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TGFβ regulation on gene expression in human embryonic stem cells

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Stjórn TGFβ á genatjáningu í stofnfrumum úr fósturvísum manna

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Ágrip

Transforming growth factor β (TGFβ) fjölskyldan gegnir veigamiklu hlutverki í fósturþroskun og ræður trúlega miklu um að viðhalda stofnfrumum úr fósturvísum (ES frumum) ósérhæfðum en einnig að beina þeim í átt að miðlagssérhæfingu. Markmið þessa verkefnis var að athuga hvaða áhrif vaxtarþættir TGFβ fjölskyldunnar hefðu á ES frumur manna (hES frumur). hES frumur voru örvaðar með mismunandi vaxtarþáttum TGFβ fjölskyldunnar til að athuga hvaða áhrif það hefði á genatjáningu- og formfræði þeirra. Á seinna stigi sérhæfingar voru sláandi hjartavöðvafrumur taldar þegar frumur voru örvaðar með mismunandi TGFβ vaxtarþáttum. Niðurstöður sýndu að BMP4 vaxtarþátturinn beinir frumum í átt að sérhæfingu á forverum hjartavöðvafruma. Til frekari staðfestingar á þessum niðurstöðum var genatjáning borin saman með hjálp örflögugreiningar á hES frumum örvuðum með ólíkum TGFβ vaxtarþáttum. Ljóst er að BMP4 eykur tjáningu margra þekktra miðlags- og hjartavöðvagena í hES frumum. TGFβ/Activin/Nodal boðleiðin viðheldur hES frumum ósérhæfðum þótt ekki sé vitað á hvern hátt. Það var því áhugavert að skoða hvernig umritunarþættir í TGFβ fjölskyldunni stjórna tjáningu þekktra og óþekktra markgena. Í þessari rannsókn var notuð ChIP (Chromatin immunoprecipitation) aðferðin sem byggist á ónæmisfellingu DNA bindipróteina eftir krosstengingu. Niðurstöður okkar sýna að umritunarþættir TGFβ fjölskyldunnar, Smads, bindast NANOG og viðhalda þannig hES frumum ósérhæfðum en nýleg grein hefur verið birt með svipuðum niðurstöðum. Í heildina tekið gefa niðurstöður okkar sterkar vísbendingar um áhrif TGFβ fjölskyldunnar á örlög hES fruma. Mikilvægast ber þó að nefna að BMP4 hvetur til myndunar hjartavöðvafruma en TGFβ/Smad2/3 tengist NANOG geninu og viðheldur þar með hES frumum ósérhæfðum.

Abstract

The Transforming growth factor β (TGFβ) superfamily plays an important role in embryonic development and probably has a major role in both pluripotency and mesodermal differentiation of human embryonic stem cells (ES cells). The goal of this project was to investigate the role of the TGFβ superfamily members in human ES cells (hES cells). This was done by stimulating human ES cells with different TGFβ superfamily members and analyzing cell morphology and gene expression. Beating cardiomyocytes were counted upon hES cell exposure to the TGFβ superfamily members. Our data show that BMP4 is a potent inducer of cardiomyocyte differentiation. To confirm these results, differential gene expression was analyzed in hES cells by comparing undifferentiated hES cells with cells treated with different growth factors of the TGFβ superfamily. BMP4, a member of the TGFβ superfamily, induced many known mesodermal genes in hES cells. The TGFβ/Activin/Nodal pathway has been indicated in maintaining hES cell self-renewal but it has not been shown by what means. Therefore, genes that are regulated by transcription factors downstream of the TGFβ signalling pathway in hES cells were analyzed. To investigate transcription factors that bind to the target genes downstream of the TGFβ/Activin/Nodal pathway in hES cells we used the Chromatin immunoprecipitation (ChIP) method. Our results indicate that TGFβ mediated Smads bind to NANOG and therefore maintain hES cell pluripotency. This is in line with very recently published data. Overall, the results have given us an insight into the role of the TGFβ superfamily in hES cell fate. Most importantly, the results show that BMP4 induces cardiomyocyte differentiation in hES cells, whereas TGFβ sustains hES cell pluripotency via Smad2/3 and NANOG interaction.

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ALK	Activin receptor-like kinase
bHLH	basic Helix-Loop-Helix
BMP	Bone morphogenetic protein
BMPR	BMP receptor
bp	base pair
ChIP	Chromatin immunoprecipitation
CM	Cardiomyocytes
DNA	Deoxyribonucleic acid
ES	Embryonic stem cell
FBS	Fetal Bovine Serum
FGF	Fibroblast growth factor
hES	human Embryonic Stem cells
ICM	Inner cell mass
Id	Inhibitor of differentiation, inhibitor of DNA binding
kb	kilo base
kDa	Kilodalton
LIF	Leukemia inhibitory factor
mES	mouse Embryonic Stem cells
Microarray	Gene expression array
PAI-1	Plasminogen activator inhibitor - 1
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
PS	Primitive streak
PS1	Phosphorylated Smad 1/5/8
PS2	Phosphorylated Smad 2/3
RNA	Ribonucleic acid
SB-431542	TGFβ antagonist
Smad	sma/Mad (small/Mothers against dpp)
STAT	Signal Transducer and Acivator of Transcription
TBP	Tata Binding Protein
TF	Transcription factor
TGFβ	Transforming growth factor beta
TβR	TGFβ receptor

I. Introduction

1. Embryonic Stem Cells

In 1970, an observation was made that an early mouse embryo grafted into adult mice produced teratocarcinomas. Teratocarcinomas are malignant multidifferentiated tumors containing population of undifferentiated cells. These undifferentiated embryonal carcinoma (EC) cells could multiply in culture (Smith, 2001). Studies of EC cells paved the way for the progress of embryonic stem cell cultures. The pluripotency of EC cells was best sustained by co-culture with embryonic fibroblasts that provided some critical nutrients or factor support. In 1981, it was reported that pluripotent mouse embryonic stem cell lines had been derived directly from mouse blastocysts. The inner cell mass of a blastocyst was isolated and plated on a culture dish with mouse embryonic fibroblast (MEF) cells, resulting in the product of the ES cells (Evans and Kaufman, 1981).

Nearly twenty years later, in 1998, the first human embryonic cells (hES) were derived and since that time a number of ES cell lines have been derived. ES cells are derived from the inner cell mass of blastocyst stage embryos derived from surplus embryos donated after informed consent from *in vitro* fertilization treatment. Embryonic stem cells are a population of self-renewing, pluripotent cells, i.e. they have the ability to form any cell type of the human body in contrast to adult stem cells that are restricted to the formation of a few cell types of one lineage (Figure 1). ES cells are defined functionally by their pluripotency and their ability to differentiate into all three primary germ layers, ecto-, meso- and endoderm of the embryo. This makes it possible to culture them on a large scale and differentiate them to study early human development and

eventually to apply them for a cell transplantation therapy (Figure 2). (Thomson *et al.*, 1998). Hence, hES cells should give us insights into developmental events that could be important in clinical areas. Our knowledge of human development is largely based on the description of a limited number of embryos of other species. In mammalian embryology the mouse is the most popular animal to study. However, when human and mouse embryology are compared, it becomes clear that the early structures including the placenta, extra embryonic membrane, and egg cylinder all differ in the two organism (Thomson *et al.*, 1998).

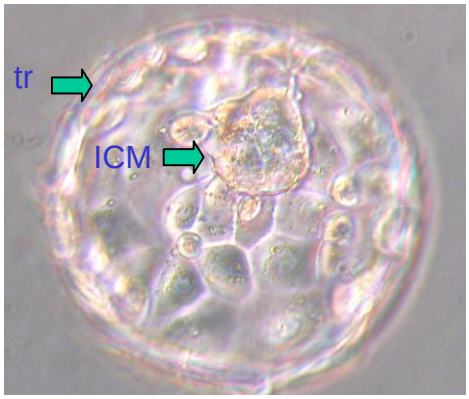

Figure 1. Human blastocyst 3 days after thawing an embryo at 8-cell stage. The trophoectoderm (tr) is separated from the inner mass (ICM) which will give rise to ES cells. These cells give rise to the embryo and are used to generate embryonic stem cells. These cells can give rise all three germ layers; ecto-, meso- and endoderm (Valdimarsdottir & Mummery, 2005).

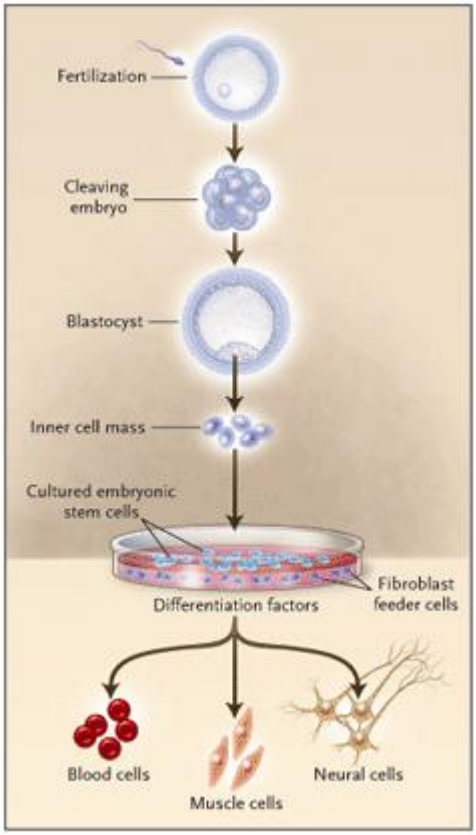

Figure 2. After fertilization embryo cleavage starts and continues at morula stage and then blastocyst stage. Inner cell mass is isolated to derive embryonic stem cells *in vitro* in a culture dish containing the necessary growth factors (Gearhart, 2004).

1.1 Maintenance of pluripotent mouse and human ES cells

In 1995 and 1996 Thomson and co-workers reported the successful derivation of embryonic stem cells in two species of primates. Two years later, 1998 the same group made their breakthrough with human embryonic stem cells (Thomson *et al.*, 1998). Comparison of mouse and human genomes shows that genes involved in reproduction have the strongest evolutionary conservation (Pera and Trounson, 2004). The maintenance of ES cell self-renewal could be expected to be similar in the human and mouse systems. However, the two systems are dissimilar for both maintaining pluripotency and differentiation in specific lineages. There are phenotypic differences

between mouse embryonic stem cells (mES) and hES cells. mES cells grow in rounded colonies and have a indistinct cell border whereas hES colonies are flatter and have more distinct cell border. mES cells are smaller in size than hES cells and show different growth regulation. Both mouse and human individual cells contain a large nucleus and a small cytoplasm (Pera and Trounson, 2004, Pera *et al.*, 2000).

Normally, hES cells are cultured on feeder cells to sustain their pluripotency and inhibit differentiation. The factors necessary for hES cell self-renewal and produced by feeder cells are unknown although basic fibroblast growth factor (bFGF), Activin A and TGFβ have been identified as important factors. For mES cells the feeder cells can be replaced by the cytokine leukaemia inhibitory factor (LIF) which acts through the gp130 receptor. LIF plays an important role in early mouse development and combination of LIF and BMP4 signals are set to maintain mES pluripotency (Amit *et al.*, 2004, Beattie *et al.*, 2005, James *et al.*, 2005). However, LIF is dispensable in hES cells. Also, BMP4 induces trophoectodermal differentiation in hES cells. The BMP antagonist, Noggin, promotes proliferation of undifferentiated hES cells through repression of BMP signals. In order to control hES self-renewal and differentiation into specific lineages it is important to identify the critical signalling pathways (Pera and Trounson, 2004, Stewart *et al.*, 2008).

Embryonic stem cells propagate indefinitely in the primitive undifferentiated state while remaining pluripotent. This has opened possibilities for using hES cells to treat diseases, for therapeutic applications and understanding differentiation in early human development. The capability to differentiate into any cell of the human body also makes it difficult to direct which pathway to undergo *in vitro*. In order to steer the differentiation

of human ES cells it is possible to apply growth factors to the cell culture. Transcription factors control the expression of multiple genes and have major effects on development. To choose what pathway a cell will undergo, i.e. either differentiation or self-renewal, depends on these growth factors (Valdimarsdottir and Mummery, 2005).

hES cell culture on feeder cells is labour intensive. Therefore, several groups have been successful in culturing hES cells in conditioned medium without feeders. The most common way is to use matrigel (BD Biosciences) as a support matrix using defined medium including bFGF (Stewart *et al.*, 2008, Klimanskaya *et al.*, 2005, Amit *et al.*, 2000).

1.2 Differentiation of hES Cells

1.2.1 Spontaneous differentiation

hES cells can differentiate into cells belonging to all three germ layers, including insulin producing cells, heart cells, nerve cells, bone cells, blood cells and liver cells. Spontaneous differentiation entails the formation of embryoid bodies (EBs) *in vitro*. EBs are three dimensional structures that resemble *in vivo* development (Figure 3). They are formed from pluripotent hES cell aggregates cultured in suspension or in hanging drops to prevent attachment. They contain a heterogenous mixture of cell types of ectodermal, mesodermal and endodermal lineages. This provides an easy *in vitro* model for studying the molecular mechanism controlling differentiation (Mummery *et al.*, 2003, Passier *et al.*, 2005, Feraud and Vittet, 2003).

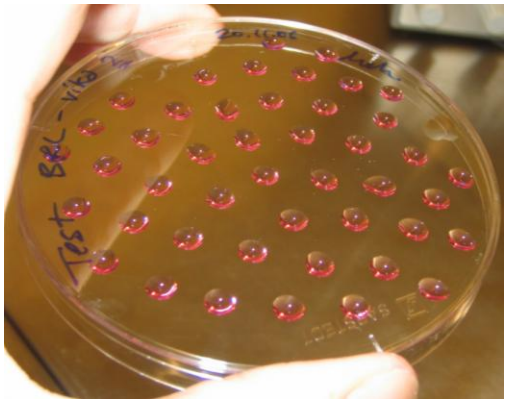

Figure 3. Embryoid bodies, EBs, are rounded collections of cells that arise when cultured in hanging drops. Embryoid bodies contain cell types derived from all 3 germ layers, ecto-, meso-, and endoderm.

1.2.2 Directed differentiation

Much effort is being put in studying which factors decide if an ES cell undergoes self-renewal or differentiation. This choice is made by known and unknown factors. In order to apply hES cells in drug screening and regenerative medicine it is essential to be able to enrich for populations of specific cell types. This can be done by directed differentiation which entails induction with co-cultures or growth factors. A good example of this is the co-culture of hES cells with a mouse endoderm-like cell line (END-2) that results in more cardiomyocytes (Passier *et al.*, 2005). Also, neuroectodermal differentiation is induced by co-culture with stroma cells with the addition of sonic hedgehog (shh) and FGF8 (Perrier *et al.*, 2004).

1.3 Transforming growth factor β (TGFβ) superfamily

Transforming growth factor β (TGFβ) plays an important role in many cell types. TGFβ is know to play a part in cell viability, regulating cell growth, differentiation, migration, proliferation, extracellular matrix deposition, apoptosis, homeostasis and response to injury or disease and embryonic development (Valdimarsdottir and Mummery, 2005, Shi and Massague, 2003). The family of TGFβ was first discovered 25 years ago. This growth factor family consists of about forty different members that are similar in structure. TGFβ is a member of a large superfamily of related growth and differentiation members, including TGFβ, Activin, Nodal and Bone Morphogenetic Proteins (BMP). The main members are classified into two groups, BMPs, which are the largest ones, and the latter group, TGFβ, Activin, and Nodal.

The TGFβ prototype transduces its signal from the membrane to the nucleus by binding to a complex of serine/threonine kinase TGFβ type I and II receptors. The type I receptor, also known as activin receptor-like kinase (ALK) acts downstream of the type II receptor to pass the phosphorylation signal to the Smad transcription factors. The Smad complex then enters the nucleus, where transcription of their target genes begins (Figure 4) (Shi and Massague, 2003, Saha *et al.*, 2008).

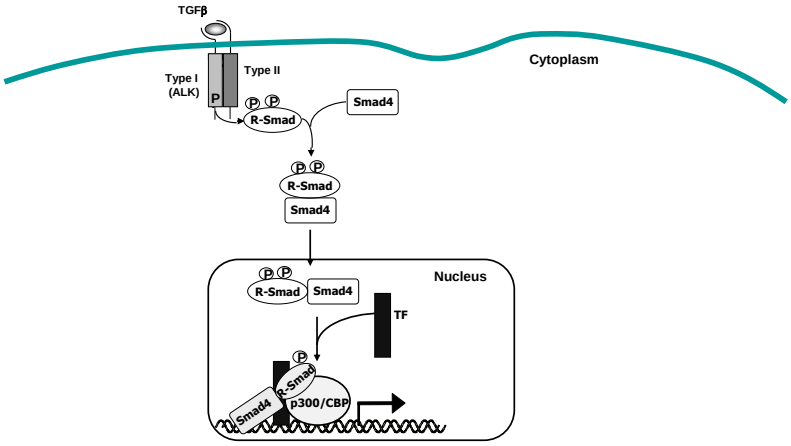

Figure 4. The TGFβ signal transduction pathway (Valdimarsdottir and Mummery, 2005).

TGFβ and Activin have high affinity for the type II receptors but BMP has higher affinity for type I receptors. The type I receptor determines the specificity of intracellular signals. In adults these receptors are constantly expressed whereas in embryos the expression is limited (Valdimarsdottir and Mummery, 2005).

The TGFβ superfamily signals through two main branches: BMP/Smad1/5 and TGFβ/Activin/Smad2/3 signalling. The BMP type I receptors ALK2, ALK3 and ALK6 phosphorylate the Smad1/5 branch. The TGFβ/Activin/Nodal signalling branch involves the activation of phosphorylation of Smad2/3 via ALK4, ALK5 and ALK7 (Figure 5) (James *et al.*, 2005, Valdimarsdottir and Mummery, 2005, Goumans *et al.*, 2003a).

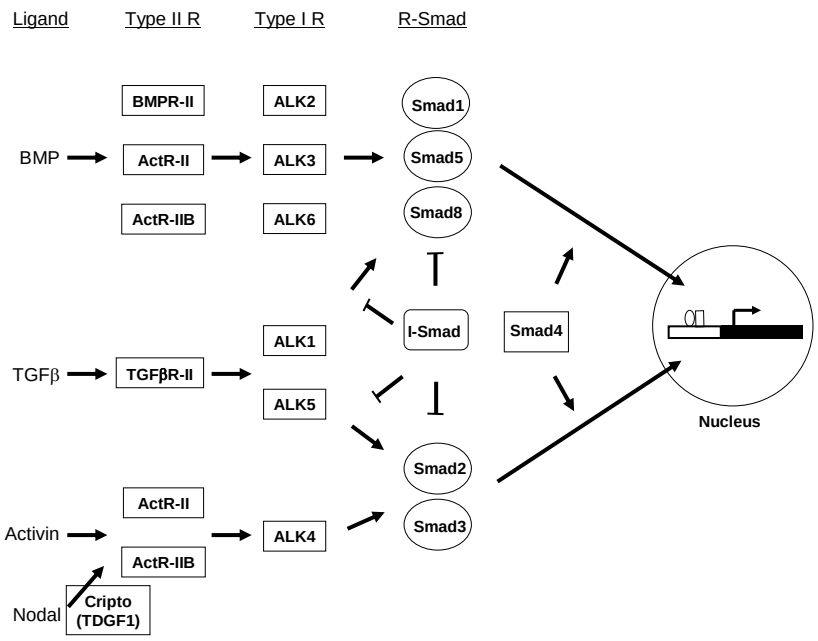

Figure 5. Divergence and convergence in TGFβ signalling through receptors and Smads (Valdimarsdottir and Mummery, 2005).

The Smads are molecules of 42-60 kDa with two highly conserved regions, Mad homology domains, MH1 and MH2. The Smads are divided into three subclasses depending on their structure and function: the receptor-regulated Smads (R-Smads), common mediator Smad (Co-Smad) and inhibitory Smads (I-Smads) (Valdimarsdottir and Mummery, 2005, Shi and Massague, 2003, Attisano and Wrana, 2000). The Phosphorylated R-Smads form complexes with common mediator Co-Smads, also known as Smad4, go to the nucleus and regulate the transcriptional activity of their target genes. R-Smads mediate the interaction of the Smad complex with DNA binding proteins (Valdimarsdottir and Mummery, 2005, Attisano and Wrana, 2000, Goumans *et al.*, 2003b). There are two inhibitory Smads, Smad6 which inhibits Smad1/5 and Smad7

which inhibits BMP and TGFβ signalling. mES cells are dissimilar to hES cells in that they have no requirement for active Smad2/3 signalling in the maintenance of pluripotency as hES cells (Figure 6) (James *et al.*, 2005).

1.3.1 The TGFβ superfamily in development

Members of the TGFβ superfamily play an important role in the earliest cell fate decisions of embryogenesis in *Xenopus*, zebra fish and chicken and mouse (Saha *et al.*, 2008, Puceat, 2007, Goumans and Mummery, 2000). Knockout mice play an important role to understanding the effect of the TGFβ superfamily in early embryogenesis. Null mutations of TGFβ superfamily ligands, receptors and intracellular signalling proteins have been created. Most of these knockout mice show embryonic lethal phenotypes due to defects in mesoderm formation, specifically cardiogenesis and angiogenesis (Table 1) (James *et al.*, 2005, Goumans and Mummery, 2000).

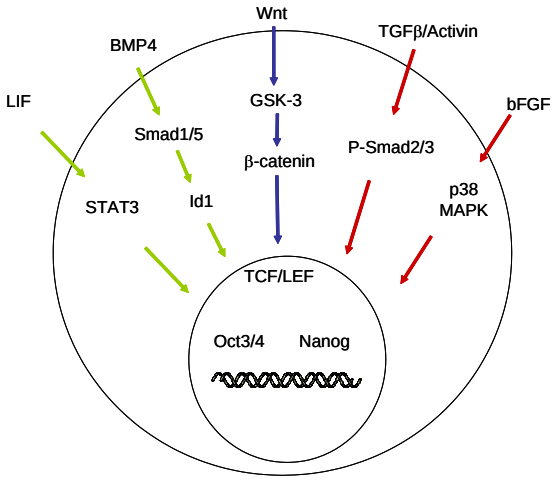

Figure 6. Signalling pathways that maintain pluripotency in ES cells. LIF and BMP enhance self-renewal in mouse ES cells (green pathways), whereas TGFβ/activin and bFGF promote self-renewal in human ES cells (red pathway. Wnt enhances self-renewal in both mouse and human ES cells (blue pathway) (adapted from Valdimarsdottir and Mummery, 2005).

Table 1. Defects in mice deficient in TGFβ signal transduction.

Genes	Phenotype	Lethality	Reference
TGFβ1	Defect in extraembryonic mesoderm differentation and haematopoiesis	E10.5	(Dickson *et al.*, 1995)
ALK1	Defect in angiogenesis and differentiation and recruitment of SMC	E11.5	(Oh *et al.*, 2000, Urness *et al.*, 2000)
ALK5	Defect in vessel formation. Impaired EC migration and prolifation and fibronectin production	E10.5	(Larsson *et al.*, 2001)
TβRII	Defect in yolk sac vasculogenesis and haematopoiesis. Distended capillary vessel formation	E10.5	(Oshima *et al.*, 1996)
Smad1	Failure in establishing chorion-allontoic circulation. Lack of VCAM-1 expression	E9.5	(Tremblay *et al.*, 2001, Lechleider *et al.*, 2001)
Smad5	Lack of normal development of the yolk sac vasculature. Irrecular distribution of blood cells and enlarged blood vessels.	E9.5-E11.5	(Chang *et al.*, 1999, Yang *et al.*, 1999)
BMP2	Heart mailformation, mailformation of the amnion/chorion	E7.5-E10.5	(Zhang and Bradley, 1996)
BMP4	Gastrulation and mesoderm formation	E7.5-E9.5	(Winnier *et al.*, 1995) (Lawson *et al.*, 1999)
BMP5/7	Retarded heart development and abnormal development of the vasculature, chorion/allontoic fusion	E10.5	(Solloway and Robertson, 1999)
ALK2	Failure in primitive streak elongation, delayed mesoderm formation and malformed visceral endoderm	E7.5-E9.5	(Gu *et al.*, 1999)
ALK3	Failure in mesoderm formation, reduced proliferation of the epiblast	E7.5-E9.5	(Mishina *et al.*, 1995)
ALK6	Defects in appendicular elements		(Yi *et al.*, 2000)
Id1/Id3	abnormal angiogenesis, forming enlarged, dilated bloodvessels, premature neural differentiation	E13.5	(Lyden *et al.*, 1999)
Nodal-/-	Failure in gastrulation and primitive streak formation. embryos show an increase in neuroectoderm differentiation suggesting that Nodal act as an inhibitor of neuroectoderm specification *in vivo.*	E7.5	(Conlon *et al.*, 1994)

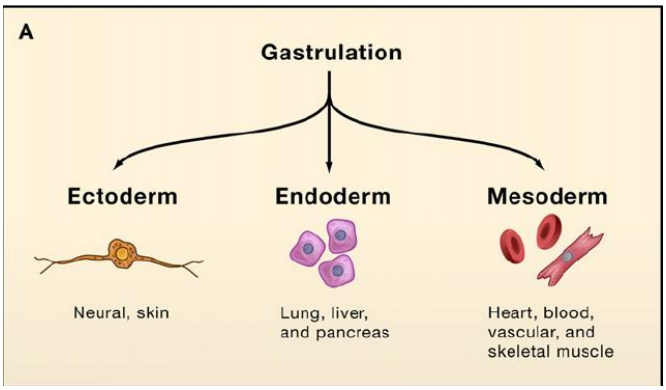

Figure 7. The derivatives of the three primary germ layers – ectoderm, mesoderm and endoderm – generated during gastrulation (Murry and Keller, 2008).

The generation of the three primary germ layers, ectoderm, mesoderm, and endoderm throughout the process of gastrulation is the most important event during embryogenesis (Figure 7). Gastrulation is marked by the formation of a structure known as the primitive streak (PS). Mapping studies and molecular analysis have shown that regions of the PS posterior, mid, and anterior differ in gene expression pattern and developmental potential. The specification of mesoderm and endoderm is not random but rather controlled with high standards of accuracy. Gene expression analysis studies have shown that members of the TGFβ family including BMP4, Nodal and members of the Wnt family are essential for these developmental steps (Conlon *et al.*, 1994, Murry and Keller, 2008, Hogan, 1996, Schier, 2003, Yamaguchi, 2001, Yang *et al.*, 2008)

Two members of the TGFβ family have been shown to induce differentiation in mouse ES cells cultured in chemically defined medium. Activin A was shown to mediate dorsoanterior mesoderm differentiation and BMP4 the formation of more posterior or ventral mesoderm (Johansson and Wiles, 1995). The BMP ligands and their downstream effectors, Smad1/5 play an essential role in determining ES cell fate, causing them to

adopt a mesodermal fate instead of differentiating into the ectodermal lineage (Johansson and Wiles, 1995, Ying *et al.*, 2003). The BMPs have been shown to phosphorylate Smad1/5 in both human and mouse ES cells (Xu *et al.*, 2005, Monteiro *et al.*, 2004). BMPs have been shown to upregulate Id proteins which in turn inhibit bHLH transcription factors, thereby blocking differentiation of mES cells. Overexpression of Id1 mimicks the effect of BMP in mES cells (Ying *et al.*, 2003, Hollnagel *et al.*, 1999). This effect has not been shown in hES cells. On the contrary, repression of BMP sustains hES cells self-renewal. Furthermore, studies have shown that BMP4 stimulation in hES cells in conditioned medium containing bFGF, promotes trophoblast differentiation (Xu *et al.*, 2005). Blocking BMP2 activity in serum does not maintain hES cell self-renewal, but instead enhances primitive endoderm differentiation (Pera *et al.*, 2004).

Studies indicate that TGFβ/Activin/Nodal signalling via Smad2/3 activation is sufficient to maintain the pluripotent state of hES cells (James *et al.*, 2005). ActivinA/Smad2/3 signalling has also been shown to induce expression of Oct4, Nanog, Wnt3, and bFGF as well as suppressing BMP signals in hES culture (Beattie *et al.*, 2005, James *et al.*, 2005, Besser, 2004).

Nodal, a member of the TGFβ superfamily induces mesoderm and endoderm formation and is critical for patterning of nervous system and determining left and right asymmetry in vertebrates. The Activin/Nodal branch involves the activation of type I receptors ALK 4/5/7. Activation of Smad2 downstream of Nodal signalling takes place in several processes in the development in the embryo. Nodal and the inhibitors of Nodal signalling, Lefty A and Lefty B are down-regulated very early upon differentiation. High expression of these genes in undifferentiated cells is maintained by phosphorylation and

activation of the transcription factor Smad2/3 (Beattie *et al.*, 2005, James *et al.*, 2005, Besser, 2004).

The genes that are regulated in both mouse and human ES cells, include members of the transforming growth factor β TGFβ signalling pathway, such as the teratoma-derived growth factor-1 (TDGF-1/Cripto) which is an EGF-CFC co-receptor for Nodal signalling, as well as Lefty-A (Lefty2 in the mouse) and Lefty-B (Lefty1 in the mouse), which are inhibitors of Nodal signalling. Nodal is highly expressed in undifferentiated hES cells and mES cells. Nodal signalling is regulated by Cripto, which forms a complex and binds TGFβ/activin/nodal (Figure 5) (Besser, 2004). Lefty A and Lefty B are the earliest genes which are involved in decreasing expression upon differentiation in non-conditioned medium. Activin/Nodal signalling in hES regulates all three genes. $Nodal^{-/-}$ embryos show an increase in neuroectoderm differentiation suggesting that Nodal act as an inhibitor of neuroectoderm specification *in vivo.* Lefty expression has been characterised in hES cells during their differentiation and has been implicated in ectoderm specification. Overexpression of Lefty resulted in a pronounced increase in neuroectoderm development. These data strongly support the hypothesis that Nodal signalling inhibits neuroectoderm specification during early hES differentiation (Puceat, 2007, Smith *et al.*, 2008).

1.4 Prominent transcription factors in ES cell self-renewal

It is essential to understand how signal transduction is activated by transcription factors that regulate target gene expression in order to maintain ES cell pluripotency or to drive differentiation into a suitable cell type. Transcription factors are proteins that bind to DNA regulatory elements and thereby, affect the initiation and efficiency of transcript. The core transcription factors which maintain both mouse and human ES cell pluripotency are NANOG, OCT4 and SOX2 (Jaenisch and Young, 2008). Binding sites for these factors are located within regulatory regions of most known genes that maintain pluripotency. Oct4, a member of the POU family of homeodomain proteins (Tomilin *et al.*, 1998) and a second, recently identified, homeodomain protein, Nanog play and important role in maintaining pluripotency and self-renewal of ES cells (Chambers *et al.*, 2003). Interestingly, OCT4 is required not only for self-renewal of cells but also for early mesodermal and cardiac cell commitment in the embryo (Puceat, 2007). SRY (sex determining region Y)-box 2, also known as SOX2 encodes a member of the SRY-related HMG-box (SOX) family. NANOG, OCT4 and SOX2 regulate many developmentally important genes. A number of other genes have been implicated as markers for pluripotency, including Cripto and UTF1 (Okuda *et al.*, 1998). However, these genes are not expressed exclusively by the inner cell mass of the blastocyst and their exact roles in ES cells still remains to be determined.

There may be a strong connection between the TGFβ signalling pathway and Nanog activity. Nanog prevents BMP -induced mesoderm differentiation of mES cells by directly binding to Smad1 and in turn blocks transcriptional activation of target genes leading to maintenance of pluripotent mES cells self-renewal (Suzuki *et al.*, 2006).

Recent studies have shown that a Smad2/3 can bind to the NANOG promoter, hence it is likely that TGFβ/Activin/Nodal plays a crucial role in sustaining Nanog promoter activity in pluripotent ES cells (Xu *et al.*, 2008).

1.5 Therapeutic potential of hES cells

Human ES cells might be used to treat diseases such Parkinson's disease, diabetes and myocardial infarction. However, there are ethical complications using human embryos. In addition, the possibility that the patient could reject the donor cells following transplantation leaves open questions of how general this procedure can be. One way to overcome these problems is to generate pluripotent cells that originate from the patient, i.e. genetically matching cells. Several different strategies have been used to induce the conversion of differentiated cells into a pluripotent state. Somatic cell nuclear transfer (SCNT) involves the injection of a somatic nucleus into an enucleated oocyte. More interestingly, introduction of only a few defined transcription factors into somatic cells can induce the production of pluripotent stem cells (iPS cells). Normal skin fibroblasts can be reprogrammed to form iPS cells using this procedure. In the first successful attempt, four different transcription factors were transferred using retroviruses. The transcription factors were Oct4, Sox2, c-Myc and Klf4 and to improve the generation of iPS cells Nanog was used as a selection marker (Figure 8) (Takahashi and Yamanaka, 2006, Okita *et al.*, 2007). This new way of generating pluripotent stem cells is constantly improving and now it is possible to use fewer transcription factors without viral infection (Huangfu *et al.*, 2008, Kim *et al.*, 2009). Also, *p53* suppression, using short-interfering RNA (siRNA), was recently shown to increase the iPS cell induction efficiency (Hong *et*

al., 2009). This approach might be used to create patient- and disease-specific stem cells for further research and even therapy. These cells can differentiate into cell types of the three germ layers *in vitro* and in to teratomas (Takahashi and Yamanaka, 2006, Rodolfa and Eggan, 2006). In this way scientists are deriving iPS cells from patient with hereditary diseases (patient specific iPS cell lines), hoping to learn more about their cause and to develop new treatments (Jaenisch and Young, 2008, Takahashi and Yamanaka, 2006, Eminli *et al.*, 2006).

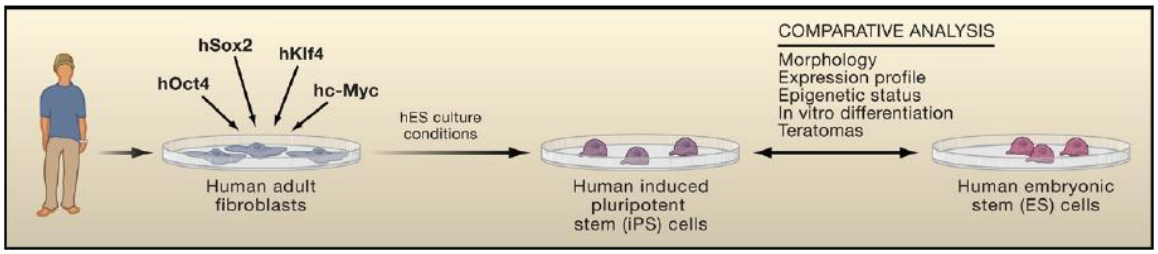

Figure 8. Transcription Factor-Induced Pluripotency (Zaehres and Schöler, 2007).

Aims of the thesis:

The Transforming growth factor β (TGFβ) superfamily plays an important role in embryonic development and probably has a major role in both pluripotency and mesodermal differentiation of human ES cells. The aim of this project was to investigate the role of the TGFβ superfamily in hES cells. This was done by stimulating hES cells with different TGFβ superfamily members and analyzing cell morphology and gene expression. Furthermore, several genes that are regulated by transcription factors downstream of the TGFβ signalling pathway in hES cells were analyzed.

The thesis is divided into three parts:

1. Characterization of human embryonic stem cells

Culture conditions for hES cell pluripotency and differentiation methods were defined. The morphology, marker expression and karyotype of the cells were determined. After optimization, hES cells were stimulated with different TGFβ superfamily members and cell fate analysed.

2. Gene expression analysis using microarrays

Differential gene expression was analyzed in hES cells by comparing undifferentiated hES cells and cells after treatment with different growth factors of the TGFβ superfamily. We used 4-Plex expression arrays from Roche NimbleGen System to compare the gene expression on whole genome microarrays.

3. ChIP (Chromatin immunoprecipitation)

The TGFβ/Activin/Nodal pathway has been implicated in maintaining hES cell self-renewal but it has not been shown by what means. Our aim was to define target genes to which transcription factors downstream of the TGFβ/Activin/Nodal pathway bind directly hES cells.

II. Material and methods

2 Cell Culture and isolation

2.1 Maintenance of undifferentiated human ES cells

hES cells were routinely cultured on a monolayer of irradiated primary mouse embryonic fibroblasts (MEFs) (ICR mouse strain). These cells can be used as a feeder layer to support the growth and maintenance of human ES cells in the undifferentiated state. 5 x 10^6 MEF cells were seeded on 8 wells of 6-wells plates (NUNC) in MEF medium (Table 2). The hES cells lines, HUES 3 and 9, obtained from Doug Melton, Howard Hughes Institute (Cowan *et al.*, 2004) were suspended in 3 ml hES medium (Table 2) and plated in 1 well of a 6-well plate on MEF cells. MEF cells were incubated in 2 ml of hES medium containing bFGF for 1 h prior to hES cell plating. 48 hours after seeding of the hES cells, the hES medium was changed every day.

Table 2. MEF medium and hES medium (Cowan *et al.*, 2004).

MEF medium	ml	**hES medium**	ml
DMEM (11960-044) (Invitrogen)	435	KO-DMEM (10829-018) (Invitrogen)	384,5
10% Australian Fetal Calf Serum (FCS) (Cambrex)	50	PenStrep 5,000U/ml 5,000µg/ml (Invitrogen)	5
Pen/Strep 5,000U/ml 5,000µg/ml (Invitrogen)	5	Gluta-Max 5U/ml (Invitrogen)	5
Gluta-Max 5U/ml (Invitrogen)	5	Non Essential Amino acids (100X) (Invitrogen)	0.5
Non Essential Amino acids (100X) (Invitrogen)	5	2-mercaptoethanol 55mM (1000X) (Invitrogen)	50
Total Volume	500	KO Serum Replacement (Invitrogen)	50
		Plasmanate (Bayer)	500
		Total Volume	
		bFGF (4ng/ml) (PeproTech)	

2.1.1 Passage of human Embryonic Stem (hES) Cells

MEF cells were pre-incubated in 2 ml hES medium 1h before passaging hES cells. Then, culture medium was removed and discarded and cells washed once with 1 ml PBS. Cells were trypsinized with 0.3 ml 0.05% trypsin/EDTA and incubated at 37°C for 1 min (hES cells round up). 2.5 ml hES medium were added to inactivate the trypsin and the supernatant was transferred to a 15 ml tube and incubate for 2 min to let MEF layer sink to the bottom while hES cells stayed in the suspension because they are smaller in size. hES cell suspension was transferred from the MEFs to a new 15 ml tube and drawn repeatedly up and down with the pipette at least 10 times to get a single cell suspension. Finally, hES cells were split in 1:6 ratio.

2.2 Karyotype analysis

When working with hES cell lines it is important to ensure that they have a normal karyotype. Chromosomal abnormalities are common in hES lines hence frequent karyotyping analysis is needed. Chromosomal abnormalities can increase rapidly with each passage. hES cells were analysed every 10^{th} passage. HUES cells were cultured in 3 wells of a 6-well multidish. It is essential to have the cells growing in an exponential state to get enough metaphase chromosomes. hES cells were cultured in hES medium with 0.1 µg/ml of Colcemid (Invitrogen) for 1-3 h at 37°C. The cells were then trypsinized and pipetted to produce single cell suspension. Cells were pelleted with centrifugation. The pellet was pipetted and resuspended in 0.075M KCl hypotonia for 7-8 minutes at 37°C.

The cells were fixed in methanol:asetic acid (3:1) for 30-60 minutes at room temperature and washed. The cells suspension was dropped to prepared glass slides. Samples were stained with Tryptan Blue Solution (0.4%). Approximately 100 metaphase nuclei were examined for each cell line. For more detailed information, see in appendix Karyotype analysis (Shao *et al.*, 2007, Heins *et al.*, 2004).

2.3 Differentiation of human ES cells in vitro

2.3.1 Generation of Embryoid bodies, EBs using the "hanging-drop" method

hES cells were trypsinized with 0.3 ml 0.05% trypsin/EDTA and incubated at 37°C for 1 min. The trypsin was inactivated with 2.5 ml differentiation medium (Table 3). hES cells were counted and cell suspension diluted in differentiation medium to give 60.000 cells/ml (=1200 cells/20 µl). 10 ml of PBS were added in a 10 cm cell culture dish to sustain humidity. 20 µl of suspension were transferred onto the lid of a cell culture dish. The lid was turned on top of the culture dish so that the drops were hanging vertically downwards. hES cells were incubated in the hanging drops for 6 days in an incubator, gassed with 5% CO_2 in air, at 37°C. After 3-5 days of culture, each hanging drop contained one EB that is comprised of various differentiated cell types.

Table 3. Differentiation medium.

Differentiation medium	ml
KO-DMEM (10829-018) (Invitrogen)	384,5
PenStrep 5,000U/ml 5,000µg/ml (Invitrogen)	5
Gluta-Max 5U/ml (Invitrogen)	5
Non Essential Amino acids (100X) (Invitrogen)	5
2-mercaptoethanol 55mM (1000X) (Invitrogen)	0.5
KO Serum Replacement (Invitrogen)	50
Plasmanate (Bayer)	50
Total Volume	500

2.3.2 Generation of Aggregates from human ES cells

24-well plates were pre-coated with 0.1% gelatin. Then, 0.5 ml of differentiation medium was added with or without growth factor into each well and 200 µl tip pipette used to transfer one EB per well. The aggregates were incubated, gassed with 5% CO_2 in air, at 37°C and medium changed every 3-4 days.

2.3.3 Differentiation on gelatinized plates

6-well plates were pre-coated with 0.1% gelatin. 2 ml were added into each well using B27/N2 differentiation medium (Table 4). hES cell clumps (1:6 ratio) were plated on the well. The aggregates were incubated, gassed with 5% CO_2 in air, at 37°C and medium changed every 3-4 days.

Table 4. B27/N2 Differentiation medium (Laflamme *et al.*, 2007).

B27/N2 Differentiation medium	ml
DMEM-F12 (1:1) glutamax (Invitrogen)	474,5
B27 supplement (Invitrogen)	10
N-2 supplement (Invitrogen)	5
PenStrep 5,000U/ml 5,000µg/ml (Invitrogen)	5
Non Essential Amino acids (100X) (Invitrogen)	5
2-mercaptoethanol 55mM (1000X) (Invitrogen)	0,5
Total Volume	500

2.3.4 Cardiomyocyte induction by hES/END-2 co-culture

hES cells were co-cultured on mitomycin treated ENDoderm like cells (END-2 cells) for 12 days in a differentiation medium without serum (Passier *et al.*, 2005). Beating cardiomyocytes were counted.

2.3.5 Western blotting

Cells were lysed in a lysis buffer with protease inhibitors and sonicated. Protein samples were separated on 10% SDS-polyactylamide gel electrophoresis and transferred to nitrocellulose filters (GE Healthcare). Blots were incubated overnight with a primary antibody against TBP (rabbit polyclonal, 1:1000, Diagenode) and a HRP-linked secondary antibody (rabbit (from Donkey) 1:5000, GE Healthcare). Detection was performed by enhanced chemiluminescence (ECL) (GE Healthcare).

2.3.6 Immunofluorescence

hES cells were cultured in chamber slides (NUNC). hES cells were fixed in 2% paraformaldehyde for 30 min, washed and permeabilised with 0.1% tritonX-100 at RT for 8 min. The cells were pre-incubated with 4% Normal Goat Serum at RT for 1 hour before incubation with primary antibody overnight against Oct4 (mouse monoclonal, 1:100, Santa Cruz Biotechnology) or Nanog (rabbit polyclonal, 1:100, Chemicon/Millipore). After wash with PBS/0.05% Tween the cells were incubated with the secondary antibody at RT for 1 hour against Cy3-conjugated anti-mouse IgG (1:250) or goat-anti-rabbit IgG2-FITC (1:200), respectively. After wash with PBS/0.05% Tween and then water, the cover slips were inbedded in Fluoromount G (Sigma) and dried overnight at RT.

2.4 Chromatin Immunoprecipitation (ChIP)

Chromatin Immunoprecipitation (ChIP) is a way to examine whether a transcription factor of interest has a direct target gene amplified by PCR for 30 cycles. For detailed information, see appendix Chromatin Immunoprecipitation. In this procedure, antibodies are used to precipitate DNA bound by transcription factors to which the antibodies are raised against. To analyze whether Smad restricted signalling proteins interact with unknown target genes in hES cells, anti-Smad1 antibodies or anti-Smad2 were used to immunoprecipitate the protein-DNA complex (Xu *et al.*, 2008) (Termen *et al.*, 2008). In short, hES cells were crosslinked with 1% formaldehyde for few minutes, harvested in PBS and nuclei extracted. Chromatin was sheared by sonication and DNA/protein complexes were precipitated with the antibodies of interest. After immunoprecipitation,

samples were amplified by PCR for 30 cycles. For detailed information, see appendix Chromatin Immunoprecipitation.

2.4.1 Reverse Transcription polymerase chain reaction (RT-PCR)

RNA was isolated using Qiagen columns and reverse transcription of the RNA into cDNA was conducted using the Superscript II reverse transcriptase (Invitrogen). RT-PCR for the various genes was performed using the conditions and primers (Biomers) detailed in Tables 5-7.

The PCR reaction was performed in 50 µl reaction volume as follows:

Table 5. The PCR reaction.

Step	Vol.	
1.	1µl	Template – ChIP sample
2.	3µl	25mM $MgSO_4$
3.	5µl	10x NH_4SO_4 PCR buffer
4.	1µl	10mM dNTP
5.	1µl	5µM forward primer
6.	1µl	5µM reverse primer
7.	0,4µl	*Taq* polymerase (5U/µl)
8.	37,6µl	Deionised sterile water
Total Volume	50µl	

The basic PCR programme was used as follows:

Table 6. The basic PCR programme.

Step		Temperature - °C	Time – min
1.	Denaturing	94	4
2.	Denaturing	94	1
3.	Annealing	60	1
4.	Elongation	72	1
5.	Go to step 2	29x	
6.	Elongation	72	5
7.	Forever	4	∞

The PCR fragment was run on a 1-2% agarose gel at 80V for approximately 40 min.

Table 7. Human primer sequences used for PCR.

PCR target	Primer	Primer sequence	Product size	PCR conditions
Nanog	hNanog F hNanog R	tgcctcacacggagactgtc tgctattcttcggccagttg	395 bp	AT=60°C
Brachyury	hBrachT F hBrachT R	atcaccagccactgcttc gggttcctccatcatctctt	155 bp	AT=56.2-60.5 °C (60.5°C)
ANF	hANF F hANF R	tctgccctcctaaaaagcaa atcacaactccatggcaaca	248 bp	AT=56°C
ARP	hARP F hARP R	caccattgaaatcctgagtgatgt tgaccagcccaaaggagaag	115 bp	AT=60°C
Smad1	hSmad1 F hSmad1 R	aacaaatctcttctgctgtcc aaccgcctgaacatctcctct	850 bp	AT=64°C
Smad2	hSmad2 F hSmad2 R	cgaaatgccacggtagaaat ccagaagagcagcaaattcc	223 bp	AT=52°C
Smad4	hSmad4 F hSmad4 R	ccatttccaatcatcctgct acctttgcctatgtgcaacc	221 bp	AT=52°C
Id1	hId1 F hId1 R	aaacgtgctgctctacgaca gattccgagttcagctccaa	152 bp	AT=51°C
Id2	hId2 F hId2 R	acgacccgatgagcctgcta tcctggagcgctggttctg	213 bp	AT=51°C
Id3	hId3 F hId3 R	tgagcttgctggacgac ccttggcatagtttggagag	571 bp	AT=51°C
PAI-1	hPAI-1 F hPAI-1 R	cctccaacctcagccagacaag cccagcccaacagccacag	222 bp	AT=59°C
C-fos	hC-fos F hC-fos R	aacgcttgttataaaagcagtggc tcagtcttggcttctcagatgc	81 bp	AT=60°C
Myo	hMyoglobin exon 2 F hMyoglobin exon 2 R	agcatggtgccactgtgct ggcttaatctctgcctcatgatg	50-100 bp (Diagenode)	AT=60°C

2.5 Purification of total RNA from hES cells

Total RNA was isolated from hES cells using RNeasy Mini kit from Qiagen. A Human reference sample was used as a control between different microarray experiments. The RNA concentration was measured on a Nanodrop spectrophotometer and integrity determined on Agilent 2100 Bioanalyzer RNA Nano chips. RNA integrity number (RIN) higher then 8.0 is necessary to get a successful gene expression data from microarray experiments. 20 µg of total RNA sample was needed for Roche NimbleGen arrays. Microarray experiments were performed by Roche NimbleGen.

2.6 Microarray gene expression

We used microarrays to determine differential gene expression in hES cells at different developmental stages. 4-Plex expression arrays (4x72K) were used to compare the gene expression on whole genome microarrays. These arrays comprise 72,000 probes per array with long-oligo probes (60mers) that increase sensitivity and specificity for gene expression analysis. Hybridization was done with fluorescently labelled cDNA, reverse transcribed from our RNA samples and relative abundance determined. We analyzed the data from the microarray gene expression study by using the ArrayStar 2.0 software from DNASTAR.

III. Results

3.1. Characterization of human embryonic stem cells

It is important to understand how embryonic stem cells undergo either differentiation or self-renewal. This choice is decided by cues from growth factors or inhibitors. It is therefore important to identify these factors and their downstream signalling machinery including transcription factors that regulate gene expression. The generation of mice lacking specific components of the TGFβ signal transduction pathway shows that TGFβ is a key player in mesodermal development (including cardiomyocytes). Hence, it is likely that TGFβ plays a role in embryonic stem cell fate, both self-renewal and differentiation.

TGFβ members regulate specific target genes that in turn regulate embryonic stem cell fate decisions. To gain more insights into the role of the TGFβ superfamily in pluripotent hES cells and their differentiation, our focus was to stimulate hES cells with different TGFβ members and analyze the cells morphologically and in terms of gene expression using differential gene expression on microarray. We also wanted to study which genes are regulated by transcription factors downstream of the TGFβ signalling pathway in hES cells. This was done by Chromatin Immunoprecipitation.

In 2006, hES culture started for the first time in Iceland. To retain pluripotent state in culture, ES cells depend on a mitotically inactive feeder cell layer (mouse embryonic fibroblast, MEFs) or feeder conditioned medium with basic FGF growth factor included in the medium. The cells secrete known and unknown factors that are crucial for pluripotent embryonic cells. HUES cultures need care and are very sensitive to any

differences. For example, MEFs need to be freshly seeded in order to secrete factors which are necessary for hES cells. Also, if old medium is used, the cells will behave differently and can change morphology and even karyotype.

We started out by characterization of the two hES cells lines, HUES 3 and HUES 9, and optimized their culture conditions and differentiation protocol. The hES cell were cultured on MEFs. The morphology, marker expression and karyotype of the hES cells was characterized. We also used another hES cell line named H1 which is among the best defined hES line and is widely used. As seen in figure 9A, using phase contrast microscopy the morphology of HUES cells is as expected. Colonies of undifferentiated hES cells cultured on MEFs are circular and discrete and individual cells have big nuclei.

To confirm the pluripotency of the HUES 3 and HUES 9 cell lines expression of markers characteristic for undifferentiated hES cells was examined. Oct4 is a POU-domain transcription factor that is essential for establishment of ES cells from the ICM. Nanog is a transcription factor that is known to be expressed in pluripotent ES cells and plays an essential role in sustaining human ES self-renewal. These pluripotent markers are strongly downregulated during differentiation (Valdimarsdottir and Mummery, 2005). The hES cells were fixed and immunofluorescently stained with Cy3 labelled Oct3/4 antibody and FITC-labelled Nanog antibody. Both pluripotent markers were highly expressed in the hES colonies only (Figure 9B and 9C).

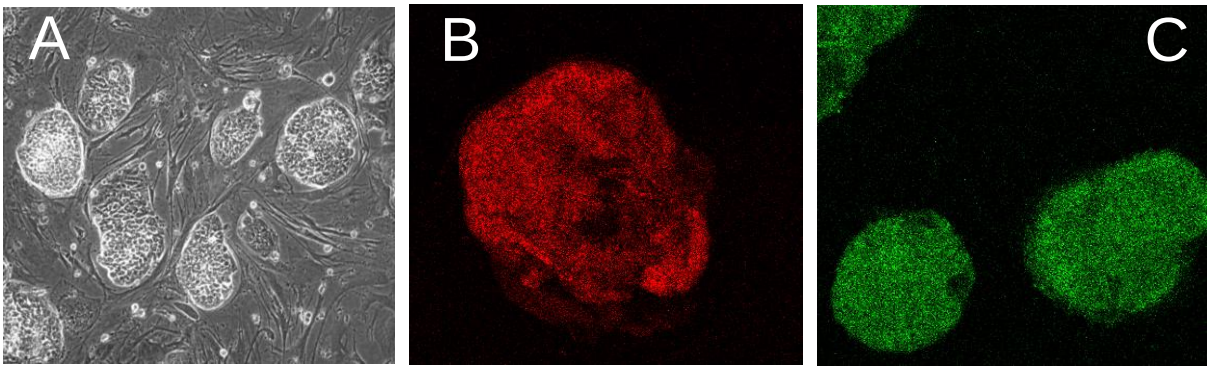

Figure 9. hES cell pluripotency. hES colonies cultured on MEF cells (**A**). hES cells immunofluorescently labelled with the pluripotent markers Oct4 (**B**) and Nanog (**C**).

hES cells are sensitive to chromosomal changes after multiple passage as the incidence of abnormal karyotype might increase during long time culture. We therefore checked the karyotype of HUES 3 and HUES 9 regularly, in our case every 10 passages as described in material and methods. Approximately 100 metaphase nuclei were examined for each cell line. Karyotype analysis revealed that HUES 3, passage 35 was abnormal with several chromosomal destructions; hence we stopped working with HUES 3 cell lines. The HUES 9 cell line appeared to be normal apart from the fact that this cell line has an inversion on chromosome 9, inv(9)(p11q12) (Table 8) (Figure 10) (Cowan *et al.*, 2004). This inversion is common in humans considered to have no deleterious or harmful effect and shows no phenotype. We therefore continued all our experiments using HUES 9 line (Garder RJM., 2004).

Table 8. Karyotype analysis on HUES 3 and HUES 9

HUES 3	Passage 35	Abnormal	HUES 3 = 50, XY,+add(12)(qter),+15, +17, +20
HUES 9	Passage 15	Normal	Invert chromosomal 9

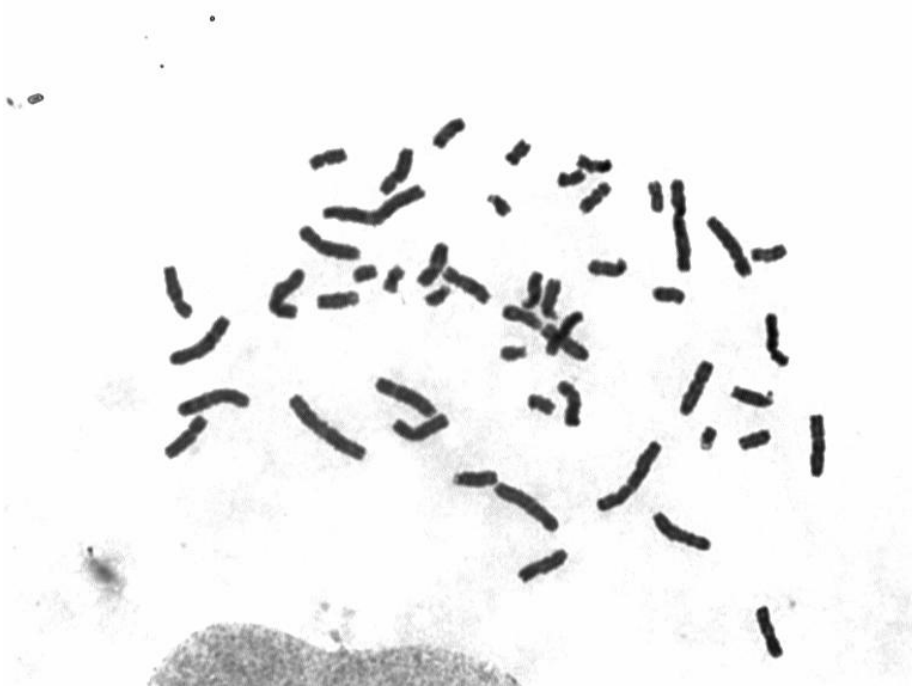

Figure 10. Karyotype analysis in human embryonic stem cells.

BMP4 stimulates hES cell differentiation

To investigate the role of the TGFβ superfamily on hES cells we stimulated the cells with the main TGFβ superfamily members, TGFβ1 (4ng/ml) and BMP4 (10ng/ml). BMPs lead to activation of Smad 1/5/8 through BMP type I and type II receptors whereas TGFβ, Activin and Nodal lead to Smad 2/3 activation through Activin type I and type II receptors. hES cells were split on MEFs (1:5 dilution) with different stimuli (Figure 11A). The TGFβ cascade is only active for 24 hours (differs between TGFβ members). Hence, the medium was replenished every day. hES cells were cultured for 3 days and morphological studies clearly indicated that BMP4 induced hES cell to undergo differentiation compared to unstimulated hES cells that maintained their pluripotent hES cell characteristics, growing in discrete colonies on MEFs. The BMP-induced

differentiation was not detected when the BMP/Smad1/5 pathway was hindered by the BMP natural antagonist Noggin (Figure 11A). TGFβ stimulated hES cells remained pluripotent (data not shown). The same hES cells were immunostained with Cy3 labelled Oct3/4 antibodies, the most common pluripotent marker for ES cells. (Valdimarsdottir, unpublished data) Unstimulated hES cells showed high expression of Oct3/4. On the other hand, hES cells subjected to BMP4 had very low Oct3/4 expression (Figure 11B) indicating that the cells have differentiated. Overall, this suggests that BMP4 is a potent hES cell differentiation inducer.

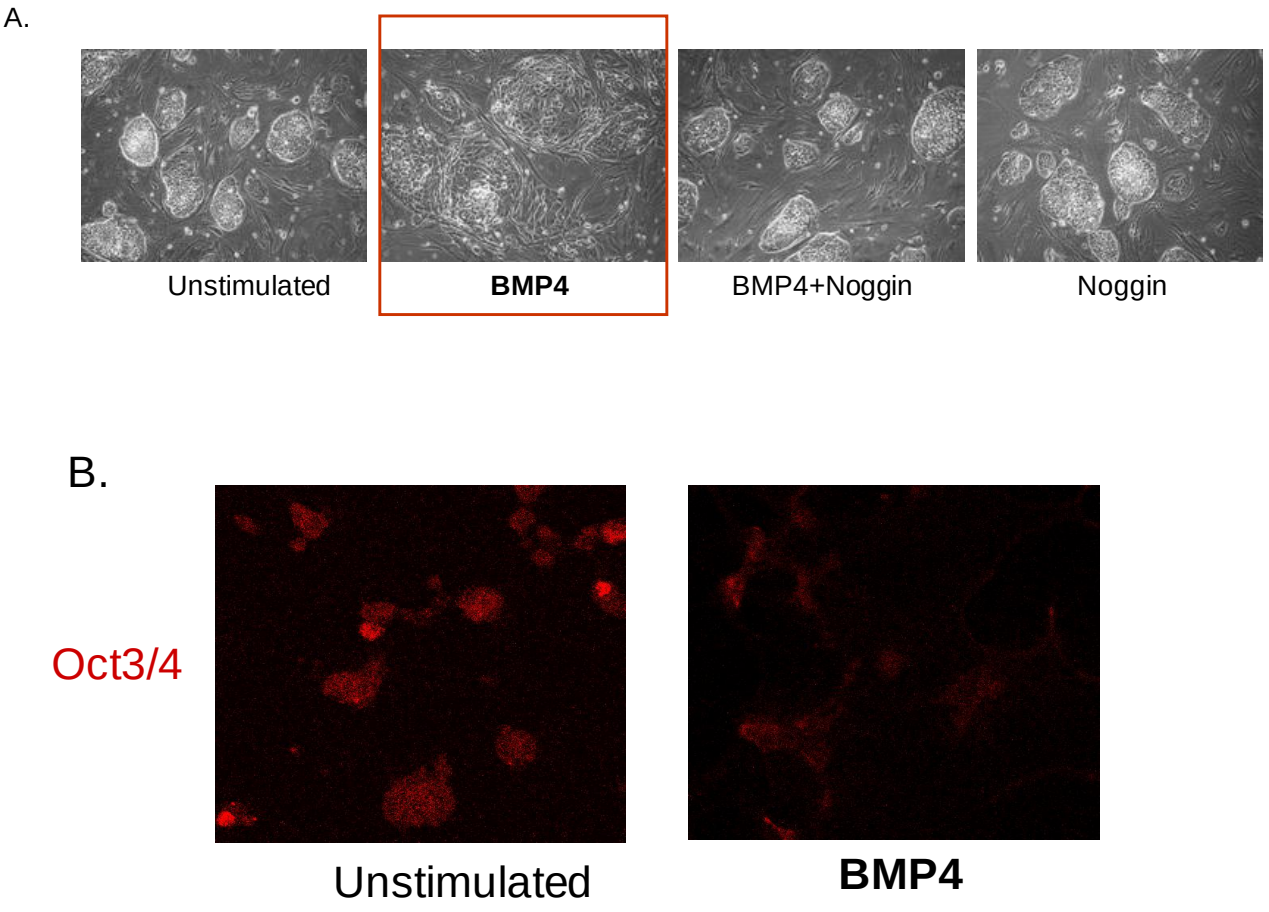

Figure 11. hES cells differentiate upon BMP4 induction. hES cells were stimulated with or without BMP4 and/or Noggin for 3 days (**A**) and the cells fluorescently labelled with Oct4. Oct4 staining disappears upon BMP4 stimulation (**B**).

BMP induces cardiomyocyte differentiation in hES cells

Because of their importance in mouse mesodermal development, especially in cardiogenesis and angiogenesis (Goumans and Mummery, 2000), the TGFβ family was studied with respect to their effects on differentiation of ES cells to cardiomyocytes. BMPs have been shown to induce the expression of Id proteins (Inhibitors of differentiation), which in turn inhibit bHLH transcription factors, thereby blocking differentiation of mouse embryonic stem cells (Hollnagel *et al.*, 1999). Overexpression of Id can mimick the effects of BMP. This effect has not been shown in hES cells and in fact Thomson and colleges have shown that repression of BMP sustains undifferentiated hES cells. Additionally, they have shown that BMP stimulation in hES cells in conditioned medium containing bFGF promotes trophoblast differentiation (Xu *et al.*, 2005). Pera and colleges reported that blocking BMP activity in serum does not maintain hES cell self-renewal, but instead enhances primitive endoderm differentiation (Pera *et al.*, 2004). Since BMP4 dramatically induced differentiation of hES cells, the question if BMP4 was a potent inducer of hES cell cardiomyocyte differentiation was addressed. In the case of hES cells we used well established co-culture of hES2-END2 cells in serum free medium (Passier *et al.*, 2005) and in case of mES cells we made use of embryoid bodies (data not shown). After 12 days of differentiation during hES2-END2 coculture the number of beating areas formed was assessed. Results showed that BMP4 is a potent inducer of cardiomyocyte differentiation of hES cells (Figure 12A). On the other hand, hES cells subjected to TGFβ and ActivinA had no effect on the number of beating cardiomyocytes compared to unstimulated cells. The BMP effect could be inhibited by the BMP natural antagonist, Noggin (Figure 12B).

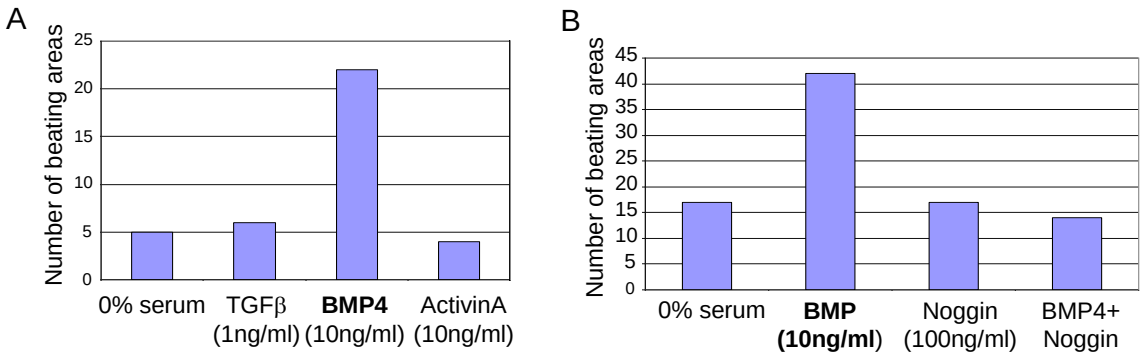

Figure 12. hES-END2 co-culture with different stimuli. Beating areas were counted after 12 days of co-cultured. BMP4 induces CM differentiation whereas Noggin inhibits it. Represented experiments in triplicate.

We wanted to exclude the END-2 cells from the hES2 co-culture as we did not entirely know what factors the END-2 cells are secreting to the hES cells. We therefore used the EB model to differentiate hES cells into beating cardiomyocytes but unfortunately the hEBs did not develop into cardiomyocytes (data not shown) even though we tried varying amounts of cells to form EBs and medium with and without serum (data not shown). Next, we seeded the hES cells on gelatinized plates in differentiation medium. This approach was not successful either (data not shown). In both cases the pluripotent hES cells differentiated into various cell types but not into beating cardiomyocytes. Various approaches to differentiation experiments are still undergoing in the lab to get around this problem.

In line with the BMP4-induced cardiomyocyte differentiation we wanted to know if different TGFβ superfamily members would regulate the expression of cardiomyocyte precursor genes and cardiomyocyte genes. We therefore determined the expression of cardiac genes in HUES 9 cells that were subjected to the TGFβ superfamily. For this we studied unstimulated cells as well as cells stimulated with TGFβ (4ng/ml) and BMP4 (10ng/ml). RNA was isolated, cDNA generated and PCR performed on cDNA samples.

T-Brachyury is one of the early mesodermal markers and is highly upregulated upon BMP4 stimulation (Figure 13A) whereas TGFβ induced hES cells do not express any T-Brachyury. The ANF (Atrial Natriuretic Factor) marker appears later in cardiac development and is also identifiable upon BMP4 stimulation. These results support the idea that BMP induces cardiomyocyte differentiation from hES cells. The expression of the majority of the TGFβ superfamily components (type I receptors and Smads) was also tested upon different stimuli but their expression was hardly affected (Figure 13B).

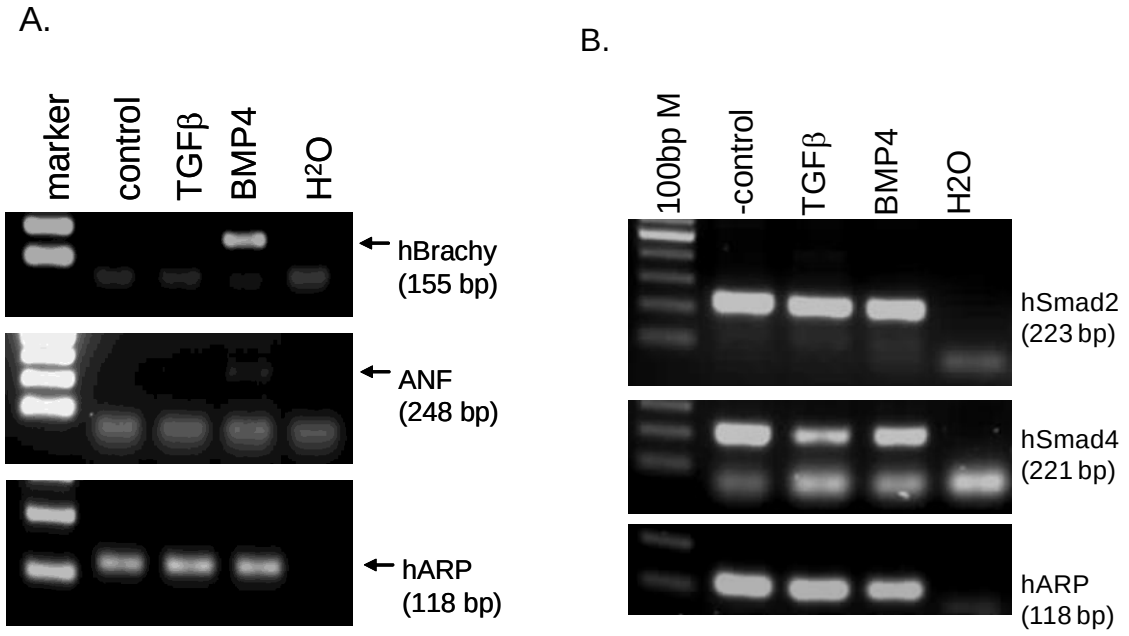

Figure 13. Mesodermal and cardiac genes are upregulated upon BMP4 induction. **A**. RT-PCR on samples after different stimuli of hES cells. hBrachyury with expected band at size 155bp. ANF with expected band at size 248bp and control hARP with expected band at size 118 bp. **B**. RT-PCR on samples after different stimuli of hES cells. Smad2 with expected band at size 223 bp. Smad4 band at size 221 bp and control hARP with expected band at size 118 bp. ANF, Atrial Natriuretic Factor.

3.2. Gene expression

BMP induces cardiomyocyte genes

We have shown that BMP4 induces differentiation of hES cells into cardiomyocyte. This is seen both morphologically and by immunofluorescent studies which showed down-regulation of known pluripotent markers. Moreover, hES cells underwent cardiomyocyte differentiation upon BMP4 stimulus as was assessed with the number of beating areas and RT-PCR of known cardiomyocyte marker genes. In order to get more insight into which genes are regulated by the main members of the TGFβ superfamily in hES cells we used gene expression analysis.

Table 9. HUES 9 cultured on MEF cells. Different stimuli.

	Samples
1	HUES 9 UD Unstimulated
2	HUES 9 UD TGFbeta (4ng/ml)
3	HUES 9 UD BMP4 (10ng/ml)
4	HUES 9 UD SB431532 (10µM)
	Human-Ref

HUES 9 cells were cultured on MEF cells in 6-well plates with and without different stimuli. This included unstimulated cells as well as cells treated with TGFβ (4ng/ml), BMP4 (10ng/ml) and the ALK4/5/7 inhibitor, SB431542 (10µM) (Table 9). Medium containing different growth factors was changed every day. After 3 days RNA was isolated from the HUES 9 cells. Each experiment was repeated 3 times. The RNA quantity was measured for each sample using the Nanodrop spectrophotometer

(Nanodrop ND-1000 spectrophotometer). It was necessary to analyze the RNA samples further using Bioanalyser (Agilent) for quality and purity control prior to use on gene microarray (Figure 14B). Each sample must have an RNA Integrity Number (RIN) at minimum 8.0. This number is used for standardization of RNA quality control as it is critical for obtaining meaningful gene expression data. The Bioanalyzer software automatically generates the ratio of the 18S to 28S ribosomal subunits of each sample (Figure 14A). All our samples had a sufficient RIN number (9.2 or higher). 20 µg of RNA sample was needed for a whole genomic chip from Roche NimbleGen. cDNA was prepared, labelled with Cy3 and hybridized to the 72K 4-Plex expression arrays from Roche NimbleGen. We used the same human reference sample as a control between experiments. Data was analyzed in ArrayStar v2.0 expression analysis software.

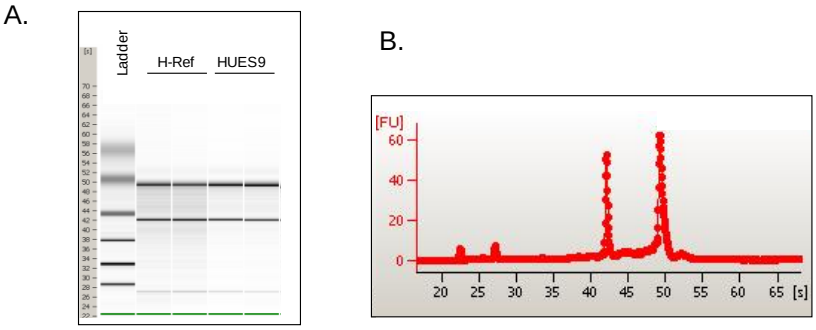

Figure 14. RNA samples from HUES 9 were analysed using Bioanalyzer. **A**. The quality and the size of the RNA analysed on a digital gel. **B.** The quality and purity of RNA samples from HUES 9 compared. RIN number is obtained after separation of the RNA on a spectrophotometer. [FU], Fluorescence Unit, [s], seconds.

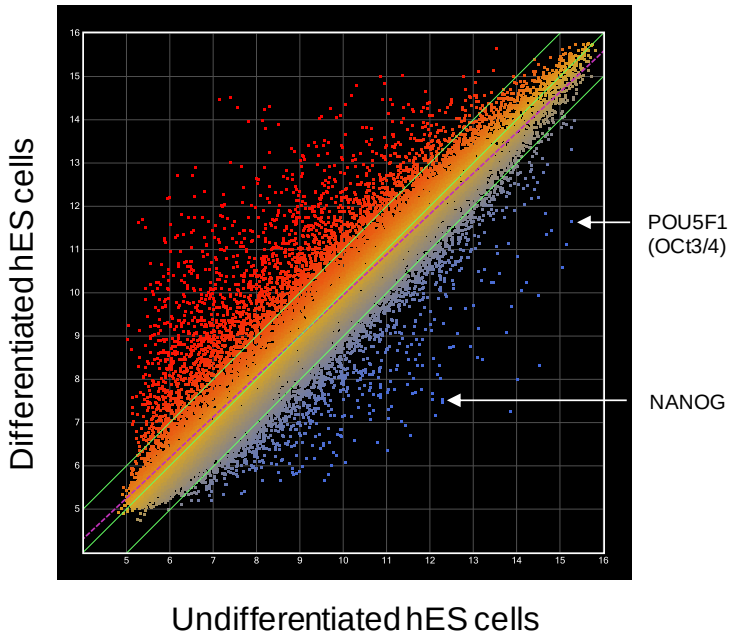

Figure 15. Scatter plot of a comparison of gene expression values (log_2) levels between undifferentiated hES cells (x-axis) and differentiated hES cells (y-axis). The arrow depicts the POU5F1 (Oct3/4) gene and Nanog gene.

Scatter plot gives a visual comparison of gene expression levels between any two datasheets (Figure 15). Each data point represents an individual gene and is plotted based on its expression levels in the selected samples. If genes lie on the line which is drawn diagonally in the middle of the plot, then these genes are equally expressed in both samples. Therefore, the expression dots with distance far away from the diagonal line are the most interesting ones.

In order to confirm that the procedure was successful we started out by comparing pluripotent hES cells to differentiated cells (hES cells were differentiated for 6 days) since pluripotent markers are expected to be rapidly downregulated upon differentiation. As shown in table 10 the majority of genes important for self-renewal maintenance are drastically downregulated after 6 days of hES cell differentiation, including NANOG, OCT3/4, TDGF1 and LEFTYB. Surprisingly, SOX2 was only downregulated by 1.3-fold

expression in the differentiated cells as compared to the untreated cells. This data indicated that the approach was reliable (Table 10).

Table 10. Comparison of gene expression in hES cells. hES cells undifferentiated vs. differentiated.

UD vs D

GenBank accession no.	Gene name	Fold change	Gene description
NM_024865	NANOG	26,548 down	Nanog homeobox
NM_002701	POU5F1	12,238 down	OCT3/4
NM_003212	CRGF	21,756 down	TDGF1 teratocarcinoma-derived growth factor 1 (Cripto)
NM_020997	LEFTY1/LEFTYB	22,237 down	Left-right determination factor 1
NM_018055	NODAL	9,734 down	Nodal homolog
NM_002164	IDO	28,048 down	Indoleamine-pyrrole 2,3 dioxygenase
NM_024504	PFM11	73,594 down	PR domain containing 14
NM_002006	FGFB	8,570 down	FGF2 fibroblast growth factor 2 (basic)

On the basis of our previous results that BMP4 induces cardiomyocyte differentiation, gene expression was compared between unstimulated hES cells and hES cells that had been subjected to BMP4 for 3 days. Table 11 shows a group of genes that were expressed at substantially higher levels in BMP4 stimulated hES cells compared to untreated hES cells. These genes include T-Brachyury, ISL-1, GATA-3, Cardiac muscle 1 alpha, HAND1, TBX3 and MIXL1 (MILD1). These genes are all important in mesodermal development although GATA-3 and MIXL1 seem to be more important in directing differentiation towards haematopoietic cells rather than towards cardiomyocytes, (Table 11. Gray box) (Puceat, 2007, Bu *et al.*, 2009, Keller *et al.*, 1993, McFadden *et al.*, 2005, Boyett, 2009, Ng *et al.*, 2005). Some of the genes that turned out to be BMP4-upregulated in the microarray analysis have been suggested by other groups to be influenced by BMP4. Mixl1 was shown to be required for BMP4-induced ventral mesoderm patterning in differentiating ES cells (Ng *et al.*, 2005). Dlx5 is a target gene of

the BMP signaling pathway and is an important regulator of both osteogenesis and dorso-ventral patterning of embryonic axis (Miyama *et al.*, 1999). Also, BMP4 is crucial for vascular tube formation acting via the FoxF1 (Astorga and Carlsson, 2007). At last, BMP4 is suggested to play two distinct and sequential roles during blood formation, initially as an inducer of mesoderm and later to specify blood via activation of Wnt signalling and CDX/HOX pathway (Lengerke *et al.*, 2008). Several genes within the BMP signalling cascade were also upregulated in BMP4 treated cells (Table 11. Purple box), i.e. BMP4, Cerberus and BAMBI.

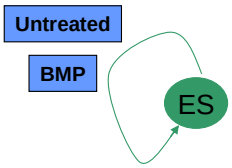

Table 11. Comparison of gene expression in hES cells. hES cells unstimulated cells vs. stimulated hES cells with BMP4. Mesodermal genes in gray box. BMP genes in purple box.

↑ BMP4

GenBank accession no.	Gene name	Fold change	Gene description
NM_003181	T	3,326 up	T, brachyury homolog (mouse)
NM_002202	ISL1	3,553 up	ISL1 LIM homeobox 1
NM_002476	AMLC	3,161 up	Myosin light chain 4, atrial, embryonic muscle
NM_005159	ACTC1	2,614 up	Actin, alpha, cardiac muscle 1
NM_004821	Thing1	6,823 up	HAND1 (Heart and Neural crest Derivatives expressed 1)
NM_153604	MYOCD	3,996 up	Myocardin
NM_005593	MYF5	2,774 up	Myogenic factor 5
NM_032638	NFE1B	3,732 up	GATA2 (GATA binding protein 2)
NM_001002295	MGC2346	3,231 up	GATA3 (GATA binding protein 3)
NM_005257	GATA6	3,094 up	GATA6 (GATA binding protein 6)
NM_031944	MIXL1 (MILD1)	2,644 up	Mix1 homeobox-like 1
NM_030775	WNT5B	3,467	Wingless-type MMTV integration site family member 5B
NM_005221	DLX5	3,735 up	Distal-less homeobox 5
NM_001430	HLF	3,158 up	EPAS1 (endothelial PAS domain protein 1)
NM_005996	UMS	3,520 up	TBX3 (T-box 3)
NM_001451	FREAC1	3,763 up	FOXF1 (Forkhead box F1)
NM_001804	CDX1	3,923 up	Caudal type homeobox transcription factor 1
NM_001265	CDX2	4,953 up	caudal type homeobox transcription factor 2
NM_001305	CPER	3,780 up	Claudin 4
NM_001884	HAPLN1	4,565 up	Hyaluronan and proteoglycan link protein 1
NM_001676	ATP12A	6,971 up	ATPase, H+/K+ transporting, nongastric, alpha polypeptide
NM_001202	BMP2B1	2,683 up	BMP4 (Bone Morphogenetic Protein 4)
NM_005454	CER1	2,505 up	Cerberus1 (cysteine knot superfamily)
NM_012342	BAMBI	2,640 up	BAMBI (BMP and activin membrane-bound inhibitor homolog

3.3. Chromatin Immunoprecipitation

Transcription factors are regulatory proteins that bind to DNA and are involved in the control of gene expression. It is well known that regulation of certain transcription factors plays a crucial role in of stem cell self-renewal and differentiation. Hence, understanding their role and targets is of central interest to stem cells research. Although, it has been unclear how hES cells maintain their pluripotency, it is known that several important molecular mechanism are involved. Pluripotent hES cells need to be cultured in medium with bFGF and increasing amount of data support the idea that pluripotency is sustained via activation of Smad2/3 by the TGFβ/Activin/Nodal pathway. Studies based on the ALK4/5/7 inhibitor, SB431542, suggested that the TGFβ/Activin/Nodal pathway is essential for hES self-renewal. However, it has been unclear how TGFβ/Activin/Nodal mediated Smad2/3 signalling in hES cells regulates target genes directly, particularly to OCT4 and Nanog (Beattie *et al.*, 2005, James *et al.*, 2005, Xu *et al.*, 2008).

We were therefore interested in unraveling how gene expression is regulated by transcription factors downstream of the TGFβ pathway. We used chromatin immunoprecipitation (ChIP) which is a good method to determine whether certain transcription factors bind to particular DNA sites. The method relies on antibodies which are used to precipitate DNA bound by the transcription factors under investigation. After the specific antibody precipitation the DNA complexes are purified and then analyzed by RT-PCR using primers specific for the candidate promoter. To test which Smads are activated downstream of the TGFβ superfamily we made use of antibodies that recognize either Smad 1/5 or Smad2/3. The antibodies used in the chromatin immunoprecipitation have been raised against unphosphorylated and phosphorylated Smad2/3 (transcription

factor (TF) activated downstream of TGFβ and ActivinA) and phosphorylated Smad1/5 (TFs activated downstream of BMP).

First we had to optimize the ChIP technique to our hES work in the lab. We used antibodies raised against the widely expressed TBP (TATA Binding Protein) and Histone3 (acetylated histone) as controls. To confirm that the TBP antibody would recognize TBP in human cells we checked cell lysates from 293T cells (human embryonic kidney cells) and hES cells by western blotting. Cell lysate was loaded on 10% SDS-PAGE gel and immunoblotted after transfer with the anti-TBP antibody. The TBP antibody recognized the TBP protein showing one band of correct size, 38kDa (Figure 16A). Serum was used as a negative control for the immunopreciptation and TBP and Histone3 as positive controls. After ChIP we performed PCR reaction as described in material and methods using 30 cycles. To analyze the PCR products, the c-fos primer set was used as a positive control as both TBP and Histone3 bind to the c-fos promoter to initiate transcription. We also tested the primers which served as negative controls, namely hsp70 primer pair specific for the Hsp70 (heat shock protein) promoter and the myoglobin exon 2 primer pair. These two genes are tissue-specific and the locus is transcriptionally silent in most of the cell lines (Figure 16B).

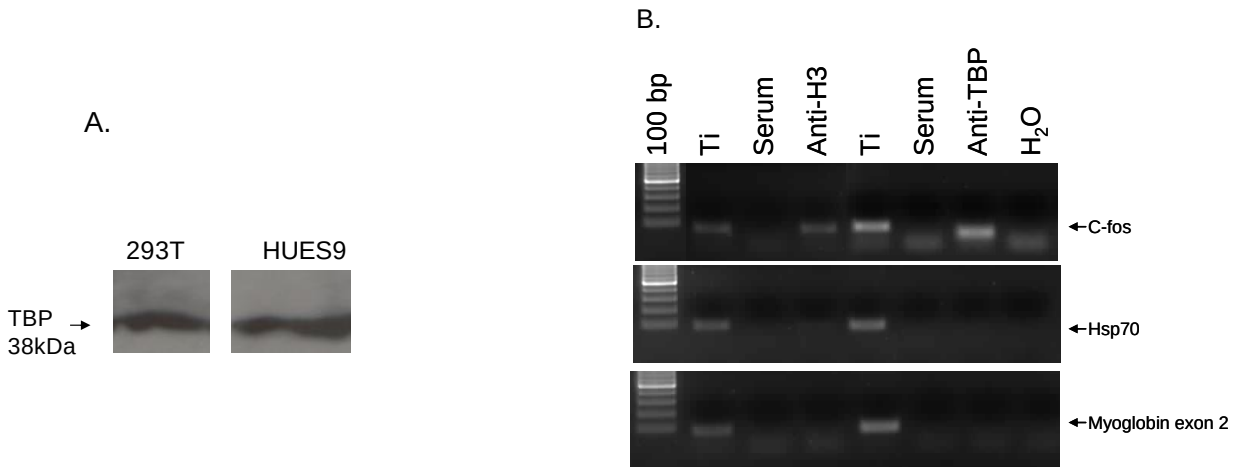

Figure 16. A. TBP protein is recognized by the TBP antibody in human cells. **B.** ChIP for Histone3 and TBP binding. HUES cells were harvested and processed for ChIP with anti-H3, anti-TBP and serum. The expression of the precipitated DNA was measured by PCR. Primers used were c-fos, Hsp70 and Myoglobin exon 2.

As seen in figure 16B, the expected band of the c-fos product of size 81 bp was seen in the samples when using the Histon3 antibody as well as in the Total input (Ti) sample. No bands were observed using the negative primers, hsp70 and myoglobin exon 2 when immunoprecipitated with Histone3 antibody. No band was detected with serum, as expected. Similar results were seen when using the TBP antibody. The optimization for the ChIP method with the antibodies, TBP and Histon3 was successful.

Next, we optimized our antibodies of interest for the ChIP (Figure 17). We tested antibodies against the transcription factors Phospho-Smad2 (activated form) and Smad2 (inactivated form) in hES cells. The protease inhibitor, PAI-1 (Plasminogen Activator Inhibitor-1), prevents ECM degradation and is known to be upregulated downstream of the TGFβ/Smad2 pathway (Goumans *et al.*, 2003b). After the ChIP with P-Smad and Smad antibodies, the PAI-1 primer set was used to detect expression in the PCR reaction.

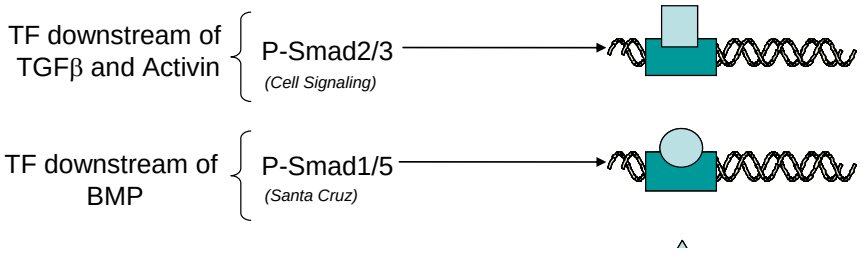

Figure 17. Antibodies of interest in ChIP. P-Smad2/3; Phosphorylated Smad2/3, P-Smad1/5; Phosporylated Smad1/5.

No bands were observed in serum and H_2O as expected (Figure 18). The expected product of PAI-1 (222bp) can be seen in the Total input as well as in the sample immunoprecipitated with anti-Smad2 and anti-Phospho-Smad2 antibodies. The PCR band was more abundant in the latter case indicating that the antibody against phosphorylated Smad2 results in better yield of the Smad2-PAI-1 complex. We therefore used Phospho-Smad2 and not Smad2 antibody in all our experiments hereafter.

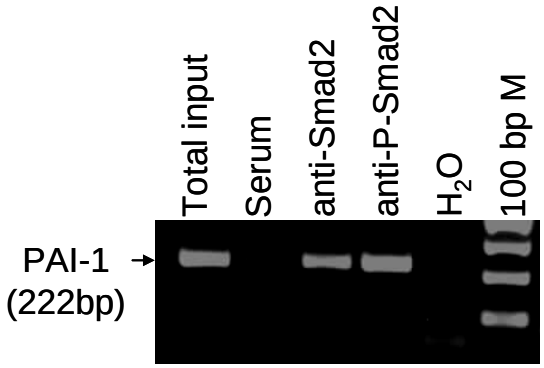

Figure 18. Smad2/3 interacts with PAI-1. Expected product band of PAI-1 at size 222 bp.

As mentioned before, the transcription factors NANOG and OCT4 are essential for maintaining both mES and hES cell pluripotency. There is also emerging evidence that the TGFβ/Activin/Nodal pathway contributes to hES cell pluripotency via Smad2/3. We were therefore interested in studying if the Smad2/3 transcription factors could directly bind to NANOG or OCT4 and thereby have a positive affect on hES cell self-renewal. We immunoprecipitated the sheared DNA from the hES cells with Phospho-Smad2 and performed PCR using Nanog specific primers to detect NANOG expression. As seen in Figure 19, the expected band at size 395 bp for NANOG can been seen in Phospho-Smad2 immunoprecipitated sample but no band appears in Phospho-Smad1 immunoprecipitated sample or in the serum sample, which is the negative control. Interestingly, a faint band appears in the Oct4 immunoprecipitated sample. Meanwhile, Thomson and co-workers published a paper showing similar results. They showed that both Smad2 and Smad1 can bind to the NANOG promoter (Xu *et al.*, 2008).

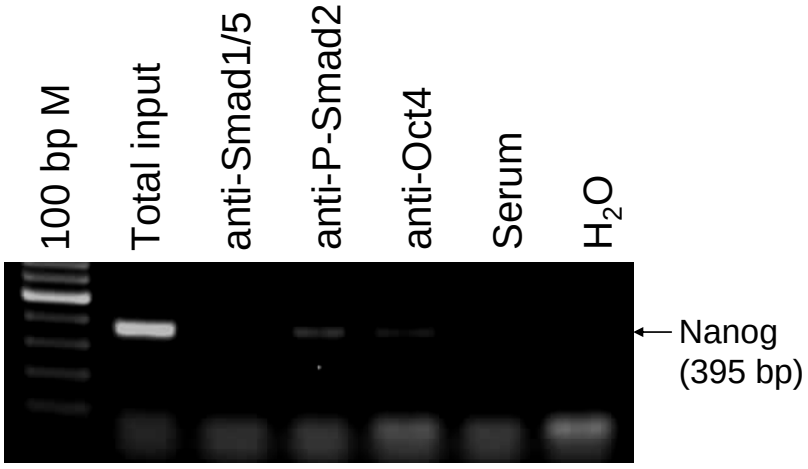

Figure 19. Smad2/3 interacts with NANOG. Expected product band of NANOG at size 395 bp.

IV. Discussion

We started out by characterization of two hES lines, HUES 3 and HUES 9, studying their morphology, marker expression and karyotypes. The morphology of the hES cell colonies was as expected. To confirm the pluripotency of the hES cell line, expression of markers characteristic for undifferentiated hES cells was examined using the well known pluripotent markers Oct4 and Nanog. These pluripotent markers were strongly downregulated during differentiation. Karyotype analysis was done on hES cell line, HUES 3 and HUES 9 where only the latter cell line was shown to be normal. Therefore, we continued all our experiment using the HUES 9 line.

We were interested in studying the role of the TGFβ superfamily in differentiation of hES cells. Knockout mice have played an important role in characterizing the TGFβ superfamily in early embryogenesis. TGFβ superfamily deficient mice show failure in mesoderm formation. Moreover, these mice point to a pivotal role of BMP and TGFβ signalling in cardiogenesis and angiogenesis. Hence, we were interested in analyzing hES differentiation into cardiomyocytes. We observed that BMP4 induces ES cell differentiation into beating cardiomyocytes and gene expression and microarray confirmed these results further showing mesodermal gene expression upon BMP induction. Noggin involvement completely inhibited the cardiomyocyte induction and instead maintained the cells in a pluripotent state. It has been suggested that Noggin promotes the differentiation of human ES cells into neuroprogenitor cells rather than cardiomyocytes (Pera *et al.*, 2004). Our data show that BMP4 induces cardiomyocyte differentiation of hES cells. Emerging data support our results. BMP2 or BMP4 under

serum free culture conditions inhibits neural differentiation and thereby support their role in the maintenance of pluripotency in mouse ES cells (Ying *et al.*, 2003). However, in human ES cells, BMP is believed to induce differentiation into extraembryonic lineages such as primitive endoderm and trophoectoderm (Xu *et al.*, 2005, Pera *et al.*, 2004). These reports reveal how difficult it is to assess the true effect of the BMPs when added at different concentrations, in the absence or presence of serum and bFGF or in the presence of feeder layer, matrigel or CellStart. Also, the switch from self-renewal to differentiation upon TGFβ related factors may be triggered by a combination of other signals induced by feeder cells or ES cells, themselves. Therefore, more experiments under standarized conditions are needed to elucidate if the different effects in mouse and human ES cells are due to the species and not the different culture conditions (Puceat, 2007). Recent reports are very much in line with our results, showing that BMP4 promotes induction of cardiomyocytes from hES cells in serum based embryoid body development (Takei *et al.*, 2009). Interestingly, we observed this effect of BMP4 on hES cells in knockout serum replacement medium but many factors in serum can affect the hES cell fate. It is suggested that the effect of BMP4 on cardiac induction has a time window restricted to the early stage of EB formation when specification into cardiac lineage occurs. The temporal window in which BMP functions in cardiomyocyte differentiation is therefore very important (Takei *et al.*, 2009, Yuasa *et al.*, 2005).

BMP2 acts via the same BMP receptors as does BMP4 and has similar effects on various cell types. BMP2 has been indicated as a potential mesodermal and cardiogenic instructor when used at low concentrations. Tomescot and co-workers (2007) found that two HUES cell lines do differentiate into cardiomyocytes upon BMP2 induction and

SU5402, which is a fibroblast growth factor receptor inhibitor. Gene induction was tested by qPCR showing that both mesodermal and cardiac genes were induced by the BMP2 morphogen in HUES-1 cells (Tomescot *et al.*, 2007).

In the mouse, the beginning of gastrulation is marked by the formation of the primitive streak (PS) in the region of the epiblast that will form the posterior end of the embryo. Molecular analysis and lineage mapping studies have defined posterior, mid and anterior regions of the PS that differ in gene expression patterns and developmental potential (Murry and Keller, 2008). Genes such as *Brachyury (T)* and *Mixl1* are expressed throughout the PS, whereas others are found in other regions. Furthermore, mapping studies have shown that the specification of distinct subpopulations of mesoderm and endoderm is not random. Different regions of the PS compose different signals that are responsible for the induction of specific lineage. Members of the TGFβ family, BMP4 and Nodal and also the Wnt family are essential for these development steps. Recent studies have shown that by using BMP4 or combination of BMP4 and Activin leads to the induction of hES cells towards subpopulations of mesoderm. This led to the proposal that different subpopulations of mesoderm can be induced by different signalling pathways (Murry and Keller, 2008, Yang *et al.*, 2008, Laflamme *et al.*, 2007).

Our microarray data from differentially induced hES cells confirmed our results on the effect of BMP induced cardiomyocytes derived from hES cells. BMP4 induced various genes that are essential for cardiomyocyte development. T-Brachyury is one of the early markers in mesodermal development and is highly upregulated upon BMP4 stimulation in hES cells whereas TGFβ has no affect. ISL-1 encodes a member of the LIM/homeodomain family of transcription factors. ISL-1 marks progenitors of the

secondary field in the early embryo (Bu *et al.*, 2009). HAND1 (heart and neural crest derivatives expressed 1) is a basic helix-loop-helix (bHLH) transcription factor which is important for mammalian heart development. Lack of Hand1 in mice results in embryonal lethality, as well as cardiac abnormalities. Therefore, Hand1 is a strong candidate for many cardiac malformations in human heart disease (McFadden *et al.*, 2005). Cardiac muscle 1 alpha, also known as ACTC1 is found in muscle tissues and defects in this gene have been associated to cardiomyopathy. MIXL1 homeobox gene is expressed in the primitive streak of the gastrulating embryo and marks cells destined to form both mesoderm and endoderm (Ng *et al.*, 2005). All parts of the cardiac conduction system have been shown to develop from the primary myocardium of the linear heart tube as an inducer of the transcription factor, Tbx3 (Boyett, 2009).

Interestingly, Cerberus and BAMBI, which are BMP inhibitors, were upregulated upon BMP4 stimulation. This could be a negative feedback loop supporting the idea that BMP4 is a transient inducer during mesoderm development and needs to be shut down within a restricted time window. Of note, ID1, 2 and 3 were not upregulated in BMP4 subjected hES cells. IDs have been identified as important BMP target genes in endothelial cells (Valdimarsdottir *et al.*, 2002). These bHLH transcription factors have also been indicated in hES cell differentiation upon BMP induction (Xu *et al.*, 2005).

Recent studies indicate that pluripotency of hES cells is improved by bFGF and TGFβ/Activin signalling. Hence, it is very interesting to unravel how these signalling pathways work on the maintenance of self-renewal and how genes are regulated thereby. We therefore stimulated hES cells with different members of the TGFβ superfamily to study which genes the R-Smads can bind to and possibly regulate. We used antibodies

that recognize either Phosphorylated-Smad1/5 or Phosphorylated-Smad2/3 in ChIP assays. Phosphorylated-Smad2 was able to bind to the NANOG and OCT4 promoters. Very resent data by Xu and co-workers showed that direct binding of TGFβ/Activin responsive SMADs (Smad2/3) to the NANOG promoter plays an important role in sustaining hES cell pluripotency. Moreover, self-renewal of human ES cells is characterized by TGFβ activation of SMAD2/3 and BMP pathway facilitates repression via SMAD1/5/8. Furthermore, they observed that both TGFβ SMAD2/3 and BMP SMAD1/5/8 can interact with the NANOG proximal promoter. These data are in line with our results although we did not detect Phospho-Smad1/5 interacting with NANOG. TGFβ and FGF signalling inhibit BMP signalling and sustain expression of the core transcription factors such as NANOG, OCT4, and SOX2 which play a role in pluripotency and promote undifferentiated proliferation of hES cells (Xu *et al.*, 2008). It will be interesting to know what are the other target genes downstream of Smad2/3 and Smad1/5 in hES cells. In other cells lines TGFβ and BMP have been shown to repress each other by inhibition of the R-Smads themselves (Goumans *et al.*, 2003b). This could also be the case in hES cells but needs further investigation. In conclusion, we show that TGFβ signalling via Smad2/3 activates a core pluripotency gene in human ES cells. Furthermore, BMP4 leads to cardiomyocyte differentiation via Smad1/5.

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Appendix

Karyotype analysis

1. hES cells are cultured in 8 wells of a 6-well plate as described.
2. Incubate the cells with 0.1µg/ml of Colcemid (Invitrogen Corparation).
3. Incubate the hES cells at 37°C for 1-3 h, until the cell line is ready for karyotype analysis.
4. Remove culture medium and retain it in a tube, use again in step 7.
5. Wash the cells once with prewarmed PBS.
6. Use 1 ml of 0.05% trypsin/EDTA in each well and incubate at 37°C for 3-5 min or longer if needed.
7. Used culture medium from step 4 to inactivate the trypsin.
8. Remove and collect MEF cells and hES cells with silicon Pasteur pipette in 15 ml tube.
9. Draw the cells repeatedly up and down with the silicon Pasteur pipette into a single cell suspension.
10. Incubate for 2 min at RT, MEF cells will fall down but hES cells will stay in the suspension because they are much smaller in size.
11. Remove the hES cells from the MEFs to 15 ml tube.
12. To get hES cells to single cell suspension, draw the cells repeatedly up and down with pipette 10 times.
13. Centrifuge the tube for 10 min at 1300 rpm and discard the supernatant.
14. Add 7 ml of 0.075M KCl hypotonia prewarmed at 37°C.

15. Resuspend the pellet carefully 4 times with Pasteur pipette.
16. Incubate hypotonia in 7-8 min at 37°C.
17. Add 1 ml of fixer (3:1 methanol/acetic acid- prepared freshly) to hypotonia and mix gently with the supernatant.
18. Centrifuge the tube for 10 min at 1300 rpm.
19. Discard the supernatant at line 0.5 ml marked on the tube.
20. Resuspend the pellet and carefully add 4-5 ml of fixer while shaking constantly.
21. Incubate the cells at RT for 30-60 min. Store fixer at 4°C.
22. Centrifuge the tube for 10 min at 1300 rpm.
23. Discard the supernatant at line 0.5 marked on the tube.
24. Wash cells with 4-5 ml of fixer.
25. Wash Pasteur pipette with the supernatant 2-4 times.
26. Centrifuge the tube for 10 min at 1300 rpm.
27. Discard the supernatant at line 0.5 marked on the tube.
28. Repeat washing steps from 30-33 if necessary.
29. After the last centrifuge step, add 500-1000 µl of fixer.
30. Clean microscope slides with 90% ethanol.
31. Let a few drops of the cell suspension fall on the slide from 25 cm height. Let the slide dry.
32. Samples are stained with Tryptan Blue Solution (0.4%). Incubate for 3 min**.**
33. Wash colour off with buffer and dry the slide.
34. Slides are ready to view in microscope and chromosomes can be counted.

The Chromatin Immunoprecipitation (ChIP) assay

Day 1:

1. hES cells were cultured in 2 wells of 6 well plates.
2. hES medium and PBS prewarmed at RT.
3. Remove and discard culture media and wash cells with PBS. Use 0.3 ml 0.05% trypsin/EDTA and incubate at 37°C for 1 min (hES cells round up).
4. Inactivate the Trypsin with 2.5 ml differentiation medium and transfer the supernatant to 15 ml tube.
5. Incubate for 1-2 min to let MEF layer sink to the bottom while hES cells will stay in the suspension because they are smaller in size.
6. Remove the hES cell suspension from the MEFs to a new 15 ml tube.
7. Centrifugation at 1000 rpm for 5 min. Wash the cell pellet once with 1 ml PBS.
8. Resuspend the cells in 1 ml PBS containing 1% formaldehyde (0.27 ml of 37% formaldehyde to 10 ml PBS) in an eppendorf tube to cross-link and incubate at RT with gentle agitation for 10 minutes.
9. Centrifuge the cells at max speed for 30 sec and resuspend the cells in 1ml PBS containing 0.125 M Glycine (0.625 ml of 2 M Glycine to 10 ml PBS) and incubate for 5 minutes at RT with gentle shaking to stop the cross-linking reaction. Centrifuge as above.
10. Resuspend the pellet with 1 ml PBS and centrifuge the cells at max speed for 30 sec.
11. Gently resuspend the cell pellet in 400µl of cold cell lysis buffer (CLB) and

incubate for 10 minutes on ice. Centrifuge at 2500 rpm for 5 minutes at 4ºC to collect nuclei.

12. Remove the supernatant and resuspend nuclei in 1 ml RIPA buffer with protease inhibitors and store on ice for 10 min, then transfer supernatant to 15 ml tube.
13. Prepare the sonicator waterbath with ice and water to cool the samples during sonication. Put clean metal rods in 15 ml tubes with spacers in sonicator. Shear the samples of chromatin using the Bioruptor™ from Diagenode (catalog # UCD-200). Samples are sonicated for 15 cycles of: [30 seconds "ON" / 30 seconds "OFF"] each. Total time is of 15 minutes. Using these conditions, the DNA is sheared to approximately 500-2000 bp fragments.
14. After the sonication, transfer the sheared chromatin to 2 ml eppendorf tubes and centrifuge at 13000 rpm for 5 minutes at 4ºC.
15. Remove the supernatant to a 2 ml eppendorf tube and add 1 ml of RIPA buffer with protease inhibitors. The final volume is 2 ml.
16. For immunoprecipitation reaction, preclear chromatin by adding 40 µl protein G-separose (GE Healthcare) and incubate for 1-2 hour at 4ºC with rotation.
17. Centrifuge the beads at 3000 rpm for 1 minute at 4ºC. Use the supernatant 500 µl chromatin per reaction as follows: total input (Ti) sample with no antibody, 3 µl of Fetal Bovine Serum sample with no antibody, and finally 5 µg of antibody of interest.
18. Add 45 µl of the homogenous protein G-Sepharose suspension per reaction.
19. Incubate over night at 4ºC with rotation.

Day 2:

20. Centrifuge the protein G-Sepharose beads at 13000 rpm for 1 min at 4ºC. Save the supernatant from the Ti sample with no antibody, on ice.
21. Remove the supernatant with antibodies and serum and wash the pellet four times with 1 ml of RIPA buffer. For each wash, vortex briefly and centrifuge at 13000 rpm for 1 minute at 4ºC. Leave the tubes undisturbed for a minute before removing the supernatant.
22. Wash the pellet similarly, once with 1 ml of IP wash buffer 2 (IPWB2) and once with 1 ml of TE pH 8.0.
23. After the last wash, centrifuge as above and remove the last traces of buffer. Elute antibody/protein/DNA complexes by adding 150 µl of IP elution buffer and shake on vortex for at least 15 minutes. Centrifuge at 14000 rpm for 1 min and remove the supernatants to clean tubes. Repeat and combine both elutions in the same tube.
24. Add 1.5 µl of RNase A (10mg/ml stock) and 18 µl of 5 M NaCl (final concentration of 0.3 M) to each sample with or without antibodies. Incubate the samples at 65ºC for 6 hours.
25. Add 9 µl of proteinase K (10mg/ml stock) (Fermentas) and incubate overnight at 45ºC.

Day 3:

26. After the de-crosslink the phenol chloroform is extracted and purified using Phase Lock Gel (PLG) spin columns. (Eppendorf, Phase Lock Gel (PLG)), Add 400 µl of Phenol Chloroform Isoamylaalcohol mixed with Tris Alkaline (65 µl in 1 ml) buffer and add 2 µl tRNA (5mg/ml stock) to each sample.
27. To precipitate the nucleic acid, add 5 µg of glycogen, 1 µl of tRNA (5mg/ml stock) and 50 µl of 3 M sodium acetate pH5.2 to each sample. Vortex well and add 1.25 ml of 100% ethanol and precipitate at -20ºC over night.
28. Centrifuge at 13200 rpm for at least 20 minutes at 4ºC and wash the pellet with 500 µl of ice-cold 70% ethanol and centrifuge at 13200 rpm for 10 min at 4°C.
29. Remove the supernatant carefully and air dry the pellets for 15 minutes or longer if needed.
30. Resuspend the pellets in 100 µl of water for the total input (Ti) and 50 µl of water for the other samples.
31. The samples are ready for further analysis by Semi Quantitative PCR.

ChIP buffers: Chromatin Immunoprecipitation (ChIP) assay

Stock:
CLB: Cell Lysis Buffer, RIPA: RIPA Lysis Buffer and IPWB2: IP wash Buffer 2, IPEB: IP Elution Buffer.
Tris HCl (pH8)
Tris HCl (pH 7.0-7.2)

5 M NaCl: 1 liter
Dissolve 292 g of NaCl in 800 ml of H_2O. Adjust the volume to 1 liter with H_2O. Dispense into aliquots and sterilize by autoclaving. Store the NaCl solution at room temperature. Store at RT. (CLB)
1 M NaCl:
100 ml of 1 M NaCl = 20 ml of 5 M NaCl and adjust the volume to 100 ml with H_2O. Store at RT. (RIPA)

10% NP-40:
10 ml NP-40 and adjust the volume to 100 ml with H_2O.
CLB: use only 2 ml to make buffer. RIPA: Use 10 ml for 1 L buffer. IPWB2: use only 5 ml to make buffer. Store at RT.

Na butyarate 1 M: 50 ml
(MW $C_4H_7NaO_2$ = 110.09) Make 50 ml stock solution. Dissolve 5.50 g of Na butyarate in a final volume of 45 ml of H_2O. Adjust the volume of the solution to 50 ml with H_2O. Sterilize the solution by passing it through a 0.22-µm filter or by autoclaving for 15 minutes (1.05 kg/cm2) on liquid cycle. Store the solution at 4°C. (CLB)

0.5 M EDTA:
pH = 8.0 1 liter
186.12 g disodium ethylenediaminetetraacetate – $2H_2O$ ($Na_2EDTA – 2H_20$, MW: 372.24) in 800 ml H_2O stir vigorously on a magnetic stirrer. Adjust to pH 8.0 with NaOH (~ 20 g NaOH pellets) and adjust volume to 1 liter H_2O. Divide into aliquots and sterilize by autoclaving. Store at RT.
Note: the disodium salt of EDTA will only solute when pH of the solution is adjusted to 8.0 by the addition of NaOH.

5 M LiCl: 100 ml
Dissolve 21.2 g of LiCl in a final volume of 90 ml of H_2O. Adjust the volume of the solution to 100 ml with H_2O. Sterilize the solution by passing it through a 0.22-µm filter or by autoclaving for 15 minutes (1.05 kg/cm2) on liquid cycle. Store the solution at 4°C.
1 M LiCl: 100 ml
Dissolve 4.24 g of LiCl in a final volume of 90 ml of H_2O. Adjust the volume of the solution to 100 ml with H_2O. Sterilize the solution by passing it through a 0.22-µm filter or by autoclaving for 15 minutes (1.05 kg/cm2) on liquid cycle. Store the solution at 4°C. (IPWB2)

NaHCO3:
Elution buffer: Dissolve 1.68 g of $NaHCO_3$ in 150 ml H_2O stir vigorously on a magnetic stirrer. 20 ml of 10% SDS mixed to the solution. Adjust volume to 200 ml with H_2O. Store at RT.

10% SDS: 1 liter
Dissolve 100 g sodium dodecyl sulfate crystals (SDS) in 900 ml H_2O. Heat to 68°C to solute the crystals. Adjust to pH to 7.2 with HCl (-50 µl). Adjust volume to 1 liter with H_2O. Dispense into aliquots and store at room temperature.
Note: the fine crystals of SDS disperse easily, wear a mask when weighing SDS and clean the weighing area and balance after use.
If SDS crystals precipitate (e.g. due to cold temperature), it can be resolved by warming the solution at 37°C.

Cell Lysis Buffer: 100 ml
Adjust volume to 80 ml with H_2O. Add 1 ml of 1 M Tris HCl (pH8), 200 µl of 5 M NaCl, 2 ml of 10% NP-40, 1 ml of 1 M Na butyrate and 350 µl of protease inhibitors. Adjust volume to 100 ml with H_2O. Sterilize by autoclaving and dispense into aliquots (15 ml tube) storage -20°C. Store the solution at 4°C.

RIPA Lysis Buffer: 1 liter
150 mM NaCl
0.1% SDS
0.5% deoxycholate
0.5% Triton X-100
50 mM Tris (pH8)
Adjust volume to 1 liter with H_2O. Dispense into aliquots; in 15 ml tubes and 50 ml tubes.
For storage:
15 ml tubes with RIPA Lysis Buffer and protease inhibitors. Store the solution at 4°C.
50 ml tubes with RIPA Lysis Buffer. Store at RT.

IP wash Buffer 2 (IPWB2): 50 ml
Adjust volume to 15 ml and add 500 µl of Tris HCl (pH 8.1)
12.5 ml of 1 M LiCl
500 µl of 0.1M EDTA
5 ml of 10% NP-40
10 ml of 5% deoxycholic acid = DOC
Adjust volume to 50 ml H_2O.

IP Elution Buffer (IPEB):
Dissolve 1.68g of $NaHCO_3$ in 150 ml H_2O stir vigorously on a magnetic stirrer. 20 ml of 10% SDS mixed to the solution. Adjust volume to 200 ml with H_2O. Store at RT.
Dispense into aliquots