biotechnology, Inc. sc-17958), anti-KRT7 (Abcam ab52870), anti-CDH1, anti-HistoneH3 pS10 (Novus Biologicals 06570), anti-pSMAD2 (Cell Signalling Technology 3108), anti-SMAD2/3 (Cell Signalling Technology 3102), anti-pSMAD1 (Cell Signalling Technology 9511), anti-SMAD1 (Cell Signaling Technology 9512), anti-pGSK3β (Cell Signalling Technology 9336), anti-GSK3β (Cell Signalling Technology 9315), anti-pAKT thr 308 (Cell Signalling Technology 9275), anti-AKT (Cell Signalling Technology 9272), anti-GAPDH (Ambion 4300). Secondary antibodies: Alexa Fluor 594 chicken anti-goat IgG, Alexa Fluor 546 goat anti-rabbit IgG, Alexa Fluor 488 goat anti-mouse IgG, Alexa Fluor 488 donkey anti-goat IgG, Alexa Fluor 594 chicken anti-rabbit IgG, Alexa Fluor 488 chicken anti-rabbit IgG (Molecular Probes, Invitrogen).

2.2.2.2: Protein extraction

Lysis buffer (25% glycerol, 0.42M NaCl, 1.5mM MgCl2, 0.2mM EDTA, 20mM HEPES) was prepared and stored at 4°C. To make a 100x stock solution of protease inhibitor (complete protease inhibitor cocktail tablets: Roche), two tablets were first dissolved in 1 ml distilled water. This working solution was added to the lysis buffer at the rate of 1 µl per 100 µl of lysis buffer (1x), just before cell lysis. The monolayer culture of cells was rinsed with pre-warmed PBS (37°C), followed

by addition of lysis buffer at a rate of 60 - 80 µl (6-well format) per well and the cells were scraped out using a cell scraper. Subsequently, the lysates were transferred to centrifuge tubes and kept on ice for further processing. To ensure effective cell lysis, the lysates were vortexed for approximately 1-2 minutes and mechanical lysis was carried out by passing the lysates through 1 ml syringe and injection needle (BD microlance 3: Becton Dickinson) several times. Then the samples were centrifuged at 10,000 xg for 10 minutes and supernatant was collected for further protein analysis.

* To avoid repeated freeze-thaw cycles and for future use, small aliquots of excess of the stock solution of protease inhibitor were prepared and stored at 4°C (not more than 2 weeks) or -20°C (not more than 12 weeks).

2.2.2.3: Protein quantification

Bradford method was adopted to quantify the Protein samples. Standard curve was prepared by using standard protein solutions (Bovine gamma globulin in concentration: 0.125mg/ml, 0.25mg/ml, 0.5mg/ml, 0.75mg/ml, 1.5mg/ml, 2mg/ml; BioRad, Hercules, CA, USA). A stock solution of bradford reagent (Bio-Rad Protein Assay; Bio-Rad, Hercules, CA, USA) was prepared by diluting it in 1x PBS at a rate of 1:5 (To be stored at 4°Cin dark for future use).

980µl of the stock solution of Bradford reagent was added to 20µl of each standard and sample in 1 ml cuvettes (Sarstedt, Nümbrecht, Germany). Then the samples were mixed properly by inversion and incubated for 5 minutes at room temperature. Afterwards, absorbance was measured at 595 nm using the Bradford programme of a spectrophotometer (Ultrospec 3100 pro: GE Heathcare, Munich, Germany). Standard curve was prepared using MS Excel by plotting standard concentration (x axis) against absorbance (y axis), followed by derivation of the equation of the line obtained. Based on this, the concentrations of all the samples were calculated.

2.2.2.4: SDS PAGE

Protein gels (10 - resoving; 5% - stacking) of 1.5 mm thickness were prepared using Bio-Rad protein gel casting apparatus. The gels of 1.5 mm thickness can accommodate a maximum of 30 µl sample. 10% (w/v) APS solution (Ammoniumperoxodisulfate; Carl Roth) was prepared by dissolving 0.1g in 1ml of distilled water. After setting up the apparatus, 10% resolving gel was prepared (2.05 ml of distilled H2O, 1.25 ml of Tris-Cl (pH 8.8), 50µl of 10% SDS, 1.65ml of 40 % acrylamide/bisacrylamide (mixing ratio 29:1: Rotiphorese® Gel 40; Carl Roth, Karlsruhe,

Germany), 25µl of 10% APS solution and 2.5µl of TEMED (Carl Roth), vortexed, to ensure proper mixing and immediately poured into the assembled gel casting chamber. Instantly, the poured gel was covered with isopropanol to make sure that the gel is evenly distributed to form a straight edge. After the resolving gel solidified, isopropanol was discarded and the gel was washed with distilled water two times. Then 5% stacking gel was prepared (1.4 ml of dH2O, 0.6 ml of Tris-Cl (pH 6.8), 25µl of 10% SDS, 0.4 ml of 40% acrylamide/bisacrylamide, 25µl of 10 % APS and 5µl of TEMED), mixed thoroughly by vortexing and poured on top of the solidified resolving gel in the gel casting chamber. Immediately, comb of 1.5 mm thickness was placed carefully on the stacking gel, avoiding air bubbles.

From each sample, 20 µg protein solution was brought to 20 µl volume using distilled water and mixed with 10 µl of 3x protein loading buffer (Supplementary protocols). The marker was prepared by mixing 7 µl (~3µg/µl) prestained marker (Broad range protein marker (6-175 kDa): New England Biolabs, Beverly, MA, USA), 13 µl of distilled water and 10 µl of 3x protein loading buffer. The prepared samples and marker were incubated at 95°C for 5min and immediately kept on ice for 1 minute before loading, to avoid formation of secondary structures. Electrophoresis was performed in 1x loading gel (Supplementary protocols) at 110 V until the dye front reached the resolving gel and then at 130 V till it reached the end of the resolving gel. The gels were then used for Western blot analysis.

2.2.2.5: Western blotting

1x transfer buffer (Supplementary protocols) was prepared well in advance and placed at 4°C for cooling. The gels were soaked in 1x transfer buffer and the buffer was discarded. Two pieces of filter papers and a piece of membrane (Amersham HybondTM ECLTM nitrocellulose membrane (GE Helthcare)) was cut, matching the size of the gel and soaked in 1x transfer buffer. Then, the components required for transfer of the proteins to the blot were assembled in the following order, on the black edge of the Bio-Rad cassette: Bio-Rad cellulose material, Filter paper, gel, nitrocellulose membrane, filter paper, Bio-Rad cellulose material. This assembly was made in a tray, with 1 x transfer buffer in it, avoiding the inclusion of any air bubbles into the assembly. Thereafter, the cassette was locked and placed in the transfer chamber, with the gel towards the anode (negative), so that during the transfer, proteins move from the gel towards the membrane. Blotting was performed in pre-cooled 1x transfer buffer at 130 V for 90 minutes.

After blotting the protein resolution and transfer was checked by staining the membrane with Ponceau Solution (Sigma-Aldrich). The membrane was shortly washed with dH2O and incubated in a blocking solution (3% low-fat milk powder in TBST (Supplementary protocols) either at room temperature for 45 minutes or overnight at 4°C with gentle shaking. After short washing with 1x TBST the membrane was incubated with primary antibody (diluted in 1% BSA in 1x TBST) with gentle shaking for 1 hour at room temperature or overnight at 4 °C. Then the membrane was washed 3 times with 1x TBST for 5 minute each, with gentle shaking at room temperature. Afterwards, the membrane was incubated with secondary antibody (diluted in 3% low-fat milk powder in 1x TBST), with gentle shaking at room temperature. Then the membrane was washed 3 times with 1x TBST for 5 minute each, with gentle shaking at room temperature.

The detection reagents (GE Healthcare) were brought to room temperature and equal volumes of detection reagent 1 and detection reagent 2 were mixed just before use (if any delay is there, place it in dark). The membrane was placed on a thin plastic film (Saran film), with the protein transferred surface on top and the detection reagent-mixture was dispensed on the membrane and incubated for 1 minute. Then the entire detection reagent on the membrane was removed by slightly lifting the film on one side, so that it is absorbed in the tissue paper which was placed on the other side of the film, next to the membrane. After discarding the tissue paper, the membrane was directly covered with foil on all four sides by avoiding air columns inside. Then the membrane was placed in a Hypercassette (Amersham). In a dark room film (Amersham Hyperfilm ECL) was exposed to the membrane and developed using the Curix 60 fim developing machine (Agfa, Cologne, Germany). Membrane stripping was performed by using the Restore western blot stripping buffer (Thermo scientific: 21059). The membranes were exposed to the stripping buffer with gentle shaking for 10-30 minutes and washed with TBST. After this, they were blocked (3% low-fat milk powder in TBST) and the same procedure was followed for incubations with primary and secondary antibodies and the steps thereafter. The details of antibodies used are listed in section: 2.2.2.2. The secondary antibodies used were ECL Mouse IgG, HRP-Linked F(ab')2 Fragment (from sheep) (1:5,000) (GE Healthcare), ECL Rabbit IgG, HRP-Linked F(ab')2 Fragment (from donkey) (GE Healthcare) and rabbit anti-goat antibody conjugated with peroxidase (1:10,000) (Calbiochem).

2.2.3: Periodic Acid-Schiff (PAS) Staining (Glyogen storage)

Periodic Acid-Schiff (PAS) Staining System (Sigma) was used to identify glycogen storage. The purpose of this method is to identify carbohydrate macromolecules through chemical reaction. In

brief, periodic acid oxidizes the glucose residues and creates aldehydes, which react with Schiff reagent producing purple colour. Cells were fixed with 4% PFA for 15min. at RT, washed with distilled water and incubated with Periodic Acid solution for 5 minutes at RT. Then washed 3 times with water and incubated for 15 minutes at room temperature with Schiff's reagent. Afterwards the cells were washed with distilled water (5 minutes), stained with Hematoxylin (2 minutes) and again washed with distilled water. The cells were assessed for the appearance of purple colour, an indicator of glycogen storage.

2.2.4: Transmission Electron Microscopy

Undifferentiated hESCs were grown on matrigel-coated Thermanox plastic coverslips (Nunc, Rochester, NY, http://www.nuncbrand.com) till they reached ~50% confluency and SU treatment was carried out for 5 days. Cells were then rinsed with pre-warmed PBS and fixed with 2.5% glutaraldehyde in 50 mM sodium cacodylate buffer (pH 7.4), supplemented with 50 mM sodium chloride for at least 30 minutes at room temperature (RT). Specimens were washed in the same buffer and postfixed for 1.5 hours in 0.5% osmium tetroxide at room temperature, followed by 0.1% tannic acid for 30 minutes and 2% uranyl acetate for 1.5 hours. Samples were dehydrated in a graded series of ethanol, embedded in Spurr's resin (Low Viscosity Spurr Kit, Ted Pella, Redding, CA, http://www.tedpella.com) and polymerized at 60°C. Ultrathin sections (70 nm) were prepared with an ultramicrotome (Reichert Ultracut E, Leica, http://www.leica-microsystem.com) and mounted on electron microscopy copper grids, 300 mesh. Sections were counterstained with uranyl acetate and lead citrate for 20 seconds. Micrographs were made with a Philips CM100 using a 1K charge-coupled device camera (Tietz Video and Image Processing Systems, Gauting, Germany http://www.tvips.com). Measurement of mitochondrial diameters was performed using the EMMENU4 software (Fastscan, TVIPS).

Results:

3.1: Experimental quality and sub grouping of samples based on correlation coefficient (Quality control)

Undifferentiated hESCs were treated for 3 hours, 1 day, 3 days and 5 days (Table 1) and total RNA was isolated for microarray analysis (Illumina). Fig. 3.1a depicts the dendrogram constructed from the pearsons correlation coefficient between each of the treatments.

Figure 3.1 a

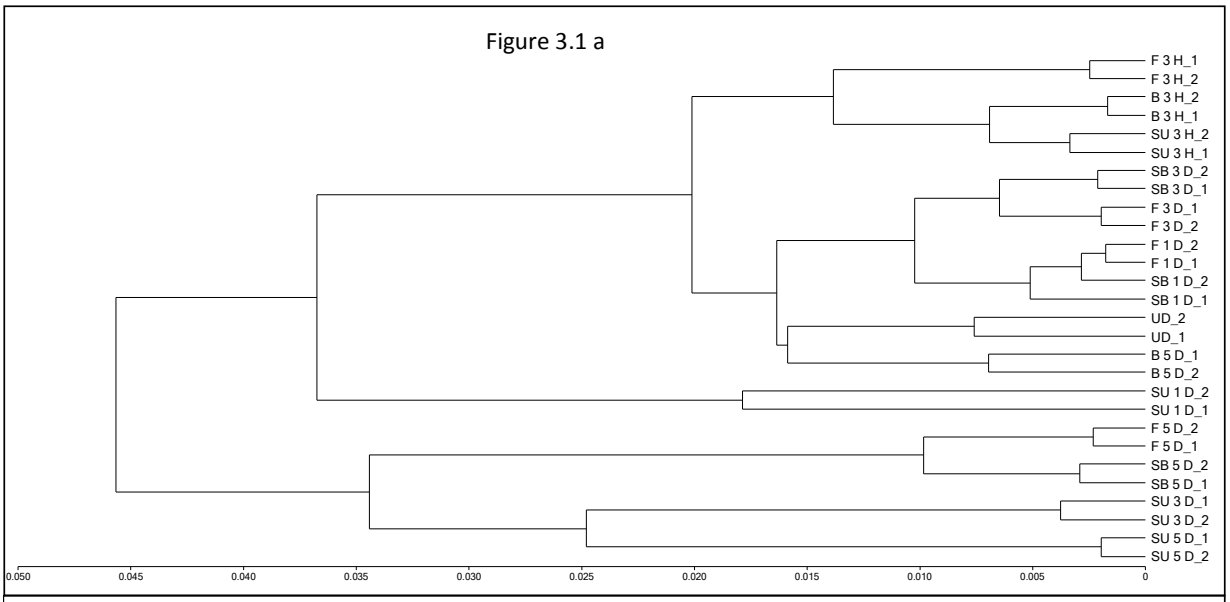


Figure 3.1 b

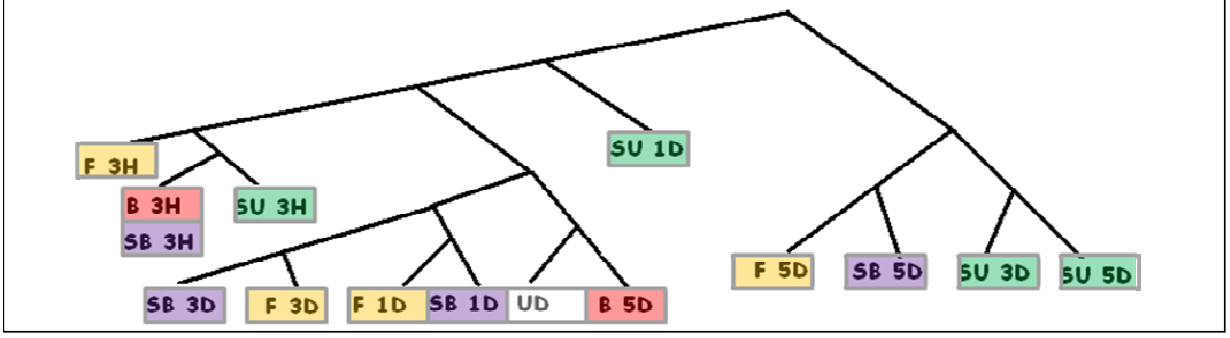


Figure 3.1: a) Dendrogram created from hierarchial clustering of all the replicates (1 and 2) of the samples (**B**: +BMP4; **SB**: +BMP4+SB431542; **F**: +BMP4+SB431542+FGF2; **SU**:

+BMP4+SB431542+SU5402), based on pearsons correlation coefficient between the normalized data of each of them; b) Simplified cartoon depicting the dendrogram. SB431542 inhibits ACTIVIN/NODAL pathway and SU5402 inhibits FGF signaling.

The clustering together of biological replicates, due to their high correlation coefficient (0.982-0.998), exhibits the reproducibility between them. 3 hours treatment of SB and B and also 3 day treatment of F and SB were not distinguishable from each other in terms of correlation coefficient of the whole data. For better understanding, based on the dendrogram, a simplified cartoon depicting a tree, with each branch representing a treatment was constructed manually (Fig. 3.1b).

All together, the samples are divided into 2 major branches, one consisting of 5 days treatments of F (+BMP4-TGF+FGF), SB (+BMP4-TGF) and SU (+BMP4-TGF-FGF) and also, 3 days treatment of SU, which clearly reflects the overall transcriptome changes brought about in the cells after these treatments. From this dendrogram, it can be understood that 5 days treatments of SB, F, SU and 3 days treatment of SU are different from all the other treatments, the SU treatments, segregated again from the other two (F 5D and SB 5D). In the same major branch, SU 1D also exists as a separate node. All the 3 hours treatments are clustered together. 5 days of BMP4 treatment (B 5D) is quite similar to that of the undifferentiated cells. 1 day and 3 days treatments of SB and F cluster together respectively.

Taken together, based on correlation coefficient, the samples can be divided into 2 major groups, one consisting of the endpoint (5 days) treatments of F, SB, SU and 3 days treatment of SU and the other, consisting of all the other samples. SU treatment segregates itself from all the other treatments of time points, earlier than 5 days, right after 1 day.

3.2: A comprehensive view of the extent of variation in the transcriptome of the cells after 5 days of treatments (end point)

The variation in the transcriptome of hESCs, after 5 days of treatment is very well visible from the dendrogram (Fig. 3.2 a), the number of regulated genes (2 fold up or down regulated) (Fig. 3.2 b) and Principal component analysis (PCA) (Fig. 3.2 c). First, a dendrogram was constructed based on the pearsons correlation coefficient between the complete data of each sample (5 days treatment). Out of this complete set of genes, to obtain the list of regulated ones, only significant genes (p value <=0.05), which were detected in at least one of the samples (UD, 5 days: (B, SB, F, SU)) was used

(11358)**.** From this, those genes were selected, which were 2 fold regulated in at least one of these samples (4449)**.** These genes were subsequently used for both Venn diagram and PCA (Fig. 3.2c).

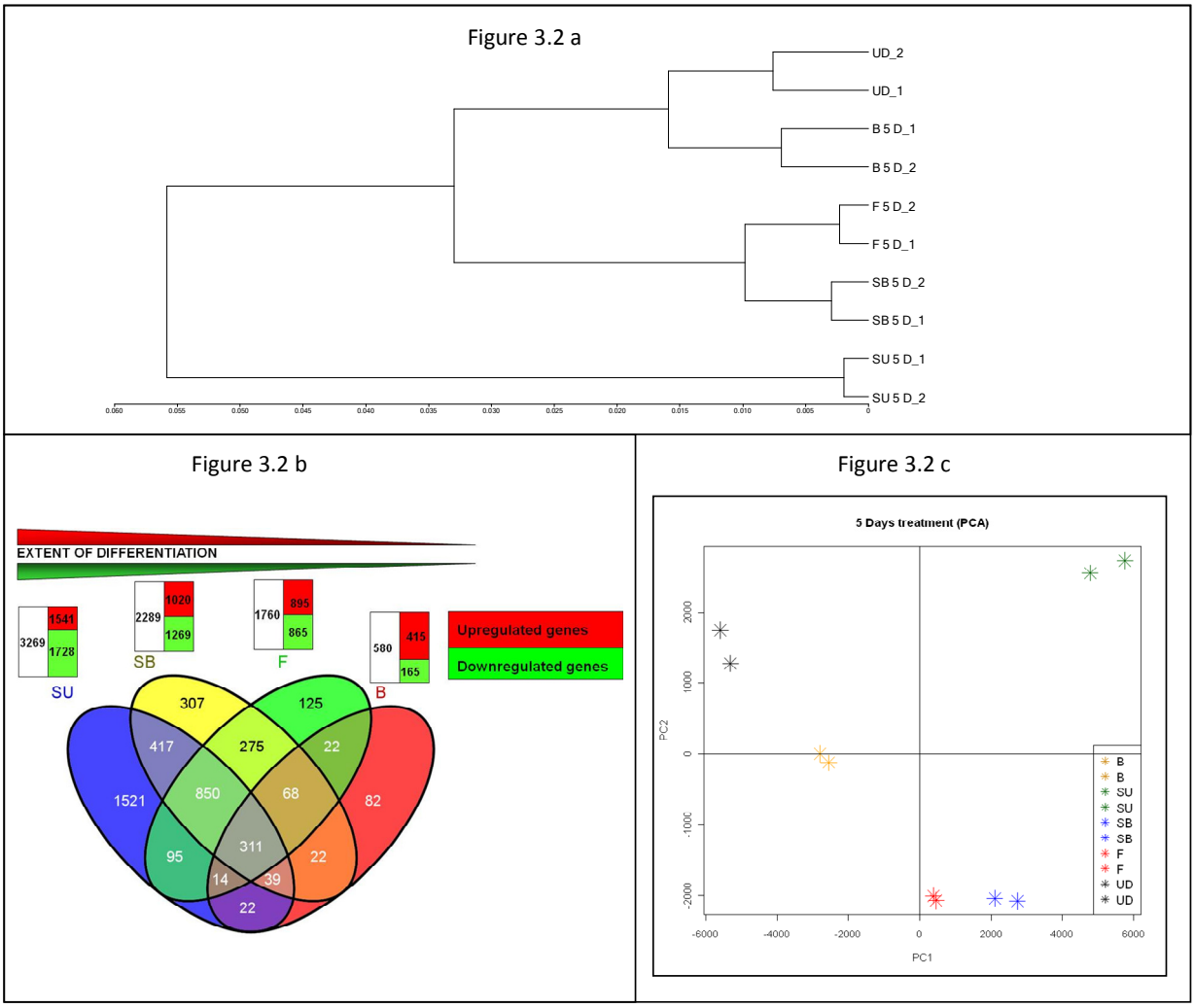


Figure 3.2: a) Dendrogram representing the correlation (pearsons correlation coeficient) between the samples after 5 days of treatment, based on the complete normalized data of each of them. b) Venn diagram depicting the overlap of the number of regulated genes in each treatment. The boxes on top of each set denotes the total number of regulated genes (at least 2 fold) (white), up-regulated genes (red) and down-regulated genes (green) in each sample after 5 days of treatment, in comparison with the undifferentiated hESCs (untreated or control). c) Principal component analysis on the set of genes, regulated (at least 2 fold) with respect to their expression in undifferentiated hESCs in at least one of the samples (B, SB, F or SU) after 5 days of treatment. PC1: Principal component 1; PC2: Principal Component 2; The color representing each sample is given in the legend of the graph.

The dendrogram (Fig. 3.2 a) shows that the treatment, SU is very different from that of the others, as this treatment separates itself from the others by forming a major distinct branch of the tree. The other major branch is further subdivided into two. The cells treated with only BMP4 (B 5 D) share a single node with that of undifferentiated cells (UD) (control or untreated), which shows that the treatment did not bring changes in the cells as much as the others. SB and F share the other node of the second major branch. The same trend is seen in the number of regulated genes (Fig. 3.2b) in each treatment, when compared to the undifferentiated cells (control or untreated). The venn diagram (Fig. 3.2 b and supplementary table:2) depicts that SU showed the maximum number of regulated genes (3269 (Up: 1541, Down: 1728)), followed by SB (2289 (1020, 1269)), F (1760(895, 865)) and B (580(415, 165)). This also shows that the extent of differentiation was the maximum in case of SU, followed by that of SB and F and the minimum was seen in the case of B.

The same set of genes, which were regulated in at least one of the treatments, when compared to undifferentiated cells was subjected to Principal component analysis (Fig. 3.2 c). PCA is a method used for reducing data dimensionality of complex data, by performing a covariance analysis between the samples. This analysis also led to similar results, but gave us more insight into the data. The first principal component (PC1), which reveals the maximum variability between the samples, shows maximum separation between SU and UD and the minimum between B and UD. SB and F fall in between. PC2, which reveals the next major variability between the samples, reveals the noticeable uniqueness of SU from SB and F, taken together. All the treatments show variability between each other in term of both PC1 and PC2, except for SB and F. They are different from each other only in terms of PC1 and do not show as much difference from each other, as shown by the others in this analysis. BMP4 treated cells (B) were the most similar to undifferentiated cells, compared to the others. But they reveal their identity by separating themselves from the undifferentiated cells (UD), through both PC1 and PC2.

Taken together, it is clear that SU treated cells are the most different from that of the undifferentiated cells and B treated cells are the least. SB and F seem to be not as much different from each other as the others in terms of the regulated genes. But, they seem to have a distinct transcription profile from that of B, SU or undifferentiated cells (UD).

3.3: Analysis of the acceleration and extent of differentiation brought about in the transcriptome during the course of each treatment (Time course analysis)

To understand the overall dynamic changes in the transcriptome of the cells during the course of each treatment, principal component analysis was conducted on the list of genes, which were regulated in at least one of the time points (3 hours, 1 day, 3 days or 5 days) for each of them.

Figure 3.3

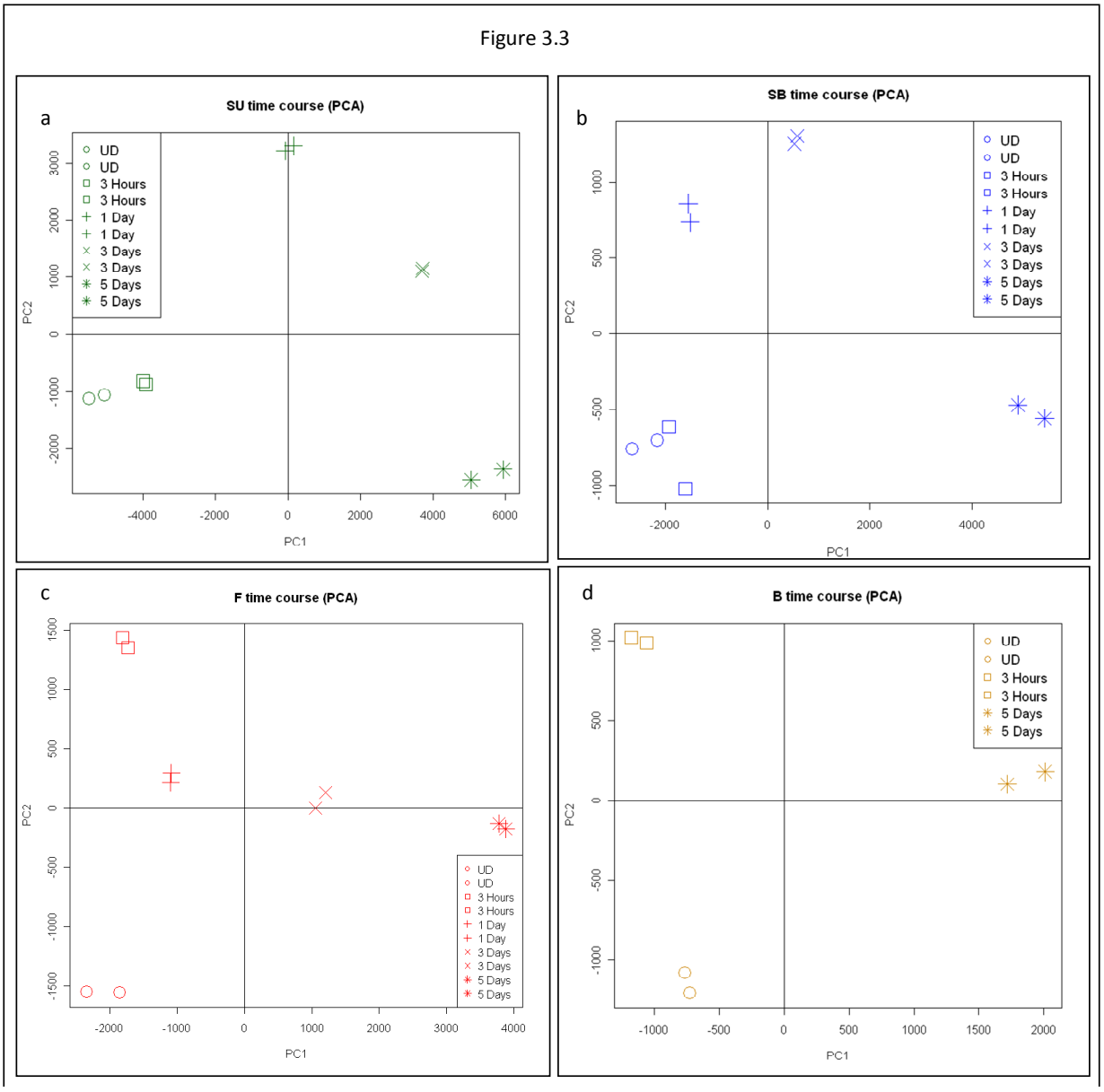


Figure 3.3: Principal component analysis on the set of genes, regulated in atleast one of the time points of each sample a) SU b) SB c) F d) B; PC1: Principal component 1; PC2: Principal Component 2; The symbol representing each time point is given in the legend of each graph.

When all the four plots (Fig. 3.3) are taken into consideration according to the degree of variability, SU (+BMP4-TGF-FGF) treatment shows (Fig. 3.3a) the maximum, followed by SB (+BMP4-TGF), F (+BMP4-TGF+FGF) and B (+BMP4). This can be easily said from the spread of the data points for each treatment along the axes for PC1 and PC2. F seems to have immediate (3 hours) major effect on the cells, followed by B and thereby SU.

In 3 hours, SB treatment did not induce noticeable transcriptome changes in the cells, but after 1 day, there are changes seen. This is visible from the way this time point has separated itself from the undifferentiated cells in terms of both the principal components. SU treatment stands out from all the treatments (SB, F, B) in 1 day and also from its own other time points (3 hours, 1 day and 3 days), as seen from the extent to which this treatment is separated from the undifferentiated cells in the plot, both in terms of PC1 and PC2. SB and F exhibit similar extent of changes at the global level after 1 day of treatment.

After 3 days of treatment, SU treated cells again change their profile from that of 1 day and start looking more closer to the 5 days treated cells, compared to the other time points. In the case of SB treatment, the cells are the most variable at this time point from that of the undifferentiated cells, in terms of both the components and also when compared to the others.

In 5 days, again there is very drastic change seen in these cells, when compared to all the other time points, as seen from the maximum separation of this treatment from the undifferentiated cells in the axis representing PC1. But these cells are not as different from the undifferentiated cells as the SU treated cells after 5 days. After 5 days of treatment of F, the cells also achieve a transcriptome, which is quite different from its 3 hours treatment and the extent of this change looks quite similar to that of SB 5D. But the unique feature of the plot for F is that from day 1 till 5 days, the extent of variation changes in these cells only in terms of PC1 and remains very similar when PC2 is taken into consideration. The extent of differentiation in B treated cells is much less, when compared to all the other treatments. Just BMP4 treated cells differentiate the least, in comparison to all the other treatments. An additional TGF βRI inhibition (SB) accelerates the differentiation process. But the synergistic activation of BMP4, along with the inhibition of TGF βRI and FGFRI (SU) accelerates

the differentiation process to the maximum and brings about a very drastic and intensive differentiation in a course of 5 days.

3.4: Influence of ACTIVIN/NODAL or FGF signaling on BMP4-induced differentiation driven lineage specification of human embryonic stem cells

BMP signalling is known to promote differentiation of hESC into trophoblast (Xu et al., 2002), extra-embryonic endoderm (Pera et al., 2004), mesoderm (Zhang et al., 2008) and hematopoietic cells (Chadwick et al., 2003).

TGFβRI inhibition of hESCs is known to drive the hES cells towards extraembryonic components neurectoderm , mesendodem, mesoderm (Greber et al., 2008; LaVaute et al., 2009; Vallier et al., 2009; Wu et al., 2008), depending on the activity of other pathways. In addition to its ability in supporting hESC self-renewal (Thomson et al., 1998), FGF signaling has been implicated in endodermal, mesodermal and neurectodermal differentiation (Ameri et al., 2010; Bendall et al., 2007; Carpenter et al., 2001; Chambers et al., 2003; Cohen et al., ; Dvorak et al., 2005; Greber et al., 2010 ; Park et al., 2004; Reubinoff et al., 2001; Schuldiner et al., 2000; Schulz et al., 2003); (Johannesson et al., 2009). Moreover, a lot of studies have shed light of the crucial role played by FGF signaling in early embryonic development and the cell fate decisions taken by the cells based on its presence or absence (Yamanaka et al., 2010; Zernicka-Goetz et al., 2009). Hence, to understand the direction towards which each treatment is directing the hESCs, we looked for the expression of lineage specific markers after the treatments (Fig. 3.4 and Supplementary Table 2).

In all the treatments (Fig 3.4 and Supplementary table: 3), except B, among the ES cell markers investigated, both *NANOG* and *MYC (c-MYC*) were the first ES cell markers, which were down-regulated, followed by the others. Down-regulation of *NANOG* is known to incline the undifferentiated embryonic stem cells towards differentiation, but this does not mark commitment and this is reversible (Chambers et al., 2007; Silva et al., 2009). Because of the earlier down-regulation of *MYC*, along with *NANOG* in all our treatments, except B, there is a possibility that *MYC* could be another major player in this predisposed state, which requires further investigation. So at this predisposed state, in which both *NANOG* and *MYC* are down-regulated and other ES cell markers like *OCT4* and *SOX2* are not yet down-regulated, the cells could be on the verge of differentiation, but not yet differentiated and could be revived. This state is reached in SU treated cells just after 3 hours of treatment, as seen by the down-regulation of *NANOG* and *MYC*, followed

by the down-regulation of the other ES cell markers. But down-regulation of *NANOG* and *MYC* is seen in SB and F treated cells only after 1 day, followed by down-regulation of the other ES cell markers, which is also slower in these cells, compared to the SU treated cells. In the B treated cells, which were showing less down-regulation of the ES cell markers including *NANOG* in comparison to the other treatments, only *MYC* was down-regulated more than 2 fold after 5 days. These results reemphasise the fact that SU treatment, in which both the self-renewal pathways are inhibited, differentiation is accelerated, which takes the cells farther away from the undifferentiated state, compared to all the other treatments and the B treated cells are the slowest in the rate of differentiation.

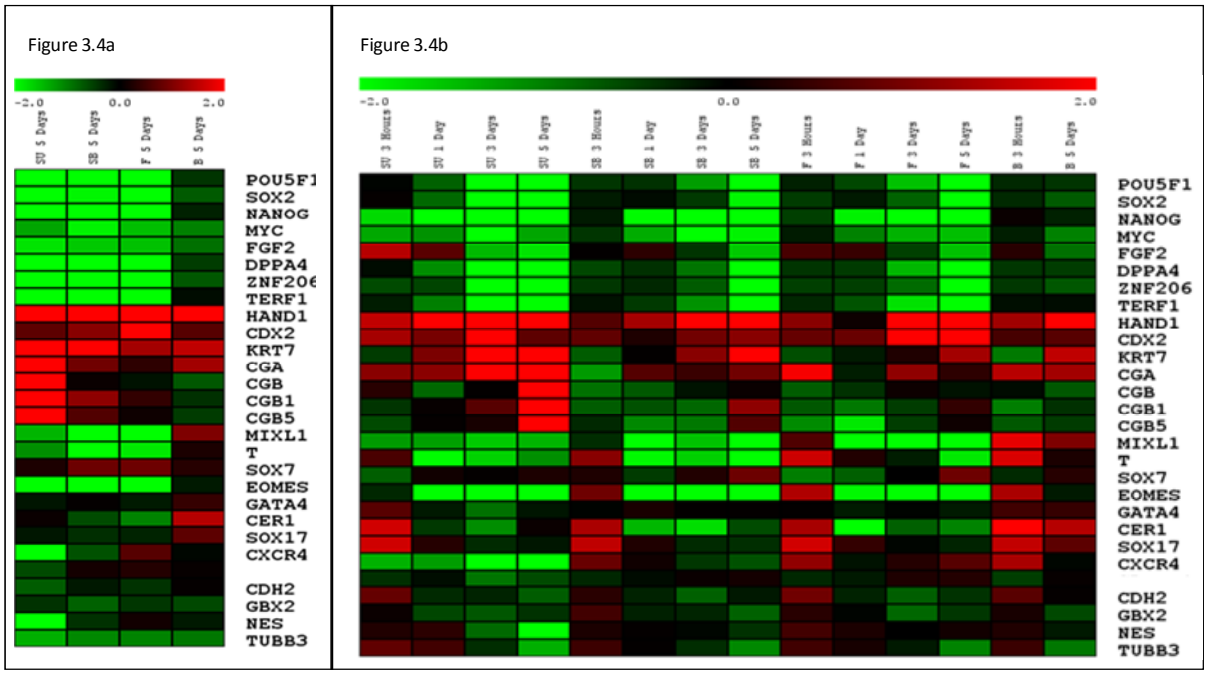


Figure 3.4: Heat map depicting the regulation of genes, with respect to the undifferentiated hESCs (control or untreated) a) after 5 days of treatments b) during the course of each treatment (3 hours, 1 day, 3 days, 5 days) (Treatments: SU (+B+SB+SU); SB (+B+SB); F (+B+SB+F); B (+B)) (B: BMP4)) Abbreviations: B: BMP4; F: FGF2; SB (SB431542): Inhibitor against TGF β superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7; SU (SU5402): Inhibitor against (FGFR1) and aFGF-induced tyrosine phosphorylation of ERK1 and ERK2)

Activation of BMP signaling and blocking of both ACTIVIN/NODAL and FGF signaling (SU) led to systematic down-regulation of the ES cell markers, *OCT4, SOX2, NANOG, MYC, DPPA4, ZNF206 and TERF1*. Both *NANOG* and *MYC* was significantly down-regulated (more than 2 fold)

right after 3 hours. *FGF2* was up-regulated by 2 fold after 3 hours and then steadily down-regulated. FGFR1 inhibition was part of SU treatment, and it was surprising that *FGF2* was up-regulated after 3 hours and started showing down-regulation only after 1 day. Except *NANOG, MYC* and *DPPA4*, which were also significantly down-regulated from 3 hours and 1 day respectively, all the 8 ES cell markers we investigated were down-regulated after 3 days. The trophectoderm or placental markers, *HAND1*, *KRT7*, *CGA*, and *CGB* were highly up-regulated. The trophectoderm marker, *CDX2* showed transient up-regulation, peaking at 3 days, by 4 fold. *CGA* showed more than 200 fold up-regulation (Log2 ratio = 7.8) and *CGB1* and 5 showed more than 30 fold up-regulation. Primitive streak formation is the first step of gastrulation towards the formation of mesoderm and endoderm. The primitive streak markers, *T* and *MIXL1* and the mesendoderm marker, *EOMES* were down-regulated throughout the treatment. The extra-embryonic endoderm marker, *SOX7* didn't show any significant regulation, when compared to the undifferentiated cells. The endoderm markers, *GATA4*, *CER1, SOX17* and *CXCR4* were all either not regulated or down-regulated all throughout, except for *CER1* and *SOX17*, which were transiently up-regulated by 3 fold after 3 hours. The neurectoderm markers, *CDH2, GBX2, NES* and *TUBB3* were all down-regulated or not significantly regulated all throughout the treatment. Taken together, the SU treatment is leading the cells through the differentiation pathway, which leads to trophectoderm or placental lineage and not to extra-embryonic endoderm or the embryonic lineages, neurectoderm or mesendoderm.

The activation of BMP signaling and blocking of both ACTIVIN/NODAL (SB) also led to systematic down-regulation of the ES cell markers. In this case also, we see that, among the ES cell markers we investigated, *NANOG* and *MYC* were the first ones to be down-regulated. But unlike SU treatment, in which they were down-regulated right after 3 hours, in this treatment, they were significantly down-regulated only after 1 day. Even the other ES cell markers exhibited a slower rate of down-regulation, when compared to the SU treated cells. In this treatment, all the 8 ES cell markers were seen to be significantly down-regulated only after 5 days, except for *MYC, NANOG* and *OCT4, TERF1* which were also down-regulated from 1 day and 3 days respectively. *FGF2* did not show any transient up-regulation in this case, like that seen in SU treated cells. Unlike SU treated cells, in SB treated cells, out of the trophoblast markers investigated, only *HAND1, CDX2, KRT7, CGB1* were significantly up-regulated after 5 days. Only *HAND1* and *CDX2* were up-regulated at earlier time points, 1 day and 3 days respectively. *CDX2* remained up-regulated after 5 days of treatment, unlike the transient expression seen in SU treatment. The primitive streak marker, T was up-regulated by 2 fold after 3 hours and down-regulated thereafter. The other primitive streak

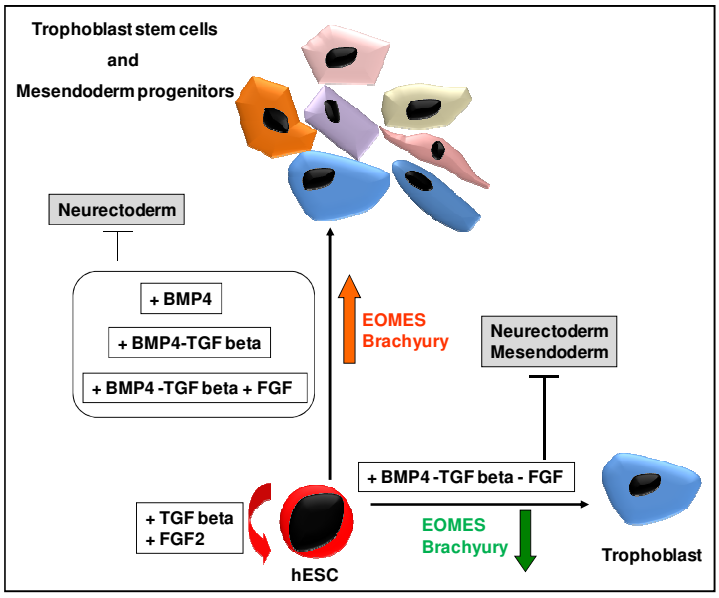
marker, *MIXL1* didn't show any up-regulation in the time points we investigated. The mesendoderm marker, *EOMES* was up-regulated after 3 hours of treatment and then down-regulated. The extra-embryonic endoderm marker, *SOX7* was up-regulated after 5 days. All the endoderm markers were either not significantly regulated or down-regulated. As seen in SU treated cells, this treatment also induced up-regulation of only *CER1* and *SOX17* (more that 2 fold) after 3 hours. None of the neurectoderm markers were seen to be up-regulated. To summarize the cell fate of SB treated cells, this treatment can support the differentiation of hESCs to both extra-embryonic components (trophectoderm and extra-embryonic endoderm) and embryonic components, mesoderm, endoderm and not to neurectoderm.

When both BMP and FGF pathways are kept active along with blocking of ACTIVIN/NODAL pathway (F), the down-regulation of ES cell markers was similar to that seen in the SB treated cells. *NANOG* and *MYC* were significantly down-regulated only after 1 day. And in this treatment also, all the 8 selected ES cell markers were seen to be significantly down-regulated only after 5 days, except for *MYC* (1 day), *NANOG* (1 day), *OCT4* (3 days) *DPPA4* (3 days) and *TERF1*(3 days), which were also down-regulated earlier. There was no transient up-regulation of FGF2 seen and like that seen in SB treated cells, it was significantly down-regulated only after 5 days, in contrast to an earlier down-regulation in SU. The trophoblast markers, *HAND1, KRT7* and *CDX2* were up-regulated after 5 days. *CGA* was seen to be down-regulated after 1 day and 5 days and up-regulated after 3 hours and 3 days. *HAND1* also showed similar behaviour, except that after 3 days of up-regulation, it remained up-regulated after 5 days too. The primitive streak marker *T* and the mesendoderm marker *EOMES* showed up-regulation after 3 hours. The extra-embryonic endoderm marker, *SOX7* was up-regulated after 5 days. Another primitive streak marker, *MIXL1* was down-regulated from 1 day onwards and didn't show any up-regulation. As seen in SB treated cells, the endoderm markers, *CER1, SOX17* and *CXCR4* were significantly up-regulated after 3 hours and consequently down-regulated from one day. To sum up, F treatment can support the differentiation of hESCs to both extra-embryonic and embryonic (mesoderm or endoderm) lineages, but not neurectoderm lineage.

The hESCs in which BMP signaling was activated (B), the down-regulation of ES cell markers was lesser, when compared to the cells treated with other treatments. *MYC* showed the maximum down-regulation of 2 fold. The trophectoderm markers, *HAND1, KRT7* and *CGA* were seen to be up-regulated by more than 2 fold after 5 days. *HAND1* and *CGA* were up-regulated right after 3 hours. The primitive streak markers, *T* and *MIXL1* were up-regulated by more than 3 fold after 3 hours and

MIXL1 remained up-regulated by 2 fold after 5 days. The mesendoderm marker, *EOMES* was also up-regulated by more than 2 fold after 3 hours. There was no significant regulation of the extra-embryonic endoderm marker, *SOX7* or the neuronal markers. The endoderm markers, *CER1, SOX17* and *CXCR4* were seen to be up-regulated after 3 hours and *CER1* remained up-regulated even after 5 days. From this, we can conclude that activation of BMP signaling supports differentiation of hESC to trophectoderm or mesendoderm lineages and not to neurectoderm lineage.

BMP signaling supports differentiation into endoderm (Wills et al., 2008), trophoblast (Xu et al., 2002) or to the extra-embryonic lineages, trophectoderm and primitive endoderm (Vallier et al., 2009), but inhibits early neuronal development (Gaulden and Reiter, 2008; Greber et al., 2007, 2008; Munoz-Sanjuan and Brivanlou, 2002; Vallier et al., 2009). The BMP signalling mediated differentiation to extra-embryonic lineages strongly increases when the TGFβ pathway (SMAD2/3) is blocked (Vallier et al., 2009). Inhibition of ACTIVIN/NODAL and FGF signaling activation promote differentiation of hESCs toward neuroectoderm (Smith et al., 2008; Vallier et al., 2009). From these studies, it can be understood that both BMP signaling and ACTIVIN/NODAL signaling do not support neuronal differentiation of hESCs, but support endodermal differentiation. Both these pathways might support differentiation into different sub-lineages of the endodermal lineage or to extra-embryonic lineages, depending on their activity, which is also dependent on other signals in the cell. This is not very clearly understood.



Our results reemphasise the known fact that BMP signalling (SB, F and SU) does not support neurectoderm differentiation. When the hESCs are exposed to these treatments, we see transient expression of both primitive streak and mesendoderm markers. We also see up-regulation of some endodermal and trophectoderm markers. From this, we understand that BMP signalling, in the presence (B) or absence of ACTIVIN/NODAL signaling (SB) or in the presence of FGF signaling (F) can support mesoderm, endodermal or extra-embryonic differentiation and does not support neurectodermal differentiation. In the SU treatment, in which BMP signaling is active and both ACTIVIN/NODAL signaling and FGF signaling are blocked, there was no up-regulation of primitive streak, mesendoderm or endodermal markers. Hence, BMP signaling, irrespective of the presence or absence of ACTIVIN/NODAL signaling, in the presence of FGF signaling, can support both endodermal and trophoblast differentiation, but in the absence of both ACTIVIN/NODAL signaling and FGF signaling, directs the hESC only to trophoblast lineage.

3.5: The synergistic activation of BMP signaling and blocking of both ACTIVIN/NODAL signaling and FGF supports differentiation of hESCs to trophoblast lineage and not to embryonic lineages

Trophoblast cells are the precursors for the formation of a functional placenta. To confirm the observation in the microarray data, that the SU treated cells were not exhibiting differentiation into embryonic lineages and only to trophoblast, we performed real time PCR validations of the lineage specific markers and the ES cells markers in the 5 days treated cells (Fig. 3.5 a; b).

The regulation of ES cells markers, *OCT4* and *NANOG* and the primitive streak marker, Brachyury (*T*) (Fig. 3.5 a) were similar to that seen in the microarray data. *NANOG* was abruptly down-regulated, followed by *OCT4*, which was more than 4 fold down-regulated only after 3 days of treatment. *T* was not significantly regulated after 3 hours, but was progressively down-regulated right from 1 day of treatment. The mesendoderm markers, *GATA6* and *SOX17* and neurectoderm markers, *PAX6* and *NCAM1* were not up-regulated at the mRNA level in these cells after the treatment (Fig. 3.5 b). The trophectoderm markers, *CGB* and *KRT7* were up-regulated in these cells after SU treatment for 5 days (Fig. 3.5 b), which proves their well-directed lineage specification towards trophoblast and not to the embryonic lineages.

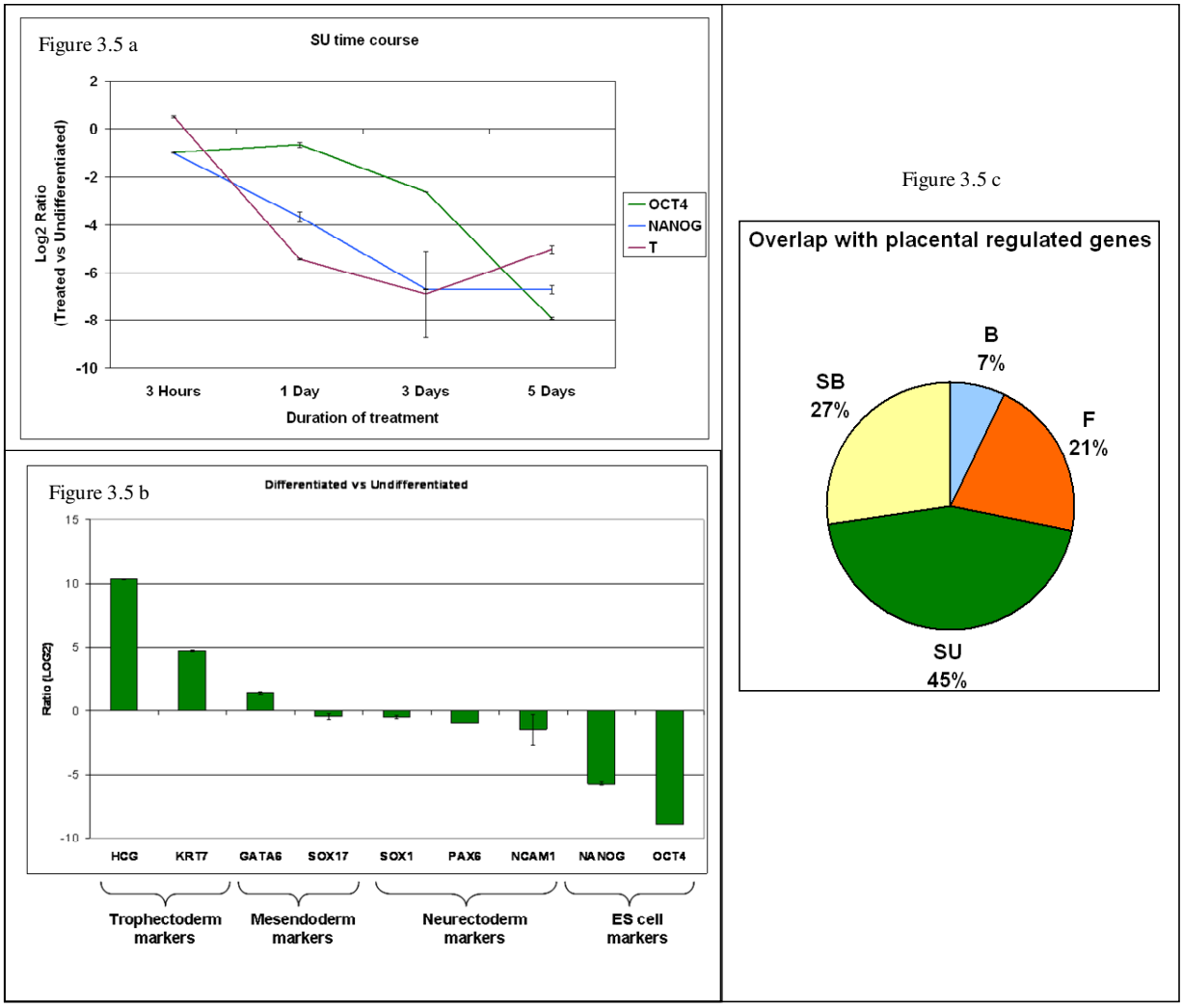


Figure 3.5: Real time PCR validation of a) *OCT4, NANOG* and *T*. x axis represents duration of treatment b) lineage specific markers. x axis represents genes and the lineages they represent have been marked below the gene names. ; y axis represents Log2 Ratio between treated and untreated samples. Normalization was carried out against *GAPDH* gene expression. C) Pie chart representing the percentage of overlap of genes regulated in each sample after 5 days, with the genes regulated in placenta, with respect to undifferentiated cells.

To reconfirm this, we looked for the overlap of the regulated genes in placental total RNA, with that of the 5 days-treatments (B, SB, SU, F), with respect to the undifferentiated cells (Fig 3.5 c and Supplementary table 4), which showed the same trend of regulation, as seen in the placenta (4911). 45% (2173) of these were also seen to be differentially regulated in SU treated cells, followed by 27% in SB (1345), and followed by 21% in F (1038). The least overlap of only 7% (355) was found

with that of B treated cells. The amount of overlap indicates that the BMP activation, along with the additional blocking of ACTIVIN/NODAL pathway (SB or F) seems to have accelerated the process of differentiation (as seen in section 3.3) towards trophoblast lineage, when compared to just BMP4 treatment (B). Previously it has been shown that the BMP signalling mediated differentiation to extra-embryonic lineages strongly increases, when the TGFβ pathway (SMAD2/3) is blocked (Vallier et al., 2009). But when FGF signalling is also inhibited (SU), differentiation process towards trophoblast is even more accentuated, as seen from the maximum overlap with the placental genes. Therefore, the synergistic activation of BMP signaling and blocking of both ACTIVIN/NODAL signaling and FGF supports differentiation of hESCs to exclusively to trophoblast lineage.

3.6: The influenceof the treatments on the expression dynamics of *OCT4* and *CDX2*

Our results till now led us to understand that synergistic activation of BMP pathway and blocking of ACTIVIN/NODAL pathway (SB) accelerate the process of differentiation (Section: 3.3 and 3.5) and in addition, when FGF signaling is also blocked, the kinetics of this is the maximum and solely directed to trophoblast lineage. The ES cell marker, *OCT4* is abundantly expressed in the ICM and the trophectoderm marker, *CDX2* in the trophectoderm of human blastocyst (Adjaye et al., 2005) and OCT4 knock-down in hESCs supports trophoblast differentiation (Babaie et al., 2007; Hay et al., 2004; Matin et al., 2004). Oct4 and Cdx2 are known to antagonize each other for the lineage diversion to ICM or trophectoderm (Niwa et al., 2000; Niwa et al., 2005; Strumpf et al., 2005). Cdx2 is required to repress Oct4 and Nanog during early development, for trophoblast specification (Strumpf et al., 2005) and recently, it has been shown that Cdx2 and Nanog also antagonize each other (Chen et al., 2009). Therefore, we studied the dynamics of OCT4 and CDX2 during the course of all the treatments.

The real-time PCR data (Fig. 3.6 a 1, 2) revealed that *OCT4* is down-regulated in all the treatments right after 3 hours of treatment. After 1 day of treatment, F and SB led to 2 and 3 fold down-regulation, respectively. After 5 days, the difference between them was increased in terms of the down-regulation, which was 9 and 13 fold respectively. But the extent of down-regulation was much higher (256 fold) in SU treated cells after 5 days than all the other treatments. There was no significant difference in the *OCT4* levels after 5 days in the BMP4 treated cells.

CDX2 (Fig. 3.6 b 1, 2) was significantly up-regulated after all the treatments, and remained up-regulated till 5 days, except in SB and SU treated cells. In line with the microarray data, in these

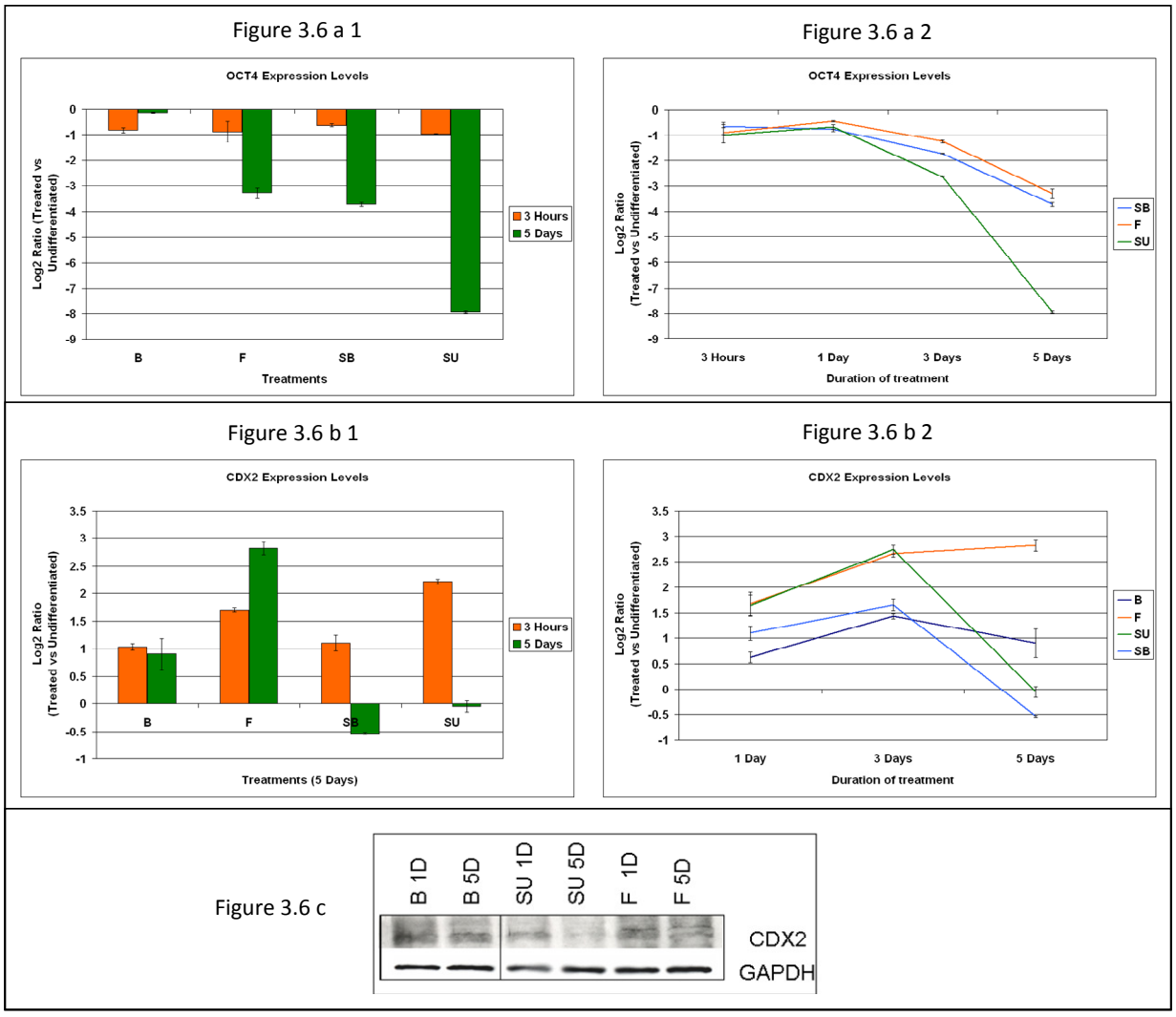


Figure 3.6: a) Real time PCR validation of *OCT4* expression in 1) all the samples (B, F, SB and SU) after 5 days of treatment. x axis represents the samples 2) F, SB, SU during the course of treatment. x axis represents the duration of treatment. b) Real time PCR validation of *CDX2* expression in 1) all the samples (B, F, SB and SU) after 5 days of treatment. x axis represents the samples 2) all the samples (B, F, SB and SU) during the course of treatment. x axis represents the duration of treatment. ; y axis represents Log2 Ratio between treated and untreated samples. Normalization was carried out against *GAPDH* gene expression. C) Western blot, representing *CDX2* protein expression in the samples, B, SU, F after 1 day (1D) and 5 days (5D) of treatment.

cells, *CDX2* expression was transient. In SU treated cells, after 3 hours, 1 day and 3 days, the real time PCR data revealed 5, 3 and 6 fold up-regulations respectively and subsequently, after 5 days, was down-regulated. CDX2 down-regulation was also visible at the protein level (Fig. 3.6 c), through western blot analysis. In SB treated cells also showed transient up-regulation, peaking at 3 days, but the extent of up-regulation was only half of that seen in SU treated cells (3 fold).

Overall, all the treatments led to the down-regulation of the ES cell marker, *OCT4*, but this was more rapid and to the maximum extent in SU treated cells. The trophoblast marker, *CDX2* was up-regulated right after 3 hours of all the treatments and remained expressed throughout 5 days, except in SB and SU treated cells, which showed only transient expression of *CDX2*. SU treated cells had the maximum overlap with placental regulated genes, followed by SB treated cells.

The placental mRNA represents highly specialized cells and the genes seen to be regulated in placenta will be those, which are functional in these highly differentiated cells, which are no more pure trophoblast stem cells.

In mouse, *Cdx2* expression is necessary for the segregation of inner cell mass and trophectoderm (Beck et al., 1995; Chawengsaksophak et al., 2004; Strumpf et al., 2005). In *Cdx2* mutant blastocysts, down-regulation of *Oct4* and *Nanog* is not seen in the outer cells and trophoblast differentiation is affected causing a failure in implantation (Strumpf et al., 2005). But down-regulation of *Cdx2* has been reported, when trophoblast stem cells are differentiated, either upon FGF4 withdrawal (Tanaka et al., 1998) or upon TGFβ treatment (Selesniemi et al., 2005). So, up-regulation of CDX2 is indeed required for driving or initiating the process of trophoblast differentiation from stem cells and also for trophoblast stem cells, but for further differentiation into more specialised cells, its down-regulation might be necessary. Taken together, both SB and SU treated cells, which had more overlap with that of the placental genes, showed a transient up-regulation of *CDX2*, peaking at 3 days and thereby down-regulating by 5 days. This could mean that both SB and SU treated cells are much farther ahead in the differentiation cascade than F and B treated cells, which still expressed *CDX2*.

3.7: Characterisation of the SU-treated cells:

3.7.1: The continuous activation of BMP signaling and simultaneous blocking of both ACTIVIN/NODAL and FGF signaling (SU) does not support differentiation into extravillous invasive cytotrophoblasts

In both mouse (Tanaka et al., 1998) and human (Baczyk et al., 2004; Ferriani et al., 1994), the FGF signaling is required for the proliferation of trophoblast stem (TS) cells and FGF-withdrawal promotes differentiation of TS cells, not inducing apoptosis (Simmons and Cross, 2005; Tanaka et al., 1998; Yang et al., 2006). The trophoblast stem cells, which are also called cytotrophoblast cells, can differentiate into either villous syncytiotrophoblast cells or invasive extravillous cytotrophoblasts (Enders et al., 2001). Invasive extravillous cytotrophoblasts are primarily responsible for the invasion of the decidua and myometrium.

Human leukocyte antigen (HLA)-G, which is a major histocompatibility gene, expressed in invasive extravillous trophoblast cells all throughout pregnancy (Chumbley et al., 1993; Goldman-Wohl et al., 2000; McMaster et al., 1995; Yelavarthi et al., 1991) and is not found in the non-invasive syncytiotrophoblast of the chorionic villi (McMaster et al., 1995). HLA-G is a marker for extravillous trophoblast (McMaster et al., 1998; Redman et al., 1984). Therefore, we checked for its expression in all the treated cells after 1 day and 5 days (Fig. 3.3 e). HLA-G was expressed at the protein level in all the samples after 1 day of treatment, but was significantly down-regulated in SU-treated cells after 5 days (Fig. 3.7.1 a, b). However, at the mRNA level, we could see upregulation of *HLA-G*. There could be a possible role of small RNAs, like microRNAs, for the absence of HLA-G protein, though there is up-regulation at the mRNA level. This needs to be investigated.

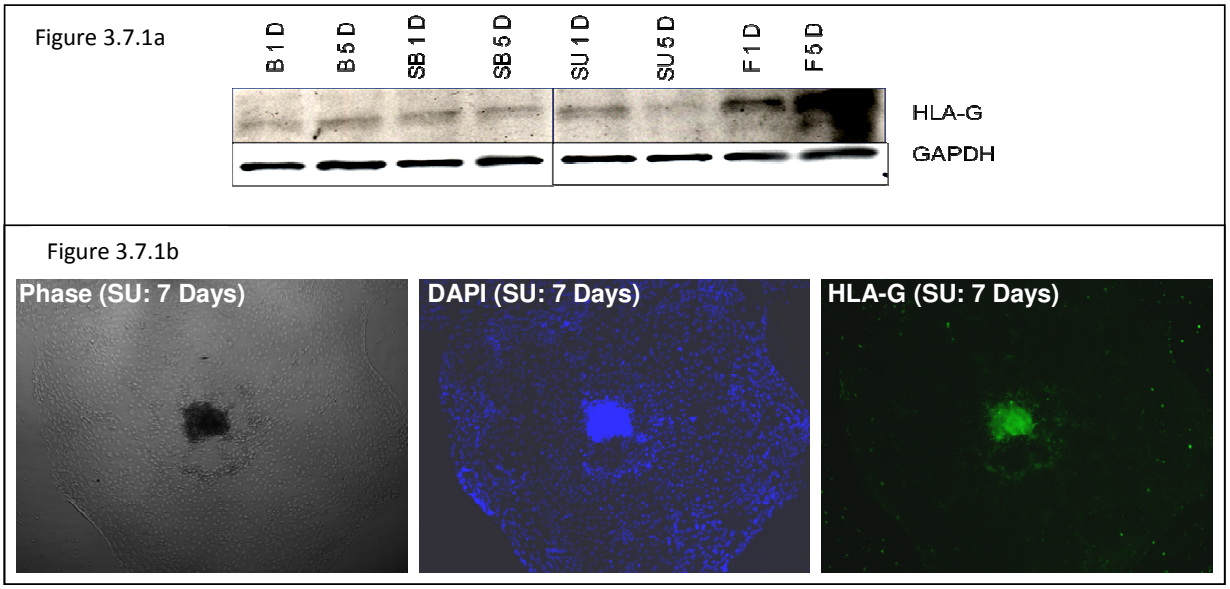


Figure 3.7.1: a) Western blot, representing HLA-G protein expression in the samples, B, SB, SU and F after 1 day (1D) and 5 days (5D) of treatment. **b)** Immunostaining for HLA-G protein expression (green) in SU treated cells for 5 days. DAPI: Blue.

It has been suggested that mouse glycogen trophoblast cells could be analogous to the human extravillous cytotrophoblasts (Georgiades et al., 2002). Like the mouse glycogen cytotrophoblast cells, the distal extravillous cytotrophoblasts in humans also contain high amounts of glycogen. Using the glycogen storage assay (Periodic Acid Schiff (PAS) staining), we checked if the SU treated cells have this property (Fig. 3.7.1 c). Very light staining was observed at few regions of some colonies, but the staining for glycogen was poor in these cells.

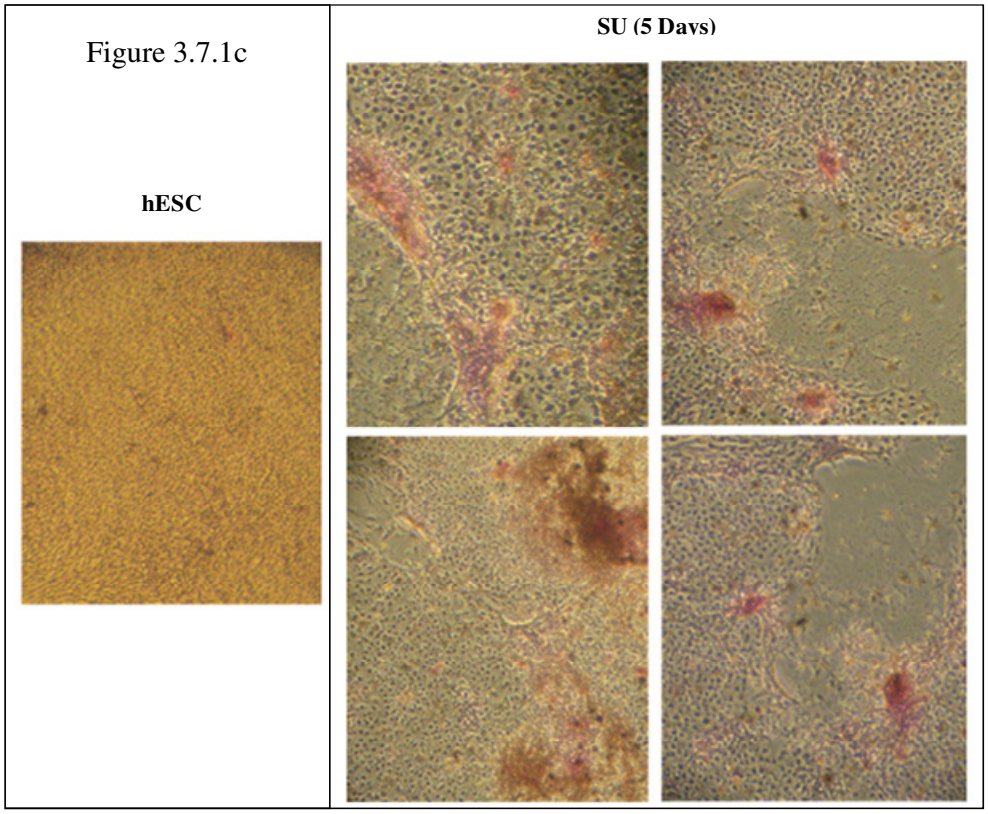


Figure 3.7.1: c) Assessment of glycogen storage using Periodic Acid Schiff (PAS) staining in human embryonic stem cells (hESC) and cells, treated with SU for 5 days.

Cytokeratin7 (KRT7 or CK7) is a specific marker for villous cytotrophoblasts (Baal et al., 2009; Cervar et al., 1999) and for human invasive trophoblasts (Handschuh et al., 2009; Tarrade et al., 2001). There are evidences that β-hCG secreting trophoblast cells are largely cytokeratin-negative (Maldonado-Estrada et al., 2004; Manoussaka et al., 2005) and the syncytiotrophoblast lining expresses little cytokeratin (Badwaik et al., 1998).

As the SU treated cells were β-hCG positive and were largely vimentin-negative, we checked for the expression of cytokeratin7 in these cells. The majority of the cells were cytokeratin7-negative, with a few exceptional tiny regions. The lack of glycogen storage and the absence of HLA-G and CK-7 protein expression suggested that 5 days of SU treatment does not induce hESCs to differentiate into invasive extravillous invasive trophoblast cells.

Figure 3.7.1 d

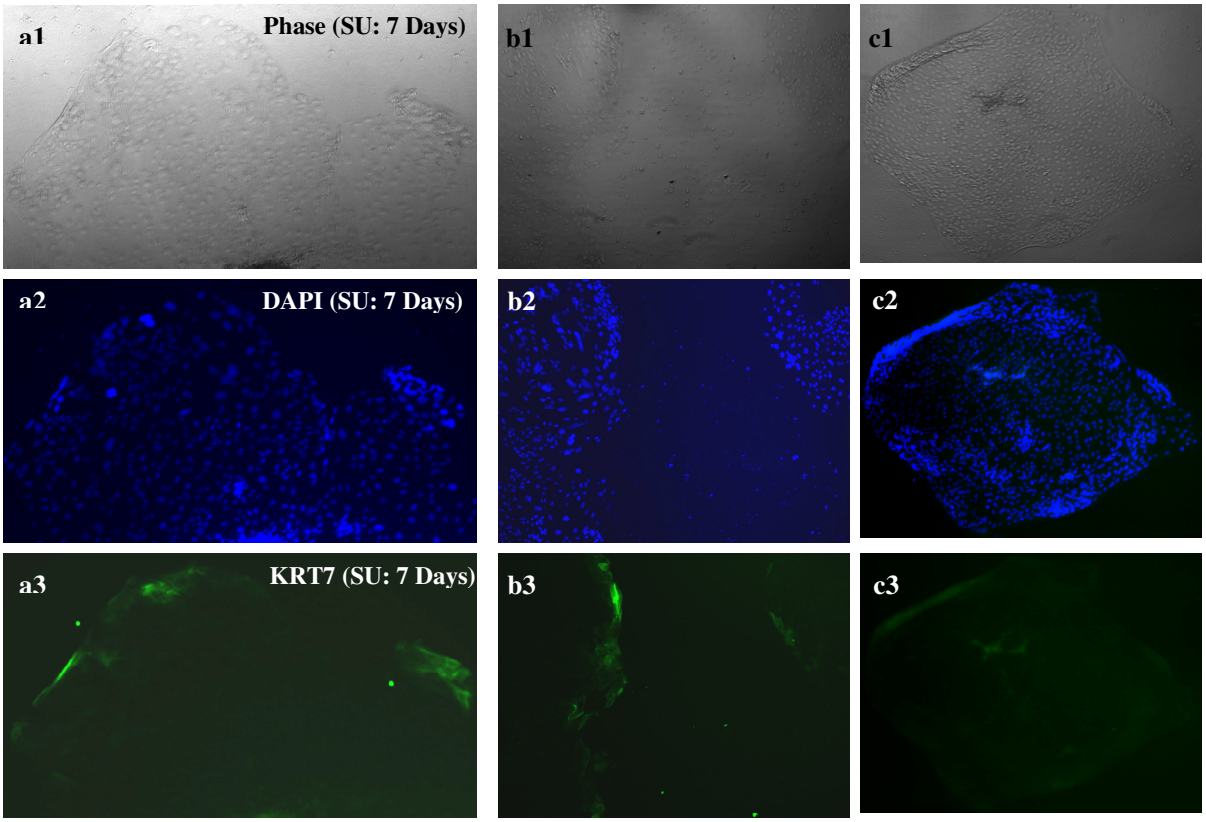


Figure 3.7.1: d) Immunostaining for CK-7 (KRT7) protein expression (green) in SU treated cells for 5 days. DAPI: Blue. A, B and C depict distinct colonies.

3.7.2: BMP pathway activation, irrespective of the presence or absence of ACTIVIN/NODAL pathways supports differentiation into mesenchymal lineage, but additional FGF blocking supports epithelial lineage

Among the two types of differentiated trophoblast cells, the syncytiotrophoblast cells remain epithelial and the extravillous invasive cytotrophoblast cells undergo epithelial-mesenchymal

transition (EMT), which infiltrate into the maternal decidual stroma and blood vessels (Charnock-Jones et al., 2004; Kingdom et al., 2000; Vicovac and Aplin, 1996; Zygmunt et al., 2003). To ascertain, if the treatments led the cells to have epithelial or mesenchymal characteristic, we checked for the expression of the epithelial marker, E-Cadherin (CDH1) and the mesenchymal marker, vimentin (VIM) after 5 days of treatment (Fig. 3.7.2).

Figure 3.7.2

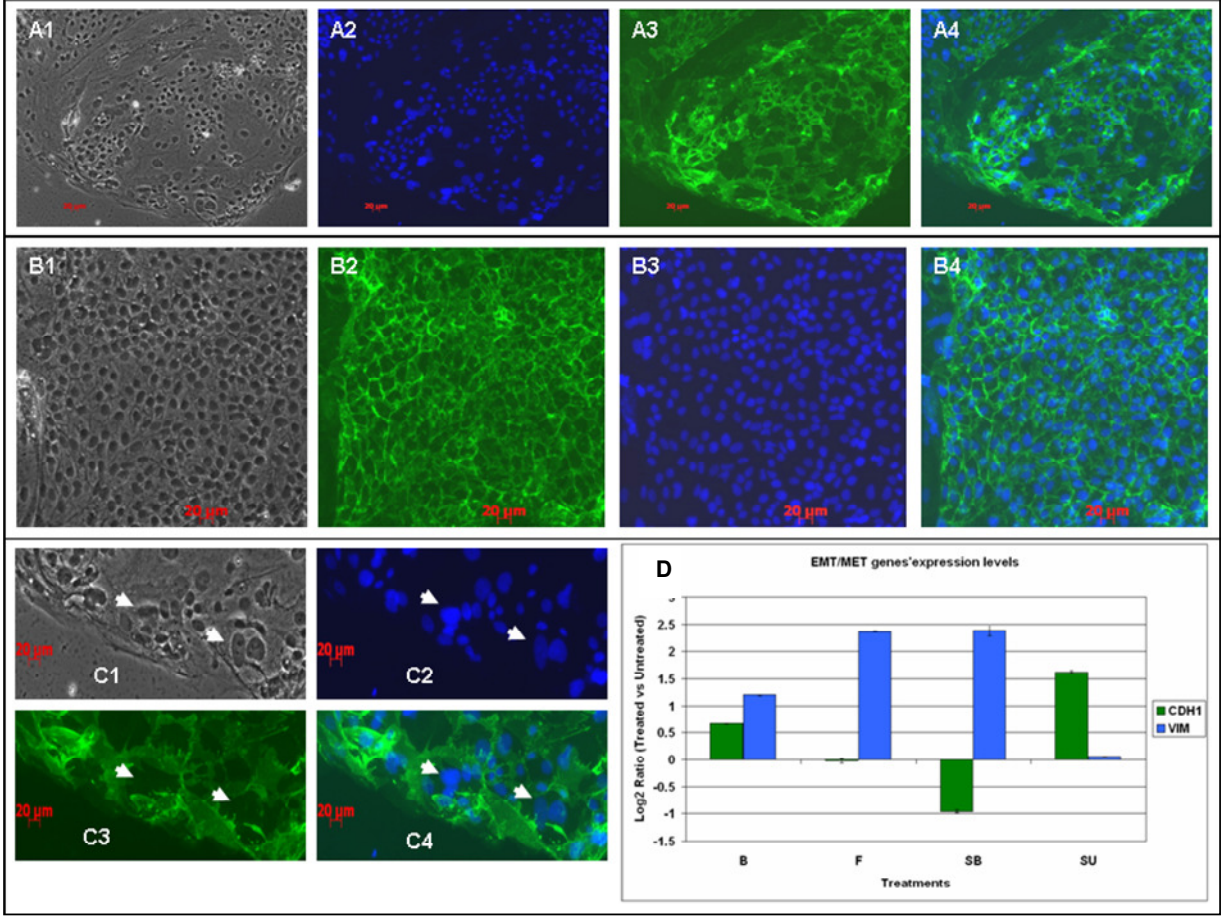
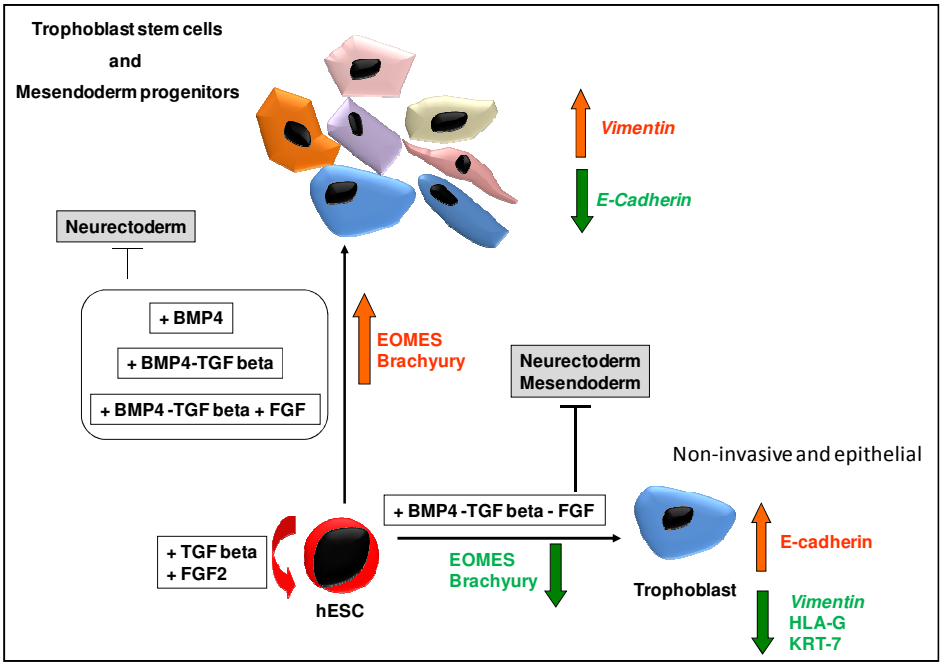


Figure 3.7.2: a, b, c) E-Cadherin (CDH1) immunostaining (blue) after SU treated cells (5 days) **a)** E-Cadherin positive cells showing different morphologies and nuclear sizes **b)** E-Cadherin positive single cells having similar morphology. **c)** E-Cadherin positive multinucleated cells. **d)** Real time PCR validation of e cadherin (*CDH1*) and Vimentin (*VIM*) in all the samples (B, F, SB, SU) after 5 days of treatment. x axis represents the samples. y axis represents Log2 Ratio between treated and untreated samples. Normalization was carried out against *GAPDH* gene expression.

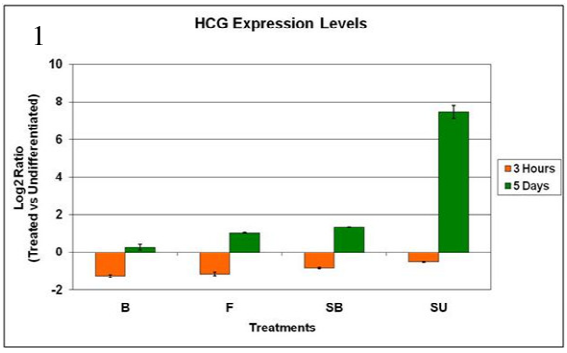
At the mRNA level (Fig. 3.7.2 D), it was seen that the epithelial marker, *CDH1* was up-regulated by 3 fold in SU treated cells after 5 days, whereas it was either down-regulated by 2 fold (SB) or not significantly differentially regulated (B, F) after the other treatments. We could also detect CDH1 protein expression in a major proportion of cells in the SU treated cells (Fig. 3.7.2 A-C). These results confirm that the SU treatment leads the cells to possess epithelial characteristic, but all the other treatments lead them to have mesenchymal characteristic after 5 days. The differentiated E-Cadherin positive cells were of various morphologies (Fig. 3.7.2 A-C), some possessing elaborate cytoplasm having huge nuclei, some having multiple nuclei and some were single-nucleated cells (B). The results till now suggest that the SU treated cells are non-invasive and epithelial trophoblast cells after 5 days of treatment.



3.7.3: SU treatment induces differentiation of hESC to non-invasive, epithelial βhCG-secreting syncytiotrophoblast in 5 days

Human chorionic gonadotrophin (hCG) is a hormone, secreted by the human placenta, which plays a very important role during early pregnancy, maintaining the corpus leuteum and progesterone production ensuring successful embryo implantation. It can be detected in the mother's serum after 8 days of ovulation (Braunstein et al., 1976). The alpha subunit (CGA) of hCG is sometimes visible in the cytotrophoblast, but the beta subunit (CGB) is essentially restricted to villous syncytiotrophoblast (Randeva et al., 2001). Pregnancy tests involve the determination of its concentration by an

Figure 3.7.3 a



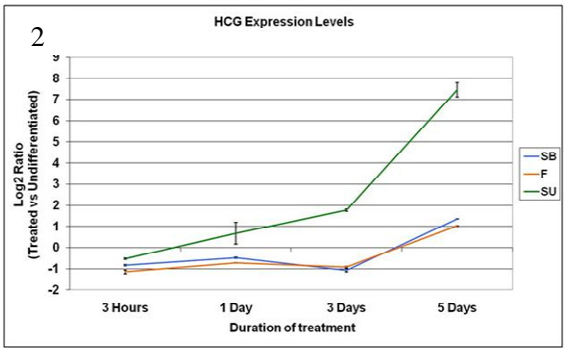


Figure 3.7.4 b

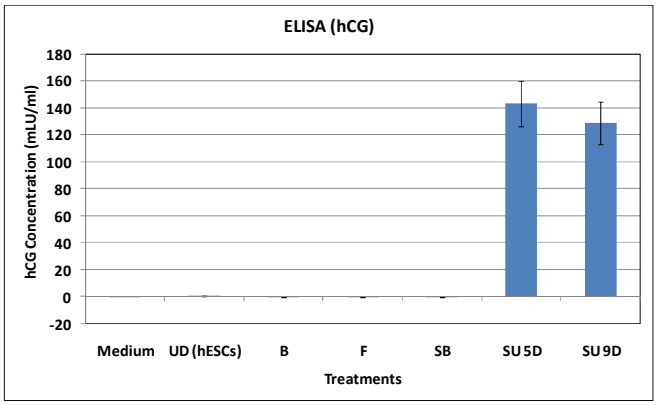


Figure 3.7. 4 c 1 2

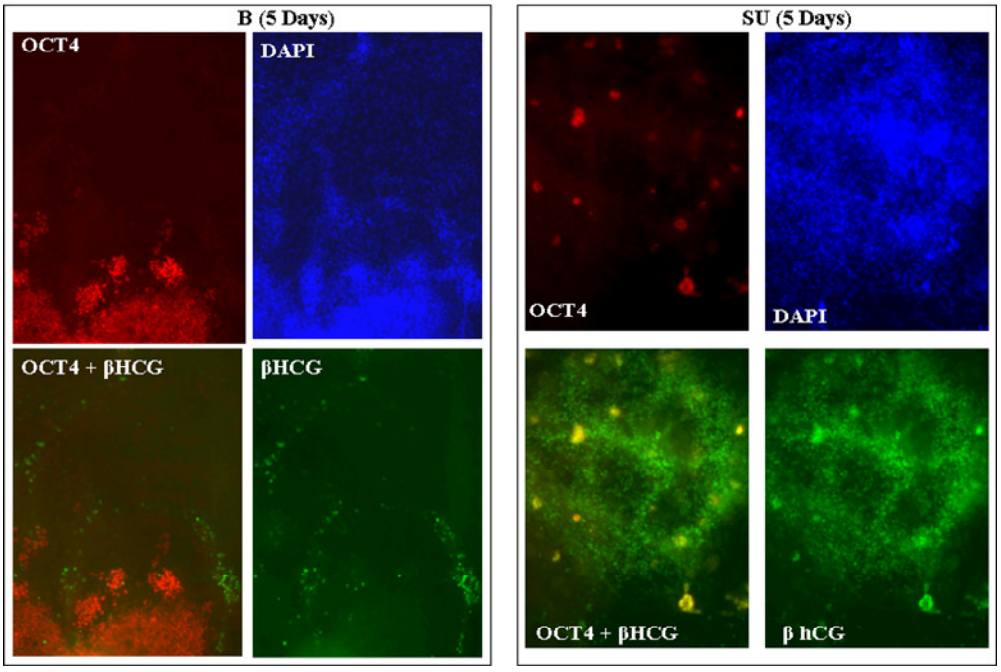


Figure 3.7.3: a) Real time PCR validation of *CGB* expression in **1)** all the samples (B, F, SB and SU) after 3 hours and 5 days of treatment. x axis represents the samples **2)** F, SB, SU during the course of treatment. x axis represents the duration of treatment. y axis represents Log2 Ratio between treated and untreated samples. Normalization was carried out against *GAPDH* gene expression. **b)** ELISA mediated estimation of concentration of βhCG in the growth media of treated cells (UD (Undifferentiated hESC), B, F, SB and SU) after 5 days of treatment. **c)** Immunostaining for βhCG (green) and OCT4 (red) protein expression in B **(1)** and SU **(2)** treated cells after 5 days. DAPI: Blue.

immunoassay (Chard, 1992). Hence, we looked for the expression of βhCG in the cells from all the treatments at mRNA and protein levels (Fig. 3.7.3).

In all the treatments, there was no significant difference in the gene regulation of the gene encoding for the placental marker βhCG, CGB till 3 days (Fig. 3.7.3 a). The B treated cells didn't show any significant difference in CGB levels after 5 days also. But it was up-regulated in F and SB treated cells by around 2 fold, whereas SU treated cells showed 150 fold up-regulation after 5 days. Through ELISA (Fig. 3.7.3 b), we couldn't detect any secreted hCG hormone in the media of B, F and SB treated cells after 5 days, but around 140 mIU/ml of hCG was detected in the media from SU treated cells (5 days). At the protein level, by immunofluorescence (Fig. 3.7.3 c), we saw that the ES cell marker, OCT4 is still heavily expressed in majority of the cells in B treated cells after 5 days, but in SU treated cells, we could only see a minority of them expressing the same. SU treated cells stained positive for βhCG very prominently.

In some colonies, βhCG staining was seen mostly at their edges (Fig. 3.7.3 d), though the colony had completely differentiated, when observed phenotypically. Hence, under our culture conditions, the trophoblast stem cell differentiation to βhCG-expressing syncytiotrophoblast, mostly initiated from the periphery of the colony, which subsequently spread towards inside. The heat map (Fig. 3.7.3 e) shows the cluster of genes, which were highly expressed after 5 days of SU treatment. The first cluster included *GREM2*, which is an antagonist of BMP signaling (details in section 3.7.5). The second cluster shows genes, which share similar profile with that of βhCG (*CGB*) gene. This cluster included the cell fusion inducing genes (*GCM1, ERVWE1, HERV-FRD*) (section 3.7.4), those involved in estrogen/progesterone biosynthesis and metabolism (*HSD17B1, HSD3B1, CYP19A1, CYP11A1*) (section 3.7.8), the tumor suppressor, *HOP (NECC1*) (section 3.7.7), etc. Taken together,

the SU treatment induces differentiation of hESC to non-invasive, epithelial βhCG-secreting trophoblast cells in 5 days.

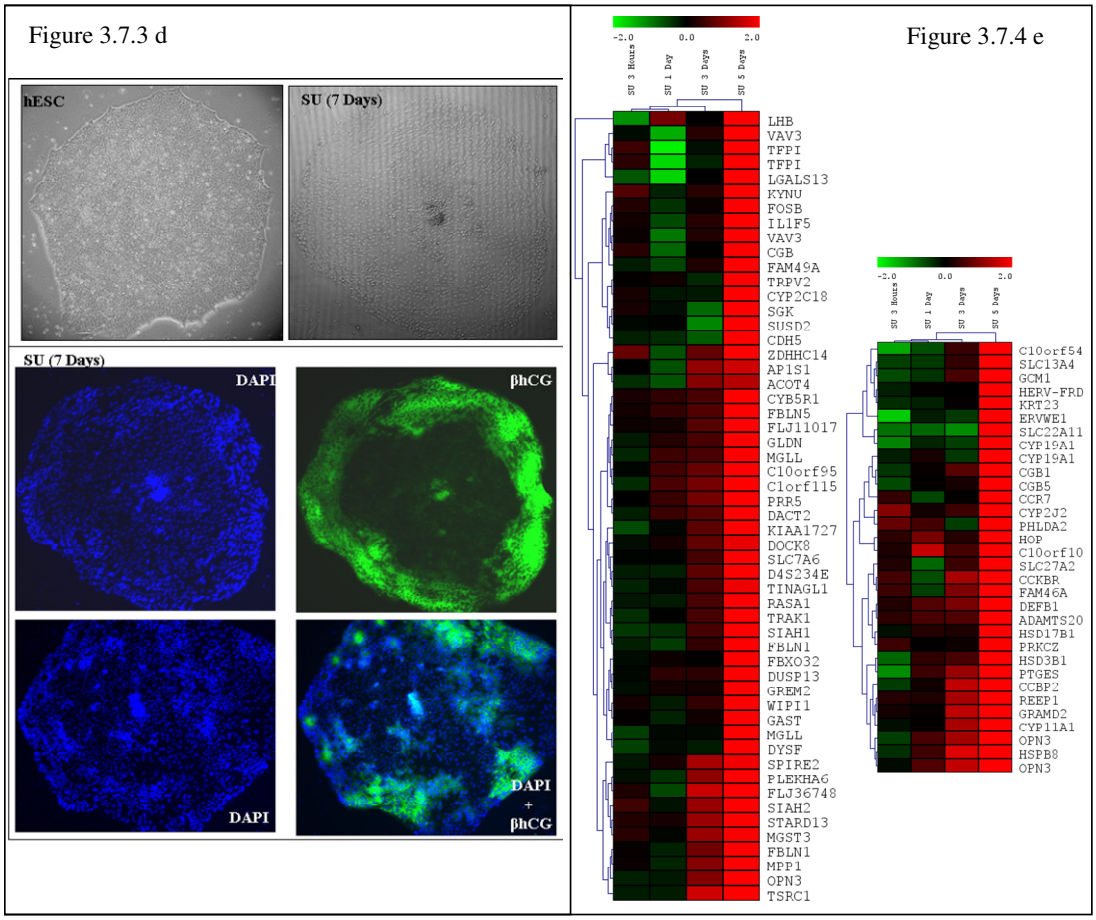
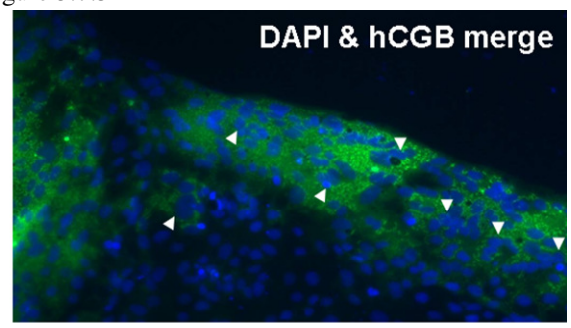


Figure 3.7.3: d) Various patterns of βhCG expressing cells in the colony. Immunostaining for βhCG (green) protein expression SU treated cells after 5 days. DAPI: Blue. Top panel shows phase contrast images of an undifferentiated hESC colony and an SU treated colony (5 days). **e)** Heat map for the genes highly expressed after 5 days of SU treatment. The second cluster shows genes, which share similar profile with that of βhCG (CGB) gene.

3.7.4: SU treatment supports the differentiation of hES cells to multinucleated syncytiotrophoblast via the induction of fusogens

The SU treated cells are E-Cadherin- positive, secreting β-hCG hormone, which led us to think of the possibility that this treatment could be supporing the hESC cells to differentiate into multinucleated syncytiotrophoblasts, which are largely epithelial and the major source of β-hCG hormone.

Figure 3.7.5 A



Arrows indicate clustered nuclei of fused cells

Figure 3.7.5 B

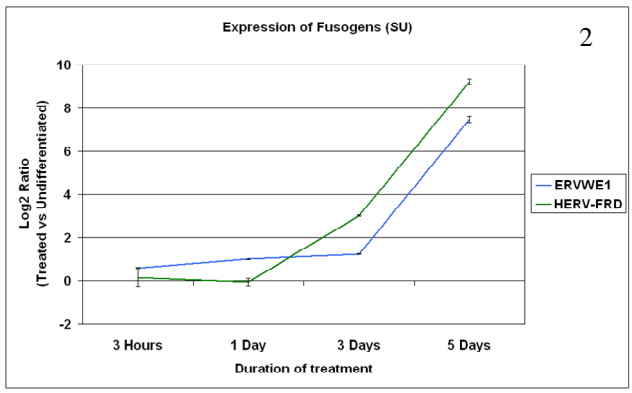
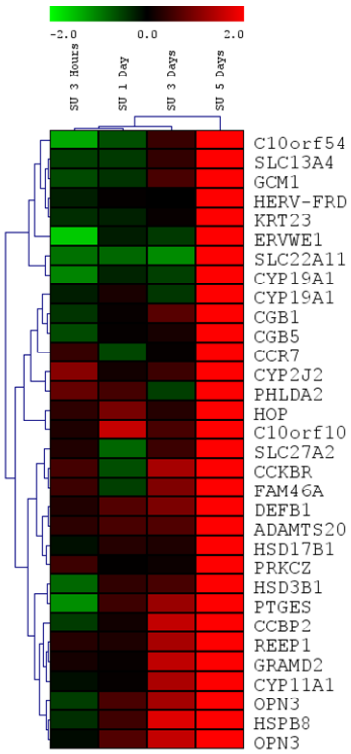


Figure 3.7.5 C



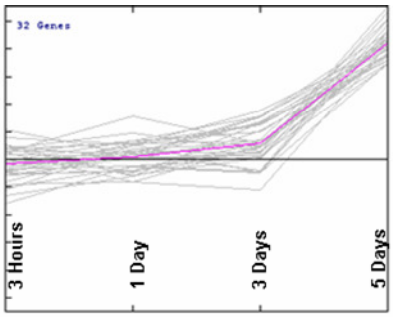


Figure 3.7.5 D

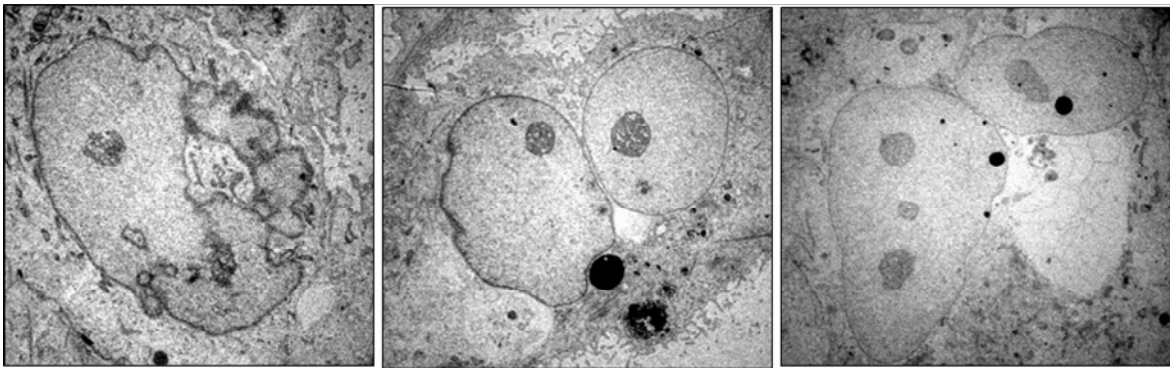
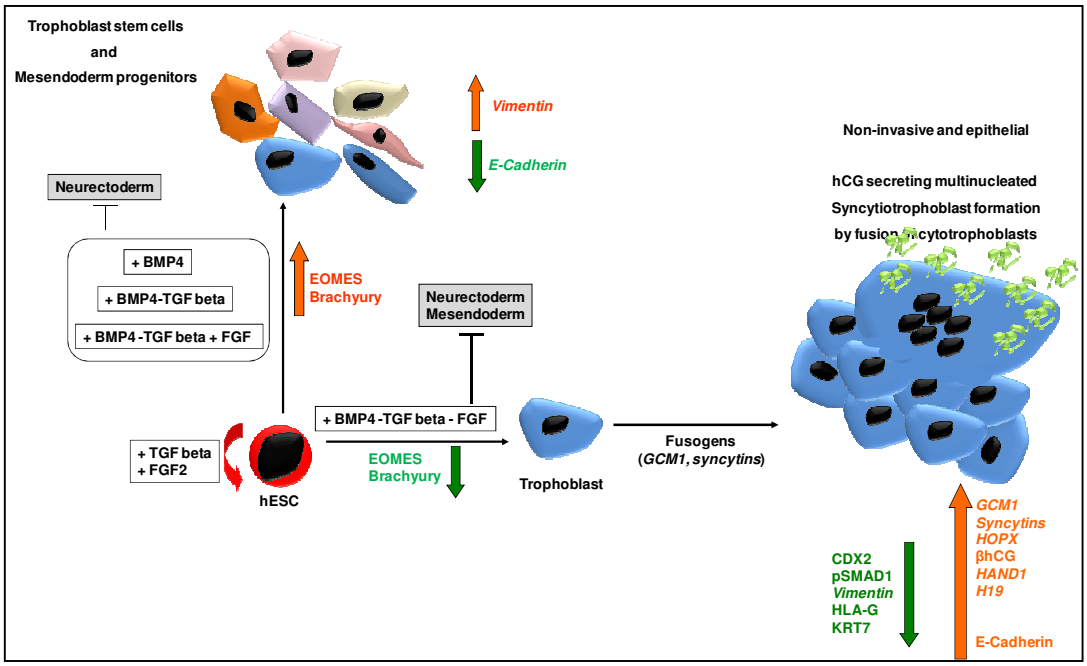


Figure 3.7.4: a) Immunostained βhCG protein expressing cells (green), showing clustered nuclei after 5 days of SU treatment. DAPI: Blue. **b)** Real time PCR validation of *ERVWE1* and *HERV-FRD* expression in **1)** all the samples (B, F, SB and SU) after 5 days of treatment. x axis represents the samples **2)** SU treated cells, during the course of treatment. x axis represents the duration of treatment. y axis represents Log2 Ratio between treated and untreated samples. Normalization was carried out against *GAPDH* gene expression. **c)** Heat map for the genes, which share similar profile with that of the fusogens, *GCM1, ERVWE1* and *HERV-FRD*. **d)** Multinucleated cells among SU treated cells (5 days), observed through electron microsopy.

A lot of genes, which were known to be expressed in syncytiotrophoblast were specifically up-regulated in SU treated cells after 5 days (Fig. 3.7.4 E and section 3.7.8). Trophoblast stem cells are known to differentiate into more specialized, multinucleated syncytiotrophoblast cells, through cells fusion events (Boyd and Hamilton, 1970; Benirschke and Kaufmann, 2000). Interestingly, the cell-fusion inducing genes, GCM1, syncytin1 (ERVWE1) (Blaise et al., 2003) and syncyntin2 (HERV-FRD) (Fig. 3.7.4 E, Fig. 3.7.4 B) (Frendo et al., 2003b) were seen to be only up-regulated in SU treated cells and not in the others (SB, F, B) (Fig. 3.7.4 B). Glial cell missing-1 (GCM1) is a placental-specific transcription factor, localised in a subset of highly differentiated villous cytotrophoblasts and syncytiotrophoblast (Baczyk et al., 2004; Nait-Oumesmar et al., 2000), regulates the expression of syncytin-1 (Yu et al., 2002) and aromatase (Yamada et al., 1999). In our time course data for SU treatment, we observed that *GCM1, ERVWE1, HERV-FRD* aromatase (*CYP19A1*) are coregulated (Fig. 3.7.4 B, Fig. 3.7.4 C). When we had a closer look at the regions positive for β-hCG, we observed clustered nuclei (Fig. 3.7.4 A, D). CDH1 is a membrane-bound protein, present at the intercellular boundaries, which disappear between fused cytotrophoblasts with syncytium formation (Alsat et al., 1996; Coutifaris et al., 1991; Douglas and King, 1990). When we had a closer look at the CDH-1 positive areas, we also found multinucleated cells, with CDH1 expression around them, but not in-between these fused cells (Fig. 3.7.2 C).

To confirm the presence of multinucleated cells, we performed electron microscopy on the SU treated cells (5 days) and in fact, we saw the presence of multinucleated cells among the SU treated cell population (Fig. 3.7.4 d). Hence, the results till now revealed that SU treatment supports the differentiation of hES cells to non-invasive, epithelial, β-hCG-secreting, fusogen induced multinucleated syncytiotrophoblast.



3.7.5: Cell cycle is disrupted in SU treated cells, but apoptosis is avoided

Apart from the two differentiated trophoblast types, extravillous and syncytiotrophoblast, at early gestation, the trophoblast has been classified into three, namely, cytotrophoblast, intermediate trophoblast and syncytiotrophoblast, based on their location and morphology (Mazur and Kurman, 1994; Benirschke and Kaufman, 1995). The proliferative capacities of various trophoblast cell types differ between each other considerably. The extravillous trophoblasts and cytotrophoblasts are highly proliferative. But the syncytiotrophoblast, which is formed by continuous fusion of cytotrophoblasts, shows no proliferation (Ichikawa et al., 1998; Wakuda and Yoshida, 1992) and is considered to represent a mitotically end-stage cell (Benirschke and Kaufman, 1995). Cell proliferation can be affected by either cell cycle arrest or apoptosis (Crocker et al., 2007; Hunter, 1993) and Cell cycle disruption, by inhibition of DNA synthesis and replication is a basic trigger for cytotrophoblast differentiation into syncytiotrophoblast (Crocker et al., 2007).

In the cells treated with SU for 5 days, cell cycle was grossly affected (Figure 3.7.5 a, b and Supplementary table 5). *CDK1* and associated cyclins, *CYCLIN A1, A2* and *B1, B2*, which drive G2 and M phase during the cell cycle, were down-regulated. Cyclin A1 and A2 are also required for S phase. *CDK2* and *CYCLIN E*, which drive the transition of cells from G1 to S phase, were up-regulated. Also, *CDK6* and *CYCLIN D3*, which are involved in G1 phase, were up-regulated. In the SU treated cells, there was also a reduction in the phosphorylation of histone H3 (H3p) (Figure 3.7.5

b), a downstream marker for chromosome condensation and entry into mitosis (Juan et al., 1998). Therefore, S, G2 and M phase were compromised in SU treated cells after 5 days, which means that normal cell division was affected in these cells.

The affected cell cycle in these cells can lead to apoptosis, through p53-mediated programmed cell death. Hence, for the differentiation of trophoblast cells to specialized cells like syncytiotrophoblast will require the shut down of this pathway, so that the cells don't turn apoptotic. The DNA-damage driven apoptosis inducing factors, *CHEK1* (Liu et al., 2000; Takai et al., 2000), *CHEK2* and *TP53* (Andreassen et al., 2001; Lanni and Jacks, 1998) were seen to be down-regulated in SU treated cells after 5 days. It was revealed from the microarray data that the targets of *TP53, MMP2* and *MMP9,* which are important for the invasive ability trophoblast (Lam et al., 2009) were not detected and down-regulated, respectively in these cells (data not shown). The modulation of p53-Mdm2 pathway is important for the development of trophoblast and its lineages (Chiu et al., 2008). Mdm2, a RING finger E3 ubiquitin ligase is essential for the degradation of p53 (Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997; Momand et al., 1992). In SU treated cells, not only *MDM2*, but also anti-apoptotic *BCL-2* and *BCL2L1* (bcl-xL) were up-regulated after 5 days and at the same time, TP*53* was down-regulated. The inhibition of apoptosis by bcl-2-related genes and loss of p53 function can act cooperatively to contribute to genetic instability (Minn et al., 1996). BCL-2 protein is expressed throughout the syncytium of normal villi and less expressed in cytotrophoblast (Ratts et al., 2000; Toki et al., 1999). *GADD45G*, a CDK1/CYCLIN B1 inhibitor (Vairapandi et al., 2002), which is highly expressed in the placenta was highly up-regulated after 5 days. Geminin, which is known to be indispensable for preventing recurring cycles of DNA replication, was down-regulated. Geminin expression is necessary to prevent endoreduplication during mammalian development (Gonzalez et al., 2006). The G1 CYCLIN/CDK inhibitors, *p57 (CDKN1C)* and *p21* were strongly up-regulated in SU treated cells, exhibiting negative regulation of cell proliferation.

To summarise, the SU treatment leads to the differentiation of hESCs to syncytiotrophoblast cells, which are no more proliferative, but are not apoptotic. The *BCL-2*-related genes and *MDM2* might be cooperating in inactivating the p53-mediated apoptosis in syncytiotrophoblast.

Figure 3.7.5 a

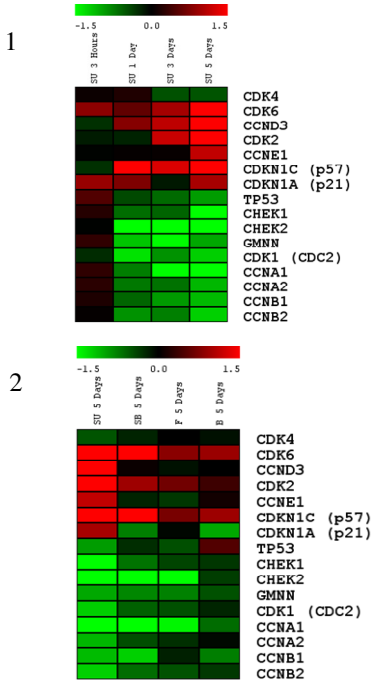


Figure 3.7.5 b

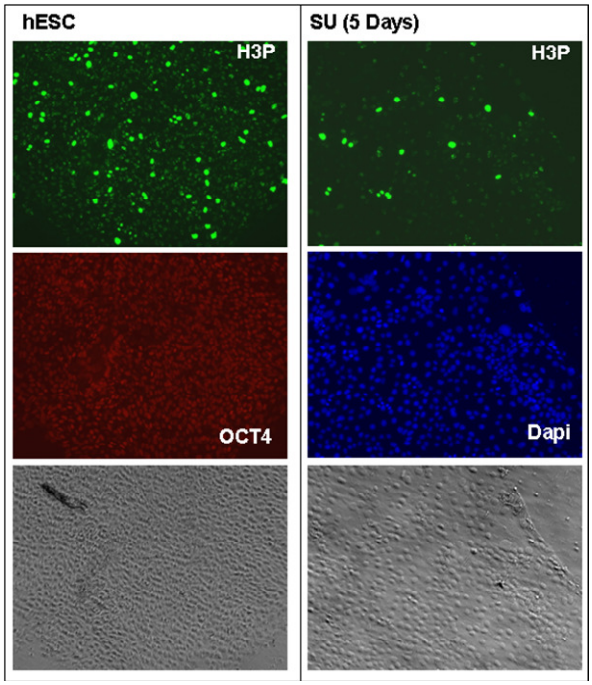


Figure 3.7.5: a) Heat map for the genes involved in cell cycle regulation. **b)** Immunostaining for phosphorylation of histone H3 (H3p) (green) in hESCs and SU treated cells after 5 days. DAPI: Blue.

When mouse trophoblast stem cells are induced to undergo endoreduplication, p57 inhibits Cdk1 which is accompanied by suppression of the DNA damage response by p21 (Ullah et al., 2008). The mouse and human placenta are comparable, as both are hemochorial placentas (Rossant and Cross, 2001), though certain differences do exist (Adamson et al., 2002). Rodent TG cells are analogous to extravillous cytotrophoblast cells of the human placenta, both are polyploid and invasive, and have similar patterns of trophoblast cell subtype-specific gene expression (Hemberger and Cross, 2001). But the SU treatment does not lead to differentiation of hESC to extravillous cytotrophoblast cells, but to syncytiotrophoblast. So syncytiotrophoblast might be adopting similar regulatory mechanism as that adopted by mouse trophoblast giant cells, to remain functional and viable, avoiding cell cycle. The differences between extravillous cytotrophoblast and syncytiotrophoblast in this mechanism need to be further investigated.

3.7.6: The status of BMP, ACTIVIN/NODAL and FGF signalling after the treatments: Autocrine signalling leads to abrogation of BMP signalling in SU treated cells after 5 days

TGF β (Amit et al., 2004; Beattie et al., 2005; Greber et al., 2008; James et al., 2005; Ludwig et al., 2006a; Vallier et al., 2005; Vallier et al., 2004; Xiao et al., 2006) and FGF2 (Beattie et al., 2005; Greber et al., 2007; Liu et al., 2006; Lu et al., 2006; Ludwig et al., 2006b; Vallier et al., 2005; Wang et al., 2007; Yao et al., 2006) mediated pathways support the maintenance hESCs. BMP signalling is known to promote differentiation of hESC into trophoblast (Xu et al., 2002), extra-embryonic endoderm (Pera et al., 2004), mesoderm (Zhang et al., 2008) and hematopoietic cells (Chadwick et al., 2003).

The transcription factors, SMAD2 and 3 are activated by TGF β (Feng and Derynck, 2005) and SMAD1, 5 and 8 are activated by BMP2/4 (Chen et al., 2004b). To understand the status of these pathways in the cells after the treatments, we conducted a western blot analysis (Fig. 3.7.6 a) and also looked for the expression changes in the downstream targets of these pathways. As already known, pSMAD2 level was high and pSMAD1 level was low or absent in hESCs. TGF β pathway was active (pSMAD2) in BMP4 treated cells, both after 1 and 5 days of treatment, which could be the explanation for the lesser extent of differentiation seen in these cells (Sections 3.1-3.3). The downstream target of SMAD2, *EOMES*, which is a mesendoderm marker, was down-regulated right after 1 day of treatment in all the treatments, except B, in which it was slightly upregulated after 1 day and then subsided. This clearly emphasises the requirement of transforming growth factor-beta/activin signaling for the activation of EOMES as seen in Xenopus (Picozzi et al., 2009). All the other SMAD2 targets, *NODAL, LEFTY1, LEFTY2* and *CER1* were downregulated (Fig. 3.7.6 b and Supplementary table: 6) in all the treatments, except for the treatment, B, in which the cells were treated with BMP4 (10 ng/ml). In this treatment, all these SMAD2 targets were upregulated after 3 hours and by 5 days, their expression levels subsided, *CER1* and *LEFTY2* still staying upregulated. *LEFTY1* was down-regulated and *NODAL* didn't show any regulation, when compared to the undifferentiated cells. The down-regulation of ACTIVIN/NODAL pathway in the treatments, SB, F and SU is evident from the western blot data, showing reduced levels of pSMAD2 and also the down-regulation of its downstream targets. But, SMAD2 phosphorylation was still active in the B treated cells after 5 days, because of which its targets do not show any difference in regulation.

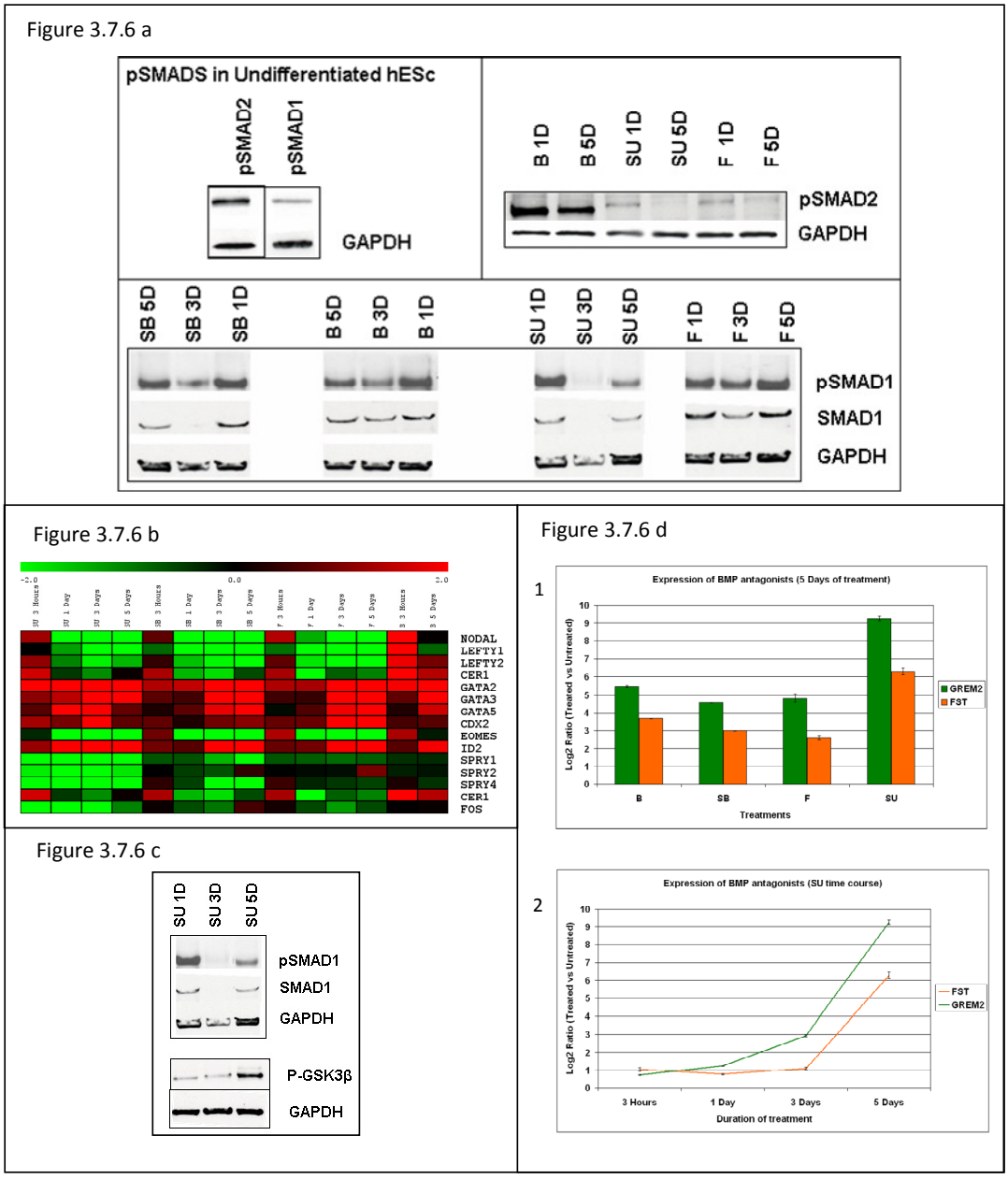


Figure 3.7.6: a) Western blot analysis for pSMAD1, pSMAD2 and SMAD1 of the hESCs and the treated cells, after 1 day (1D) and 5 days (5D) of treatment (B, SB, SU, F). * Due to loading problem, the 3 days (3D) time point was not taken into consideration for the analysis. **b)** Heat map for the genes targeted by ACTIVIN/NODAL (NODAL, LEFTY1, 2, CER1), BMP (GATA 3, 5, CDX2, EOMES, ID2) and FGF signaling (SPRY1, 2, 4, CER1, FOS). **c)** Western blot analysis for phospho SMAD1, phospho GSB3β, SMAD1 and GAPDH of the lysates from SU treated cells after 1 day (1D), 3 days (3D) and 5 days (5D). **d)** Real time PCR validation of *GREM2* and *FST* expression

in **1)** all the samples (B, F, SB and SU) after 5 days of treatment. x axis represents the samples **2)** SU treated cells, during the course of treatment. x axis represents the duration of treatment. y axis represents Log2 Ratio between treated and untreated samples. Normalization was carried out against *GAPDH* gene expression.

The BMP4-induced pSMAD1 levels remained high in the cells of all the treatments, after 1 and 5 days, except in the SU-treated cells. In SU treated cells, pSMAD1 level was high after 1 day, but drastically reduced after 5 days. This was unexpected, as all the treatments involved the activation of BMP pathway and fresh BMP4 was provided to the cells every day, with fresh media. Both microarray (data not shown) and real-time PCR data revealed much higher up-regulation of the TGF β signalling antagonist, Follistatin *(FST)* (Harland and Gerhart, 1997) and BMP signalling antagonist, Gremlin2 *(GREM2)* (Hsu et al., 1998; McMahon et al., 2000; Shi et al., 2001; Topol et al., 2000) in SU treated cells than all the other treatments (B, SB and F). Follistatin is also known to bind other ligands, including inhibin, BMPs 2, 4, 6, 7, 11, and 15, and myostatin, with lower affinities (Abe et al., 2004; Canalis et al., 2003; Gumienny and Padgett, 2002). Compared to the undifferentiated cells, after 5 days of treatment, Follistatin was up-regulated by 64 fold (Log2value = 6) in SU-treated cells, whereas, it was only 8 fold (Log2value = 3) up-regulated in B, SB and F. *GREM2* was up-regulated by 500 fold (Log2value = 9) in SU-treated cells, whereas, it was only 30 fold (Log2value = 6) up-regulated in B, SB and F. The phosphorylation of SMAD1 protein at the linker region by GSK3β (Fuentealba et al., 2007; Sapkota et al., 2007) causes degradation of SMAD1 (Sieber et al., 2009)**.** We observed increased level of pGSK3β after 5 days of SU treatment, which could also be a reason for lower level of pSMAD1 after 5 days of SU treatment. Taken together, either autocrine signalling mediated high expression of BMP antagonists, GREM2 and FST, or linker phosphorylation of SMAD1 by pGSK3β could be causing the reduced level of pSMAD1 in SU treated cells after 5 days.

The SMAD1/5/8 targets, *ID2, CDX2, GATA2, 3* and *5* were upregulated (Fig. 3.7.6 b and Supplementary table: 6) till 3 days and thereby down-regulated, except for *ID2, GATA 2* and *3*, which remained expressed after 5 days of SU treatment. *GATA 2* and *3* play a very crucial role in trophoblast differentiation (Home et al., 2009; Kim et al., 2009; Ng et al., 1994) and are critical for the expression of Cdx2 (Home et al., 2009) and human syncytin (Cheng and Handwerger, 2005). The down-regulation of ID2 is crucial for cytotrophoblast development (Janatpour et al., 2000) and extravillous trophoblast differentiation (Fisher, 2000; Quenby et al., 1998). CDX2 is a trophoblast

stem cell marker, which is important for the differentiation of hESC to trophoblast, was upregulated till 3 days and then downregulated. The *GATA* factors and *ID2* remained upregulated in the other treatments (B, SB, and F). *CDX2* remained highly upregulated in the treatment F, in which both BMP and FGF pathways are kept active and the ACTIVIN/NODAL pathway is blocked. This must be due to the continuous activation of ERK pathway in these cells, which is known to stimulate the expression of cdx2 (Gotoh, 2009). All the SMAD1 downstream targets examined were induced in all the treatments. But, specifically in SU treatment, the trophoblast marker, *CDX2* showed transient expression, which coincided with reduction in pSMAD1 levels and *GATA2, 3* and *ID2,* which are known to be important for trophoblast development remained upregulated. The differentiation of trophoblast stem cells to syncytiotrophoblast might require the downreguation of *CDX2* and *GATA2, 3* and *ID2* might be important, not only for trophoblast stem cells, but also for syncytiotrophoblast.

The FGF pathway target genes, *SPRY1, 2, 4* and *FOS* (Fig. 3.7.6 b and Supplementary table: 6) were abruptly down-regulated in SU treated cell, right after 3 hours of treatment. In SB treated cells, *SPRY2* and *FOS* were down-regulated after 1 day and 3 days, but didn't show any change in regulation after 5 days, when compared to the undifferentiated cells. In F-treated cells, all the FGF targets were downregulated, except for *SPRY2,* which was slightly up-regulated, which could be due to the exogenous activation of FGF signaling through FGF2 in this treatment. These results show that FGF signaling was indeed, very effectively disrupted in SU treated cells, might be to a lesser extent in SB and F treated cells, but was still active in the B treated cells.

3.7.7: Tumor Suppressor-encoding genes are induced after 5 days of SU treatment

The tumor suppressor gene, HOPX, a candidate choriocarcinoma suppressor gene (Asanoma et al., 2003), which suppresses proliferation of cancer cells (Yamaguchi et al., 2009) was upregulated by almost 140 fold in SU treated cells after 5 days (Fig. 3.7.7 1, 2), in contrast to 16 to 36 fold in the other treatments. In humans, HOP is expressed exclusively in syncytiotrophoblast and not in extravillous trophoblasts, which are the mouse orthologues of TG cells (Asanoma et al., 2007) and the outcome of its transfection into choriocarcinoma cell lines suggested differentiation of choriocarcinoma cells to syncytiotrophoblasts (Asanoma et al., 2003).

As mentioned in section 3.3, the epithelial cell type marker, E-Cadherin (*CDH1*), which is also a tumor suppressor, was specifically only upregulated in SU treated cells, in contrast to the other treatments, which induced the mesenchymal marker, vimentin (*VIM*).

Figure 3.7.7 a

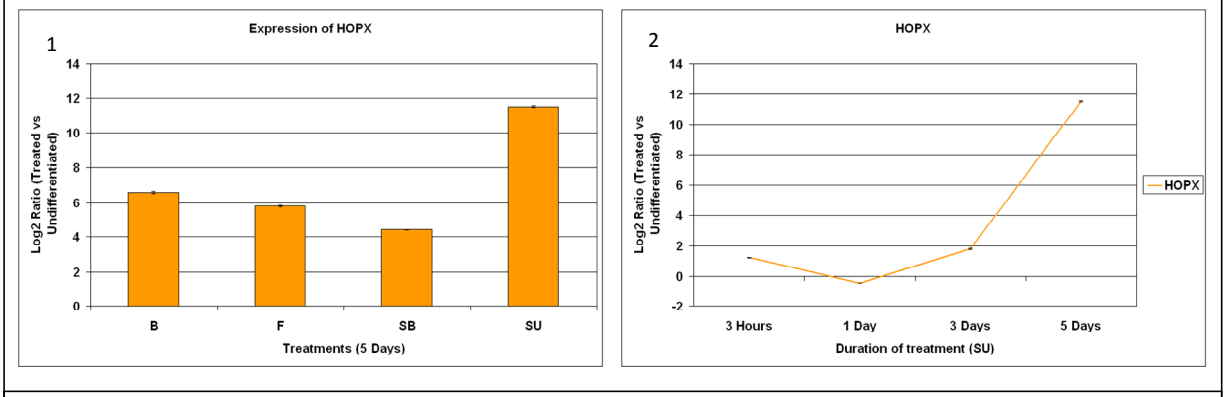


Figure 3.7.7 b

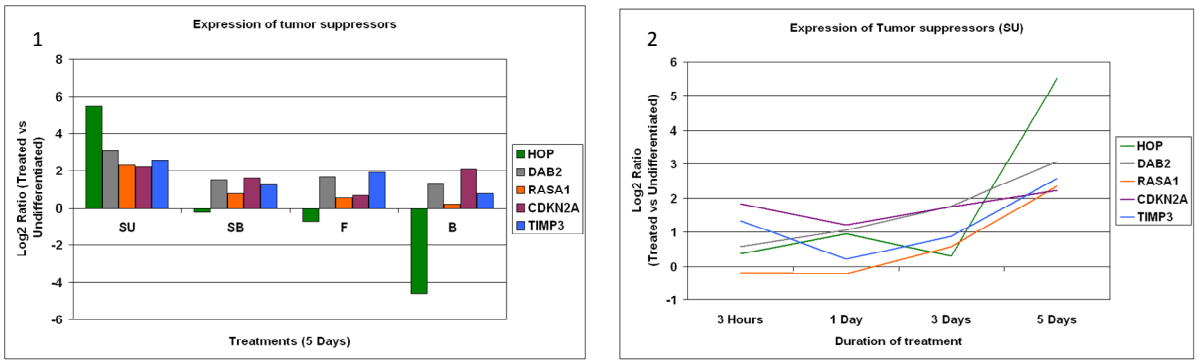
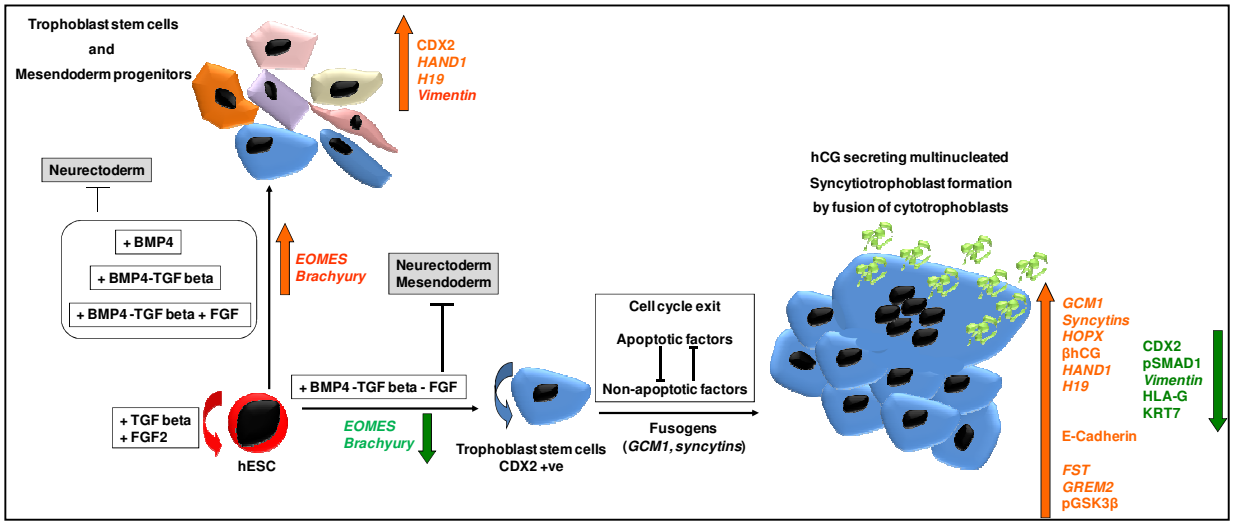


Figure 3.7.7: a) Real time PCR validation of *HOPX (NECC1 or HOP*) expression in **1)** all the samples (B, F, SB and SU) after 5 days of treatment. x axis represents the samples **2)** SU treated cells, during the course of treatment. x axis represents the duration of treatment. y axis represents Log2 Ratio between treated and untreated samples. Normalization was carried out against *GAPDH* gene expression. b) Relative expression of tumour suppressors (Normalised microarray data) in **1)** all the samples (B, F, SB and SU) after 5 days of treatment. x axis represents the samples **2)** SU treated cells, during the course of treatment. x axis represents the duration of treatment. y axis represents Log2 Ratio between treated and untreated samples.

Other known potential tumor suppressors, *TIMP3, RASA1* and *DOC-2* (Fig. 3.7.7 b and Supplementary table: 7) were also upregulated in SU treated cells after 5 days. Tissue inhibitors of metalloproteinases (TIMPs) regulate extracellular matrix (ECM) degradation by matrix metalloproteinases (MMPs) throughout embryogenesis. TIMP3 can regulate epithelial cell proliferation by inhibiting MMP activity (Gill et al., 2006). As mentioned before, *MMP2* and *MMP9*, which are important for the invasive ability trophoblast (Lam et al., 2009) were not detected and

down-regulated, respectively in these cells (data not shown) in SU treated cells after 5 days. Rho GTPase-activating protein (Rho-GAP or RASA1), is a potent tumor suppressor, which is commonly inactivated in several human cancers (Yang et al., 2009). The information in public databases, NCBI and UniProt suggested that RASA1 is abundantly expressed in placenta and in placental villi, detected only in the trophoblast layer (cytotrophoblast and syncytiotrophoblast). High levels of DOC-2 (hDab2) are found in normal trophoblast cells in culture and normal trophoblast tissues, than in either choriocarcinoma cell lines or gestational trophoblastic disease tissues and therefore, suppression of DOC-2 may play an important role in the development of gestational trophoblastic diseases (Fulop et al., 1998a).

The abnormal functioning of the placenta has been known to cause very serious complications, the most common being preeclampsia and fetal intrauterine growth restriction (IUGR). Hypermethylation of tumour suppressor genes, including E-Cadherin and TIMP3, which can cause their reduced expression, has been associated with hydatidiform mole and choriocarcinoma that could subsequently develop into gestational trophoblastic diseases (Xue et al., 2004). Altered expression of tumor suppressor gene expression has been identified in pre-eclamptic placentas (Heikkila et al., 2005). Taken together, the SU treatment supports differentiation of hESCs into syncytiotrophoblast, which shows high expression of tumor suppressors, *NECC1, E-Cadherin, TIMP3, RASA1 and DOC-2*. Out of these, *NECC1, DOC-2, E-Cadherin* and *TIMP3* have already been reported to have aberrant expression in placental disorders, like choriocarcinoma or gestational trophoblastic disease. Therefore, this SU-mediated differentiation of hESC to syncytiotrophoblast could be used to get more insights into the pathology of human placental abnormalities.



3.7.8: Endocrine functions and genes expressed in SU treated cells after 5 days, which correspond with that in human syncytiotrophoblast or placenta

Cytotrophoblast cells possess proliferative capacity and their continuous fusion maintains syncytiotrophoblast, which is a continuous epithelial layer (Ivorra et al., 2002; Kumpel et al., 2008), terminally differentiated, possessing no regenerative capacity (Crocker et al., 2007; Huppertz et al., 1999a). Cell cycle disruption, by inhibition of DNA synthesis and replication is a basic trigger for cytotrophoblast differentiation into syncytiotrophoblast (Crocker et al., 2007). With the increasing age of placenta, there are changes seen in the trophoblastic purine metabolism **(aivio et al., 1989)**. Purine metabolism, cell cycle, glycolysis/gluconeogenesis was among the pathways representing the majority of the down-regulated genes. At the same time, some of the known functional pathways in the placenta, like epithelial cell signaling, p53 signaling (Levy et al., 2000; Yamauchi et al., 2007), PPAR signaling (Barak et al., 1999; Barak et al., 2008) and fatty acid metabolism (Rakheja et al., 2002; Shekhawat et al., 2003) were seen to be active in SU treated cells, which are also active in the placenta. PPAR signaling has a crucial role to play in placenta and recent studies suggest that activation of PPAR signaling induced up-regulation of hCG alpha and beta subunit transcript levels and protein secretions in Villous cytotrophoblasts, whereas caused their down-regulation in extravillous cytotrophoblasts (Handschuh et al., 2009).

GnRH pathway is important for stimulating hCG hormone and has an autocrine/paracrine regulation of hCG biosynthesis (Cheng et al., 2000; Siler-Khodr et al., 1986). In turn, hCG stimulates the synthesis and secretion of progesterone from the corpus luteum, which is required for early establishment and maintenance of pregnancy (Shanker et al., 1998). The genes encoding ADM (adrenomedullin) and DHRS9 (dehydrogenase/reductase (SDR family) member 9), which are associated with progesterone biosynthesis and metabolism, were up-regulated in SU treated cells after 5 days. ADM is up-regulated upon progesterone production (Thota and Yallampalli, 2005) and is also known to be involved in progesterone biosynthesis (Gene ontology). ADM mRNA is localized to syncytiotrophobland the extravillous cytotrophoblastasts and its regional increase in preeclampsia can cause reduced placental perfusion (Gratton et al., 2003). DHRS9 has a role to play in progesterone metabolism (Zhang et al., 2009).

After 5 days, there were indications of active estrogen biosynthesis in SU treated cells, as seen from the up-regulation of the genes encoding the key enzymes, *STS (s*teroid sulfatase (microsomal), isozyme S)*, CYP19A1* (cytochrome P450, family 19, subfamily A, polypeptide 1)**,**

HSD3B1(hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1) and *HSD17B1*and also, one of its inducers, Corticotropin-releasing hormone (*CRH*). The estrogen-related receptors, *ESRRA* and *ESRRG* were also up-regulated. *ESRRG* is extremely highly expressed in the placenta and type 1 isoform is exclusively expressed in the placenta (Takeda et al., 2009).

Table 3.7.8 a

Pathways (up-regulated genes)	Count	P Value
Epithelial cell signaling in Helicobacter pylori infection	21	0.00
PPAR signaling pathway	20	0.00
p53 signaling pathway	19	0.00
Focal adhesion	37	0.00
Glycan structures - degradation	8	0.04
ECM-receptor interaction	16	0.04
Fc epsilon RI signaling pathway	14	0.05
MAPK signaling pathway	37	0.05
Phosphatidylinositol signaling system	14	0.06
ErbB signaling pathway	15	0.06
GnRH signaling pathway	16	0.07

Table 3.7.8 b

SYMBOL	Placenta	SU	F	B	SB	P value
CRH	8.62	4.32	2.36	1.75	4.57	0.00
STS	3.63	2.41	-2.36	-2.97	0.81	0.00
HSD17B1	8.47	3.85	-0.94	-1.81	-0.73	0.00
HSD3B1	9.21	3.74	-0.90	-0.89	-0.33	0.00
CYP19A1	7.17	3.46	-0.73	-2.44	-1.59	0.00
CYP19A1	8.57	4.86	-2.23	-1.82	-0.41	0.00
ESRRA	1.74	1.45	-0.32	0.23	-0.19	0.00
ESRRG	3.98	2.87	0.27	0.24	0.84	0.00
ADM	3.93	0.95	-1.29	0.38	-0.62	0.00
DHRS9	3.36	2.34	-0.01	-0.20	-0.14	0.00
ACOX1	1.02	1.59	-0.36	-0.25	0.18	0.00
AKR1C2	-4.39	1.88	1.38	0.15	1.26	0.00
HPGD	4.02	1.63	1.07	2.54	1.25	0.00
PTGES	3.78	3.80	-0.08	0.40	0.44	0.00
INSL4	6.69	4.54	0.10	-0.63	-0.63	0.00
PGF	7.16	6.17	1.11	0.63	0.43	0.00
PLAC2	3.43	2.02	0.51	-0.24	0.65	0.00
P11	4.29	1.04	-0.12	-1.45	0.08	0.00
PSG4	10.20	2.51	-3.36	-1.49	-1.21	0.00
TAC3	2.57	1.25	0.39	0.15	-0.03	0.00
TEAD3	1.07	1.42	0.35	-0.21	0.67	0.00
TFCP2L1	1.53	2.55	-0.60	-0.03	-0.93	0.00
CYP11A1	7.08	3.99	0.11	0.30	0.37	0.00

Table 3.7.8 c

Pathways (Down-regulated genes)	Count	P Value
Cell cycle	72	0.00
Oxidative phosphorylation	78	0.00
Lysine degradation	35	0.00
Purine metabolism	85	0.00
Ubiquitin mediated proteolysis	76	0.00
Aminoacyl-tRNA biosynthesis	27	0.00
Pyruvate metabolism	29	0.00
Valine, leucine and isoleucine degradation	30	0.00
Ribosome	57	0.01
Citrate cycle (TCA cycle)	21	0.01
Glycolysis / Gluconeogenesis	36	0.02
Pyrimidine metabolism	50	0.02
Caprolactam degradation	12	0.02
Biosynthesis of steroids	17	0.02
Propanoate metabolism	22	0.03
Parkinson's disease	15	0.03
Fructose and mannose metabolism	26	0.03
Alanine and aspartate metabolism	21	0.04
Methionine metabolism	13	0.04

Table 3.7.8: a) Pathways included among the up-regulated genes in SU treated cells after 5 days. **b)** Pathways included among the down-regulated genes in SU treated cells after 5 days**. c)** Regulated genes showing functional significance of human syncytiotrophoblast or placenta in SU treated cells

(5 days). The ratios are in Log2 (Placenta or Treated (SU, F, B, SB) vs untreated (undifferentiated hESCs)). Red: up-regulated by atleast 1.9 fold. Green: down-regulated by atleast 1.9 fold.

The synthesis of estrogen increases progressively during pregnancy and become more rapid during the late phases (Mesiano, 2001) and selective inhibition of estradiol in placenta could control the timing of labor (Thomas et al., 2004). Syncytiotrophoblast is the main site of estrogen synthesis during pregnancy, the precursors for which are C19 androgen dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS) (Mesiano, 2001). HSD3B1 is a key steroidogenic enzyme that catalyzes the initial step in the conversion of circulating DHEA to steroids like androstenedione and progesterone (Thomas et al., 1989). Thereby, androstenedione is converted by placental aromatase and HSD17B1to estradiol (Thomas et al., 1989). An interaction between the Corticotropin-releasing hormone (CRH) and estrogen exists in syncytiotrophoblast of the human placenta (Ni et al., 2004). CRH is synthesized and secreted endogenously in syncytiotrophoblast, where the presence of CRH receptors has also been reported (Florio et al., 2000). CRH stimulates estrogen biosynthesis and also increases the mRNA levels of the key enzymes required for this, STS, CYP19A1, and HSD17B1 in human placenta

CRH is also known to stimulate prostaglandin production (Jones and Challis, 1989). *ACOX1* (acyl-Coenzyme A oxidase 1, palmitoyl), *HPGD* (hydroxyprostaglandin dehydrogenase 15-(NAD)) and *AKR1C2* (aldo-keto reductase family 1, member C2), which are associated with lipid and prostaglandin metabolism were up-regulated. prostaglandin E synthase (*PTGES*) was also seen to be up-regulated after 5 days of SU treatment and this gene is involved in both prostaglandin metabolism and antimicrobial humoral response. Prostaglandin E synthase (PGES) has is known to be present in syncytiotrophoblast (Fujikura and Mukai, 2007). The up-regulation of these genes emphasizes the fact that prostaglandin and lipid metabolizing pathways are active in these cells.

In placenta, INSL4 (insulin-like 4) is gene expression is highest in the syncytiotrophoblast and lesser in , villous stroma and intermediate trophoblast (Laurent et al., 1998). The correlation of INSL4 levels in the amniotic fluid correlate with that of the levels of chorionic gonadotrophin suggesting a common regulatory pathway for the production of these hormones in syncytiotrophoblast (Millar et al., 2005).

Both placental genes, *GCM1* (glial cells missing homolog 1) and *PGF* (placental growth factor) were up-regulated in SU treated cells after 5 days. GCM1 is expressed in cyto- and syncytiotrophoblast

(Nait-Oumesmar et al., 2000) and can transcriptionally regulate *PGF* (Chang et al., 2008), syncytin (*ERVWE1*) (Yu et al., 2002) and *aromatase* (Yamada et al., 1999), and its expression is decreased in preeclampsia (Chen et al., 2004a) and hypoxic trophoblasts (Knerr et al., 2005). Other than the role of promoting extravillous trophoblast proliferation (Athanassiades and Lala, 1998), PGF functions as a survival factor for trophoblasts (Desai et al., 1999; Zhou et al., 2002) and also relaxes human placental vessels (Szukiewicz et al., 2005), contributing to vascular development and function in the placenta.

The gene Placenta specific-2 (*PLAC2*), whose function in placenta is not clear, was also up-regulated in SU treated cells.

PP11or ENDOU (endonuclease, polyU-specific) was up-regulated upon SU treatment and is abundantly expressed and localized in syncytiotrophoblast in human term placenta (Inaba et al., 1980) and possesses endoribonuclease activity, through which it could be involved in protecting the fetus through modulating the processes, like protection from viruses or degradation of floating fetal DNA or RNA (Laneve et al., 2008).

Out of the 11 human pregnancy-specific glycoproteins (PSG) which are mainly synthesized by placental syncytiotrophoblasts (Teglund et al., 1995), only *PSG4* was detected and up-regulated after 5 days of SU treatment. Increased levels of pregnancy specific glycoproteins (PSGs) and hCG have been associated with the placental pathological status of preeclampsia (Merviel et al., 2001; Vaiman et al., 2005). But the specific up-regulation of only PSG4 in the SU treated cells hint towards some special role for this glycoprotein in β-hCG secreting epithelial syncytiotrophoblast, which needs further investigation.

Another gene, *TAC3* (tachykinin 3) was highly expressed in SU treated cells after 5 days. TAC3 or NKB is expressed by the outer syncytiotrophoblast (Page et al., 2006) and plays a role in maintaining high placental blood flow in normal pregnancy (Laliberte et al., 2004) and in regulating fetal placental vascular tone (Brownbill et al., 2003). In pre-eclampsia , elevation of TAC3 expression causes increased circulating levels of the protein (Page et al., 2006). Due to its high expression in placenta at term and preterm labor, its role in parturition has also been suggested (Torricelli et al., 2007).

The transcription factors, *TEAD3, GATA2* and *GATA3* were upregulated in SU treated cells. TEAD3 is upregulated during the differentiation of cytotrophoblasts to syncytiotrophoblast in vitro and is

explicitly expressed in differentiated syncytiotrophoblast of the human term placenta (Jacquemin et al., 1998). In cooperation with a GATA-like protein, it regulates HSD3B1, which is one of the key enzymes involved in estrogen and progesterone synthesis (Peng et al., 2004). GATA 2 and 3 play a very crucial role in trophoblast differentiation (Home et al., 2009; Kim et al., 2009; Ng et al., 1994).

CYP11A1 (P450scc) initiates biosynthesis of all steroid hormones in the human placenta. The transcription factor CP2-like 1 (TFCP2L1 or LBP9) mRNA expression is restricted to syncytiotrophoblasts from both first-trimester and full-term placenta (Henderson et al., 2008) and is required for the stimulation of CYP11A1 in human placental JEG-3 cells (Huang and Miller, 2005).

To conclude, BMP activation, along with the inhibition of ACTIVIN/NODAL and FGF signaling supports the differentiation of hESCs to trophoblast lineage and when this treatment is continued for 5 days, the cells adopt the more specialized, syncytiotrophoblast differentiation. The SU-treated syncytiotrophoblast cells exhibit various functional aspects, like the activation of GnRH pathway, which induces hCG secretion, PPAR signaling, estrogen, steroid, progesterone and prostaglandin biosynthesis and metabolism etc. The up-regulation of various tumor suppressors and other genes, reported to be associated to placental disorders, like pre-eclampsica in these cells, also makes them suitable for invitro study to understand the pathology of these human placental abnormalities, which could provide direction for the pre-clinical development of rational therapeutics.

3.8: The inhibition of FGF pathway supports differentiation of hESC to hCG secreting trophoblast cells and additional BMP activation and ACTIVIN/NODAL accelerate the process

The results till now revealed that BMP signaling, in the absence of both ACTIVIN/NODAL signaling and FGF signaling (SU treatment), directs the hESC only to trophoblast lineage and not to embryonic lineages. But, BMP signalling, in the presence (B) or absence of ACTIVIN/NODAL signaling (SB) or in the presence of FGF signaling (F) can support mesodermal, endodermal or extra-embryonic differentiation and does not support neurectodermal differentiation. From this, we hypothesised that the additional blocking of FGF signaling might be responsible for driving the hESCs solely to trophoblast lineage. To confirm this, with the same growth conditions, we only blocked the FGF signaling pathway, using the FGFRI inhibitor, SU5402 (SUO) and kept this treatment active for 5 days. Along with this, we also maintained the SU treatment and two other treatments, in which additionally BMP signaling was, activated (BSU) or The SMAD2/3 branch was blocked (SBSU). After 5 days of treatment, we checked for the presence of hCG hormone in the

media through ELISA (Fig. 3.8 a). hCG was detected in the media from all the treatments, which involved the inhibition of FGFRI (SU, BSU, SBSU and SUO). The real time PCR data also revealed up-regulation of βhCG encoding *CGB* by 64 fold (Fig. 3.8 b).

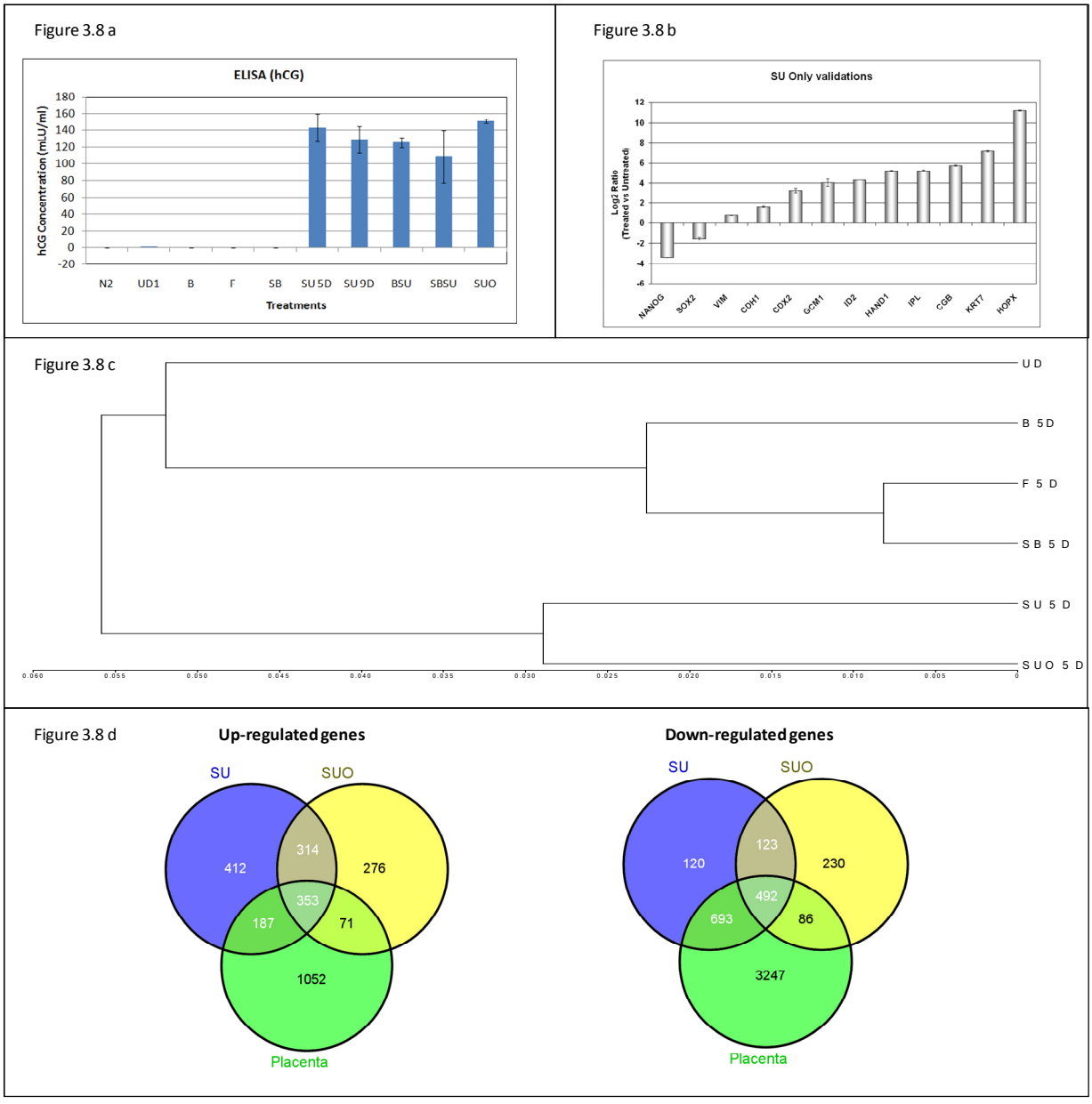


Figure 3.8: a) ELISA mediated estimation of concentration of βhCG in the growth media of treated cells (UD (Undifferentiated hESC), SU and SUO) after 5 days of treatment. **b)** Real time PCR validation of ES cell markers (OCT4, NANOG), EMT/MET genes (VIM, CDH1), trophoblast

markers (CDX2, GCM1, ID2, HAND1, IPL, KRT7) and tumour suppressor (HOPX or HOP or NECC1). x axis represents genes. y axis represents Log2 Ratio between treated and untreated samples. Normalization was carried out against GAPDH gene expression. **c)** Hierarchial clustering of the whole normalized data from all the samples after 5 days of treatment. d) Venn diagrams representing the overlap in the number of up- or down-regulated genes in placenta and 5 days of treatments, SU and SUO. The regulated list was obtained using a cut-off of atleast 2 fold and detection p value, less than 0.01.

As seen in the media from SU treated cells, we could detect hCG hormone in the media from SUO treated cells. There were some differences in terms of gene expression between these two treatments. The trophoblast marker, *CDX2* was more than 5 fold up-regulated in the SUO treated cells. In SU treated cells, *CDX2* has shown transient expression and was seen to be down-regulated, after 5 days, when compared to the previous treatment. The mesenchymal marker, *Vimentin* was not significantly regulated, when compared to the undifferentiated cell, but the epithelial marker, *CDH1* was up-regulated by more than 3 fold, showing the epithelial behavior of these cells. *GCM1*, which is required for driving the cells towards cell fusion, was also up-regulated by 16 fold in these cells. The microarray data revealed up-regulation of the fusogens, *ERVWE1* and *HERV-FRD*. Therefore, blocking of FGF signaling in hESCs induce fusogens. As seen in SU-treated cells, the trophoblast markers, *ID2, HAND1, IPL* and *KRT7* were also upregulated, when only FGF signaling was inhibited (SUO). The tumor suppressor, *HOPX (NECC1)* was upregulated by more than 2000 fold in these cells. Taken together, it can be concluded that inhibition of FGF signaling in hESCs leads to their differentiation into epithelial hCG hormone secreting trophoblast cells, in which trophoblast markers, fusogens and the tumor suppressor, *HOPX* are induced in 5 days.

The transcriptome analysis (Fig. 3.8 c) using microarrays revealed that SUO treated cells, in which FGFR1 was inhibited, shares the closest transcriptome with that of SU treated cells, which had in addition, BMP signal activation along with ACTIVIN/NODAL signal inhibition. Though the two treatments had similar influence on the transcriptome of the cells, in terms of morphological changes observed, the morphology of SU treated cells changed much more drastically than that of SUO treated cells. This could be because of the effect of additional activation of BMP signaling and inhibition of ACTIVIN/NODAL signaling included in the SU treatment. Additionally, SU treated cells had greater overlap of the regulated genes, with that of the placenta mainly in terms of the down-regulated genes (Fig. 3.8 d). Approximately, 80% of the down-regulated genes in SU treated

cells overlapped with that of placenta, but SUO treated cells had only 60% overlap. Out of the upregulated genes, approximately 40% of them overlapped with that of placenta in both SU and SUO treated cells. Taken together, the inhibition of FGF pathway supports differentiation of hESC to hCG secreting trophoblast cells and additional BMP activation and ACTIVIN/NODAL accelerate this process of differentiation.

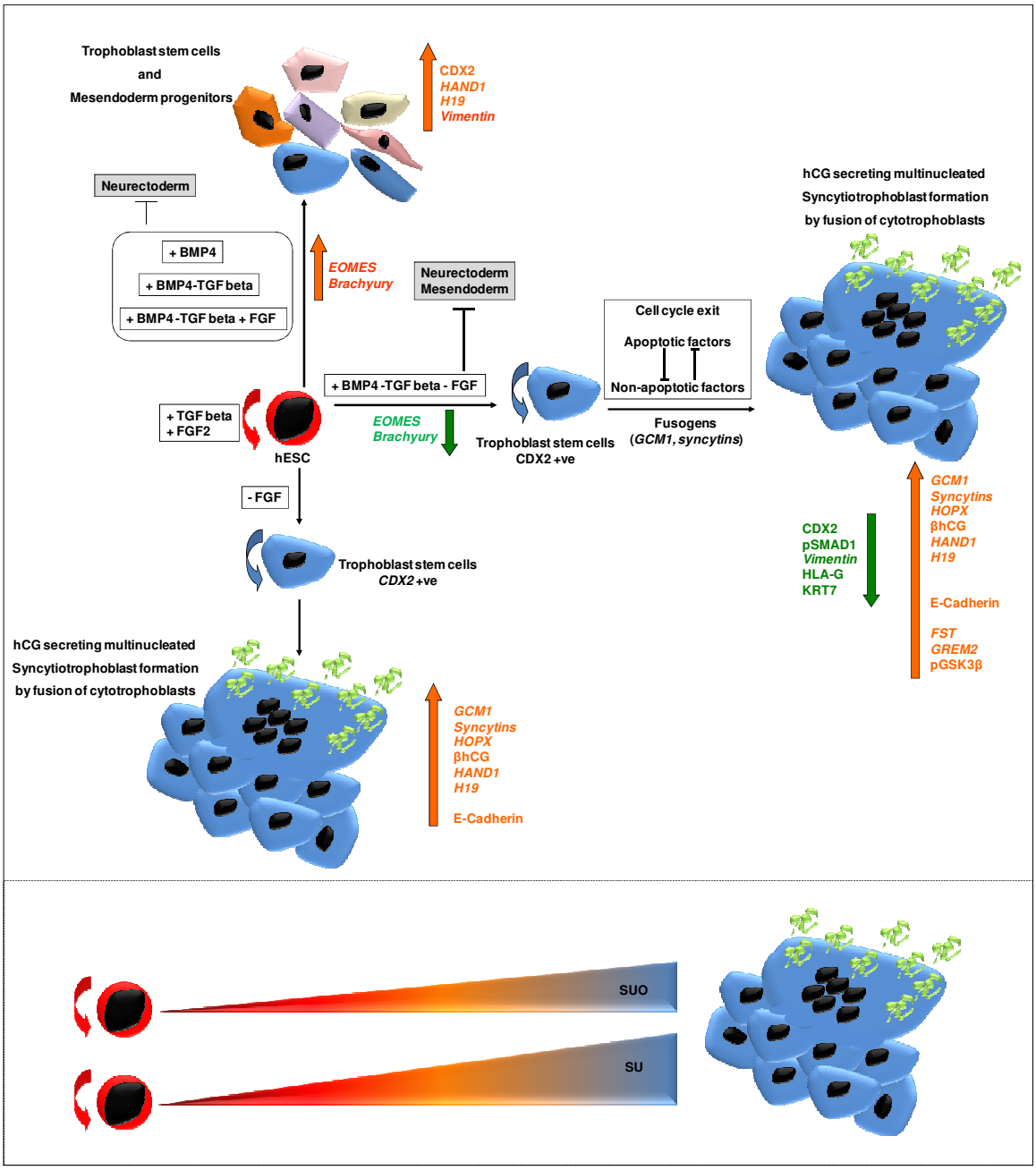


Figure 3.9: A model representing all the results and observations from this project in a gist. (Red arrows: Up-regulation; Green arrows: Down-regulation; Nuclei are black in colour; hCG secretion is depicted as green structures inside and around the multinucleated syncytiotrophoblast) BMP signaling supports differentiation of hESCs to embryonic lineages, namely mesoderm and endoderm, which has been concluded from the transient up-regulation of mesendoderm markers, EOMES and T (Brachyury) and also various other lineage specific markers. The treatments involving activation of BMP signaling prevent hESCs from entering the differentiation towards neurectodermal lineage. Inhibition of FGF signaling (-FGF) supports differentiation of hESCs to the extraembryonic trophoblast lineage and induces cell fusion events via the induction of fusogens such as GCM1 and Syncytins, which are instrumental in the formation of βhCG hormone secreting multinucleated syncytiotrophoblast, possessing epithelial characteristics (E-cadherin positive). In conjunction with the inhibition of FGF signaling, when another self-renewal supporting pathway of hESCs, namely ACTIVIN/NODAL signaling is inhibited and BMP signaling is activated (+BMP-TGF-FGF), this differentiation process is accelerated. There was enhanced similarity in terms of transriptome regulation with that of placenta, in SU-treated cells (+BMP-TGF-FGF) than SUO-treated cells (-FGF). The SU treatment induces cell fusion events and causes cell-cycle-exit, without causing p53-mediated apoptosis, in which the activation of MDM2 and a balance between apoptotic and anti-apoptotic factors might be instrumental. In a gist, BMP signaling activation or an additional inhibition of ACTIVIN/NODAL signaling either with or without exogenous FGF signaling activation supports differentiation of hESCs to embryonic mesendoderm and extraembryonic trophoblast lineages. Inhibition of FGF signaling, either alone or in conjunction with BMP signaling activation and ACTIVIN/NODAL signaling inhibition induces hESC differentiation to epithelial, βhCG hormone secreting multinucleated syncytiotrophoblast, the latter treatment inducing an accelerated differentiation process.

Discussion

Activation of BMP signaling has the potential to direct the hESCs to multiple lineages, like trophoblast (Xu et al., 2002), extra-embryonic endoderm (Pera et al., 2004), mesoderm (Zhang et al., 2008) and hematopoietic cells (Chadwick et al., 2003). For the cells to narrow down to a specific lineage from this broad range of possibilities, will require cues from other signalling pathways, which can have synergistic or antagonistic effects on each other, strongly influencing the lineage choices made by the cells. This lineage choice, made based on the interaction of other pathways with BMP signalling can control the degree of activation of the signal as well as the downstream actions of this pathway. Therefore we set out to understand the influence of ACTIVIN/NODAL (TGFβ) and FGF signalling on the lineage choices made by hESCs, when BMP signalling is activated exogenously. Both ACTIVIN/NODAL (TGFβ) and FGF signalling have been proven to play crucial roles in lineage specification during gastrulation in various organisms.

As BMP, ACTIVIN/NODAL (TGFβ) and FGF signalling pathways can have variable roles to play for directed lineage specification, depending on the signalling environment, their quantitative effects can be measured using lineage specific markers and the differentiation state of the cells. The presence of BMP signalling in all the treatments kept the cells from entering neuronal differentiation(Gaulden and Reiter, 2008; Greber et al., 2008; Munoz-Sanjuan and Brivanlou, 2002; Vallier et al., 2009) though in some of the treatments, the ACTIVIN/NODAL pathway was blocked, the inhibition of which can support neurectodermal (Smith et al., 2008; Vallier et al., 2009) and trophoblast differentiation (Wu et al., 2008). A reciprocal feedback loop exists between ACTIVIN/NODAL and BMP signalling, in which the former has an inhibitory effect on BMP mediated trophoblast differentiation and the inhibition of the same induces BMP4 (Wu et al., 2008). It has also been reported that BMP signalling mediated differentiation to extra-embryonic lineages strongly increases, when ACTIVIN/NODAL pathway is blocked (Vallier et al., 2009). Our results compliment these findings. When only BMP signalling (B) was activated, after duration of 5 days, the extent of differentiation was minimal, compared to all the other treatments (F, SB, SU). In addition, when ACTIVIN/NODAL signalling was blocked, the extent of differentiation was much higher and directed more towards trophoblast lineage, as seen from the results from principal component analysis and overlap with placental genes. hESCs exposed to all the treatments, except for the one, in which FGF signalling was blocked, showed differentiation potential of the cells

towards both extra-embryonic and embryonic mesendoderm lineages, but not neurectoderm lineage. Additionally, the positive effect on BMP-mediated differentiation by inhibition of ACTIVIN/NODAL signalling to extra-embryonic lineages was accentuated and accelerated with the additional inhibition of FGF signalling. The non-regulation of extra-embryonic endoderm marker, SOX7 and the continuous down-regulation of mesendoderm markers, *T* and *EOMES* showed that the cells were entering neither of these two lineages nor neurectoderm lineage. But these cells had an increased overlap with placental genes and other lineage specific markers such as *GATA6, SOX17, SOX1,* etc. (Fig. 3.5 b) were either down-regulated or their expression remained unchanged. Therefore, BMP-mediated differentiation, involving the inhibition of both ACTIVIN/NODAL and FGF signalling incline the hESC differentiation towards trophoblast in an accelerated way and decrease or hinder their differentiation tendency towards both embryonic and extraembryonic lineages.

Because of the earlier down-regulation of *MYC*, along with *NANOG* before the down-regulation of other ES cell markers, like *OCT4* in the treatments involving BMP activation and ACTIVIN/NODAL inhibition, we propose the possibility of *MYC* to be a second gatekeeper of pluripotency. Previously, *NANOG* down-regulation has been identified to incline the undifferentiated ES cells towards differentiation, a reversible predisposed state (Chambers et al., 2007; Silva et al., 2009). We propose the loss of *NANOG* and *MYC* to be the initial signs of losing pluripotency, which can define the "Gold standard" of stem cell quality, which requires further investigation.

In mouse, both Cdx2 and Eomes are responsive to FGF signalling in cultured TS cells (Cross, 2000; Rossant and Cross, 2001; Russ et al., 2000; Tanaka et al., 1998) and their differentiation into either syncytiotrophoblast or trophoblast giant cells is associated with down-regulation of these genes and up-regulation of transcription factors, specific to the respective cell type (Cross, 2000; Hughes et al., 2004; Selesniemi et al., 2005; Tanaka et al., 1998). Upon the activation of BMP signalling and inhibition of both ACTIVIN/NODAL and FGF pathways, CDX2 was transient up-regulation and *EOMES* showed continuous down-regulation. This led us to the possibility that these cells have lost the trophoblast stem cell (TS) state and assumably entered a more differentiated trophoblast state. The trophoblast stem cells, which are also called cytotrophoblast cells, can differentiate into either villous syncytiotrophoblast cells or invasive extravillous cytotrophoblasts (Enders et al., 2001). A detailed study of these cells, in which BMP signalling was activated and both ACTIVIN/NODAL and FGF signalling were blocked, led us to rule out their possibility of being extravillous trophoblast

based on the lack of its characteristics, namely, HLA-G (Fig. 3.7.1 A, B) and KRT7 protein expression (Fig. 3.7.1 C) and glycogen storage (Fig. 3.7.1 D) in these cells. Because of the up-regulation of various syncytiotrophoblast-specific genes and pathways, in addition to βhCG hormone secreting ability, we concluded that differentiation is towards epithelial syncytiotrophoblast lineage. These results drew us to the conclusion that the activation of BMP signalling and inhibition of both ACTIVIN/NODAL and FGF pathways, supports differentiation of hESCs to epithelial syncytiotrophoblast and does not support extravillous cytotrophoblast differentiation.

Our results unfold the dynamics of *CDX2* and *EOMES* during human ESC differentiation into syncytiotrophoblast. *EOMES* was transient up-regulation in all the treatments, except for the one, in which FGF signalling was blocked, where it was continuously down-regulated. This is in contrast to its function in mouse, in which it has been shown to have an important role in trophoblast differentiation and is also a downstream target of ACTIVIN/NODAL signalling in mouse and Xenopus (Brennan et al., 2001; Ryan et al., 1996). In hESCs, when both FGF and ACTIVIN/NODAL signalling are blocked, though there is continuous down-regulation of *EOMES*, they differentiate into syncytiotrophoblast. From this, we conclude that *EOMES* might not be essential for hESC differentiation into syncytiotrophoblast.

CDX2 is required for initiation of trophoblast differentiation of ES cells and maintaining TS cell fate with respect to the inner cell mass (Beck et al., 1995; Chawengsaksophak et al., 2004; Strumpf et al., 2005). CDX2 expression was transient in the treatments involving BMP activation and ACTIVIN/NODAL inhibition, but remained up-regulated all throughout, when FGF signalling was kept active. Cdx2 expression is dependent on FGF4-induced MEK-ERK pathway in mouse TS cells (Murohashi et al.), but our results showed that *CDX2* was transiently up-regulated, even when FGF signalling was blocked or not blocked exogenously. Therefore, even in the absence of FGF signalling, *CDX2* is up-regulated transiently during BMP activation- and ACTIVIN/NODAL inhibition- mediated differentiation of hESCs to syncytiotrophoblast. There was no alteration in the cell fate in the trophectoderm or no loss of Cdx2 expression upon the treatment of mouse embryos with FGFR/MEK inhibitors, which was thought to be attributed to the production of FGF4 by the undifferentiated ES cells in the ICM as a direct response to Oct4/Sox2 regulation (Yamanaka et al., 2010). In another study done on ACTIVIN/NODAL inhibition driven trophoblast differentiation of hESCs, both transient expression of *CDX2* and down-regulation of *EOMES* have been reported (Wu et al., 2008). Our results, along with these known facts, suggest the conservation of the dependence

of CDX2 expression sustenance on FGF signalling in both mouse and human and its down-regulation upon differentiation to syncytiotrophoblast, marked by the up-regulation of syncytiotrophoblast-specific genes such as βhCG encoding *CGB, GCM1, ERVWE1*, etc. During hESC differentiation, activation of CDX2 is supported by BMP activation and ACTIVIN/NODAL inhibition and does not require FGF signalling, but the same is required for its sustained expression in both mouse and human.

We did not investigate the necessity of EOMES in the differentiation of hESCs to extravillous trophoblast, which has invasive capacity and is mesenchymal. TGFβ1 mediated ACTIVIN/NODAL signalling is involved in epithelial to mesenchymal transition (Bhowmick et al., 2001). Therefore, the possibility of the requirement of ACTIVIN/NODAL signalling and/or EOMES for differentiation of hESCs to extravillous trophoblast cannot be ruled out and needs to be investigated.

Because of the huge impact on hESC differentiation in terms of both lineage specification and speed of differentiation, we looked for the influence on these cells when only FGF signaling was blocked. Upon mere FGF inhibition, both trophoblast and syncytiotrophoblast specific markers, including fusogens were up-regulated and βhCG hormone was also secreted by the cells after 5 days (Fig. 3.7.3). But in addition to FGF inhibition, when BMP signaling was activated and ACTIVIN/NODAL pathway was blocked, morphological changes were seen earlier and also the extent of up-regulation of many of the markers was much higher, showing enhanced effectiveness. The results revealed that upon FGF inhibition, hESCs are directed towards trophoblast differentiation, more specifically syncytiotrophoblast and their tendency of differentiation towards both embryonic lineages and extraembryonic endoderm are reduced or affected. The epithelial syncytiotrophoblast differentiation potential is enhanced by BMP activation and ACTIVIN/NODAL inhibition.

FGF signaling is known to promote the differentiation of mouse ES cells (Burdon et al., 1999; Wilder et al., 1997; Ying et al., 2003), whereas in HESCs, FGF2 or FGF4 support maintenance of pluripotency (Greber et al., 2007; Mayshar et al., 2008; Thomson et al., 1998; Vallier et al., 2005; Xu et al., 2005). During early embryonic development, cell fate decisions can be influenced based on the presence or absence of FGF signaling (Yamanaka et al.,2010 ; Zernicka-Goetz et al., 2009) and there have been also reports on the role of this pathway in differentiation of hESCs. FGF-2 has been shown to induce development of ectodermal and mesodermal cells from pre-differentiated hESCs and to support hESC differentiation into neural lineages (Bendall et al., 2007; Carpenter et al., 2001; Cohen et al., ; Dvorak et al., 2005; Park et al., 2004; Reubinoff et al., 2001; Schuldiner et al., 2000;

Schulz et al., 2003; Zhang et al., 2001). A recent study also throws light on its conserved ability to inhibit neuroectodermal commitment of EpiSCs and hESCs (Greber et al., 2010). FGF signaling has also been shown to support endodermal differentiation of hESCs (Ameri et al., 2010; Johannesson et al., 2009). Therefore, the role of FGF signaling is permissive, rather than unidirectional as its action is dependent on the evolving signaling environment, which can influence its activity in a synergistic or antagonistic way to direct the differentiation process or maintain pluripotency.

The influence of FGF pathway related changes, influencing lineage specification in mouse has been studied in more detail than human and many of these functions could be conserved between the species, though differences cannot be ruled out. During mouse blastocyst maturation, lineage specification towards epiblast and primitive endoderm occurs in an FGF/MAP kinase signal-dependent manner, sustained inhibition of which keeps primitive endoderm formation at bay and excessive activation promotes the same (Yamanaka et al., 2010). Inhibition of FGFR/ERK/GSK3 signaling blocked primitive endoderm formation (Nichols et al., 2009). Hindrance to FGF or ERK pathway restricts the differentiation potential of ES cells and resists their neural and mesodermal induction potential (Kunath et al., 2007). In mouse, FGF4 has been detected in primitive streak in addition to some of the mesendoderm and neurectoderm lineages (E7.5–E8.5) and during the early stage of gastrulation, FGF4 expression is restricted to primitive streak (Niswander and Martin, 1992). EMT is essential for mesoderm migration and morphogenesis at the primitive streak, which is controlled by FGFR1 that promotes the same through the control of Snail and E-Cadherin expression (Ciruna and Rossant, 2001). Experiments with FGF4 deficient mice have shown that cavitation, initial allocation of ICM cells and compaction during preimplantation development do not require FGF signaling (Chai et al., 1998). But, it is required for ICM proliferation in vitro, cell division in extraembryonic ectoderm in preimplantation embryos and postimplantation development (Chai et al., 1998; Feldman et al., 1995). Fgf4 null embryos didn't develop endoderm (Chai et al., 1998) and degenerate shortly after uterine implantation (Feldman et al., 1995). Disruption of Fgf4 signaling antagonises neural and mesodermal induction in ES cell (Kosaka et al., 2009). In mouse embryos, made with dominant negative FGF receptor (dnFGFR), cell division was affected, though there was no increase in cell death (Chai et al., 1998). These findings emphasize the crucial role of FGF signaling in primitive streak, mesoderm migration and morphogenesis, endoderm formation and neural development, all of which are affected in its absence, leading to rapid postimplantation degeneration. Extraembryonic ectoderm formation can occur in the absence of FGF signaling in mouse embryos without causing apoptosis, though its requirement for proliferation and growth of the

same has been shown. These findings also highlight the requirement of FGF signaling for cell proliferation during early development.

FGF4 (Tanaka et al., 1998) and TGFβ maintain long-term continuous TS cell proliferation (Erlebacher et al., 2004) and Nodal and FGF4 directly act on extraembryonic ectoderm to inhibit differentiation of trophoblast stem cells (Guzman-Ayala et al., 2004). Inactivation of MEKK4, which is a signaling hub for FGF4 activation of JNK, in trophoblast stem cells show a preferential differentiation to spongiotrophoblast and syncytiotrophoblast in mouse (Abell et al., 2009). Though it is required for the proliferation and growth of extraembryonic ectoderm, its absence neither affects its formation nor increases apoptosis (Chai et al., 1998). In our experiment, the treatments involving the inhibition of FGF signaling showed the maximum inclination towards trophoblast differentiation and also showed decreased tendency towards the formation of both embryonic lineages and extraembryonic endoderm.

Our results emphasise the importance of the presence of FGF signaling in the differentiation of human embryonic stem cells to the embryonic lineages and extraembryonic endoderm and this could be evolutionarily conserved in mouse and human. Our results show that under defined culture conditions (N2B27), upon the inhibition of FGF signaling, hESCs adopt an inclination for differentiation towards non-apoptotic cell cycle arrested trophoblast cells, harbouring various functions of the syncytiotrophoblast including βhCG hormone secretion. This effect is accentuated by the additional activation of BMP signaling and inhibition of ACTIVIN/NODAL signaling, which also show enhanced epithelial characteristics, native to syncytiotrophoblast. Our in vitro study suggest that the presence or absence of FGF pathway can play a very crucial role in the maintenance and lineage specification of hESCs and its role can be influenced by the signaling environment in and around these cells.

hESC differentiation to syncytiotrophoblast through BMP activation and inhibition of ACTIVIN/NODAL and FGF pathways, used as a model system to study human syncytiotrophoblast:

FGF inhibition supports hESCs to differentiate in the direction of extraembryonic trophoblast lineage. In addition, when BMP signalling was activated and ACTIVIN/NODAL signalling was blocked, the direction towards epithelial trophoblast, the syncytiotrophoblast was accentuated, as seen from the increased overlap with placental genes such as βhCG encoding CGB, GCM1, etc.

Therefore, we studied this differentiation process in detail, to get more insights into the result of BMP activation and inhibition of both ACTIVIN/NODAL and FGF pathways in hESCs.

Trophoblast stem cells can differentiate into either villous syncytiotrophoblast cells or invasive extravillous cytotrophoblasts (Enders et al., 2001). The transient up-regulation of CDX2, which is associated with the differentiation of trophoblast stem cells to further differentiated cell types, syncytiotrophoblast or trophoblast giant cells (Cross, 2000; Hughes et al., 2004; Selesniemi et al., 2005; Tanaka et al., 1998) led us to check for the cell type specific markers and characteristics, to ascertain the exact direction of differentiation.

The cells in which BMP was activated and both ACTIVIN/NODAL and FGF signaling were blocked, were positive for the syncytiotrophoblast specific subunit (Randeva et al., 2001) βhCG both at mRNA (*CGB*) and protein levels and also secreted the βhCG hormone. hCG is an autocrine and paracrine regulator, which can stimulate the differentiation of cytotrophoblasts, that make less hCG, to syncytiotrophoblast in human placenta (Shi et al., 1993; Yang et al., 2003). These cells were negative for the extravillous trophoblast marker HLA-G (Chumbley et al., 1993; Goldman-Wohl et al., 2000; McMaster et al., 1995; Yelavarthi et al., 1991) and a villous cytotrophoblast (Baal et al., 2009; Cervar et al., 1999) and human invasive trophoblast – expressed gene (Handschuh et al., 2009; Tarrade et al., 2001), KRT-7 at the protein level. The up-regulation of these genes at the mRNA level, but significant reduction or absence at protein level could be due to the role of small RNAs like microRNAs, which can cause translational repression. Moreover, β-hCG secreting syncytiotrophoblast cells or lining are known to be largely cytokeratin-negative (Badwaik et al., 1998; Maldonado-Estrada et al., 2004; Manoussaka et al., 2005). In addition, these cells also showed poor glycogen storage ability, a characteristic attributed to them from their analogous mouse counterpart, the glycogen trophoblast cells (Georgiades et al., 2002). The syncytiotrophoblast sustains epithelial characteristics and the extravillous invasive cytotrophoblast cells undergo epithelial-mesenchymal transition (EMT), which infiltrate into the maternal decidual stroma and blood vessels (Charnock-Jones et al., 2004; Kingdom et al., 2000; Vicovac and Aplin, 1996; Zygmunt et al., 2003).

The positive regulation of the epithelial marker, E-Cadherin at both mRNA and protein levels, along with no significant regulation of the mesenchymal marker, Vimentin (*VIM*) showed that these cells were epithelial. An explanation for this could be that both FGFR1 (Ciruna and Rossant, 2001) and TGFβ1 mediated ACTIVIN/NODAL signalling (Bhowmick et al., 2001), which promote epithelial

to mesenchymal transition are blocked in this treatment. Activin A could promote invasion of the first-trimester cytotrophoblasts (Bearfield et al., 2005) and extravillous cytotrophoblasts from first-trimester explants (Caniggia et al., 1997b). Many processes in the syncytiotrophoblast formation and functions, like cell fusion events (Fulton et al., 1981; Ramos et al., 2008), ion channels and transport (Montalbetti et al., 2005), endocytosis for nutrient transport (Fuchs and Ellinger, 2004), require extensive cytoskeletal reorganization which is associated to actin cytoskeleton human syncytiotrophoblast (Smith et al., 1977) and a majority of genes involved in the same were induced upon this treatment. Moreover, these cells were exhibiting the activity of many genes, processes and pathways, which are known to be functional in syncytiotrophoblast. All these results strengthened the fact that BMP activation, along with the inhibition of both ACTIVIN/NODAL and FGF pathways support differentiation of hESCs only to epithelial βhCG-secreting syncytiotrophoblast lineage and does not support extravillous trophoblast lineage.

From the clustering results of microarray data, we have discovered genes, which shared similar profile with that of the βhCG encoding genes, *CGB, CGB1, CGB5, and CGB7* (Figure 3.7.4 c, Figure 3.7.4 e). They included the transcription factors, *GCM1* and *HOP (NECC1)*, the fusogens, *ERVWE1* and *HERV-FRD*, the genes involved in steroid synthesis and metabolism, *HSD3B1, HSD17B1*, cytochrome P450 genes, *CYP19A1, CYP11A1, CYP2J2* etc. *GCM1* (Baczyk et al., 2004; Nait-Oumesmar et al., 2000), syncytin-1 (*ERVWE1*) (Frendo et al., 2003a), syncytin-2 (*HERV-FRD*) (Blaise et al., 2003) are responsible for cell fusion events during the differentiation of trophoblast stem cells into multinucleated syncytiotrophoblast. Steroid synthesis and metabolism, which is a subset of xenobiotic metabolism, βhCG hormone production and secretion, decreased or no proliferation, are some of the properties, native to multinucleated syncytiotrophoblast. Because of their similar expression profile during the differentiation process and the already known transcription regulation of syncytin-1 (Yu et al., 2002) and aromatase (Yamada et al., 1999) by GCM1, there is a possibility that all these functions are inter-regulated, in which the genes in these clusters (Figure 3.7.4 e; Figure 3.7.4 c) might be some of the contributing entities. Also, as *GCM1* and *HOP* are transcription factors, they might be playing a major role in the regulation of many of these co-regulated genes bestowing these functions in human syncytiotrophoblast.

The transcription factor, HOP is a tumor suppressor gene, exclusively expressed in syncytiotrophoblast and not in extravillous trophoblasts (EVT) in humans, whereas in mouse, it is expressed in trophoblast giant (TG) cells which are the orthologues of human EVT, and

spongiotrophoblast cells (Asanoma et al., 2007). The diverse localization shows the diverse functions, HOP can have in mouse and human placenta. The exact role of this gene in placenta has not been deciphered yet. But its ability of induction of syncytiotrophoblast formation (Asanoma et al., 2003) and its specific expression in syncytiotrophoblast give clues to its supportive action in human syncytiotrophoblast. Inactivation of Hop in mice by homologous recombination results in heart failure and lethality (Chen et al., 2002b; Shin et al., 2002). HOP negatively regulates SRF-dependent transcriptional activation by recruiting histone deacetylase (HDAC) and can form a complex that includes HDAC2 (Kook et al., 2003). In SU treated cells, HOP showed very abrupt up-regulation after 5 days (Figure 3.7.8 a 1, 2), which was coinciding with the appearance of syncytiotrophoblast specific functions and the down-regulation of *HDAC1* and *HDAC2* (microarray data). There is evidence of suppression of *Cdx2* promoter by the Hdac complex, along with Sall4 to prevent trophoblast differentiation in mouse (Yuri et al., 2009). From the microarray data (data not shown), down-regulation of *SALL4* was also revealed. HDAC inhibition has been shown to support differentiation of murine trophoblast stem cells to syncytiotrophoblast (Maltepe et al., 2005). This suggests a possible role of HOP in regulating chromatin remodelling during differentiation of hESCs to syncytiotrophoblast and its tumor suppressor ability might be contributing to the known absence of proliferation of syncytiotrophoblast (Ichikawa et al., 1998; Wakuda and Yoshida, 1992).

Due to the specific up-regulation of members of SNARE interactions in vesicular transport pathway which is involved in membrane fusion (Hu et al., 2003; McNew et al., 2000; Weber et al., 1998), we propose its role in syncytiotrophoblast differentiation and also suggest the role of BMP activation and inhibition of both ACTIVIN/NODAL and FGF pathways in the activation of this pathway.

Syncytiotrophoblast is the non-proliferative layer of the placenta, which is formed by continuous fusion of cytotrophoblasts (Ichikawa et al., 1998; Wakuda and Yoshida, 1992) and is thought to represent mitotically end-stage cell (Benirschke and Kaufman, 1995). When BMP signaling was activated and both ACTIVIN/NODAL and FGF pathways were blocked for 5 days in hESCs, the cells showed a cell cycle profile, in which S, G2 and M phase were compromised, as seen from the down-regulation of the respective CDKs and cyclins (Figure 3.7.5 a, b and Supplementary table 5). GADD45G, a CDK1/CYCLIN B1 inhibitor (Vairapandi et al., 2002), which is highly expressed in placenta also showed up-regulation, exhibiting the abrogation of mitosis. This was emphasised with a clear reduction in the phosphorylation of histone H3 (H3p) (Figure 3.7.5 b), a downstream marker for chromosome condensation and entry into mitosis (Juan et al., 1998). The G1 CYCLIN/CDK

inhibitors, *P57 (CDKN1C)* and *p21* were strongly up-regulated in SU treated cells, exhibiting negative regulation of cell proliferation. The expression of CDK inhibitors, p16, p21 and p57 have been observed in term placental tissue, indicating a reduction in cytotrophoblast stem cell population and a inclination for syncytiotrophoblast formation (Genbacev et al., 2000). *CDK2* and *CYCLIN E*, involved in G1-S phase transition were upregulated and also *CYCLIN E*, being a predominant cyclin in villous trophoblast and its possible role in trophoblast terminal differentiation has been suggested before (DeLoia et al., 1997). From these results, we conclude that in hESCs, when BMP signaling was activated and both ACTIVIN/NODAL and FGF pathways were blocked, their differentiation to syncytiotrophoblast involves cell cycle arrest, resulting in reduced cell-proliferation.

Cell cycle arrest can initiate apoptosis. Both the apoptotic (*BAD, BAX, and BID*) and anti-apoptotic (*BCL-2 and BCL2L1 (bcl-xL)*) Bcl-2 family members were seen to be up-regulated in a span of 5 days treatment. BCL-2 protein is expressed in syncytiotrophoblast and syncytial knots, suggesting protection from apoptosis (Quenby et al., 1998; Ratts et al., 2000; Toki et al., 1999). Bcl-2 related proteins act as the arbiters of cell survival by regulating apoptosis through the inhibition of adapters needed for the activation of caspases, that decimate the cell and in many cases, the balance between apoptotic and anti-apoptotic competing activities determines cell fate (Adams and Cory, 1998). Higher activity of apoptosis inducers, caspases 3, 6, 8 and 9 has been reported in mononucleated cytotrophoblasts (Yusuf et al., 2002). Caspase 8 has been sighted in just highly differentiated cytotrophoblasts prior to fusion and not in syncytiotrophoblast and its role in escorting the fusing cell content including the nucleus into the syncytiotrophoblast has been suggested (Gauster et al., 2009). Out of the caspases, only caspase 6 showed up-regulation. The cells, which are treated in-vitro for 5 days, will have a heterogeneous population, harbouring mononucleated cytotrophoblasts, fusing cytotrophoblasts, multinucleated, syncytiotrophoblasts, etc. As caspases have been shown to be active in the cytotrophoblasts which are on the verge of syncytiotrophoblast differentiation and the microarray data is representative of the whole cell population, the region-specific expression can be diluted and might not be disclosed from this data. The G1 CYCLIN/CDK inhibitor, *p21*, which showed up-regulation, also plays a role in apoptosis inhibition it is expressed in immature trophoblast and is not found in choriocarcinoma cells (Quenby et al., 1998; Stahle-Backdhal et al., 1995). The DNA-damage driven apoptosis inducing factors, *CHEK1* (Liu et al., 2000; Takai et al., 2000), *CHEK2* and *p53* (Andreassen et al., 2001; Lanni and Jacks, 1998) showed down-regulation. Loss of p53 function and inhibition of apoptosis by bcl-2-related genes can cooperatively contribute to genetic instability (Minn et al., 1996). Our data revealed the modulation of p53-Mdm2 pathway,

through the up-regulation of *MDM2*, which is essential for the degradation of p53 and the down-regulation of p53 and its target genes, *MMP2* and *MMP9*, which are crucial for the invasive capacity of trophoblast (Lam et al., 2009). The p53-Mdm2 pathway is important for the development of trophoblast and its lineages (Chiu et al., 2008). Thus, the differentiation of hESCs to syncytiotrophoblast involves cell cycle arrest, but viability is maintained through a balance between apoptotic and anti-apoptotic competing activities involving the activation of *BCL-2, BCL2L1, p21, CASPASE 6* and *MDM2* and inactivation of the *CHEK1, CHEK2* and *p53*.

Though BMP signalling was exogenously activated in these cells for a span of 5 days, there was a reduction in pSMAD1 levels and its targets, *CDX2* and *GATA5*. ACTIVIN/NODAL inhibition induces BMP4 expression in hESCs (Wu et al., 2008) which can induce CDX2 expression (Xu et al., 2002). Cdx2 inhibition causes down-regulation of *Bmp4* mRNA expression (Murohashi et al.). So, the down-regulation of *CDX2* might be one of the reasons for the reduction in BMP4 induced SMAD1 phosphorylation or the latter could be the reason for CDX2 down-regulation. In addition, we saw up-regulation of BMP antagonist, *GREM2* and increased phosphorylation of GSK3β, which can cause degradation of SMAD1 protein, through phosphorylation of its linker region (Fuentealba et al., 2007; Sapkota et al., 2007; Sieber et al., 2009). *GREM2* was also among the cluster of genes, co-regulated with the βhCG encoding gene, *CGB* (Figure 3.7.4 e). Hence, after 5 days differentiation of hESCs to syncytiotrophoblast, the cells' autocrine machinery is activated to defend the exogenously induced BMP signaling possibly through the production of *GREM2*, GSK3β phosphorylation or CDX2 down-regulation. But from our results, it is clearly seen that though BMP signaling initially supports the differentiation of hESCs to trophoblast, BMP signaling is negatively modulated during further differentiation to syncytiotrophoblast in humans.

Though the SMAD1 levels were reduced, its targets, *GATA2* and *3*, which are critical for the expression of Cdx2 (Home et al., 2009) and human syncytin (Cheng and Handwerger, 2005) remained up-regulated (Home et al., 2009; Kim et al., 2009; Ng et al., 1994). Another BMP signalling target gene *ID2*, which has a role in cytotrophoblast development (Janatpour et al., 2000) and extravillous trophoblast differentiation (Fisher, 2000; Quenby et al., 2005) was expressed. There could be a possibility that either a lower extent of BMP activation is enough for the expression of these genes or their induction-role might be taken over by some other BMP or SMAD1 – independent mechanism, which needs to be investigated.

There was an induction of an ACTIVIN antagonist, Follistatin (*FST*) (Harland and Gerhart, 1997), in these cells after 5 days of treatment. Follistatin is also known to bind other ligands, including inhibin, BMPs 2, 4, 6, 7, 11, and 15, and myostatin, with lower affinities (Abe et al., 2004; Canalis et al., 2003; Gumienny and Padgett, 2002). Follistatin is expressed in both cytotrophoblast and the syncytiotrophoblast (Muttukrishna et al., 1996) and there is there is a continuous rise in its levels throughout pregnancy (Fowler et al., 1998). It is localized in syncytial cells of placental villi at term and in chorionic cells (Petraglia et al., 1994). From the work done on zebra fish and NUCC-3 choriocarcinoma cell line, it is known that follistatin is capable of affecting hCG production (Shi et al., 1994; Wang and Ge, 2003) and also of reversing activin A induced hCG and progesterone release (Petraglia et al., 1994). hCG and forskolin can strongly increase the expression of follistatin through cAMP-PKA signaling (Wang and Ge, 2003) and also stimulate activin A expression (Pang and Ge, 2002). From the up-regulation of both follistatin and βhCG ecoding genes, and the cells' βhCG secreting ability, there seems to be an autoregulatory loop for the controlled expression of hCG and follistatin in human syncytiotrophoblast.

The abnormal functioning of the placenta has been known to cause very serious complications, the most common being preeclampsia and fetal intrauterine growth restriction (IUGR). Altered expression of tumor suppressor gene expression has been identified in pre-eclamptic placentas (Heikkila et al., 2005) and gestational trophoblastic diseases (Fulop et al., 1998a; Fulop et al., 1998b). Other than the above mentioned tumor suppressors, *HOP* and *E-Cadherin*, which can also have other functions in syncytiotrophoblast, there was an up-regulation of other known potential tumor suppressors, *TIMP3, RASA1* **(**Yang et al., 2009**)** and *DOC-2* in these cells after 5 days of differentiation. HOP is not found in choriocarcinoma cell lines and loss of its expression is involved in malignant conversion of placental trophoblasts (Asanoma et al., 2004). Hypermethylation of *E-Cadherin* and TIMP3, causing their reduced expression, has been associated with hydatidiform mole and choriocarcinoma that could subsequently develop into gestational trophoblastic diseases (Xue et al., 2004). TIMP3 can regulate epithelial cell proliferation by inhibiting MMP activity (Gill et al., 2006) and *MMP2* and *MMP9* were not detected and down-regulated, respectively in these cells. The public databases, NCBI and UniProt *RASA1* is amply expressed in placenta and in placental villi, detected only in the trophoblast layer (cytotrophoblast and syncytiotrophoblast). Based on the high expression of *DOC-2 (hDab2)* in normal trophoblast cells and normal trophoblast tissues, than in either choriocarcinoma cell lines or gestational trophoblastic disease tissues, it is thought to play an important role in the development of gestational trophoblastic diseases (Fulop et al., 1998a). Taken

together, high expression of tumor suppressors known to be functional in normal placenta and their aberrant expression, linked to placental disorders, like choriocarcinoma or gestational trophoblastic disease are up-regulated upon hESC differentiation to syncytiotrophoblast. Therefore, the in vitro differentiation of hESCs to syncytiotrophoblast, through BMP activation, in conjunction with the inhibition of ACTIVIN/NODAL and FGF pathways could be instrumental to get more insights into the pathology of human placental abnormalities.

Conclusion

BMP signaling supports differentiation of hESCs to embryonic lineages, namely mesoderm and endoderm, which has been concluded from the transient up-regulation of mesendoderm markers, EOMES and T (Brachyury) and also various other lineage specific markers. The treatments involving activation of BMP signaling prevent hESCs from entering the differentiation towards neurectodermal lineage. Inhibition of FGF signaling (-FGF) supports differentiation of hESCs to the extraembryonic trophoblast lineage and induces cell fusion events via the induction of fusogens such as GCM1 and Syncytins, which are instrumental in the formation of βhCG hormone secreting multinucleated syncytiotrophoblast, possessing epithelial characteristics (E-cadherin positive). In conjunction with the inhibition of FGF signaling, when another self-renewal supporting pathway of hESCs, namely ACTIVIN/NODAL signaling is inhibited and BMP signaling is activated (+BMP-TGF-FGF), this differentiation process is accelerated. There was enhanced similarity in terms of transriptome regulation with that of placenta, in SU-treated cells (+BMP-TGF-FGF) than SUO-treated cells (-FGF). The SU treatment induces cell fusion events and causes cell-cycle-exit, without causing p53-mediated apoptosis, in which the activation of MDM2 and a balance between apoptotic and anti-apoptotic factors might be instrumental. In a gist, BMP signaling activation or an additional inhibition of ACTIVIN/NODAL signaling either with or without exogenous FGF signaling activation supports differentiation of hESCs to embryonic mesendoderm and extraembryonic trophoblast lineages. Inhibition of FGF signaling, either alone or in conjunction with BMP signaling activation and ACTIVIN/NODAL signaling inhibition induces hESC differentiation to epithelial, βhCG hormone secreting multinucleated syncytiotrophoblast, the latter treatment inducing an accelerated differentiation process.

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PUBLICATIONS

1. Genome-wide expression profiling reveals distinct clusters of transcriptional regulation during bovine preimplantation development in vivo. **Proc Natl Acad Sci U S A. 2008** Dec 16;105(50):19768-73.
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Supplementary Information I: Protocols

S.1: MEF culture medium (500 ml)

450ml of DMEM (High Glucose, Gibco, Invitrogen)

50ml of FBS (10% (v/v), Biochrom AG, Berlin)

5ml of 200mM L-glutamine (1/100 (v/v), Gibco, Invitrogen)

5ml of Penicillin-Streptomycin (1/100 (v/v), Gibco, Invitrogen)

Mix all components and filter (Corning, 0.22µM, PAS)

S.2: MEF freezing medium

10% of DMSO (Sigma)

40% of DMEM (High Glucose, Gibco, Invitrogen)

50% of FBS (Biochrom AG, Berlin)

S.3: hESC Medium (Unconditioned Medium:UM)

400ml of KnockoutTM DMEM (Gibco, Invitrogen)

100ml of KnockoutTM Serum Replacement (20% (v/v), Gibco, Invitrogen)

5ml of 200mM L-glutamine (1/100 (v/v), Gibco, Invitrogen)

5ml of Penicillin-Streptomycin (1/100 (v/v), Gibco, Invitrogen)

5ml of Non-Essential Amino Acids (1/100 (v/v), Gibco, Invitrogen)

35µl of 0.14 M β-Mercaptoethanol (Sigma), diluted before 1:10 in PBS (filter)

Mix all components and filter (Corning, 0.22µM, PAS)

S.4: hESC freezing medium

10% of DMSO (Sigma)

40% of KnockoutTM DMEM (Gibco, Invitrogen)

50% of KnockoutTMSerum Replacement (Gibco, Invitrogen)

S.5: Dispase solution

Dispase (5g, Gibco, Invitrogen) was dissolved in 10ml of KnockoutTM DMEM (Gibco,

Invitrogen) and placed in water bath for 15 minutes to dissolve completely before filtration.

Then solution was filtered (0.2μM Acrodisc Syringe Filters, Pall Corporation) and 500μl (100x) aliquots were frozen in −20°C. Working solution was prepared by dissolving 500μl in 50 ml of KnockoutTM DMEM and aliquots were placed into −20°C.

S.6: β-Mercaptoethanol solution for hESCs media

14.3M β-Mercaptoethanol (Sigma) was diluted 1:10 in PBS, filtered and stored at −20°C in 40μl aliquots. Aliquots were thawed and used immediately.

S.7: Defined (N2B27) medium (DM) for hESC culture (50 ml)

47ml of DMEM/F12 (Hyclone)

0.5ml of N2 Supplement (100x, Invitrogen)

1ml of B27 Supplement minus Vitamin A (50x, Invitrogen)

340μl of BSA (Bovine Albumin Fraction V, 7.5%, Gibco, Invitrogen)

0.5ml of 200mM L-glutamine (1/100 (v/v), Gibco, Invitrogen)

0.5ml of Penicillin-Streptomycin (1/100 (v/v), Gibco, Invitrogen)

0.5ml of Non-Essential Amino Acids (1/100 (v/v), Gibco, Invitrogen)

3.5μl of 0.14 M β-Mercaptoethanol (Sigma), diluted before1:10 in PBS (filter)

Mix all components and filter (Corning, 0.22μM, PAS)

S.8 Buffers for SDS-PAGE gel electrophoresis

Resolving Buffer

1.5M Tris-HCl pH 8.8:

180g Tris base (121g/mol)

add 900ml dH2O

adjust pH8.8 with 37% HCl (approx. 26ml)

ad 1000ml dH2O Stacking Buffer

0.5M Tris-HCl pH 6.8: 60g Tris base (121g/mol)

ad 900ml dH2O

adjust pH6.8 with 37% HCl (approx. 47ml)

ad 1000ml dH2O

3x Loading Buffer (Sample Buffer)

3x SDS-PAGE SB: 9.375ml Stacking Buffer

17.2ml 87% glycerol

15ml 10% SDS

some grains bromophenol blue

ad 47.5ml dH2O

store at RT

working solution: 950µl (47.5ml) 3x Loading Buffer

50µl (2.5ml) β-Mercaptoethanol

store at −20°C

10x Running Buffer

10x SDS-PAGE RB: 250mM Tris base (121g/mol) : 30.3g

1.92M Glycine (75g/mol) : 144.1g

100ml 10% SDS

ad 1000ml dH2O

S.9: Buffers for Western blotting

10x Transfer buffer: 250mM Tris base (121g/mol) ! 30.3g

1.92M Glycine (75g/mol) ! 144.1g ad 1000ml dH2O

1x Transferbuffer: 100ml 10x Transferbuffer

200ml Methanol

ad 1000ml dH2O

1M Tris-HCl pH7.6: 120g Tris base (121g/mol)

ad 900ml dH2O

adjust pH7.6 with 37% HCl

ad 1000ml dH2O

1x TBS: 8g Sodium Chloride

20ml 1M Tris-HCl pH7.6

ad 1000ml dH2O

1x TBST: 1000 ml 1x TBS

1 ml Tween 20

Blocking solution: 3% milk powder ! 1.5g

ad 50ml 1x TBST

S.10: DAPI solution preparation

Stock solution:

Mix 10 mg of DAPI (Molecular Probes, Cat# D-1306) in 2 ml of Dimethylformamide (DMF) to dissolve. It may take some time to completely dissolve. Aliquot and store at –20 ºC.

Working Solution (100ng/ml or 300 nM in PBS):

Mix 2 µl of DAPI stock solution in 100 ml of PBS. Store this solution at 4 ºC in brown bottle or wrapped with aluminum foil to protect from light (to achieve stronger staining, use 4 µl stock solution or reduce PBS amount to 50 ml).

Supplementary Information II

Supplementary Table 1: Pearsons correlation coefficient between all the samples

	Placenta_1	Placenta_2	UD_1	UD_2	SU 3H_1	SU 3H_2	SU 1D_1	SU 1D_2	SU 3D_1	SU 3D_2	SU 5D_1	SU 5D_2	F 3H_1	F 3H_2	F 1D_1	F 1D_2
Placenta_1	1,000															
Placenta_2	0,997	1,000														
UD_1	0,558	0,560	1,000													
UD_2	0,565	0,567	0,992	1,000												
SU 3H_1	0,563	0,565	0,987	0,978	1,000											
SU 3H_2	0,563	0,565	0,984	0,978	0,997	1,000										
SU 1D_1	0,569	0,572	0,946	0,955	0,949	0,952	1,000									
SU 1D_2	0,566	0,567	0,961	0,954	0,967	0,966	0,982	1,000								
SU 3D_1	0,575	0,577	0,939	0,932	0,944	0,942	0,961	0,974	1,000							
SU 3D_2	0,574	0,576	0,939	0,933	0,943	0,942	0,963	0,974	0,996	1,000						
SU 5D_1	0,600	0,603	0,918	0,914	0,932	0,930	0,946	0,956	0,975	0,977	1,000					
SU 5D_2	0,600	0,603	0,920	0,915	0,933	0,932	0,945	0,955	0,973	0,976	0,998	1,000				
F 3H_1	0,562	0,564	0,975	0,970	0,984	0,984	0,959	0,971	0,950	0,951	0,940	0,941	1,000			
F 3H_2	0,561	0,563	0,974	0,969	0,983	0,983	0,959	0,971	0,949	0,951	0,940	0,942	0,998	1,000		
F 1D_1	0,568	0,570	0,991	0,985	0,991	0,990	0,960	0,973	0,951	0,951	0,934	0,935	0,981	0,981	1,000	
F 1D_2	0,570	0,571	0,990	0,985	0,990	0,989	0,959	0,972	0,951	0,951	0,934	0,934	0,982	0,980	0,998	1,000
F 3D_1	0,575	0,576	0,985	0,981	0,981	0,979	0,963	0,974	0,964	0,964	0,941	0,940	0,971	0,970	0,992	0,992
F 3D_2	0,575	0,577	0,984	0,980	0,981	0,980	0,964	0,974	0,964	0,965	0,942	0,942	0,973	0,972	0,992	0,992
F 5D_1	0,582	0,585	0,967	0,964	0,963	0,961	0,954	0,963	0,973	0,974	0,955	0,954	0,952	0,952	0,974	0,973
F 5D_2	0,584	0,586	0,967	0,964	0,963	0,962	0,954	0,961	0,971	0,972	0,954	0,953	0,952	0,951	0,974	0,973
B 3H_1	0,566	0,568	0,983	0,980	0,993	0,995	0,955	0,965	0,943	0,943	0,932	0,934	0,989	0,989	0,989	0,989
B 3H_2	0,568	0,571	0,982	0,982	0,991	0,994	0,956	0,963	0,940	0,941	0,930	0,932	0,988	0,989	0,989	0,988
B 5D_1	0,578	0,580	0,987	0,985	0,983	0,982	0,959	0,967	0,958	0,959	0,945	0,946	0,972	0,972	0,986	0,984
B 5D_2	0,570	0,575	0,982	0,982	0,977	0,977	0,957	0,962	0,954	0,955	0,941	0,942	0,968	0,971	0,979	0,976
SB 3H_1	0,572	0,574	0,991	0,986	0,994	0,994	0,956	0,969	0,947	0,947	0,932	0,933	0,985	0,985	0,994	0,994
SB 3H_2	0,564	0,569	0,980	0,978	0,988	0,989	0,952	0,960	0,940	0,940	0,929	0,931	0,982	0,985	0,984	0,982
SB 1D_1	0,562	0,565	0,989	0,981	0,989	0,988	0,959	0,973	0,953	0,953	0,935	0,936	0,979	0,980	0,994	0,993
SB 1D_2	0,565	0,567	0,991	0,985	0,990	0,990	0,963	0,975	0,953	0,953	0,935	0,936	0,981	0,981	0,998	0,997
SB 3D_1	0,568	0,569	0,983	0,976	0,981	0,979	0,962	0,976	0,969	0,968	0,943	0,942	0,967	0,967	0,988	0,987
SB 3D_2	0,563	0,565	0,983	0,976	0,980	0,978	0,961	0,975	0,969	0,968	0,942	0,942	0,966	0,967	0,987	0,986
SB 5D_1	0,580	0,583	0,950	0,947	0,952	0,951	0,949	0,955	0,974	0,975	0,963	0,961	0,939	0,940	0,959	0,957
SB 5D_2	0,577	0,580	0,952	0,948	0,953	0,952	0,947	0,955	0,972	0,974	0,963	0,961	0,938	0,939	0,960	0,958

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Supplementary Information II: Tables

Supplementary Table 1 continued…

	F 3D_1	F 3D_2	F 5D_1	F 5D_2	B 3H_1	B 3H_2	B 5D_1	B 5D_2	SB 3H_1	SB 3H_2	SB 1D_1	SB 1D_2	SB 3D_1	SB 3D_2	SB 5D_1	SB 5D_2
Placenta_1																
Placenta_2																
UD_1																
UD_2																
SU 3H_1																
SU 3H_2																
SU 1D_1																
SU 1D_2																
SU 3D_1																
SU 3D_2																
SU 5D_1																
SU 5D_2																
F 3H_1																
F 3H_2																
F 1D_1																
F 1D_2																
F 3D_1	1,000															
F 3D_2	0,998	1,000														
F 5D_1	0,986	0,987	1,000													
F 5D_2	0,986	0,986	0,998	1,000												
B 3H_1	0,979	0,981	0,961	0,961	1,000											
B 3H_2	0,978	0,980	0,959	0,960	0,998	1,000										
B 5D_1	0,987	0,987	0,984	0,984	0,983	0,982	1,000									
B 5D_2	0,978	0,979	0,976	0,975	0,980	0,980	0,993	1,000								
SB 3H_1	0,986	0,986	0,968	0,968	0,995	0,994	0,987	0,982	1,000							
SB 3H_2	0,974	0,975	0,959	0,958	0,993	0,993	0,979	0,985	0,991	1,000						
SB 1D_1	0,989	0,989	0,971	0,970	0,988	0,986	0,987	0,983	0,992	0,986	1,000					
SB 1D_2	0,991	0,991	0,972	0,972	0,990	0,988	0,987	0,981	0,994	0,985	0,997	1,000				
SB 3D_1	0,995	0,994	0,984	0,983	0,977	0,975	0,987	0,981	0,984	0,973	0,990	0,990	1,000			
SB 3D_2	0,994	0,992	0,983	0,982	0,977	0,974	0,988	0,982	0,983	0,974	0,990	0,989	0,998	1,000		
SB 5D_1	0,972	0,972	0,990	0,989	0,950	0,949	0,976	0,972	0,955	0,951	0,961	0,959	0,976	0,976	1,000	
SB 5D_2	0,972	0,972	0,991	0,990	0,950	0,948	0,977	0,971	0,956	0,950	0,960	0,960	0,976	0,976	0,997	1,000

Supplementary Table 2: Commonly regulated (minimum of 2 fold up or down) genes, upon all the treatments (B, SB, SU, F): 311 genes

AASS	APOA1	CASQ2	DACT1	FLJ22746	H2AFY	LCP1	MGC35212	PKP2	SLC7A2	UPK2
ABCA1	APOA2	CCDC3	DCN	FLJ35767	HAK	LEF1	MGC52057	PLEKHQ1	SLCO2A1	USP28
ABCA3	APOA4	CD200	DDC	FLJ42461	HAND1	LGP2	MIXL1	PPARG	SMARCA2	VAV2
ABCG2	APOB	CDH10	DKFZP686A01247	FLJ46831	HCFC1R1	LHFP	MMP28	PPP2R2C	SPTLC3	VGLL1
ABCG4	ARHGAP28	CEBPA	DLK1	FLRT3	HMGCS2	LHX1	MPZL1	PRKCE	SRC	VSNL1
ACP5	ARHGDIB	CHGA	DLX3	FOLR1	HOP	LMO3	MSX2	PRKCH	ST3GAL1	VTCN1
ACPP	ARID5B	CHN2	DLX5	FOXA2	HOXB2	LOC130576	MXRA5	PRKD1	STEAP2	WNT4
ACSBG1	ART3	CLC	DOK4	FRMD6	HPGD	LOC144501	MXRA8	PROK2	SVEP1	WNT5A
ACSM3	ARTN	CLDN14	DPYSL4	FUT8	HSPA4	LOC161931	MYC	PRSS12	TACSTD2	YPEL2
ACTA1	ASB9	CMKOR1	DSC2	FXYD7	HSZFP36	LOC285016	MYH2	PSTPIP2	TBX3	ZDHHC22
ACTG2	ASXL1	CNKSR3	EDG7	FZD8	HTR3A	LOC388419	NAALAD2	REEP2	TEX11	ZNF467
ACTL6B	ATBF1	CNTN4	EFNA1	GABRA5	ID2	LOC57228	NBPF20	RELN	TF	ZNF483
ADAMTS1	ATP2B1	COBLL1	EFNB2	GAL	IGFBP3	LOC57228	NR2F2	RET	TFAP2A	ZNF503
ADAMTS9	ATP2B4	COL3A1	EGFL9	GALNT9	IGFBP7	LOC90355	NRIP1	RHOBTB3	TGIF	
ADRA1B	ATP5G2	COL4A5	ELAVL3	GATA2	JAZF1	LOC91461	NRP1	RNASEL	TLN2	
AFAP1L2	ATP6V1B1	COMMD3	EPAS1	GATA3	KCNF1	LRP1B	NUDT15	SAMD3	TMEM54	
AFP	BCL6	CREG1	EPB41L3	GBP2	KCNJ13	LRP8	OBFC2A	SCUBE3	TNFRSF19	
AHNAK	BMF	CRH	EPHA4	GLB1	KDELC2	LRRC20	P2RY2	SDCCAG33	TNFSF4	
AHNAK	BNC1	CRTAC1	EPN3	GPR126	KIAA0746	LRRC32	PALLD	SELS	TP73L	
ALDH3B2	BTBD11	CSF2RA	EYA2	GPR176	KIAA1914	LRRC56	PAQR8	SEMG1	TPM1	
AMOT	C12orf46	CSF3R	FAM123A	GPR177	KIFAP3	LY6E	PARVA	SERPINA1	TRIM22	
ANKHD1	C1orf94	CTGF	FAM80A	GPR56	KLK1	MAB21L2	PDE4C	SETBP1	TRIM55	
ANKRD15	C20orf100	CXCL14	FAM89A	GRHL2	KLKB1	MAMDC2	PDE4D	SLC15A3	TSPAN14	
ANKRD19	C20orf42	CXCL5	FER1L3	GRP	KRT19	MDFIC	PDE6G	SLC1A3	TTC10	
ANKRD38	C20orf75	CXCR7	FGF13	GSTA2	KRT7	MDK	PDZD4	SLC40A1	TTC3	
ANKRD43	C8orf4	CYP27B1	FGG	GUCY1A3	KRT8	MEIS2	PHKB	SLC6A15	TTR	
ANKS1A	CAMK2N1	CYP2F1	FLJ13841	GYPE	KYNU	MEST	PIPOX	SLC7A10	UBE2I	
ANXA1	CAPN11	DAB2	FLJ22471	H19	LCK	MGC26856	PITX1	SLC7A14	UGT2B7	

Supplementary Table 3: Lineage tracing: Expression of lineage-specific markers in comparison to that in control (Undifferentiates hESCs): Log2 Ratio (Treatment vs Control) (Microarray data)

			SU				SB				F				B		
SYMBOL	**DEFINITION**	**Placenta**	**3 H**	**1 D**	**3 D**	**5 D**	**3 H**	**1 D**	**3 D**	**5 D**	**3 H**	**1 D**	**3 D**	**5 D**	**3 H**	**5 D**	**P value**
Embryonic stem cell																	
POU5F1 (OCT4)	POU domain, class 5, transcription factor 1 (POU5F1), transcript variant 1.	-9,65	-0,04	-0,77	-3,14	-6,57	-0,42	-0,39	-1,24	-3,66	-0,26	-0,56	-1,51	-3,34	-0,32	-0,42	1,01E-10
SOX2	SRY (sex determining region Y)-box 2 (SOX2).	-8,60	0,05	-0,83	-3,33	-5,91	-0,21	-0,09	-0,45	-1,94	-0,47	-0,21	-0,77	-2,01	-0,33	-0,71	3,44E-11
NANOG	Nanog homeobox (NANOG).	-5,54	-1,72	-3,53	-4,74	-3,84	-0,21	-1,97	-2,61	-5,38	-0,50	-1,90	-3,04	-4,88	0,09	-0,26	1,05E-08
MYC (c-Myc)	v-myc myelocytomatosis viral oncogene homolog (avian) (MYC).	-0,28	-1,32	-1,15	-2,26	-1,31	-0,42	-1,35	-2,10	-1,91	-0,21	-1,02	-1,40	-1,50	-0,24	-1,01	2,80E-04
FGF2	fibroblast growth factor 2 (basic) (FGF2).	-4,41	1,44	0,71	-1,46	-1,80	0,03	0,36	-0,41	-1,58	0,57	0,44	-0,51	-1,51	0,32	-0,88	1,12E-09
DPPA4	developmental pluripotency associated 4 (DPPA4).	-8,70	-0,09	-1,11	-2,83	-3,95	-0,56	-0,38	-0,91	-2,80	-0,41	-0,46	-1,44	-2,61	-0,43	-0,48	8,43E-11
ZNF206	zinc finger and SCAN domain containing 10 (ZSCAN10).	-9,82	-0,58	-0,53	-3,01	-7,57	-0,43	-0,31	-0,72	-2,87	-0,50	-0,49	-0,84	-2,47	-0,43	-0,68	3,88E-09
TERF1	telomeric repeat binding factor (NIMA-interacting) 1 (TERF1), transcript variant 1.	-4,96	-0,23	-1,00	-2,58	-3,06	-0,14	-0,45	-1,15	-2,32	-0,31	-0,66	-1,78	-2,39	-0,11	-0,10	3,33E-09

Extraembryonic endoderm																	
SOX7	SRY (sex determining region Y)-box 7 (SOX7).	2,11	-0,74	0,14	0,04	0,23	0,25	-0,46	0,27	0,86	-0,99	-0,75	0,00	0,86	-0,33	0,31	7,52E-06
Trophectoderm																	
HAND1	heart and neural crest derivatives expressed 1 (HAND1).	-1,82	1,53	2,63	4,30	4,31	0,66	1,30	2,81	4,89	1,08	0,12	2,27	4,46	1,26	4,34	1,55E-15
CDX2	caudal type homeobox 2 (CDX2).	-2,96	1,31	0,94	2,05	0,74	0,77	0,25	0,90	1,08	0,80	0,83	2,07	2,41	0,56	0,69	1,84E-06
KRT7	keratin 7 (KRT7).	5,05	-0,47	0,98	3,17	4,60	-0,73	0,04	1,08	2,15	-0,61	-0,20	0,26	1,29	-0,95	1,51	0,00E+00
CGA	glycoprotein hormones, alpha polypeptide (CGA).	8,64	1,08	1,09	2,23	7,79	-1,21	0,67	0,46	0,89	1,95	-0,24	1,14	0,36	1,45	1,28	7,77E-16
CGB	chorionic gonadotropin, beta polypeptide (CGB).	5,45	0,32	-0,81	0,03	2,57	-0,89	-0,69	-0,15	0,09	-0,73	-0,39	0,11	-0,14	-0,09	-0,69	0,00E+00
CGB1	chorionic gonadotropin, beta polypeptide 1 (CGB1).	7,64	-0,41	0,07	0,69	5,09	-0,73	-0,64	-0,81	1,13	-0,68	-0,95	-0,47	0,40	-0,99	-0,38	0,00E+00
CGB5	chorionic gonadotropin, beta polypeptide 5 (CGB5).	8,67	-0,59	0,05	0,19	5,33	-0,32	-1,07	-0,94	0,65	-1,10	-1,83	-0,48	0,12	-0,70	-0,46	9,99E-15
Primitive streak																	
MIXL1	Mix1 homeobox-like 1 (Xenopus laevis) (MIXL1).	-3,18	-1,22	-1,26	-1,59	-1,42	-0,35	-4,72	-1,53	-2,29	0,63	-4,35	-2,91	-2,38	1,82	1,00	1,79E-10
T	T, brachyury homolog (mouse) (T).	-2,95	0,59	-1,98	-1,65	-1,13	1,08	-1,96	-1,63	-2,95	1,61	0,27	-0,24	-1,83	1,71	0,22	1,62E-08
Mesendoderm																	

EOMES	eomesodermin homolog (Xenopus laevis) (EOMES).	-4,90	-0,31	-4,01	-4,08	-3,37	0,88	-2,33	-4,90	-4,90	1,38	-1,96	-3,33	-4,90	1,35	-0,20	3,25E-11
Endoderm																	
GATA4	GATA binding protein 4 (GATA4).	0,26	0,70	-0,20	-0,89	-0,17	-0,03	0,21	0,03	0,04	0,04	-0,22	-0,04	-0,21	0,51	0,43	4,65E-02
CER1	cerberus 1, cysteine knot superfamily, homolog (Xenopus laevis) (CER1).	-1,55	1,64	-0,44	-1,11	0,09	1,34	-1,44	-1,74	-0,59	1,32	-2,15	-0,76	-1,03	2,13	1,43	3,89E-06
SOX17	SRY (sex determining region Y)-box 17 (SOX17).	2,44	1,63	0,30	-0,33	-0,17	1,51	0,26	-0,34	-0,37	1,65	0,41	-0,04	-0,28	1,53	0,74	2,11E-08
CXCR4	chemokine (C-X-C motif) receptor 4 (CXCR4), transcript variant 2.	2,52	-1,40	-1,23	-2,02	-2,45	0,83	0,14	-0,43	-0,64	1,15	-0,14	0,30	0,69	1,33	-0,05	3,47E-14
Neurectoderm																	
CDH2	cadherin 2, type 1, N-cadherin (neuronal) (CDH2).	-4,89	0,79	-0,32	-0,28	-0,73	0,34	-0,27	-0,75	-0,19	0,89	-0,27	-0,74	-0,40	0,70	0,05	2,93E-11
GBX2	gastrulation brain homeobox 2 (GBX2).	-0,61	0,10	-0,55	-0,68	-0,39	0,52	-0,25	-0,28	-0,76	0,32	-0,03	-0,81	-0,43	0,20	-0,57	1,49E-02
NES	nestin (NES).	-1,29	0,26	0,35	-0,83	-2,46	0,22	0,04	-0,05	-0,39	0,53	0,22	0,00	0,15	0,26	-0,20	2,29E-08
TUBB3	tubulin, beta 3 (TUBB3).	-2,15	0,77	0,52	-0,36	-1,38	0,48	0,04	-0,35	-1,07	0,51	0,19	-0,20	-1,03	0,47	-0,94	5,09E-08

Supplementary Table 4: Overlap with placenta (Pie chart)

a) Total number of significantly regulated genes (minimum 2 fold up or down; p value<=0.05)

	Numbers		
	Up	Down	Total
Placenta	2782	3537	6319
B	415	165	580
F	895	865	1760
SU	1541	1728	3269
SB	1269	1020	2289

b) Overlap of regulated genes with the same trend as that in placenta

	Numbers			Percentages		
	Up	Down	Total	Total	Up	Down
B	239	116	355	7,23	10,83	4,29
F	441	597	1038	21,14	19,98	22,08
SU	910	1263	2173	44,25	41,23	46,71
SB	617	728	1345	27,39	27,96	26,92
	2207	2704	4911			

Supplementary Table 5: Expression of Cyclins and CDKs in comparison to that in control (Undifferentiates hESCs): Log2 Ratio (Treatment vs Control) (Microarray data): Red coulor indicates up-regulation and green colour indicates down-regulation

PROBE_ID	SYMBOL	SU 3 H	SU 1 D	SU 3 D	SU 5 D	F 3 H	F 1 D	F 3 D	F 5 D	B 3 H	B 5 D	SB 3 H	SB 1 D	SB 3 D	SB 5 D	P value
ILMN_1689001	CDK4	0,08	0,19	-0,48	-0,50	0,15	0,25	0,15	0,00	0,08	-0,10	0,13	-0,06	0,16	-0,21	2,28E-04
ILMN_1802615	CDK6	0,83	0,58	0,98	2,02	0,43	-0,09	0,22	0,82	0,53	0,91	0,29	0,05	0,00	1,67	5,80E-10
ILMN_1668721	CCND3	-0,29	0,79	1,12	1,78	-0,25	0,39	0,57	-0,10	-0,21	0,00	-0,60	0,47	0,54	0,07	4,87E-10
ILMN_1665559	CDK2	-0,16	-0,20	1,19	1,78	-0,10	-0,05	0,35	0,67	-0,24	0,37	-0,34	0,20	0,48	0,94	1,11E-16
ILMN_2374425	CCNE1	-0,02	0,04	0,05	1,14	-0,11	0,23	0,16	-0,32	0,01	0,12	-0,35	0,36	0,15	-0,21	2,00E-12
ILMN_1718565	CDKN1C (p57)	-0,29	2,61	1,30	3,81	-0,91	-0,04	0,58	0,71	-0,88	0,93	-0,78	0,31	1,03	1,71	1,54E-11
ILMN_1784602	CDKN1A (p21)	0,90	0,75	-0,14	0,99	0,28	0,25	0,21	-0,04	-0,57	-1,00	-0,12	0,11	-0,47	-0,75	3,81E-08
ILMN_1779356	TP53	0,49	-0,43	-0,63	-0,92	0,26	-0,17	-0,19	-0,47	0,53	0,49	0,56	-0,01	-0,39	-0,27	4,75E-04
ILMN_1664630	CHEK1	0,23	-0,64	-0,59	-1,79	0,09	0,13	-0,05	-0,41	0,06	-0,32	-0,16	0,21	0,00	-0,68	2,67E-11
ILMN_2395236	CHEK2	-0,01	-1,56	-2,19	-2,13	-0,48	-0,53	-1,22	-1,53	-0,59	-0,36	-0,50	-0,39	-0,83	-1,79	1,28E-08
ILMN_1720114	GMNN	0,29	-1,17	-1,49	-0,99	0,06	0,20	-0,34	-0,76	-0,03	-0,48	-0,15	-0,25	-0,56	-0,80	7,73E-10
ILMN_1747911	CDK1 (CDC2)	-0,26	-1,34	-0,86	-1,22	-0,28	-0,20	-0,21	-0,47	-0,38	-0,22	-0,68	-0,13	-0,52	-0,57	3,63E-09
ILMN_2157099	CCNA1	0,28	-0,75	-2,07	-1,81	-0,41	-0,42	-1,40	-1,45	-0,27	-0,64	-0,10	-0,09	-1,13	-1,98	4,36E-09
ILMN_1786125	CCNA2	0,26	-0,71	-0,67	-1,08	0,10	0,17	-0,11	-0,28	0,07	-0,06	-0,15	0,25	0,01	-0,46	8,28E-10
ILMN_1712803	CCNB1	0,18	-0,60	-0,91	-1,10	-0,09	0,09	-0,38	-0,19	-0,20	-0,74	-0,17	0,16	-0,11	-1,23	1,02E-04
ILMN_1801939	CCNB2	0,04	-0,86	-0,75	-1,23	-0,23	-0,05	-0,40	-0,49	-0,35	-0,33	-0,44	0,01	-0,18	-0,64	5,85E-08
ILMN_1685398	BAD	-0,02	0,57	0,68	1,69	-0,57	0,04	0,15	0,85	-0,54	-1,09	0,68	0,82	0,28	0,72	9,10E-03
ILMN_2246956	BCL2	0,29	0,03	2,18	2,44	-0,63	0,32	-0,04	0,48	-0,37	-0,48	-0,10	0,28	0,36	1,36	#######
ILMN_1742410	BCL2L1	0,34	0,34	0,31	1,03	0,23	-0,15	-0,31	-0,47	0,36	-0,29	0,34	-0,17	-0,36	-0,07	1,09E-04

Supplementary Table 6: Expression of SMAD 2/3, SMAD 1/5/8 and FGF target genes in comparison to that in control (Undifferentiates hESCs): Log2 Ratio (Treatment vs Control) (Microarray data)

			SU				SB				F				B	
SYMBOL	**DEFINITION**	**Placenta**	**3 H**	**1 D**	**3 D**	**5 D**	**3 H**	**1 D**	**3 D**	**5 D**	**3 H**	**1 D**	**3 D**	**5 D**	**3 H**	**5 D**	**P value**
SMAD2/3 targets																	
NODAL	nodal homolog (mouse) (NODAL).	-4,30	1,25	-2,63	-3,19	-3,45	0,73	-2,09	-3,32	-3,96	1,42	-1,38	-3,20	-4,06	2,27	0,05	5,55E-16
LEFTY1	left-right determination factor 1 (LEFTY1).	-3,33	0,01	-1,27	-2,67	-2,01	-0,82	-2,91	-3,19	-3,79	-0,89	-2,76	-2,55	-5,65	3,06	-0,78	2,22E-16
LEFTY2	left-right determination factor 2 (LEFTY2).	2,59	1,14	-1,04	-1,91	-1,45	0,40	-1,53	-1,96	-3,08	0,76	-1,82	-2,75	-2,83	1,86	0,93	3,00E-10
CER1	cerberus 1, cysteine knot superfamily, homolog (Xenopus laevis) (CER1).	-1,55	1,64	-0,44	-1,11	0,09	1,34	-1,44	-1,74	-0,59	1,32	-2,15	-0,76	-1,03	2,13	1,43	3,89E-06
SMAD1/5/8 targets																	
GATA2	GATA binding protein 2 (GATA2).	5,55	2,09	3,48	3,91	4,90	1,40	1,53	3,03	3,40	1,37	1,33	2,96	3,06	1,94	2,08	2,55E-15
GATA3	GATA binding protein 3 (GATA3), transcript variant 2.	4,80	1,11	1,48	4,04	4,37	0,72	0,53	2,57	3,69	0,50	0,44	2,00	3,47	0,76	2,43	0,00E+00
GATA5	GATA binding protein 5 (GATA5).	-2,65	0,68	2,64	1,99	0,80	0,23	0,73	1,84	2,34	-0,08	0,63	1,92	2,44	0,26	1,63	6,53E-09
CDX2	caudal type homeobox 2 (CDX2).	-2,96	1,31	0,94	2,05	0,74	0,77	0,25	0,90	1,08	0,80	0,83	2,07	2,41	0,56	0,69	1,84E-06
EOMES	eomesodermin homolog (Xenopus laevis) (EOMES).	-4,90	-0,31	-4,01	-4,08	-3,37	0,88	-2,33	-4,90	-4,90	1,38	-1,96	-3,33	-4,90	1,35	-0,20	3,25E-11

ID2	inhibitor of DNA binding 2, dominant negative helix-loop-helix protein (ID2).	-0,16	1,20	1,99	2,29	2,05	0,90	0,69	2,01	2,73	0,82	0,91	1,98	2,83	0,69	1,93	2,11E-09
FGF targets																	
SPRY1	sprouty homolog 1, antagonist of FGF signaling (Drosophila) (SPRY1), transcript variant 1.	-0,73	-2,69	-2,79	-3,78	-3,51	-0,66	-0,64	-1,45	-2,09	-0,67	-0,74	-0,84	-0,74	-0,38	-0,34	1,06E-12
SPRY2	sprouty homolog 2 (Drosophila) (SPRY2).	-1,50	-1,87	-2,68	-2,54	-1,83	0,02	-0,49	-0,64	0,39	-0,02	-0,08	-0,11	0,94	-0,25	-0,15	3,17E-10
SPRY4	sprouty homolog 4 (Drosophila) (SPRY4).	-0,33	-3,45	-5,49	-4,65	-3,06	0,36	-0,51	-2,20	-3,50	0,46	-0,28	-0,43	-0,53	0,22	-0,39	2,81E-13
CER1	cerberus 1, cysteine knot superfamily, homolog (Xenopus laevis) (CER1).	-1,55	1,64	-0,44	-1,11	0,09	1,34	-1,44	-1,74	-0,59	1,32	-2,15	-0,76	-1,03	2,13	1,43	3,89E-06
FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog (FOS).	3,23	-2,57	-2,00	-2,31	-1,03	0,10	-0,61	-0,92	0,55	0,16	-0,57	-1,23	-0,58	0,02	0,08	5,88E-15

Supplementary Table 7: Expression of Tumor Suppressors in comparison to that in control (Undifferentiates hESCs): Log2 Ratio (Treatment vs Control) (Microarray data)

			SU				SB				F				B		
SYMBOL	**DEFINITION**	**Placenta**	**3 H**	**1 D**	**3 D**	**5 D**	**3 H**	**1 D**	**3 D**	**5 D**	**3 H**	**1 D**	**3 D**	**5 D**	**3 H**	**5 D**	**P value**
HOP	homeodomain-only protein (HOP), transcript variant 1.	6,40	0,37	0,94	0,30	5,51	0,37	0,12	0,28	-0,21	-0,16	0,75	0,92	-0,75	0,54	-4,60	6,66E-16
DAB2	disabled homolog 2, mitogen-responsive phosphoprotein (Drosophila) (DAB2).	6,17	0,58	1,05	1,76	3,07	0,17	0,47	0,54	1,49	0,44	0,31	0,71	1,66	0,43	1,32	6,11E-15
RASA1	RAS p21 protein activator (GTPase activating protein) 1 (RASA1), transcript variant 1.	3,24	-0,18	-0,21	0,59	2,34	-0,48	0,17	0,43	0,77	-0,23	0,07	0,25	0,53	-0,27	0,19	0,00E+00
CDH1	cadherin 1, type 1, E-cadherin (epithelial) (CDH1).	-0,13	0,03	1,49	1,67	1,40	-0,10	0,23	0,85	1,47	0,21	0,08	0,54	0,95	-0,12	0,58	8,74E-12
TIMP3	TIMP metallopeptidase inhibitor 3 (Sorsby fundus dystrophy, pseudoinflammatory) (TIMP3).	8,23	1,34	0,22	0,88	2,58	1,02	0,92	1,10	1,26	0,92	0,68	1,77	1,93	0,84	0,77	0,00E+00