Function of the TGFβ superfamily in human embryonic stem cell vascular development

Svala H. Magnús

A thesis submitted for the degree of Master of Science
University of Iceland
Faculty of Medicine
School of Health Science

HÁSKÓLI ÍSLANDS

Hlutverk TGFβ stórfjölskyldunnar í þroskun stofnfruma úr fósturvísum manna í æðaþelsfrumur

Svala H. Magnús

Ritgerð til meistaragráðu í læknisfræði

Umsjónarkennari: Eiríkur Steingrímsson

Leiðbeinandi: Guðrún Valdimarsdóttir

Aðrir í Meistaranámsnefnd: Pétur Henry Petersen

Læknadeild

Heilbrigðisvísindasvið Háskóla Íslands

Febrúar 2013

Function of the TGFβ superfamily in human embryonic stem cell vascular development

Svala H. Magnús

Thesis for the degree of Master of Science

Supervisor: Eiríkur Steingrímsson

Advisor: Guðrún Valdimarsdóttir

Other members of masters committee: Pétur Henry Petersen

Faculty of Medicine

School of Health Sciences

February 2013

Prentun: Háskólaprent

Reykjavík, Ísland 2013

Ágrip

Stofnfrumur hafa verið mikið í sviðsljósinu undanfarin ár, sérstaklega stofnfrumur úr fósturvísum manna (hES frumur, human embryonic stem cells). Merkilegar rannsóknir hafa verið stundaðar á þeim í rúman áratug og hefur afrakstur þeirra gefið vonir um nýja möguleika á sviði læknisfræðinnar. Þessar rannsóknir veita ómetanlega innsýn inn í fósturþroska mannsins og möguleikann á því að síðar verði hægt að nota þessar frumur til lækninga á sjúkdómum. Miklar vonir eru bundnar við stofnfrumur í tengslum við sjúkdóma svo sem Parkinsons, sykursýki, Alzheimer, sem og hjarta- og æðasjúkdóma.

Mikilvægt er að geta stýrt því hvort stofnfrumur viðhaldi sér eða sérhæfist í ákveðnar frumugerðir líkamans. TGFβ stórfjölskyldan (Transforming Growth Factor β) hefur mikilvægu hlutverki að gegna og ræður líklega miklu um örlög ES fruma. Því er nauðsynlegt að öðlast betri skilning á virkjun boðleiðarinnar. „Knock-out“ tilraunir í músum hafa sýnt fram á að stórfjölskyldan gegnir veigamiklu hlutverki í fósturþroskun í átt að hjarta- og æðaþelsfrumum.

Í þessu verkefni var leitast eftir að varpa betra ljósi á hlutverk TGFβ stórfjölskyldunnar í sérhæfingu hES fruma í æðaþelsfrumur. hES frumur voru örvaðar með ýmsum vaxtarþáttum og hindrum stórfjölskyldunnar og áhrifin á æðaþelssérhæfingu greind með aðferðum svo sem PCR, flúrljómandi ónæmislitunum og western blottun. Einnig var sett upp aðferð sem einangrar „vascular“ frumur úr stóru þýði mismunandi sérhæfðra fruma. hES frumur voru merktar með anti-CD31 mótefni, þekkt kenniprótein fyrir æðaþelsfrumur, og þær einangraðar. Auk samskonar greiningaraðferða og áður, voru framkvæmd próf á þessum forverafrumum til að meta getu þeirra til að mynda pípulaga byggingar á matrigeli.

Niðurstöður mínar leiddu í ljós að TGFβ vaxtarþátturinn virðist stuðla að hES frumusérhæfingu í átt að „vascular“ frumum. Í frumukúlum (embryoid bodies) ýtir TGFβ undir tjáningu á TGFβ genum sem fyrirfinnast aðeins í „vascular“/æðaþelsfrumum. Einnig örvaði TGFβ tjáningu á „vascular“ genum svo sem CD31 (PECAM-1).

Mér tókst að setja upp aðferð á rannsóknastofunni til að einangra hES ættaðar „vascular“ frumur (CD31 jákvæðar frumur). Æðaþelseiginleikar þessara fruma voru rannsakaðir og kom í ljós að þær höfðu getu til að mynda pípulaga byggingar á matrigeli *in vitro* sem svipar til æðamyndunar *in vivo*. Að auki tjáðu þær alfa smooth muscle actin (αSMA) sem bendir til að þessar CD31+ forvera „vascular“ frumur hafa þá hæfni til að sérhæfast enn frekar í æðaþelsfrumur eða sléttvöðvafrumur. Áhugaverðar niðurstöður gáfu til kynna að Bone Morphogenetic Protein 9 (BMP9) sé langöflugast til þess að ýta undir pípumyndun CD31 jákvæðra fruma í gegnum Smad1/5 virkjun og Id1 tjáningu. Þessar frumur tjáðu ekki lengur αSMA og má því leiða líkum að því að BMP9 eigi þátt í að sérhæfa „vascular“ frumur í þroskaðar æðaþelsfrumur.

Abstract

For the past few years there has been much focus on stem cell research, specifically human embryonic stem cells (hES). For more than a decade important research has been pursued, bringing hope and new possibilities in regenerative medicine. These studies provide invaluable insight into early human development and open the possibility for using embryonic stem cells to treat degenerative diseases, such as Parkinson, diabetes, Alzheimer and cardiovascular diseases.

It is very important to improve the steering of hES cell differentiation towards different cell types of the human body. The TGFβ superfamily (Transforming Growth Factor β) plays important role on ES cell destiny, hence it is essential to understand its molecular mechanisms and regulation. Knock-out studies in mice have shown that the TGFβ superfamily plays a pivotal role in early differentiation towards cardiomyocytes and endothelial cells.

In this project we wanted to elucidate what effect the TGFβ superfamily members have on hES cell differentiation towards endothelial cells. hES cells were treated with members of the TGFβ superfamily and the effect analyzed with various biological assays such as PCR, immunofluorescent staining and western blotting. A method to sort vascular cells from a large population of differentiated cells was established in our laboratory. hES cells were labelled with CD31 antibody, a known vascular marker, and isolated. In addtition to previously mentioned analysis, the ability of the sorted vascular cells to form tubes on matrigel was evaluated.

My results suggest that the TGFβ growth factor promotes hES cell commitment towards vascular cells. During embyoid body differentiation, TGFβ induced expression of TGFβ genes knowing to have a specific role in endothelial cells during angiogenesis. Besides, TGFβ upregulated vascular genes such as CD31 (PECAM-1).

I succeeded in setting up a differentiation method to isolate hES derived vascular cells (CD31 positive cells). Endothelial properties of these cells were investigated. My data show that the CD31+ cells had the potency to form tube like structures on matrigel *in vitro*, a biological assay that mimics blood vessel formation *in vivo*. Also, these vascular cells were able to express the alpha smooth muscle marker (αSMA). Having these two properties suggests that these cells are vascular progenitors having the ability to further mature either into endothelial cells or smooth muscle cells. One of the most interesting results indicate Bone Morphogenetic Protein 9 (BMP9) being by far the strongest inducer of the tube formation in hES derived CD31+ cells via Smad1/5 activation and Id1 upregulation. The BMP9 stimulated cells did not express αSMA anymore and we can therefore decuce that BMP9 promotes the differentiation of these vascular precursors towards mature endothelial cells.

Acknowledgments

This project was initiated at Læknagarður and was funded by project grant from the Icelandic Center for Research, Rannís.

First and foremost, I would like to thank my supervisor Guðrún Valdimarsdóttir for giving me the great opportunity to work under her supervision. Her endless patience, encouragement and unbelievable care for her students is admirable. I would like to thank her for her support, guidance and supervision through the years and during the writing of this thesis.

My master committee, Eiríkur Steingrímsson and Pétur Henry Petersen, I would like to give thanks to for proof-reading this thesis and for useful comments.

Jói Frímann, my great friend and fellow student. Not only did I make a great friend, you really made this time unforgettable with your endless humor and a great way of telling stories. You were always there whenever I needed a good talk and uplifting.

Arna and Lena, thank you for teaching me everything you knew when I started. You were great and patients instructors. Helga and Anne thank you for kind- and helpfulness. Thank you all so much for all the fun lunch brakes and dinner parties. We sure were a good GV-Unit team !

Thanks to Sævar Ingþórsson and Hulda Rún Bertu- og Jónsdóttir in SCRU for all your help. Sævar, for always taking the time to assist with technical problems. Hulda and Sævar, for your help with AutoMACS. To my coworkers on the 4th and 5th floor, I thank you for your assistance and friendship for the past two and a half year.

To my parents, Hilmar and Systa, thank you for your invaluable and endless support. Thank you for always encouraging me and for believing in me even when I didn't myself. Thank you for being my rock during my time of studying. Without your great parenting and enormous amount of love this degree would never have been possible.

To my little family, my fiancé Davíð and our son Hilmar Björn I give special thanks. Thank you Davíð for always having the right answers, knowing what to do when I get stressed, and for taking the time to listen to me preparing my lectures. Thank you for patience during the writing of this thesis, your calmness and endless love and support. Hilmar Björn you are the little bright light of my life. Thank you for showing me the right priority in life and making all this worth it.

Table of contents

Figures

Tables

Abbreviations

ALK	Activin receptor-like kinase
AVM	Abnormal blood vessel formation
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
caALK	Constitutively active ALK receptor
CD31	Platelet endothelial cell adhesion molecule 1 (PECAM-1)
EB	Embryoid body
EC	Endothelial cell
ECM	Extra cellular matrix
ES	Embryonic stem cell
FACS	Fluorescent activated cell sorting
hES	Human embryonic stem cell
HHT	Hereditary hemorrhagic telangiestasia
HSC	Heamatopoietic stem cell
ICM	Inner cell mass
Id1	BMP target
iPS	Induced pluripotent stem cell
MACS	Magnetic activated cell sorting
MEF	Mouse embryonic fibroblast
mES	Mouse embryonic stem cell
Noggin	Inhibits BMP signalling by blocking binding sites of both types of receptors, Type I and Type II
PAH, PPH1	Primary pulmonary arterial hypertension
R-Smad	Receptor-regulated Smad
SB431542	Inhibitor of TGFβ type I receptor
SMC	Smooth muscle cell
TGFβ	Transforming growth factor beta
VE-Cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor

1 Introduction

Vessels are very important for vascular blood supply to all tissues in the human body. To control the exchange of oxygen and nutrients between surrounding tissues and blood stream, endothelial cells (ECs) play a crucial role lining the blood vessels. During development, ECs organize themselves into tubes, then stimulate development and growth of the connective tissue cells, forming a surrounding layer of smooth muscle cells covering the blood vessel wall. Signals from surrounding tissue attract ECs to invade, making vascular blood supply not only a necessity for both non-malignant tissues but for tumours as well, making them an interesting therapeutic target (Valdimarsdottir, 2004).

Stem cells are the underlying principle of life and contribute to the genesis, growth and maturation of all higher organisms (Sng & Lufkin, 2012). Throughout our lifespan stem cells generate new stem cells either by dividing or they become more specialized cells, such as muscle or brain cells (Passier & Mummery, 2003). The possibility to treat diseases such as diabetes and Parkinson's with cell replacement therapies was made available with the establishment of human embryonic stem cells (hES) cells. To be able to use embryonic stem cells (ES) for that purpose, it is crucial to understand intracellular molecular pathways that regulate both self-renewal and differentiation of ES cells (Sng & Lufkin, 2012; Valdimarsdottir & Mummery, 2005). hES cells should improve our understanding in developmental biology and provide information concerning tissue replacement, disease and drug testing. The field of stem cell medicine is rapidly growing and further experiments on hES cells will link us closer to treat or even prevent human diseases (Thomson et al., 1998).

1.1 Stem Cells

Stem cells are primitive cells in all organisms that have the unique ability to either self-renew indefinitely or differentiate into other cells of particular organism (Passier & Mummery, 2003). They are often divided into two groups. First the ES cells which are derived from the inner cell mass (ICM) of the blastocyst. Secondly, tissue specific stem cells, which are involved in tissue replacement and repair. These tissue specific stem cells (also known as adult stem cells), give rise to cell types already present in the surrounding tissue (Gilbert, 2003; Passier & Mummery, 2003).

Besides being able to divide and self-renew, stem cells can also differentiate. Totipotent stem cells are those which can form all cells of the body, including eggs, sperm and extra embryonic tissues. Stem cells that can differentiate into all cell types except germ cells and do not have the ability to form extra embryonic tissues are referred to as pluripotent. Multipotent stem cells are cells such as haematopoietic stem cells (HSC) that can only differentiate into limited types of cells. These cells are usually found in a specialized tissue and only form cells restricted to that tissue. Unipotent cells can only differentiate into one cell type (Chambers & Smith, 2004; Mummery, 2011).

1.2 Embryonic stem cells

Embryonic stem cells are pluripotent cells derived from an early embryo. The first ES cells were isolated and established from pre-implanted mouse embryos more than 30 years ago (Passier & Mummery, 2003). The study of teratomas and teratocarcinomas, spontaneous tumours derived from ES cells of the testis or ovaries both in mice and humans, led to the discovery of embryonal carcinoma cells. In the developing testis or ovaries a germ cell may not differentiate properly and can form a tumour during puberty. If the tumour is benign or non-malignant it is called teratoma, but teratocarcinoma if it is malignant. Within these tumours the lack of organization leads to development of random tissues such as bone, cartilage, muscle, tooth-like structures and hair (Mummery, 2011; Valdimarsdottir & Mummery, 2005). Based on earlier work on teratocarcinomas and mouse ES cells (mES cells), the first hES cell line was derived from human blastocyst in 1998 (Thomson et al., 1998).

Human embryonic stem cells are derived from the zygote, during assisted fertilization. After informed consent and approval, surplus embryos are donated for research (Cowan et al., 2004; Valdimarsdottir & Mummery, 2005). The embryos are cultured upto the blastocyst stage where there are two distinctive tissues present. The sphere-shaped blastocyst is composed of an outer layer, the trophoectoderm, which gives rise to extraembryonic tissues such as the placenta. The ICM gives rise to all three germ layers of the embryo, the ectoderm, mesoderm and endoderm (Smith, 2001; Valdimarsdottir & Mummery, 2005). Around days 5-8 of development the ICM is carefully isolated from the blastocyst and plated onto a layer of mouse embryonic fibroblasts (MEFs) or feeder cells. The feeder cells are essential for ES cell proliferation and they inhibit ES cell differentiation (Figure1) (Reubinoff et al., 2000).

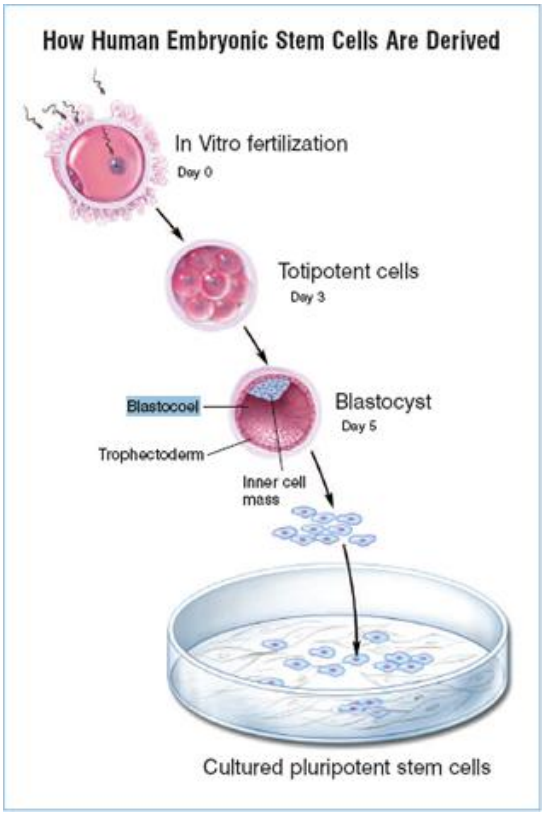


Figure 1. Isolation of human embryonic stem cells.
The inner cell mass of the blastocyst is removed and plated onto a culture plate previously coated with MEFs. These cells are pluripotent cells that can give rise to all cells of the three germ layers (Winslow, 2001).

1.2.1 Maintenance of hES cells

Culturing conditions for ES cells vary between species. The cells are placed on a culture plate or a culture bottle containing medium with all the essential nutrients for the cells such as sugars, amino acids and minerals (Figure 2). The medium needs to be refreshed every day and the cell culture is kept in a 37°C incubator. The growing cells must be diluted or passaged regularly or while they still have round and dense looking colonies and before they become confluent, meaning before they cover the whole well in a culture plate (Mummery, 2011).

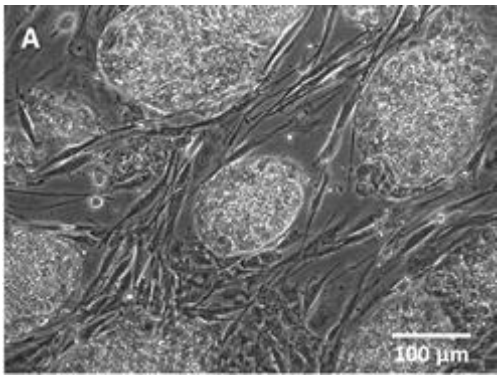


Figure 2. Human embryonic stem cell colonies cultured on MEFs.

During passaging, enzymatic dissociating solutions such as trypsin are commonly used, but more gentle enzymes can substitute for trypsin, such as collagenase, dispase or TrypLE and the cell culture is replated onto a new culture plate or used for experiments.

1.2.2 Factors sustaining ES cell pluripotency

To routinely culture and maintain undifferentiated state of mES cells, they need to be cultured either on feeder cells or in conditioned medium supplemented with leukemia inhibitory factor (LIF). Initially, a feeder layer was considered essential to maintain the pluripotent state of mES cells, but in 1988 it was demonstrated that the feeder cells secreted the LIF factor and that it was sufficient to replace the feeder layer with conditioned medium containing LIF (Passier & Mummery, 2003; Smith, 2001). The LIF cytokine binds to a heterodimer formed by LIF-R receptors and the co-receptor gp130. This signal activates JAK/stat3 signalling, the activated Stat3 is sufficient to maintain undifferentiated proliferation of mES cells (Cartwright et al., 2005; Xu et al., 2005).

This however does not apply to hES cells. In order to promote hES cell self-renewal and pluripotency, they are cultured on MEFs which supply hES cells with various growth factors and a layer to grow on. It has not been fully characterized which factors the MEFs release into the hES medium. The MEFs are mitotically inactivated or they have been treated with irradiation to prevent cell division. In addition to MEFs, hES medium has to be supplemented with basic Fibroblast Growth Factor (bFGF), these conditions are sufficient to maintain hES cells in their pluripotent state (Xu et al., 2005). The transcription factors Nanog, Oct4 and Sox2 also play an important role in keeping hES cells in their pluripotent state (Chambers & Smith, 2004; Silva et al., 2009).

1.2.2.1 Feeder-free conditions

Human embryonic stem cell lines require culture on feeder cells and/or in conditioned medium obtained from human cells. To minimize the possibility of transmitting infectious disease from other animals or species, feeder-free and serum-free ES cell culture conditions are very important if stem cell therapies are ever to become a reality (Mannello & Tonti, 2007).

hES cells can be maintained pluripotent without the support of feeder cells, on Matrigel or laminin in medium conditioned by MEFs or in fully defined culture medium, mTeSR. Feeder-free culture conditions of hES cells, show maintenance in normal karyotype, stable proliferation rate, high telomerase activity and they can form all three germ layers *in vitro* (Xu et al., 2001).

1.3 Differentiation of hES cells

When a cell starts to differentiate and specialize, the ability to proliferate decreases. The more differentiated the cell is, the less capable it is to divide. If hES cells are kept in the right conditions for differentiation they should be able to differentiate into one of more than 200 cell types present in the human body. It varies between germ layers how easily hES cells can be induced to differentiate towards each layer (Mummery, 2011). During later stages of development, the endoderm forms the inner lining of the gastrointestinal tract, the skin and the neural crest are formed by the ectoderm and the mesoderm forms for example blood cells, cardiomyocytes, and vascular- and smooth muscle cells (Figure 3) (Gilbert, 2003). hES cells are most easily induced to differentiate into ectoderm, then mesoderm, whereas cells of the endoderm seem to be much harder to produce. For unknown reasons

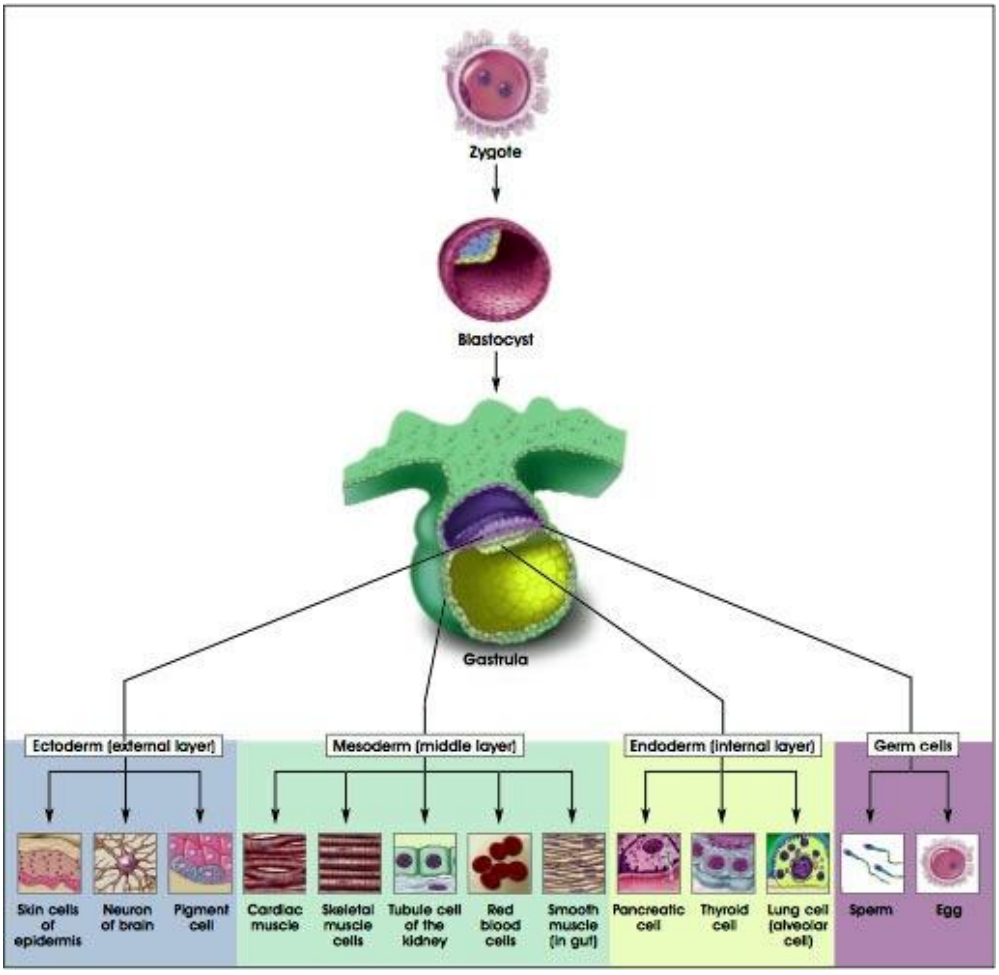


Figure 3. Origin of the three germ layers.
Early human development, showing origin of various cells types of the human body (Winslow, 2001).

the efficiency of cell types produced varies between stem cell lines (Mummery, 2011). Differentiation of hES cells can be achieved by two main methods; Spontaneous differentiation and directed differentiation (Passier & Mummery, 2003).

1.3.1 Spontaneous differentiation

Spontaneous differentiation is a differentiation method that is not directed towards any certain lineage of cells, resulting in a diverse mixture of cell types. Studies on hES cell differentiation indicate that independently-derived hES cells differ between themselves and are therefore likely to have unique characteristics. External factors like culture conditions and passage number are not the only factors that must be taken into account. Intrinsic factors have an influence on lineage commitment of hES cells (Pal et al., 2009).

3D culture systems such as embryoid bodies (EBs) in suspension or hanging drops are spontaneous differentiation models were the EBs form cells from all three germ layers (Figure 4). For the hanging drop method, hES cell colonies are dissociated from the feeder layer and certain number of cells are used for the formation of each embryoid body, depending on lineage or cell type of interest (Maltsev et al., 1994). Using spontaneous EB differentiation method in suspension, differentiation efficiency is low. For example EC differentiation yield is from 1% to 3%, (Kane et al., 2010).

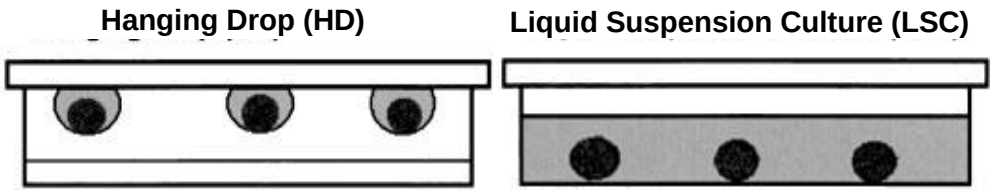


Figure 4. Spontaneous differentiation of hES cell embryoid body formation.
Hanging drop method showing cultivation of hES cells in a drop of medium mimicking blastocyst formation (left panel). Suspension method depicting hES cells cultured on non-adherent surface (right panel). Figure modulated from (Dang et al., 2002).

Because hES cells form a heterogeneous population of different cell types, it diminishes the possibility of using them as a potential source of cells of interest for cell replacement therapies. Currently, the knowledge about how to differentiate, support proliferation and maturation of hES cells into different cell types is still limited. However, several attempts have been made to direct hES cells towards certain lineages and cell types (Pal et al., 2009; Passier & Mummery, 2003).

1.3.2 Directed differentiation

A few methods can be used to direct differentiation towards a certain lineage. Well known and widely used directed differentiation method is serum and growth factor supplementation in 2D monolayer cultures (Kane et al., 2010). Gene manipulation and co-culturing with other cells are also frequently used methods. hES cells were co-cultured with visceral-endoderm like cell line, END-2 to raise the odds of cardiomyocyte differentiation (Mummery et al., 2003). The final cultures however, are always a heterogeneous mixture of different cell types. To obtain a pure population of cells, usable for regenerative medicine, one way is to induce and select the desired cells by FACS (fluorescent activated cell sorting) or MACS (magnetic activated cell sorting) methods (Levenberg et al., 2010; Passier & Mummery, 2003).

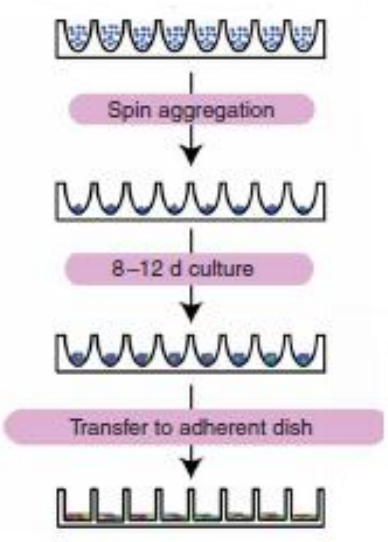


Figure 5. Directed differentiation of hES cells.
A known number of undifferentiated hES cells are used in each well in a defined serum-free medium to induce differentiation of the desired cell type (Ng et al., 2008).

Embryoid bodies in suspension are commonly used to direct differentiation towards a specific lineage, adding lineage specific growth factors to the medium. EB size seems to play a crucial role in reproducibility and to be able to control cell type specific differentiation (Burridge et al., 2007; Ungrin et al., 2008). Several research groups have reported new mechanism to control EB size using forced aggregation (Figure 5). Ng and colleagues described a method for differentation of hES cells, using a known number of undifferentiated cells in each well of a 96 well plate and a defined serum-free medium. To be able to use hES cells for clinical purposes, it is important to use pathogen-free neutral medium (Ng et al., 2008).

1.4 Endothelial cells

In recent years a constantly growing interest has been for exploring the possibility of using ECs for therapeutic purposes. This has led scientists to exploring hES cells as a potential source for endothelial progenitor cells (Figure 6) (Levenberg et al., 2007). All regenerated tissues are dependent on revascularization. The possibility to generate vascular cells from hES cells might improve regenerative medicine in many ways. This might treat diseases in regard to EC dysfunction or improve tissue transplantation and help rebuild ischemic tissues (Levenberg et al., 2002; Wang et al., 2007).

ES cell-derived endothelial cells can also be beneficial in regard to other research fields, such as human embryogenesis. They can be used as a model for studying early vasculogenesis and they might shed a light on mechanisms involved in normal developmental cell fate decisions. Moreover they might lead to therapeutic approaches for many diseases such as vascular- and heart diseases or cancer (Cleaver & Melton, 2003; Jacquemin et al., 2006; Lammert et al., 2001). Understanding EC signalling or regulatory mechanisms in developing vasculature or adult vessels is still in its earliest stages (Levenberg et al., 2007).

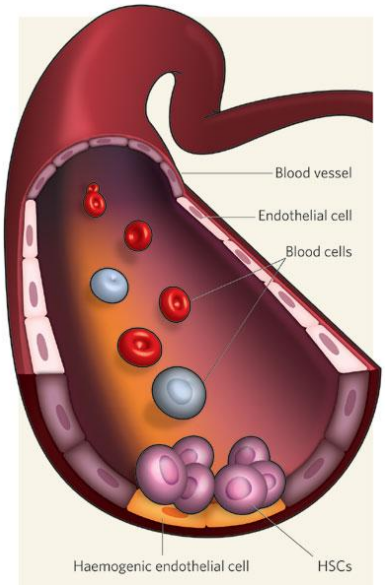


Figure 6. Endothelial cells.
Endothelial cells line the interior surface of vessels and form an interface between blood circulation and the rest of the vessel wall (Yoshimoto & Yoder, 2009).

1.4.1 Blood vessel formation

Vasculogenesis and angiogenesis are commonly known as the two crucial processes contributing to the development of the vasculature (Jain, 2003). In early development, vasculogenesis occurs when mesenchymal cells differentiate into haemangioblasts that form blood islands. The inside of the blood islands differentiates into haemotopoietic precursors and the outer cells differentiate into ECs which in turn form the primary vascular plexus (Figure 7A) (Flamme et al., 1997; Luttun et al., 2002; Pepper, 1997). The hypothesis is that HSCs and ECs originate from a common precursor, the heamangioblast, because of their proximity and development (Bollerot et al., 2005; Carmeliet, 2003).

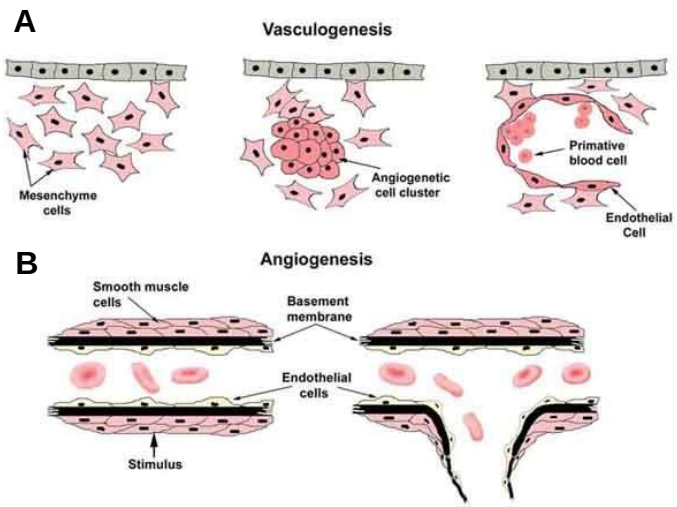


Figure 7. Vascular development.
(A) Vasculogenesis is blood vessel formation occurring by a *de novo* production of endothelial cells, whereas (B) angiogenesis is the formation of new blood vessels form pre-existing once (Chinoy, 2003).

The process of new vessels sprouting from pre-existing ones is known as angiogenesis (Figure 7B) (Carmeliet, 2000; Risau, 1997), which mostly takes place during development and adulthood (Ausprunk & Folkman, 1977; Risau, 1997). Angiogenesis is divided into two phases, the activation and resolution phase (Figure 8). In the activation phase, ECs can proliferate and migrate into the

extracellular space and form new capillary sprouts because of increased vascular permeability and membrane degradation. ECs quit proliferation and migration in the resolution phase, and start reconstructing the basement membrane and promote vessel maturation. At this point mesenchymal cells are recruited, which differentiate into pericytes and smooth muscle cells surrounding the new vessel (Carmeliet, 2000; Folkman & D'Amore, 1996; Risau, 1997).

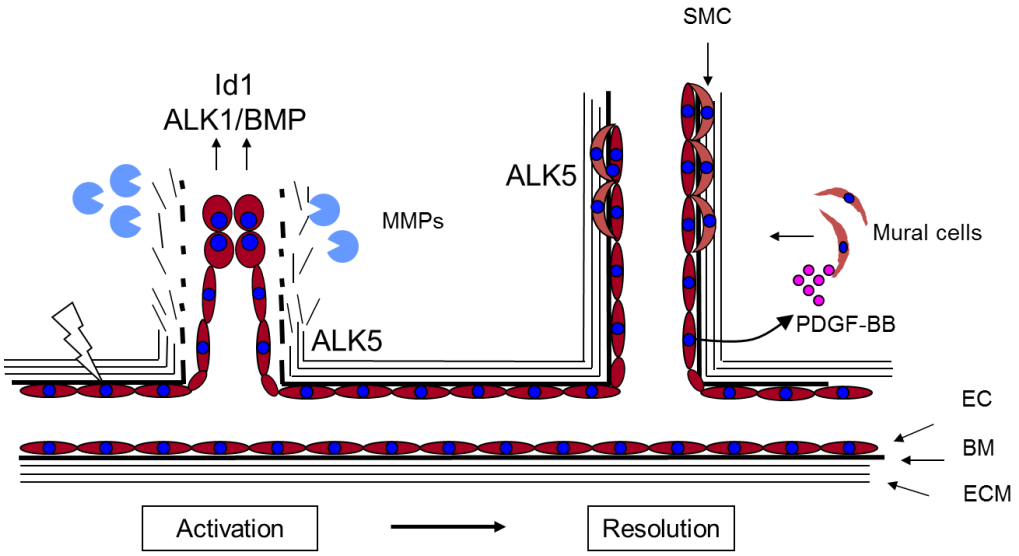


Figure 8. Activation and Resolution phase.
In the activation phase ECs proliferate and migrate into the extracellular space and form new capillary sprouts. In the resolution phase ECs quit proliferation and start reconstructing the basement membrane and promote vessel maturation (Valdimarsdottir, 2004).

A complex network of blood vessels is composed of arteries, capillaries and veins that provide all tissues of the body for oxygen and nutrients. ECs line the interior of blood vessels forming an interface between circulating blood and the rest of the vessel wall. Lack of either oxygen or nutrients triggers activation of angiogenesis by secretion of growth factors. ECs switch to the tip cell state while others form stalk cells (Figure 9). Tip cells start to migrate at the end of the stalk cells, which proliferate and form tubes (Geudens & Gerhardt, 2011; Herbert & Stainier, 2011; Larrivee et al., 2009). Tissue hypoxia is the main driving force behind angiogenesis, which activates secretion of vascular endothelial growth factor (VEGF) and other cytokines, quiescent ECs become reactivated by the VEGF signal. To initiate sprouting, cells need to break out of the vessel wall, degrade basement membrane, change cell shape, proliferate and invade the surrounding tissue while staying connected to vessel network (Blanco & Gerhardt, 2012). The ECs that lead the sprouting are known as endothelial tip cells. They get their characterization by the position, their long and dynamic filopodia and their migratory properties. The tip cells lead the sprouting towards the VEGF source and they make new connections between different sprouts to generate connected and functional vascular network (Gerhardt et al., 2003; Isogai et al., 2003). Following the tip cells are the proliferative endothelial stalk cells, establishing adherent and tight junctions to support sprout elongation (Dejana et al., 2009; Gerhardt et al., 2003). The vascular lumen formation initiates blood flow, increases tissue oxygenation which reduces secretion of VEGF and other cytokines, supporting the establishment of

quiescence. For vessel maturation and stabilization mural cells (vascular smooth muscle cells and pericytes) are recruited and extracellular matrix (ECM) rebuilt (Blanco & Gerhardt, 2012).

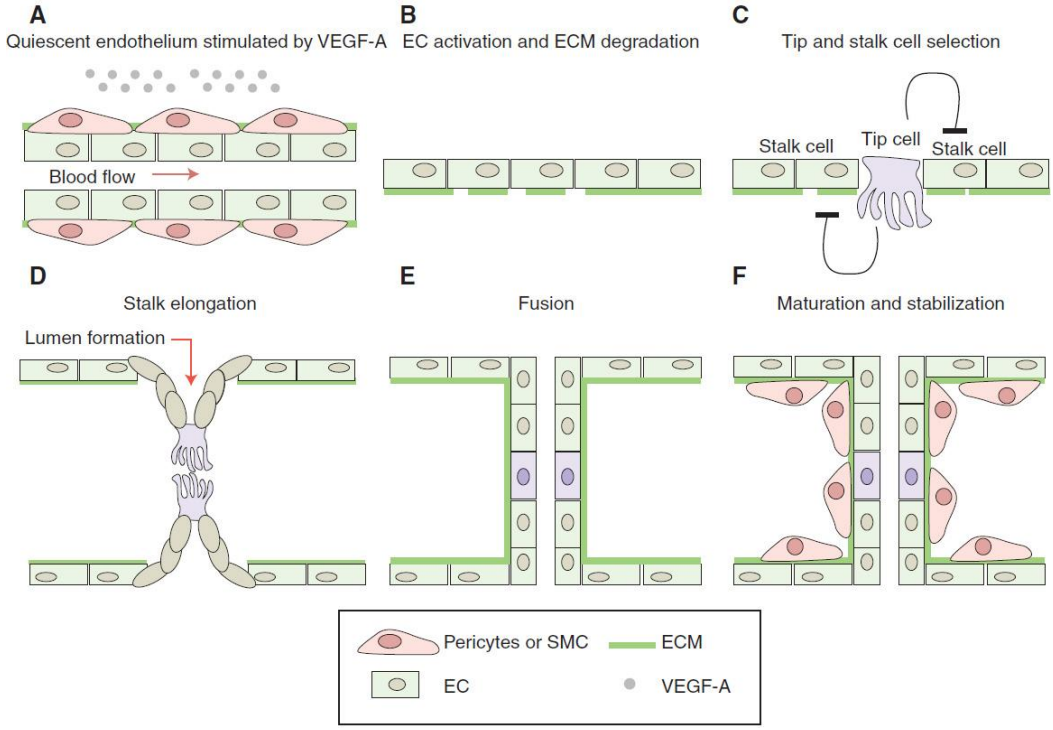


Figure 9. Tip and stalk cells.
Tip endothelial cells lead sprouting and make new connections between sprouts to generate vascular network. Stalk cells establish adherent and tight junction to support sprout elongation (Blanco & Gerhardt, 2012).

1.4.2 Isolation of hES derived endothelial cells

The process of hES cells developing towards ECs is thought to have various differentiation stages. To characterize vascular-endothelial differentiation of hES cells, both *in vitro* and *in vivo* assays have been used. Multiple markers can been used, such as VE-Cadherin (vascular endothelial caderin), PECAM1 (platelet endothelial cell adhesion molecule, also known as CD31), CD34, Flk1 (VEGFRII) and their ability to take up Dil-labeled acetylated low-density lipoprotein (Dil-Ac-LDL). For mature EC identification, vWF (von Willebrand factor), eNOS (endothelial nitric oxide synthase) and E-selectin proteins have been used. To select and purify ECs from hES culture, two main approaches are commonly used. First, selecting EBs for specific cell-surface markers and second, supplementing feeder layer or the medium with various growth factors. These two methods are thought to differ substantially in regard to the source of the cells and the cell line itself. It could also have an effect whether undifferentiated cells or differentiated EBs are used, the age of the EBs and the type of growth factors supplemented (Levenberg et al., 2007).

1.4.3 Are endothelial- and smooth muscle cells derived from the same progenitor ?

Vascular diseases are the leading cause of death worldwide. No known marker exists to isolate endothelial progenitors from hES cells at different stages of development. This restrains the ability to define and investigate the hES derived endothelial cells (Kirton & Xu, 2010; Levenberg et al., 2007; Yoder, 2009). ECs are thought to share progenitors with HSCs. Both express CD34, and they share a similar mesodermal origin during embryonic development. Therefore CD34+ cell population must be heterogeneous (Asahara et al., 1997; Wang et al., 2004; Zambidis et al., 2005). Later it was demonstrated that CD34+CD31+ cells were able to give rise to both ECs and SMCs. If grown in serum-free medium containing growth factors (BMP4, VEGF and FGF2) it promoted the cells first to differentiate into a pool of HSC expressing cells (CD34+CD31-), developing into a mixture of HSCs and ECs (CD34+CD31+) and finally a population of ECs (CD34-CD31+) (Bai et al., 2010). At different time points both CD31 and CD34 are expressed in haematoendothelial differentiation, but CD31 is normally associated with more mature EC phenotype and not in SMCs. It has been demonstrated that hESC-ECs and hESC-SMCs, derived from the same CD34+ vascular progenitor cell population, show great difference highlighting their independent contribution in the structure and function of mature vasculature (Hill et al., 2010). Endothelial precursors have been isolated based on the expression of CD31, CD34 as well as CD133 (Prominin-1) (Ferreira et al., 2007; Rufaihah et al., 2010; Wang et al., 2007).

1.4.4 Driving force of mesodermal differentiation

What growth factors are responsible for hES cells ability to differentiate into mesodermal lineage is not well known. BMP does not apper to be the only driving force of mesoderm as FGF has also been shown to promote mesodermal specification and proliferation (Vallier et al., 2009; Yang et al., 2008). The combination of these two factors has great effects on the outcome of the differentiation pathways. BMP4 and FGF alone were not sufficient to promote hES cells towards mesodermal differentiation, but with addition of VEGF it greatly increased mesodermal determination (Bai et al., 2010).

Nourse and colleagues compared a few different endothelial differentiation methods. When EBs were treated with VEGF, it promoted expression of the endothelial markers CD31 (PECAM-1) and VE-Cadherin without inducing expression of haematopoietic markers (Nourse et al., 2010). Other groups do not belief VEGF to promote hESC-EC differentation, nor do they see CD31 expression in network structures (Boyd et al., 2007; Nakagami et al., 2006). Many different approaches and differentiation methods have been used to differentiate ECs from hES cells. For regenerative use of hESC-EC, a large-scale production is needed.

1.5 The TGFβ superfamily

The Transforming Growth Factor beta (TGFβ) superfamily consist of over 40 members, including bone morphogenetic proteins (BMPs), activins, nodal and growth and differentiation factors (GDFs)

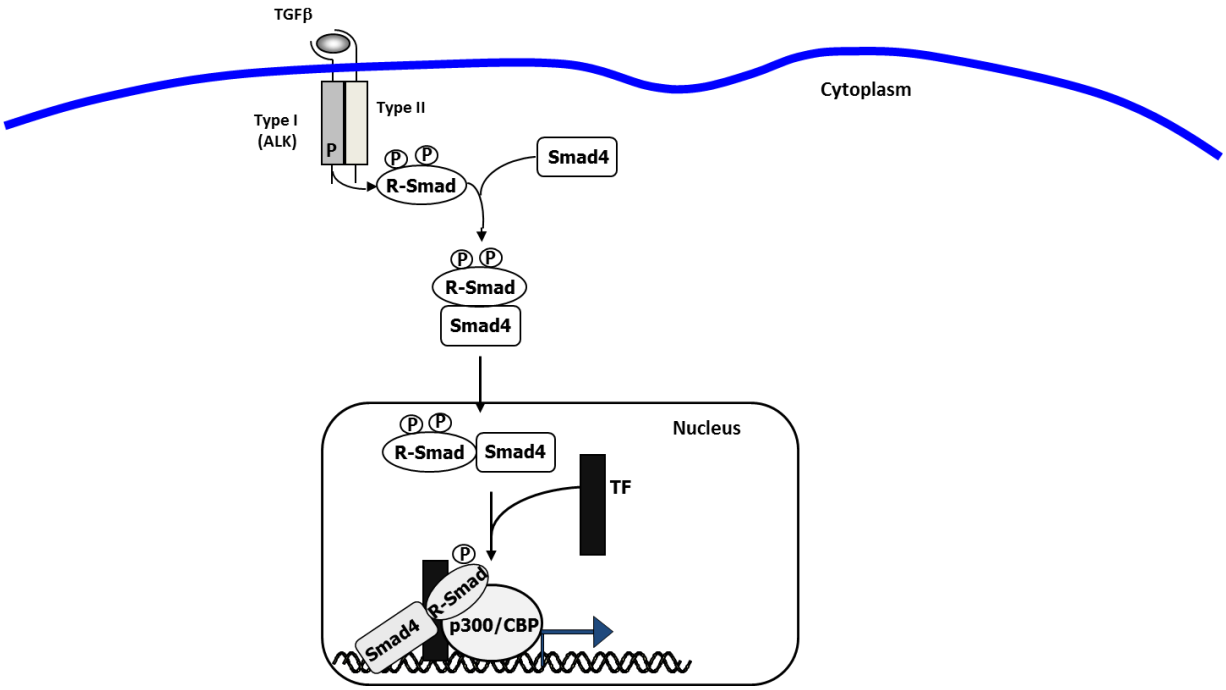


Figure 10. TGFβ superfamily signalling pathway (Valdimarsdottir, 2004).

(Massague, 2000). They act throughout the body, affecting most cell types and play an important role in the maintenance of self-renewal and pluripotency of both mouse and human ES cells (Watabe & Miyazono, 2009). TGFβs act as growth inhibitors in most cell types, they induce apoptosis for epithelial cells but are also known to stimulate the production of extracellular matrix proteins. Activins play critical roles in the induction of dorsal mesoderm during early embryogenesis and Nodals in the anterior patterning and formation of left and right asymmetry. The members of the superfamily all play their part in cell growth, differentiation, migration and apoptosis *in vivo* (Massague, 1990; Miyazawa et al., 2002). TGFβ transduces signals from the membrane to the nucleus by binding to a heteromeric complex of serine/threonine kinase receptors known as TGFβ type I (TβRI) and type II (TβRII) receptors (Figure 10). The type I receptor, also known as the activin receptor-like kinase (ALK) acts downstream of the type II receptor and propagates the phosphorylation signal through specific downstream mediators, the Smads (Heldin et al., 1997; Massague, 2000; Piek et al., 1999). The Smads enter the nucleus where they activate transcription of their target genes (Valdimarsdottir & Mummery, 2005).

There are two major pathways involving Smad proteins that are activated by members of the TGFβ superfamily (Miyazono et al., 2001). The cytokines of the superfamily can be classified into two subfamilies depending on which Smad signalling pathway they activate (Miyazawa et al., 2002), the TGFβ and the BMP subfamilies. There are three subclasses of Smads depending on their structure and function, namely the receptor-regulated Smads (R-Smads), the common-mediator Smad (Co-Smad) and inhibitory Smads (I-Smads) (Macias-Silva et al., 1996; Souchelnytskyi et al., 1997). R-Smads undergo conformational change to be able to form complex with Co-Smads after phosphorylation by the type I receptor. Receptors type I and II pair in many different ways, therefore the combinations of type I receptors and R-Smads are highly restricted. This specificity in pairing is based on the complementary of the L45 loop in the type I kinase domain and the L3 loop in the C-

terminal of the MH2 domain of R-Smads. Even though loop L3 differs only in two amino acids between Smad2/3 and Smad1/5/8, it is enough to distinguish between different R-Smads (Valdimarsdottir & Mummery, 2005). Two inhibitory Smads have been identified in mammals, Smad6 and Smad7. Smad6 is thought to preferentially inhibit phosphorylation of BMP Smads whereas Smad7 acts as inhibitor of TGFβ subfamily Smads (Itoh et al., 1998; Souchelnytskyi et al., 1998).

1.5.1 TGFβ subfamily

Each member of the TGFβ superfamily binds to a unique combination of type I and II receptors (Figure 11). TGFβ type II receptor is constitutively active and serves as a specific receptor for TGFβ. Upon

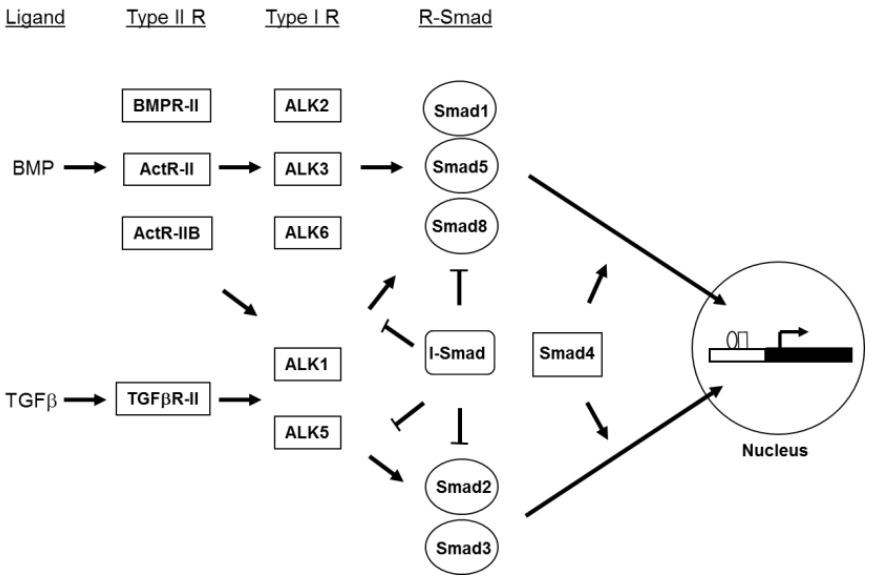


Figure 11. TGFβ superfamily signalling through receptors and Smads (Valdimarsdottir, 2004).

binding, TGFβ ligand recruits the TGFβ type II receptor and type I receptor, also known as the activin receptor-like kinase 5 (ALK5), that is phosphorylated by the type II receptor (Franzen et al., 1993; Reissmann et al., 2001; Wrana et al., 1994), whereas activin signals through ALK4, both resulting in the phosphorylation of Smad2 and Smad3 (Chen & Massague, 1999; Derynck & Zhang, 2003; Oh et al., 2000; Shi & Massague, 2003; ten Dijke et al., 1994; Yamashita et al., 1995).

1.5.2 BMP subfamily

Originally the BMPs were thought to induce bone and cartilage tissues *in vivo* (Wozney et al., 1988). Now it is known that they have diverse effects on various cells and induce ventral mesoderm during early embryogensis (Miyazawa et al., 2002). BMPs also regulate tooth, hair, skin, kidney, muscle, haematopoietic and neuronal development as well as maintaining iron metabolism and vascular homeostasis *in vivo* (Miyazono et al., 2010).

These family members are subdivided into subgroups based on their similarity of primary amino acid sequence. First group is BMP2/4, second BMP5/6/7/8 and finally BMP9/10 (Suzuki et al., 2010).

The BMPs signal through ALK2, ALK3 and ALK6 causing phosphorylation of Smad1, Smad5 and Smad8 (Figure 11) (Chen & Massague, 1999; Derynck & Zhang, 2003; Oh et al., 2000; Shi & Massague, 2003; ten Dijke et al., 1994; Yamashita et al., 1995).

1.5.3 BMP9-ALK1 mediated effect in angiogenesis

BMP9 and BMP10 have been shown to bind to ALK1 and Endoglin in ECs, which causes phosphorylation of Smad1/5/8 (Brown et al., 2005). However, the regulation of BMP9-ALK1 signalling in ECs has not been fully understood. ALK1 receptor is a specific endothelial type I receptor, which BMP9 is known to bind to with high affinity. Interestingly, TGFβ is also thought to bind to ALK1. Whether either of these bindings result in proliferation and migration of ECs is still unclear. TGFβ has been shown to bind to ALK5 causing phosphorylation of Smad2/3, but studies have also confirmed activation of ALK1-Smad1/5/8 pathway leading to activation of EC proliferation and migration (Goumans et al., 2002).

Suzuki and colleagues examined the role of BMP9 both in vasculo- and angiogenesis. They demonstrate that BMP9 induces *ex vivo* vascular formation, proliferation of *in vitro* cultured MESEC (Mouse embryonic stem cell derived endothelial cells) cells and *in vivo* angiogenesis (Goumans et al., 2002; Suzuki et al., 2010). Inconsistent with Suzuki‘s findings, BMP9 has been reported to inhibit proliferation and migration of endothelial cells (David et al., 2007; Scharpfenecker et al., 2007). Scharpfenecker and colleagues showed that BMP9 does bind to ALK1 with high affinity and phosphorylates Smad1/5/8 and induces Id1 transcription. However, they find BMP9 to inhibit bFGF stimulated proliferation and migration of BAECs (bovine aortic endothelial cells) and to block VEGF induced angiogenesis. BMP10 is the ligand most related to BMP9 based on protein sequence comparison. In the absence of BMP9, BMP10 is believed to be able to substitute for BMP9 resulting in inhibition of proliferation and migration of endothelial cells. However BMP10 binds with lower affinity to ALK1 than BMP9 and is mainly expressed in the murine heart (David et al., 2007). Another group showed that blocking BMP9 with neutralizing antibody in newborn mice induced retinal vascular density. Injection of the extracellular domain of ALK1 to BMP9-KO mice showed impaired vascularization of the retina, supporting the importance of ALK1 and suggesting another ligand for ALK1 (Ricard et al., 2012). Whether BMP9 and BMP10 mediate vascular effect similarly still has to be clarified (Cunha & Pietras, 2011).

TGFβ has also been shown to bind to ALK1, but only in the presence of ALK5, suggesting that TGFβ regulates the activation state of proliferation and migration of endothelial cells through a fine tuning of these two receptors (Goumans et al., 2002). Synergistic effects of BMP9 and TGFβ has been elucidated on endothelial cell growth and sprouting via ALK1 (Cunha & Pietras, 2011).

The inconsistency between results from different research groups is still considerable. The reason might be that the possible crosstalk between pathways is not fully understood or that the effects of BMP9 stimulation and ALK1 signalling regulation is cell type dependent (Larrivee et al., 2012; Suzuki et al., 2010). The role of BMP9 and BMP10 is known to some extent in angiogenesis. However there is not much known about the effect in vasculogenesis.

1.6 Vascular defects in TGFβ superfamily deficient mice

Most of current knowledge about endothelial molecular regulation and differentiation is derived from mouse embryonic developmental studies. The mouse system can be predictive of the human system, but at the same time it is well known that regulation of vascular differentiation differs between mouse and human ES cells. Therefore a better understanding is needed of the human regulatory system in order to promote endothelial differentiation for regeneration and clinical therapies (Xu et al., 2005; Zambidis et al., 2005). The importance of the TGFβ superfamily members in angiogenesis has been demonstrated with knock-out studies in mice, which show their crucial role in development and disease (Goumans & Mummery, 2000).

Before and around gastrulation, *BMP4* is expressed in extraembryonic ectoderm. At later stages of gastrulation it is also detected in mesoderm. If mice lack the *Bmpr1a* and *Bmpr2* genes they die by day 9.5 because of mesodermal formation defects during gastrulation, showing little or no mesodermal formation and no expression of the early mesodermal marker *Brachyury T* (Beppu et al., 2000; Lawson et al., 1999; Mishina et al., 1995). BMP2/5/7 deficient mice show malformation in development of the amnion and chorion as well as retarded heart development (Goumans & Mummery, 2000).

When TβRI (ALK5) is absent it leads to defects in yolk sac vessel formation and haematopoiesis (Goumans & Mummery, 2000). TGFβ1 knockout mice die during midgestation due to abnormal vascular development and they suffer from hyperdilated, leaky vessels (Goumans & Mummery, 2000). Knockout of *Alk1* and *Eng* genes causes abnormal angiogenesis, enlargement of major vessels, lack of capillary network and defects in differentiation and recruitment of smooth muscle cells (Li et al., 1999; Oh et al., 2000; Urness et al., 2000). *Smad1* deficient mice fail to establish chorion-allantoic circulation, and disruption of the *Smad5* gene shows lack of normal vascular structures of the yolk sac, dilation of blood vessels and irregular distribution of blood vessels (Chang et al., 1999; Lechleider et al., 2001; Tremblay et al., 2001; Yang et al., 1999). Id1, a downstream member of the BMP subfamily, is important for regulation of angiogenesis. It is required for proliferation and invasiveness of ECs during angiogenesis. Mutant mice show abnormal angiogenesis with enlarged and dilated blood vessels (Table 1) (Lyden et al., 1999).

Table 1. Defects in TGFβ ligand, receptor and Smad deficient mice.

Gene	Phenotype	Lethality	Reference
BMP4	Mesodermal formation defects during gastrulation	E9.5	(Lawson et al., 1999; Winnier et al., 1995)
BMP2/5/7	Developmental malformation of the amnion and chorion, as well as retarded heart development	E7.5-10.5	(Solloway & Robertson, 1999; Zhang & Bradley, 1996)
TGFβ1	Abnormal vascular development	E10.5	(Dickson et al., 1995)
ALK5	Yolk sac vessel formation and haematopoiesis defects	E10.5	(Larsson et al., 2001)
ALK1	Abnormal angiogenesis, enlargement of major vessels, lack of capillary network and defect in differentiation and recruitment of smooth muscle cells	E11.5	(Oh et al., 2000; Urness et al., 2000)
Endoglin	Abnormal angiogenesis, enlargement of major vessels, lack of capillary network and defect in differentiation and recruitment of smooth muscle cells	E10-11.5	(Arthur et al., 2000; Bourdeau et al., 1999; Li et al., 1999)
Smad1	Failure in establishing chorion-allantoic circulation.	E9.5	(Lechleider et al., 2001; Tremblay et al., 2001)
Smad5	Lack of yolk sac vascular structures, dilation and irregular distribution of blood vessels.	E9.5-11.5	(Chang et al., 1999; Yang et al., 1999)
Id1	Abnormal angiogenesis with enlarged and dialted blood vessels	E13.5	(Lyden et al., 1999)

1.7 Regenerative medicine

Vascular defects can cause many types of human diseases. One of those diseases is Hereditary hemorrhagic telangiectasia (HHT) also known as Osler-Weber-Rendu disease. It is a genetic disorder which is mostly caused by mutations in the gene for either *ALK1* or *Endoglin*. It is characterized as abnormal blood vessel formation (AVM) in the nose, oral cavity, gastrointestinal tract and often in organs such as the lung, liver and brain. It can lead to nosebleeds, acute and chronic digestive tract bleeding, resulting in severe ischemic injury or stroke. (Suzuki et al., 2010).

Another genetic disorder is primary pulmonary arterial hypertension (PAH or PPH1), caused by a stenosis of precapillary pulmonary arteries. An increased pulmonary artery pressure and right ventricular systolic pressure is caused by proliferation of endothelial and smooth muscle cells in the

pulmonary arteries. More than 70% of patients with familial PAH have a mutation in the *BMPR2* gene, but the mutation alone is not sufficient to initiate the process of the disease (Lane et al., 2000). Mutations in the *ALK1* gene have also been linked to some patients with PAH. The BMP signalling pathway may thus play an important role in endothelial and smooth muscle cells with regard to maintaining vascular homeostasis in the pulmonary arteries.

The components of the TGFβ superfamily affect many cellular processes through different pathways. Interruption of these pathways can lead to many different diseases, including cancer. To give an example, to date, three different biological ALK1 inhibitors have been reported from pharmaceutical companies. Preclinical tumor studies in mice show that blocking ALK1 diminishes tube formation *in vitro*, delays tumor growth *in vivo*, and decreases blood flow in tumors (Cunha & Pietras, 2011; Waite & Eng, 2003). During early stages of tumor development TGFβ is known to act as a suppressor but at the later stages it promotes growth (Ikushima & Miyazono, 2010). Thus, for cancer treatments it is of great importance to consider inhibitors for some TGFβ components, because blocking the signalling might be helpful depending on the stage of the disease. With regard to endothelial cells, affecting the BMP9/10 pathway could also be promising, both for cancer and vascular disorders, such as PAH and HHT.

1.8 iPS cells

For the past 10 years cardiovascular research has largely focused on embryonic stem cells and cardiomyocyte and vascular progenitor cells for use in regenerative medicine. However the use of embryonic stem cells has always been controversial and we still face the problem of immune rejection after transplantation. Few years ago Yamanaka and colleagues introduced a method which reprogrammes ES cells using defined factors to create induced pluripotent stem cells (iPS) cells. By viral expression of Oct4 and Sox2, combined with either Klf4 and c-Myc or LIN28 and Nanog, human iPS cells have been generated from multiple cell types (Figure 12) (Takahashi et al., 2007; Yamanaka, 2008a, 2008b; Yu et al., 2007). Human iPS cells are thought to be both molecularly and functionally similar to hES cells, and one way to overcome ethical and immune rejection is to generate iPS cells from the patients own cells (Soldner et al., 2009; Ye et al., 2009). However, before iPS cells can be used for clinical purposes, there are several issues that need to be addressed. So far most of the iPS cells made are based on lenti- or retroviruses, which could cause genetic mutations. A better knowledge of what genes are involved in development and differentiation would make it possible to develop new and more improved techniques for human iPS cell derivation. Furthermore, properties of differentiated human iPS cells must be compared to their natural counterparts *in vivo* (Li et al., 2011).

Human iPS cells have already been differentiated to various cell types including cardiomyocytes and vascular cells (Choi et al., 2009; Zhang et al., 2009). In the nearest future, many studies will have developed and fully explored derivation of vascular progenitor cells from pluripotent stem cells and iPS cells for regenerative use (Kane et al., 2011).

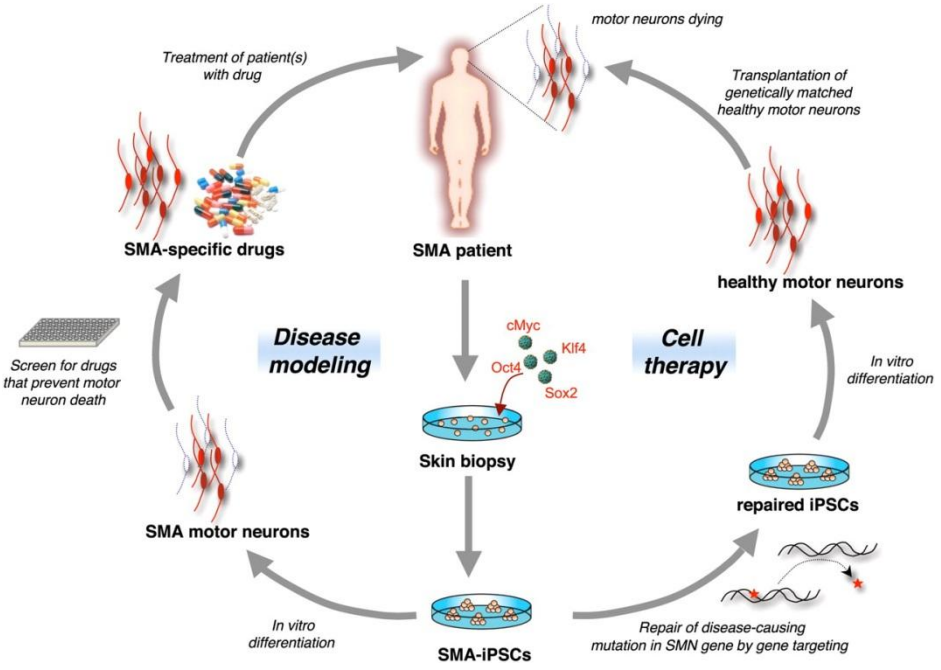


Figure 12. Potential applications of induced pluripotent stem cells (Stadtfeld & Hochedlinger, 2010).

2 Project aim

Human embryonic stem cell-derived endothelial cells have great potential for vascular research and for cell transplantation or regenerative medicine. Several research groups have succeeded in differentiating hES cells into endothelial cells by various differentiation methods. However none of these methods possess the ability to produce hES derived endothelial cells with enough efficiency for clinical application. The aim of this project was to analyze hES cells as an *in vitro* model for human vascular development and to improve currently used techniques to make hES derived vascular cells more efficient. Since gene ablation studies have shown a pivotal role of the TGFβ superfamily in cardiovascular development, its effect on a biological and molecular level in hES cell vascular commitment was studied.

The thesis is divided into three main parts:

1. Effect of BMP4 on embryoid body vascular outgrowth.

Embryoid bodies were formed from human embryonic stem cells and cultured in either differentiation medium or EGM-2 alone, or supplemented with BMP4, a member of the TGFβ superfamily. EBs capability to migrate and form outgrowths was evaluated. Using adenoviral infection and in turn performing tube like formation assays, activation of the BMP branch and EBs ability to form tubes on a gel substrate was evaluated.

2. The effect of the TGFβ superfamily on hES cell vascular differentiation.

Embryoid bodies were formed using the SpinEB method and stimulated or inhibited for 10 days with different members of the TGFβ superfamily. Gene expression was determined in cells undergoing differentiation, using Q-PCR.

3. Isolation, expansion and sprouting of hES derived vascular cells

Embryoid bodies in suspension were made from hES cells and cultured in differentiation medium. A cell sorting technique using the MACS method was established in our laboratory to sort CD31+ cells, a population of vascular cells. Various biological assays were performed to assess the CD31+ cell population. The CD31+ cells were subjected to different members of the TGFβ superfamily and their ability to form tubes on gel substrate and protein expression was evaluated.

3 Materials and methods

3.1 Cell culture

3.1.1 Mouse embryonic fibroblasts (MEF cells)

A monolayer of irradiated primary mouse embryonic fibroblasts was used as a feeder cells to support growth and maintenance of hES cells in undifferentiated state. MEF cells (granted from ATCC-LGC Standards and Cell Systems) were seeded onto 0.1% gelatin coated 6-well culture plates in MEF medium (Table 2), 5 x 10^5 cells per well, and incubated for 24 hours before hES seeding.

MEF cells were also used to collect conditioned medium (CM). The MEF cells secrete various metabolites, growth factors and extracellular matrix proteins, which the hES cells need in order to facilitate cell growth and pluripotency. Roughly 4 ml of "stripped" hES medium (Table 3) was applied to each well containing only MEF cells. The medium was then collected every 24 hours (from now termed CM medium) and replaced with either fresh stripped hES medium, or seeded with hES cells using hES medium.

Table 2. MEF medium.

MEF medium		
	Stock concentration	Final Concentration
Dulbecco's modified eagle medium, DMEM (11960-044) (Invitrogen)		
Fetal Calf Serum (Sigma-Aldrich)		10%
Penicillin (Invitrogen)	5000 U/ml	50 U/ml
Streptomycin (Invitrogen)	5000 µg/ml	50 µg/ml
GlutaMAX (Invitrogen)	200 mM	2 mM
Non-Essential Amino Acids (Invitrogen)		100x dilution

3.2 Maintenance of undifferendiated hES cells

In this study four hES cell lines were used. The first one was HUES9 obtained from Douglas A. Melton at Howard Hughes Medical Institute, Harvard University (Cowan et al., 2004). The second line used was H1 cells from WiCell (Thomson et al., 1998) and the third and fourth lines were HES2 and HES3 from ES Cell International, ESI (Reubinoff et al., 2000). Pluripotent hES cells were maintained on MEFs in 6 well culture plates, using hES medium. hES medium was supplemented with 10 ng/ml basic Fibroblast Growth Factor (bFGF) and CM or mTeSR (Table 4) medium to accelerate cell growth. Before use, the CM was filter-sterilized using 0.22 µm filter. hES cells were then split every 3-4 days and hES medium replaced daily.

Table 3. hES medium.

hES medium			
		Stock concentration	Final Concentration
Stripped hES medium	Dulbecco's modified eagle medium, DMEM/F-12 + GlutaMAX (31331-028) (Invitrogen)		
	KO serum replacement (Invitrogen)		20%
	Non-Essential Amino Acids (Invitrogen)	100x	100x dilution
	Penicillin (Invitrogen)	5000 U/ml	50 U/ml
	Streptomycin (Invitrogen)	5000 µg/ml	50 µg/ml
	GlutaMAX (Invitrogen)	200 mM	2 mM
	2-Mercaptoethanol (Invitrogen)	55 mM	77 µmol/ml
ADD			
hES medium	Conditioned medium (CM) or mTeSR1		10%
	bFGF	100 ng/µl	10 ng/ml

Table 4. mTeSR medium.

mTeSR medium	
mTeSR™1 Basal Medium (StemCell Technologies)	400 ml
ADD	
mTeSR™1 5x Supplement	100 ml
• **Bovine Serum Albumin**	
• **rh bFGF**	
• **rh TGFβ**	
• **Lithium Cloride**	
• **Pipecolic acid**	
• **GABA**	

- Concentrations not available

mTeSR medium (Table 4) is a feeder-independent maintenance medium used for hES cells. It is a defined serum-free medium and does not require addition of other growth factors.

3.2.1 Passage of human embryonic stem cells

hES medium was pre-warmed up to 37°C (Table 3). Medium was removed and cells washed once with 1 ml 1x PBS. The cells were dissociated using 0.5 ml TrypLE (Invitrogen) and incubated at 37°C for 2-3 minutes. The TrypLE was then inactivated with 1.5 ml hES medium and transferred to 15 ml

tube and then centrifuged for 5 minutes at 1000 rpm. The cell pellet was resuspended in hES medium by pipetting 5-10 times, or until single cell suspension. The cell suspension was then split in 1:6 to 1:12 ratio.

3.3 Feeder-free cell culture

MEF feeder culture for hES cells is not practical for all experiments. For those experiments, the hES cells were feeder depleted and instead cultured on Matrigel (1:100 dilution). The Matrigel used was a Growth Factor Reduced BD Matrigel^{TM} Matrix from BD Biosciences. It is a gelatinous protein mixture, secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells, that resembles the extracellular environment.

When hES cells are grown without feeders, they were cultured in either 50% hES medium and 50% CM or mTeSR combined with 10ng/ml bFGF to guarantee their pluripotency.

3.4 Differentiation of hES cells

In this study two differentiation methods were used. The first was by generating embryoid bodies in suspension and the second was the SpinEB method. Both methods involve spontaneous differentiation. Methods can be directed into different lineages with addition of stimulants and/or inhibitors. Various growth factors and inhibitors (Table 5) were used that affect the TGFβ and BMP pathways and facilitate mesodermal differentiation.

Table 5. Stimulants and inhibitors.

Substrate	Concentration	Effect	Supplier
TGFβ low	0.5 ng/ml	Stimulant of the TGFβ pathway	PeproTech
TGFβ high	5 ng/ml	Stimulant of the TGFβ pathway	PeproTech
BMP4	10 ng/ml	Stimulant of the BMP pathway	PeproTech
BMP9	1 ng/ml	Stimulant of the BMP pathway	R&D Systems
BMP10	10 ng/ml	Stimulant of the BMP pathway	R&D Systems
VEGF	10 ng/ml	Stimulant of the TGFβ pathway	PeproTech
ActivinA	10 ng/ml	Stimulant of the TGFβ pathway	PeproTech
SB-431542	10 µM	Inhibitor of the TGFβ pathway	Tocris
Noggin	300 ng/ml	Inhibitor of the BMP pathway	PeproTech

3.4.1 Embryoid bodies in suspension

This method was used both for individual experiments only and as a forerunner for longer experiments.

hES cells were grown on MEFs in 6 well tissue culture plates. The cells were dissociated into clumps using 1ml of 1mg/ml dispase (Invitrogen) diluted in DMEM/F12 medium and incubated by

gentle shaking for 15 minutes at 37°C. The dispase was then inactivated with hES medium, the cells scraped off the plate and placed in 15 ml tubes and then centrifuged at 800 rpm for 4 minutes. The cells were resuspended gently in differentiation medium (F12 medium supplemented with 1.3 % A-Monothioglycerol (α-MTG)), and clumps transferred to 12-well low attachment plates (BD-Falcon) and growth factors and inhibitors added.

3.4.2 SpinEB method

To promote differentiation of hES cells when exogenous growth factors were added, the SpinEB method was implemented, using a defined serum free medium, BPEL medium (Bovine Serum Albumin (BSA) Polyvinylalcohol Essential Lipids) (Table 6) (Ng et al., 2008).

Day 0:

hES cells ($2.3x10^6$) were split using normal passaging procedure (see 3.2.1) onto matrigel coated plates in 50/50 medium (50% CM and 50% hES medium) with 10 ng/ml bFGF. Cells were stored overnight at 37°C or until 70-90% confluent.

Day 1:

100 µl of sterile H_2O was added to 36 outer wells of a sterile 96-well V-shaped low-attachment plate to humidify the EBs during differentiation. Medium was aspirated and the cells rinsed once with 1x PBS. Then cells were dissociated with TrypLE to generate single-cell suspension. To inactivate the TrypLE, hES medium was added and the cell suspension transferred to 15 ml tubes and centrifuged for 5 minutes at 1000 rpm. The cells were resuspended and diluted to total number of cells needed, supplemented with growth factors

- Optimum cell number is 3000 cells/well
- $1.8x10^5$ cells needed in 6 ml BPEL medium for each 96 well plate

Then 100 µl hES cell suspension was aliquot into each well of the 96 well plate, then centrifuged at 1100 rpm for 2 minutes at 4°C to induce formation of the Spin EBs and incubated at 37°C. Every other day medium was changed until embryoid bodies were harvested at days 5, 7 and 10 for analysis.

Table 6. BPEL medium.

Compound	Stock concentration	Amount	Final concentration
Iscove's modified Dulbecco's medium (IMDM) (Invitrogen)	1x	86.2 ml	
Ham's F-12 nutrient mixture (Invitrogen)	1x	86.2 ml	
Deionized BSA (Sigma-Aldrich)	10%	5 ml	2.5 mg/ml
Polyvinylalcohol (PVA) (Sigma-Aldrich)	5%	5 ml	
Linoleic acid (Sigma-Aldrich)	10.000x	20 µl	100 ng/ml
Linolenic acid (Sigma-Aldrich)	10.000x	20 µl	100 ng/ml
SyntheChol (Sigma-Aldrich)	7.200x	28 µl	2.2 µg/ml
A-Monothioglycerol (α-MTG) (Sigma-Aldrich)	1.30% (13µl í 1 ml IMDM)	600 µl	~400 µM
Rh Insulin-transferrin-selenium ethanolamine sol. (Sigma-Aldrich)	100x	2 ml	
Protein-free hybridoma mixture II (PFHMII) (Invitrogen)	1x	10 ml	5%
Ascorbic acid 2 phosphate (Sigma-Aldrich)	5 mg/ml	2 ml	50 µg/ml
GlutamaxI (Invitrogen)	200 mM (100x)	2 ml	2mM
Penicillin/Streptomycin (Invitrogen)	5000 µl/ml / 5000 U/ml (100x)	2 ml	50 µg/ml / 50 U/ml

3.5 Cell separation methods

3.5.1 Magnetic-activated Cell Sorting (MACS)

5, 7 and 10 days after differentiation with either embryoid bodies in suspension or SpinEBs, the EBs were dissociated. The single cells suspension was labeled with anti-CD31 antibody (Miltenyi Biotec) and separated from the cell mixture by Magnetic-activated Cell Sorting (MACS) according to protocol by Levenberg et al (Levenberg et al., 2010).

EBs were harvested to a 15 ml tube/s, using collagenase type IV solution (Invitrogen) (1mg/ml into DMEM/F12 nothing added medium). The tube/s were then centrifuged for 4 minutes at 800 rpm and the supernatant aspirated. 2-3 ml of Collagenase B solution was added into the tube/s (Roche) (1 mg/ml into DMEM/F12 nothing added medium), and incubated for 2 h (flick the tube/s regularly).

After 2 hours the tube/s were centrifuged for 3 minutes at 1680 rpm, the supernatant removed and the cells washed once with 5 ml 1x PBS. The tube/s were then again centrifuged for 3 minutes at 1680 rpm, the supernatant aspirated and 2 ml of TrypLE added and incubated at 37°C for 5-10 minutes (flick tube/s regularly). To dissociate the cells properly into single cell suspension we used 1000 µl pipette and pipetted up and down a few times. To inactivate the TrypLE, 2 ml of 5% FBS was added and the cell suspension passed through a 40 mm cell strainer into new 15 ml tube/s. Then the tubes

were centrifuged for 5 minutes at 1680 rpm and the supernatant aspirated. The cells were resuspended in 5-10 ml 5% FBS and counted using hemocytometer. Next the cells were centrifuged at 4°C for 5 minutes at 1680 rpm and the supernatant removed, and the cells resuspended in 300 µl 5% FBS. Now the cells were labeled by adding 100 µl FcR blocking sol and 100 µl anti-CD31 microbeads (Miltenyi Biotec) into the 15 ml tube/s and the cell suspension was kept on ice for 30 minutes. The cells were centrifuged at 1680 rpm for 5 minutes, the supernatant removed and the cells rinsed with 5-10 ml FBS. Finally the cells were centrifuge at 4°C for 5 minutes at 1680 rpm, the supernatant aspirated (repeat for higher purity)* and the cells resuspended (up to $1x10^8$) in 500 µl MACS buffer (Appendix A) and pipetted thoroughly.

From here it was either done manually:

- ✓ LS columns rinsed with 3 ml MACS buffer – cells suspension added
- ✓ Unlabeled cells which pass through collected
- ✓ Column washed 3x with 3 ml MACS buffer
- ✓ Column placed over a new collection tube
- ✓ 5 ml MACS buffer added
- ✓ Plunger applied firmly to flush out magnetically labeled cells
- ✓ For higher purity cell fraction, steps repeated from *

Or in an autoMACS:

- ✓ Miltenyi Biotec – see manual for autoMACS pro separator miltenyibiotec.com

After isolation, the vascular cells are then cultured in medium conditions (Table 7) with or without growth factors that support specific differentiation and expansion pathways.

Table 7. EGM-2 medium.

EGM-2 medium
EBM-2 medium (CC-4176) (Lonza)
10% **Fetal calf serum (Sigma-Aldrich)**
Hydrocortisone (Lonza)
hFGF (Lonza)
VEGF (Lonza)
R3-IGF (Lonza)
Ascorbic acid (Lonza)
hEGF (Lonza)
GA-1000 (Lonza)
Heparin (Lonza)

3.5.2 Dynabeads cell sorting

After dissociating the EBs to single cells suspension, the cells were marked with magnetically labelled CD31 antibody and sorted with dynabeads. This is more gentle way of cell sorting and more practical for endothelial cell isolation.

The medium was aspirated and the cells were washed in PBS. To dissociate the cells, 0.5 ml of TrypLE was added for a few minutes and then 1.5 ml of MACS buffer to inactivate the TrypLE. Cells were then harvested and centrifuged at 1000 rpm for 5 minutes. The cell pellet was resuspended in 1 ml of MACS buffer, 30 µl of Dynabeads (Miltenyi Biotec) added to the cell solution and incubate for 30 min at 4°C and flicked every 10 minutes. The cell suspension was washed twice with 3 ml MACS buffer, centrifuged at 1000 rpm for 5 minutes and resuspended in 5 ml MACS buffer. Tube/s were placed on magnet for 1 minute and the medium aspirated. Tube/s were then remove from magnet and cells resuspended in 5 ml MACS buffer. The tube/s were then again placed on the magnet for 1 minute, the buffer aspirated and the cells resuspended in 4 ml PBS. The cell suspension was centrifuge for 5 minutes at 1000 rpm, the PBS removed and the cells resuspend in appropirate medium. For endothelial cell sorting, EGM-2 medium was used with and without growth factors that support vascular differentiation.

3.6 RNA isolation

RNeasy Mini Kit from Qiagen was used for RNA isolation. The kit is designed to purify RNA from small amounts of starting material, all RNA molecules longer than 200 nucleotides are purified and ready to use with this RNeasy procedure. All RNA isolation was performed on SpinEBs (see. 3.4.2) after 5, 7 and 10 days in culture.

Cells were harvested and centrifuged for 5 minutes at 1000 rpm and then the supernatant was carefully removed. To disrupt the cells, Buffer RTL was added (see appropriate volume of RTL buffer

in Table 8). 10 µl of β-mercaptoethanol (β-Me) has to be added to each ml of Buffer RTL. Vortex or pipette to mix.

Table 8. Volume of Buffer RTL.

Numer of cells	Volume of Buffer RTL
< 5 x 10^6	350 µl
5 x 10^6 – 1 x 10^7	600 µl

The lysate was homogenized by vortexing for one minute (or pipette with 20 gauge needle at least five times to mix). One volume of 70% EtOH was added and the solution mixed by pipetting. 700 µl were transferred to an RNeasy spin column in a 2 ml collection tube and centrifuged for 30 s at 10.000 rpm, the flow-through was discarded. Next 700 µl of Buffer RW1 were added to the column, which was centrifuged for 30 s at 10.000 rpm and the flow-through in the collection tube was discarded. 500 µl buffer RPE were added and the column centrifuged for 30 s at 10.000 rpm and the flow-through discarded from the collection tube. To the column 500 µl were added of buffer RPE, which was centrifuged for 2 minutes at 10.000 rpm and the flow-through from the collection tube was discarded. The RNeasy spin column was then centrifuged for 1 minute at full speed and the flow-through discarded from the collection tube. Finally, the column was place in a new 1.5 ml collection tube and 30 – 50 µl of RNease-free water added to the column, which was centrifuged for 1 minute at 10.000 rpm. After isolation, RNA concentration was measured using NanoDrop spectrometer. Samples were stored in -80°C until further use.

3.7 cDNA synthesis

For cDNA synthesis SuperScript™ III First Strand Synthesis Systems for RT-PCR (Invitrogen) was used. This system is optimized to synthesize first-strand cDNA from purified total RNA.

X µl of RNA sample was used and RNase-free water was filled up to 10 µl final sample amount. Next 10 µl of total RNA was mixed with 10 µl of 2x RXN (RT Reaction Mix) and incubate for 5 minutes at 65°C and then let stand on ice for 1 minute. 2 µl of RT enzyme mix was added (1:10 of total sample volume) and incubated for 30 - 50 minutes at 50°C. The reaction was terminated at 85°C for 5 minutes and then cooled on ice. Finally, 1 µl of RNase H (2U/µl) was added and incubated at 37°C for 20 minutes. Samples were stored at -20°C until further use.

3.8 Adeonviral infection of hES cells

hES cells were infected with adenoviruses expressing constitutively active (ca) ALK1, ALK2, ALK5, Id1 and LacZ (kindly provided by Kohei Miyazono) using a multiplicity of infection (MOI) of 100. Optimized infection efficiency of the adenoviruses had previously been verified. Once cells had

reached 70% confluency they were infected with previous viruses and the following day the hES cells were dissociated for embryoid body formation and PCR analysis.

3.9 PCR and RT-PCR techniques

3.9.1 Normal PCR

PCR reaction was executed with primers for the housekeeping gene hARP (Biomers), (Table 11) when cDNA synthesis was complete. A standard PCR solution (Table 9) and program (Table 10) was used for the amplification.

3.9.2 Quantitative real-time PCR (qRT-PCR)

Real-time PCR was performed on diluted samples and 15 µl reactions executed using Maxima SYBR green/ROX qPCR master mix (Fermentas). The procedure was carried out according to manufactures protocol using the following primers (Table 11). The control gene used was hARP. After the run, the 7500 Software v2.0 (Applied Biosystems) gave a Ct value for each sample. The Ct values were then used to calculate relative quantification values, or fold difference, relative to the housekeeping gene.

Table 9. PCR reaction solution.

Reaction solution	Volume (µl)
$MgCl_2$ (Invitrogen)	1.5
10x DreamTaq Buffer (Invitrogen)	2.5
dNTP Mixture, 2 mM (Invitrogen)	0.5
Forward primer (Biomers, 100 pmol/µl)	0.5
Reverse primer (Biomers, 100 pmol/µl)	0.5
Nuclease-free water (Invitrogen)	18.5
cDNA	1
Dream Taq DNA Polymerase (Invitrogen)	0.06

Table 10. Standard PCR program.

Step	Temperature (°C)	Time
1	94	30 sec
2	94	20 sec
3	60	1 min
4	72	1 min
Cycle for step 2 for x more times		
5	72	5 min
6	4	∞

Table 11. Primers used.

Species	Primer	Sequence	Product	PCR conditions
***Human**	hARP f 569	caccattgaaatcctgagtgatgt	115 bp	AT = 60°C
	hARP r 684	tgaccagcccaaaggagaag		
Human	VE-Cadherin f	tggagaagtggcatcagtcaacag	118 bp	AT = 57°C
	VE-Cadherin r	tctacaatcccttgcagtgtgag		
***Human**	Endoglin f	cccgcaccgatccagaccactcct	192 bp	AT = 60°C
	Endoglin r	tgtcacccctgtcctctgcctcac		
***Human**	CD31 f	atcatttctagcgcatggcctggt	159 bp	AT = 60°C
	CD31 r	atttgtggagggcgaggtcataga		
Human	KDR f	cctctactccagtaaacctgattggg	219 bp	AT = 60°C
	KDR r	tgttcccagcatttcacactatgg		
Human	Id2 f	acgacccgatgagcctgcta	213 bp	AT = 51°C
	Id2 r	tcctggagcgctggttctg		
Human	Id3 f	tgagcttgctggacgac	571 bp	AT = 51°C
	Id3 r	ccttggcatagtttggagag		
Human	vWF f	ttccagaatggcaagagagtg	345 bp	AT = 56°C
	vWF r	tgagttggcaaagtcataagg		
Human	ALK1 f	gagtccagtctcatcctgaaagc	544 bp	AT = 57°C
	ALK1 r	ctcttgaccagcacattgcg		
***Human**	Id1 f	aaacgtgctgctctacgaca	152 bp	AT = 51°C
	Id1 r	gattccgagttcagctccaa		
Human	CD34 f	tgaagcctagcctgtcacct	200 bp	AT = 56°C
	CD34 r	cgcacagctggaggtcttat		
Human	Tie-2 f	atcccatttgcaaagcttctggctggc	512 bp	AT = 58°C
	Tie-2 r	tgtgaagcgtctcacaggtccaggatg		
Human	SMA f	cctcccttgagaagagttacgagttg	447 bp	AT = 56°C
	SMA r	agaggagcaggaaagtgttttagaagc		
***Human**	ALK1 f	atctgagcagggcgacac	61 bp	AT = 60°C
	ALK1 r	actccctgtggtgcagtca		
***Human**	Calponin f	cgcccacaaccaccacgcacacaa	203 bp	AT = 56°C
	Calponin r	ccgccagtctgctctctccaaactctaa		

*primers were also used for qRT-PCR.

3.9.3 Western Blotting

Cells were lysed in a mix of lysis and sample buffer and sonicated (Bioruptor) (Appendix A). Then the samples were boiled at 95°C for 10 minutes. Samples were loaded on 10% or 12.5% SDS PAGE gels and the proteins migrated through the gel at different speeds (120 mA for 2.5 hours). The proteins in the gel were transferred onto a nitrocellulose membrane in a wet transfer system at 400 mA for 2 hours. Next steps where either done using wet transfer or using the dry iBlot method as explained below (Figure 13).

Wet transfer: The nitrocellulose membrane was preincubated for 30 minutes in 5% milk diluted in TBS-T at room temperature. Primary antibodies (Table 12) were mixed with TBS-T containing 3% milk and incubated on a shaker overnight at 4°C. Following incubation the membranes were washed in TBS-T and then incubated with secondary antibodies (Table 12) on a shaker for 1 hour at room temperature, ending with a TBS-T wash 3 times. Finally, the membranes were treated with ECL Western Blotting Detection System (GE Healthcare) and developed in a dark room with Dürr Med Medicine 260 developing device.

iBlot dry transfer: This new technique is much faster than the wet transfer even though in principle they are very similar. After electrophoresis, the gel was placed on top of the bottom buffer matrix which had been covered with a PVDF membrane. Next a wet filter paper was placed on top of the gel, then the top buffer matrix and last a sponge was placed on top of the filter. The device was closed and the transfer allowed to take place for 5:40 minutes. The nitrocellulose membrane was preincubated in TBS for 1 hour at room temperature. Primary antibodies (Table 12) were mixed with TBS-T containing 5% BSA and incubated on a shaker overnight at 4°C. Following incubation the membranes were washed in TBS-T and then incubated with secondary antibodies (Table 12) diluted in TBS-T and 0.01% SDS on a shaker for 1 hour at room temperature, ending with a 3 times TBS-T wash.

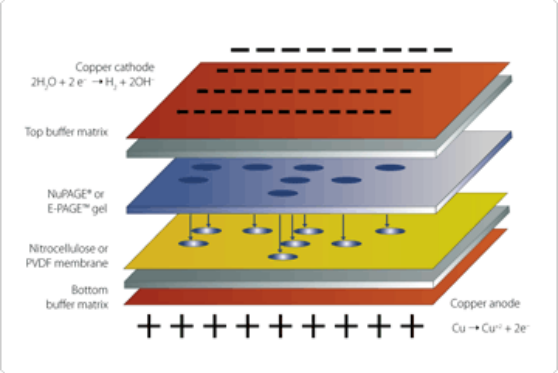


Figure 13. Western blotting method.
(According to Invitrogen protocol)

Table 12. Antibodies used for Western Blotting.

Antibody against	Cat.No	Origin	Type	Dilution	Supplier
Actin	MAB1501R	Mouse	Primary	1:20000	Millipore
Phospho-Smad1	S463/465	Rabbit	Primary	1:1000	Cell Signaling
Phospho-Smad2	S465/467	Rabbit	Primary	1:1000	Cell Signaling
Id1	sc-488	Rabbit	Primary	1:500	Santa Cruz Biotechnology
HRP-Anti-Mouse	NA931	Sheep	Secondary	1:10000	GE Healthcare
HRP-Anti-Rabbit	NA934	Donkey	Secondary	1:10000	GE Healtcare
IRDye 680LT	926-68021	Rabbit	Secondary	1:20000	Li-Cor
Irdye 800CW	926-32210	Mouse	Secondary	1:20000	Li-Cor

3.9.4 Immunofluorescent staining

Various optimizations were done in order to optimize paraformaldehyde and Goat Serum concentrations. Different incubation and permablilization time periods were also tested. The conclusion was to fix cell cultures with 2% paraformaldehyde in Phosphate Buffered Saline (PBS) for 30 minutes, followed by 8 minute incubation in Triton X-100 in PBS to permeabilze the cell membranes. The two first steps were performed at room temperature.

Samples were preincubated in PBS and 4% Goat Serum. Primary antibodies (Table 13) mixed with PBS containing 4% Goat Serum were added and incubated on a shaker overnight at 4°C. Then samples were washed 3x in PBS/0.05% Tween and incubated with fluorescently labeled secondary antibodies (Table 13) for 1-2 hours in the dark. Samples were rinsed with PBS/0.05% Tween and again rinsed shortly with dH_2O containing nuclei stain (Topro3). Then the chamber slides were mounted with coverslips using Fluoromount (Sigma-Aldrich) for preservation of the fluorescent signal. Immunoflourescence was visualized using a confocal Zeiss LSM 5 Pascal laser scanning microscope.

Table 13. Antibodies used for immunofluorescent staining.

Antibody against	Cat.No	Origin	Type	Isotype	Dilution	Supplier
PECAM-1	M0823	Mouse	Primary	IgG1	1:100	Dako
Id1	sc-488	Rabbit	Primary	IgG	1:50	Santa Cruz Biotechnology
VE-Cadherin	550878	Mouse	Primary	IgG1	1:250	BD Pharmingen ™
Endoglin	555746	Mouse	Primary	IgG1	1:250	BD Pharmingen ™
Phospho-Smad1	S463/465	Rabbit	Primary	IgG	1:100	Cell Signaling
Phospho-Smad2	S465/467	Rabbit	Primary	IgG	1:100	Cell Signaling
Actin	MAB1501R	Mouse	Primary	IgG1	1:1000	Millipore
CD34	130-046-702	Mouse	Primary	IgG2a	1:100	Miltenyi Biotec
αSMA	Ab7817	Mouse	Primary	IgG2a	1:200	Abcam
KDR	FAB357P	Mouse	Primary	IgG	1:100	R&D Systems
Cy3 anti-mouse	115-165-146	Goat	Secondary	IgG	1:250	Jackson ImmunoResearch
Alexa 488 anti-rabbit	A11070	Goat	Secondary	IgG	1:1000	Invitrogen
IgG	120-000-288	Goat	Secondary	IgG	1:500	Miltenyi Biotec
Topro3	T3605	-	Nucleic	-	1:500	Invitrogen

3.9.5 Acetylated low density lipoprotein (ac-LDL) assay

This assay is characteristic of endothelial cells. It can be used to distinguish endothelial cells from for instance smooth muscle cells and fibroblasts.

Cells were incubated with 10 µg/ml ac-LDL conjugated to Alexa Fluor 488 (Invitrogen) diluted in culture medium for 4 hours at room temperature. Control cells were incubated with fresh medium only, and both conditions rinsed 3x with PBS. The cells were fixed with 4% paraformaldehyde for 8-10 minutes and then rinsed 3x with PBS and once with dH2O containing Topro3.

Then the chamber slides were mounted with coverslips using Fluoromount (Sigma-Aldrich) for preservation of the fluorescent signal. Immunoflourescence was visualized using a confocal Zeiss LSM 5 Pascal laser scanning microscope.

3.9.6 Anti-BMP9

To investigate the role of endogenous BMP9 in early vascular development, neutralizing anti-BMP9 antibody (0.1 µg/ml) was used from R&D systems.

hES cells were counted (35000 cells per chamber) and preincubated with neutralizing anti-BMP9 antibody for 30 minutes in EGM-2 medium containing 2.5% serum. Cells were then split onto matrigel

coated chamber slides (8 wells) and treated in medium containing various growth factors for about 24 hours. The cells were then rinsed once in 1x PBS and fixed with 4% paraformaldehyde. The cells were then immunofluorescently stained according to previously described method (see. 3.9.4). Then the chamber slides were mounted with coverslips using Fluoromount (Sigma-Aldrich) for preservation of the fluorescent signal. Immunoflourescence was visualized using a confocal Zeiss LSM 5 Pascal laser scanning microscope.

4 Results

4.1 The effects of TGFβ superfamily in vascular development

Our knowledge of vascular development in humans is mainly based on ablation studies in mice. However, vascular differentiation kinetics differ between mouse and human ES cells, meaning that the mouse system is not necessarily predictive of the human system. Because the TGFβ superfamily is very important in early mouse mesodermal development, we wanted to study its effects and how downstream transcription factors regulate target genes that are important in differentiation of hES cells into the vascular lineage. Because of the discrepancy in mouse and human ES cells, as well as within human ES cells themselves, it is important to unravel the TGFβ superfamily's role in human ES cells.

Much is known about what regulates later stages of vascular development, i.e. angiogenesis. Much less is known about the mechanism regulating *de novo* formation of the vasculature. We therefore wanted to improve the enrichment and enhancement of hES derived endothelial precursors and to study their potential to make tube-like structures by stimulation or inhibition by different members of the TGFβ superfamily.

4.1.1 Effect of BMP4 on spontaneous differentiation

Several studies have explored the endothelial potential of hES cells in general, mainly by using spontaneous differentiation. Embryonic stem cells form embryoid bodies which recapitulate the development of all three germ layers including early vascular events (Feraud et al., 2001; Itskovitz-Eldor et al., 2000; Risau et al., 1988). In mice, the vasculature arises from the mesoderm, and it is suggested that one of its regulatory signals is BMP4 (Baron, 2001; Winnier et al., 1995). Recent paper by Boyd et al indicates that BMP4 can enhance the formation and outgrowth of an immature vascular system in human embryonic stem cell-derived embryoid bodies (EB). Vascular outgrowths are a network of cells similar to capillary-like structures formed by mature endothelial cells (Boyd et al., 2007). With that in mind, we wanted to compare the effect in equivalent differentiation medium to Boyd's and in well established endothelial growth medium on vascular outgrowth from human EBs.

To determine the effect of BMP4 in human vascular development, hES cell colonies grown on MEFs in H1 medium were dissociated for embryoid body formation and differentiation. EBs were cultured for 5 days on low attachment culture plates in differentiation medium with and without BMP4 (10 ng/ml) (Figure 14). EBs from both conditions were moved onto matrigel substrate and cultured for additional 5 days either in differentiation medium or EGM-2 medium with and without BMP4.

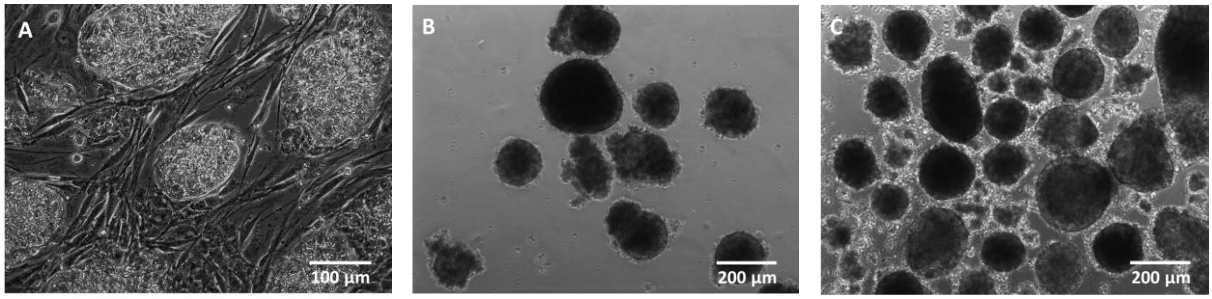


Figure 14. Morphology of hES cells and embryoid bodies.
(A) Undifferentiated hES cell colonies on MEF feeder layer. (B) Untreated EBs in suspension at day 4. (C) BMP4 (10 ng/ml) treated EBs in suspension at day 4.

As seen on the flowchart in Figure 15, EBs grown in differentiation medium without treatment with BMP4 did not develop any outgrowth. Within 2-3 days the cells began to migrate out of the EB along the surface when supplemented with BMP4, consistent with Boyd's results. EBs culture in EGM-2 medium without supplementation of BMP4 resulted in outgrowths (black arrows). However, additional supplementation of BMP4 to EGM-2 medium resulted in abnormal hyper outgrowth (red arrow). Because the EGM-2 medium contains various growth factors possibly including BMP4, the baseline is already inducing vascular outgrowth. Therefore, the addition of BMP4 might result in hyper abnormal outgrowths.

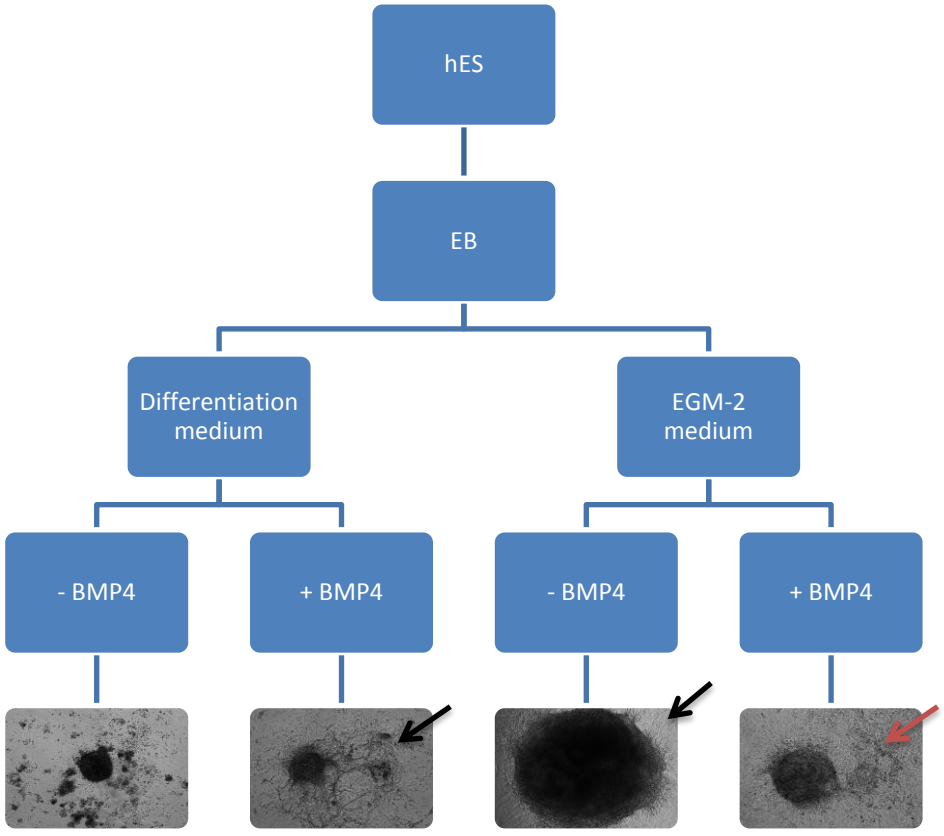


Figure 15. Differentiation flow chart of human embryoid body outgrowth.
hES cells were cultured in either differentiation medium or EGM-2 medium with or without 10 ng/ml BMP4 to observe outgrowth.

Since we can not be absolutely certain that the BMP signalling cascade is continuously active during this differentiation process we decided to use a more robust setup by overexpressing BMP type I receptor, ALK2 and the BMP4 downstream target Id1. We infected hES cells with LacZ (control), and with constitutively active (ca) ALK2 and Id1 adenoviral constructs. When hES cells had reached 70 % confluency they were infected and the day after the cells were harvested for western blot analysis. The western blot results confirm the overexpression of caALK2 and Id1, with anti-HA and anti-Id1 antibodies (Figure 16, A and B). Parallel, infected embryoid bodies were seeded onto matrigel coated wells in differentiation medium for tube like formation (Figure 16C). Morphology images show that the caALK2 (b) and Id1 (c) overexpressed human EBs develop vascular outgrowths (red arrows) much more potently than the LacZ control (a).

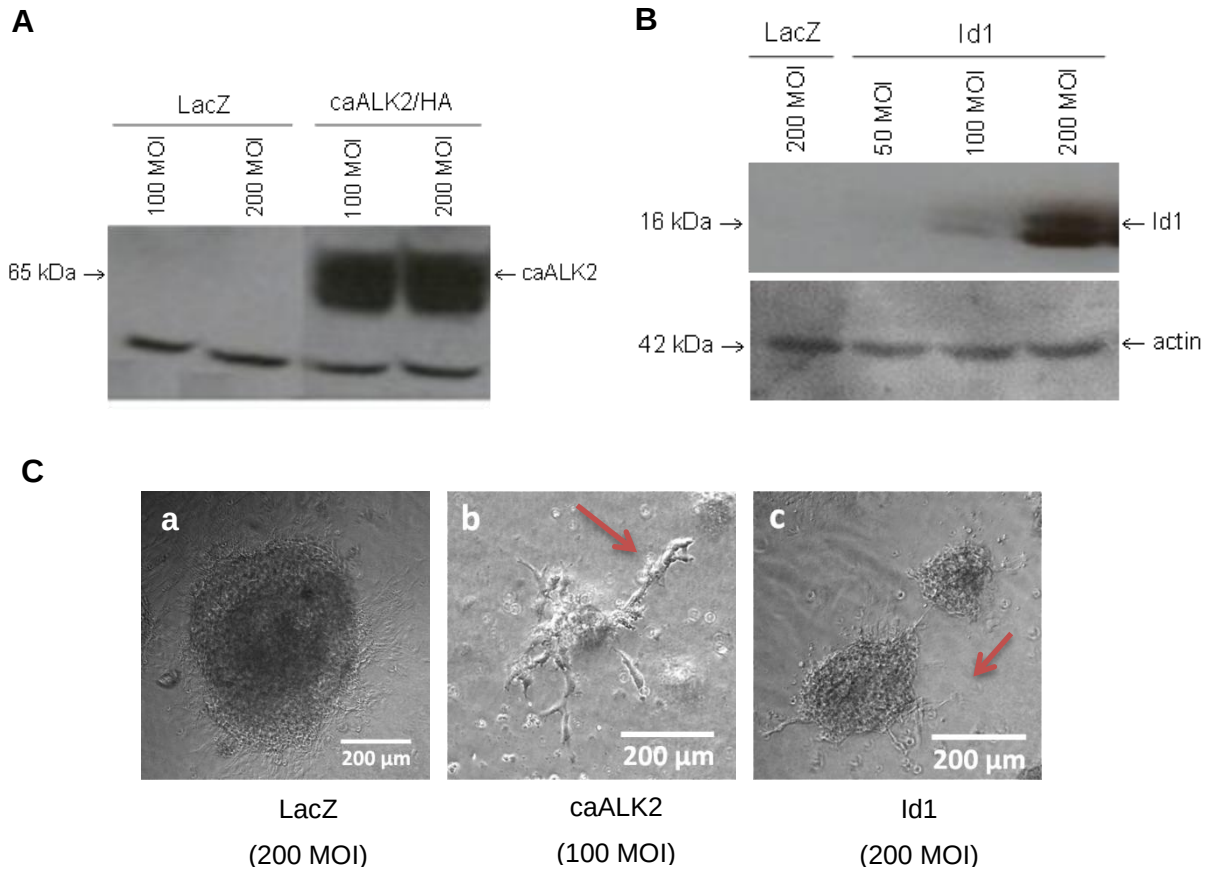


Figure 16. BMP signalling pathway induces vascular outgrowth from embryoid bodies via ALK2 and Id1.
ES cells were infected with adenoviral constructs containing LacZ, caALK2/HA and Id1. The day after, the cells were harvested for western blot analysis to confirm the overexpression of caALK2 (A) and Id1 (B) with anti HA and anti-Id1. (C) When hES cells were infected with previously used adenoviral constructs and seeded onto matrigel, they showed the ability to form tube-like structures.

It is important to verify that the human EB outgrowths are real vascular outgrowths. Hence, human EBs need to be immunofluorescently stained to observe vascular markers. After cultivation of EBs under various culture conditions, the whole EBs were immunofluorescently stained to study protein distribution. As can be seen from Figure 17 the stainings were rather unpredictable. Primary

antibodies used for protein assessment were anti-Id1 and anti-Endoglin, secondary antibodies used were Alexa Fluor 488 (green) and Cy3 (red). No specificity from either growth factors or antibodies could be observed. It was difficult to stain the whole EBs as we could not be certain that the staining had reached the core of the EB. Therefore, as mentioned in materials and methods, optimization for immunofluorecense staining was performed. EBs were permeabilized in different concentrations at different time intervals. Instead of using a combination of PBS and goat serum for secondary antibody stainings, we used IF buffer (Appendix A). However, we were not satisfied with whole EB immunofluorescent stainings using previous protocol. Also, it was challenging to keep the EBs fixed on the chamber slides after transferring them from culture plates. Therefore we decided to attempt using protocol published by Ng et al and assess vascular gene expression during EB development (Ng et al., 2008).

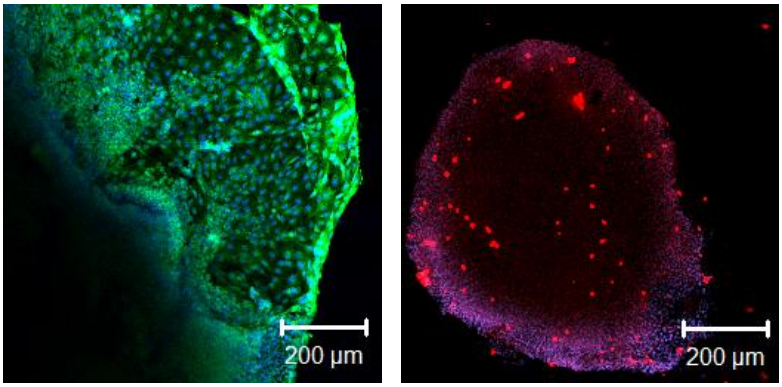


Figure 17. Immunofluorescent staining of EB generated in BPEL medium.
(A) Id1 expression was mostly observed in outgrowths of BMP4 stimulated EBs. Id1 expression was detected using anti-Id1. (B) Anti-Endoglin antibody detected Endoglin expression on BMP4 treated EB surface. Secondary antibodies used were anti-mouse Cy3 (red) and anti-rabbit Alexa Fluor 488 (green).

4.2 Upregulation of vascular markers induced by the TGFβ superfamily

In 2008 Ng et al developed a method that uses recombinant protein-based, animal product-free medium in which hES cells are aggregated by centrifugation to form EBs (Ng et al., 2008). The importance of this method is twofold. Firstly, to initiate aggregation, a known number of undifferentiated hES cells is used in each well of a 96 well round bottomed low attachment plate, resulting in reproducible formation of EBs uniform in size in all wells (Costa et al., 2007). Secondly, the method uses of defined medium that is neutral and therefore suitable for analyzing the effects of exogenously added growth factors (Johansson & Wiles, 1995). We carried out Ng‘s protocol (Materials and methods 3.4.2) to direct differentiation towards endothelial lineage, using various stimulants. hES colonies were harvested and optimum cell number for mesodermal differentiation used, i.e. 3,000 cells per well. The 96 well plate was then centrifuged to initiate forced EB formation (Figure 18).

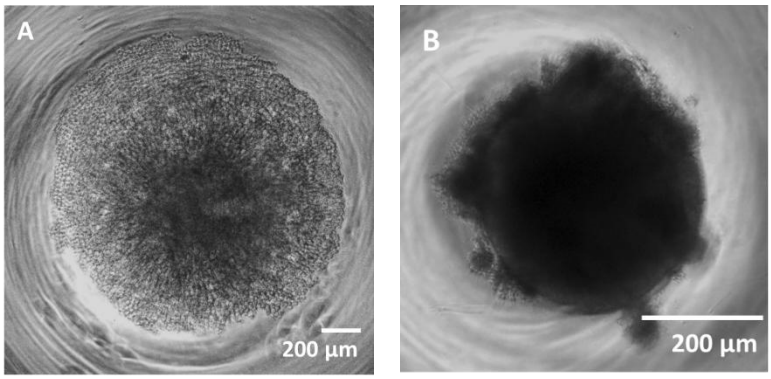


Figure 18. Embryoid bodies generated in BPEL medium using the SpinEB method.
(A) Morphology images of undifferenatiated hES cells at day 0 right after centrifugation.
(B) More compact Spin embryoid bodies at day 2.

Because of the controversy in regard to which growth factors induce mesodermal differentiation and the proliferation and migration of vascular cells, we were interested in studying what vascular genes and TGFβ endothelial specific genes were expressed upon different stimulation or inhibition with TGFβ superfamily members. To our knowledge, the effects of few of these growth factors have not been elucidated in human vasculogenesis. Both BMP9 and BMP10 have been shown to bind to the endothelial specific receptor ALK1 in endothelial cells, causing phosphorylation of Smad1/5/8 (Brown et al., 2005). Whether this binding causes inhibition or induction of vascular formation is still unclear and inconsistent between research groups as previously mentioned.

Table 14. Growth factors used for differentiation.

Growth factors	Concentration
TGFβ	5 ng/ml
BMP4	10 ng/ml
BMP9	1 ng/ml
BMP10	10 ng/ml
VEGF	10 ng/ml
ActA	10 ng/ml
SB-431542	10 µM
Noggin	300 ng/ml

For relative expression studies, a SpinEB experiment was carried out. The EBs were cultured for 5 and 10 days on low attachment culture plates in chemically defined medium and treated with different members of the TGFβ superfamily or their inhibitors (Table 14). The cells were harvested, RNA isolation performed and cDNA made. All experiments for each gene were repeated three times and each experiment performed in triplicate.

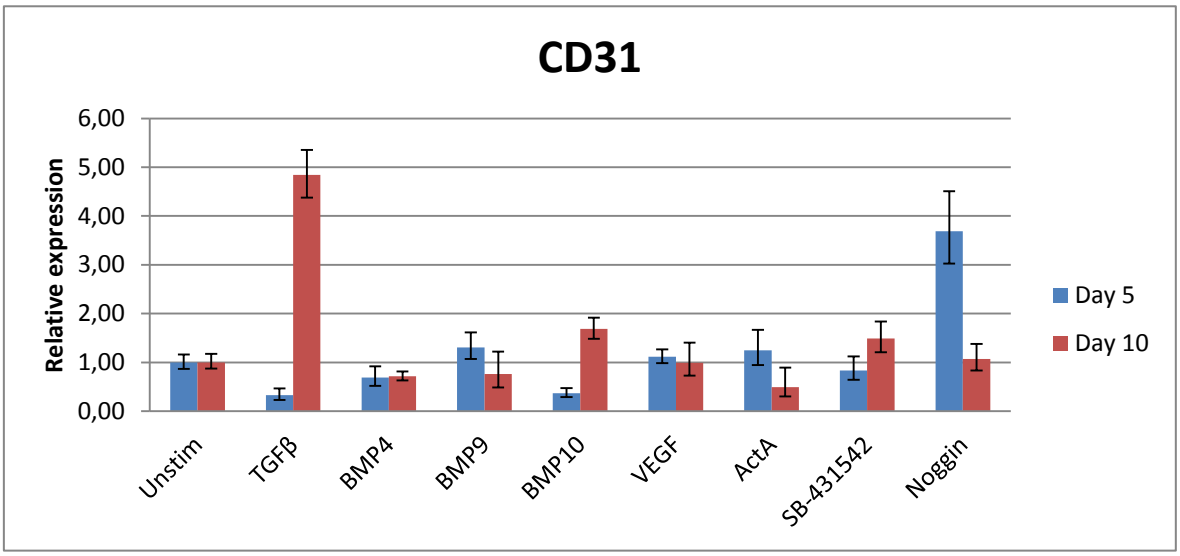


Figure 19. TGFβ and Noggin upregulated the vascular marker CD31 (PECAM-1) during hES cell differentiation.
hEBs were treated with various growth factors of the TGFβ superfamily for 5 and 10 days, and unstimulated cells used as a control. CD31 expression was assessed with qRT-PCR. $P < 0.05$. Error bars, s.d. of experimental values performed in triplicate.

We assessed the expression of several vascular and smooth muscle cell markers as well as TGFβ components that have been reported to have a pivotal role in blood vessel formation. CD31 is a cell surface protein that can be found on various cell types, especially endothelial cells and is involved in angiogenesis. As seen in Figure 19, TGFβ highly induced the vascular marker CD31, almost 5 fold expression, whereas the other ligands tested did not. Noggin is the inhibitor of BMP4 and SB-431542 the inhibitor of the TGFβ branch of the superfamily. Inhibiting the BMP branch with Noggin for the first 5 days also upregulated CD31 expression close to 3.7 fold. When the TGFβ cascade was inhibited, no CD31 upregulation was detected, which is consistent with our main results. Other ligands tested did not have much effect on CD31 expression.

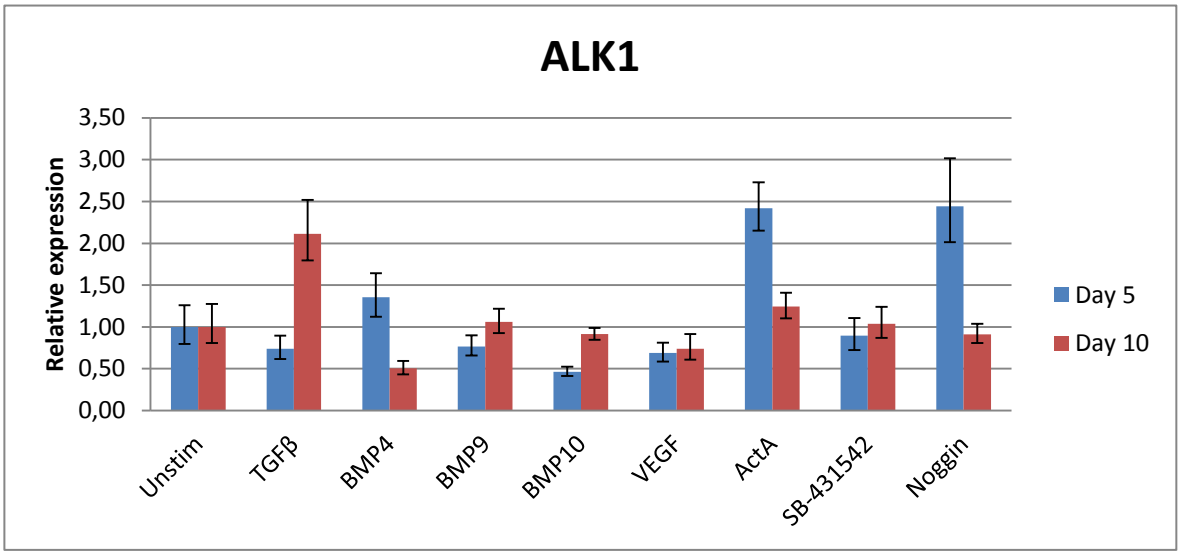


Figure 20. Ligands of the TGFβ branch upregulate the endothelial marker ALK1 during hES cell differentiation.
EBs were treated with various stimulants and inhibitors for 5 and 10 days, and unstimulated cells used as a control. ALK1 expression was assessed with qRT-PCR. $P < 0.05$. Error bars, s.d. of experimental values performed in triplicate.

Next I assessed the expression of the endothelial specific TGFβ type I receptor ALK1. Again, the TGFβ branch of the superfamily highly upregulated ALK1. Ligands belonging to the TGFβ branch of the superfamily induced more ALK1 expression than the other ligands. Stimulation with both TGFβ and ActivinA upregulated ALK1 expression the most, along with Noggin inhibition for the first 5 days. TGFβ upregulated up to 2 fold expression of ALK1 and both ActA and Noggin induced nearly 2.5 fold expression. As before, the BMPs did not have much effect upregulating ALK1 (Figure 20).

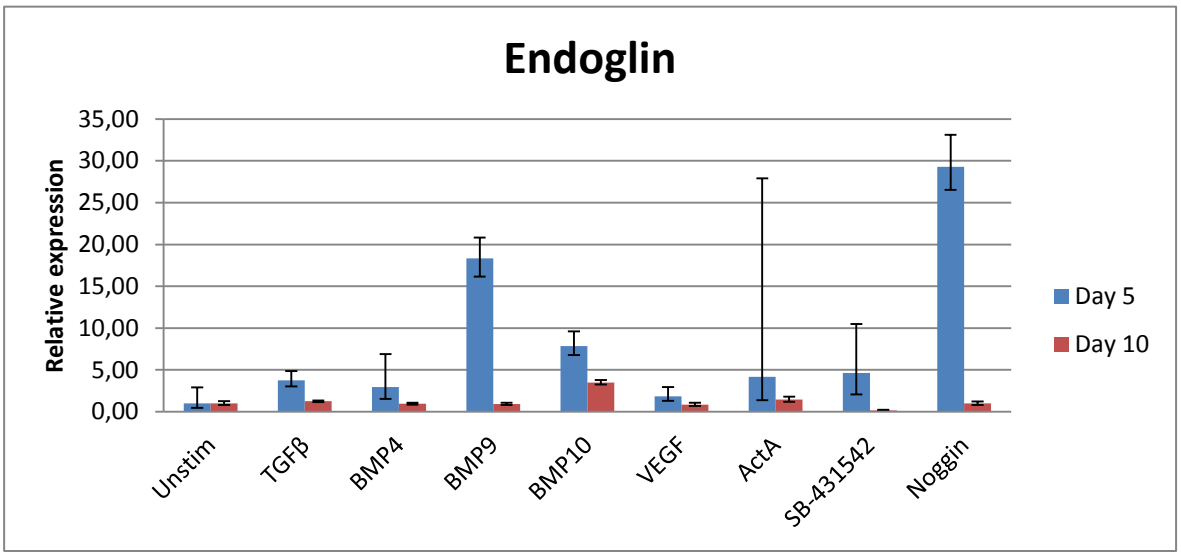


Figure 21. BMP9 and Noggin upregulate Endoglin during hES cell differentiation.
EBs were treated with various stimulants and inhibitors for 5 and 10 days, and unstimulated cells used as a control. Endoglin expression was assessed with qRT-PCR. $P < 0.05$. Error bars, s.d. of experimental values performed in triplicate.

The endothelial TGFβ type III co-receptor Endoglin is more upregulated by BMP9 and BMP10 than the TGFβ branch (Figure 21). Endoglin is located on the cell surface and is part of the TGFβ receptor complex. This receptor has a role in the development of the cardiovascular system and in vascular remodeling. BMP9 and BMP10 stimulation upregulate potently the highest Endoglin expression, as well as early inhibition with Noggin. Both BMP9 and Noggin induce Endoglin stronger than other ligands. BMP9 induced over 18 fold expression of Endoglin and Noggin more than 29 fold. Of note, although Noggin inhibits BMP4, it does not inhibit BMP9 or BMP10. Other ligands tested did not have much effect on Endoglin expression.

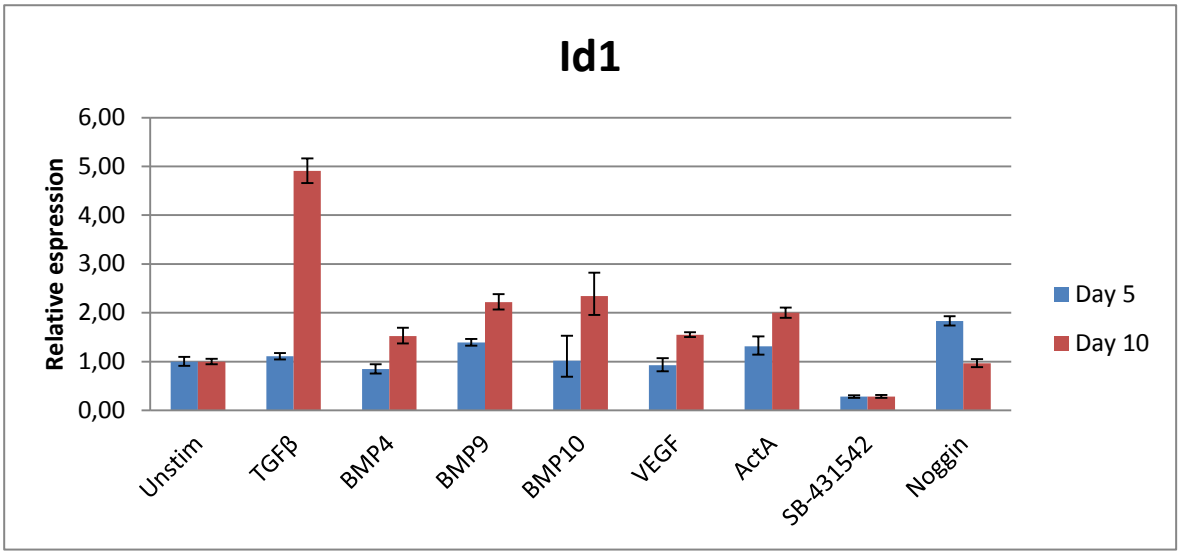


Figure 22.TGFβ upregulates Id1 during hES cell differentiation.
EBs were treated with various stimulants and inhibitors for 5 and 10 days, and unstimulated cells used as a control. Id1 expression was assessed with qRT-PCR. $P < 0.05$. Error bars, s.d. of experimental values performed in triplicate.

Id1 is a known BMP target. BMP4/9 and 10 upregluate Id1 but TGFβ resulted in 5 fold upregulation upon stimulation on day 10 during EB differentiation (Figure 22). To our surprise, TGFβ was more potent inducer of Id1 then the BMP ligands. This could be explained by data reported by Goumans and colleagues that TGFβ can induce the ALK1/Smad1/5/8/Id1 cascade in ECs. By day 10, the vascular genes needed for endothelial phenotype are already present and TGFβ can therefore work through both ALK1 and ALK5 (Goumans et al., 2002). Other ligands tested did not have much effect on Id1 expression.

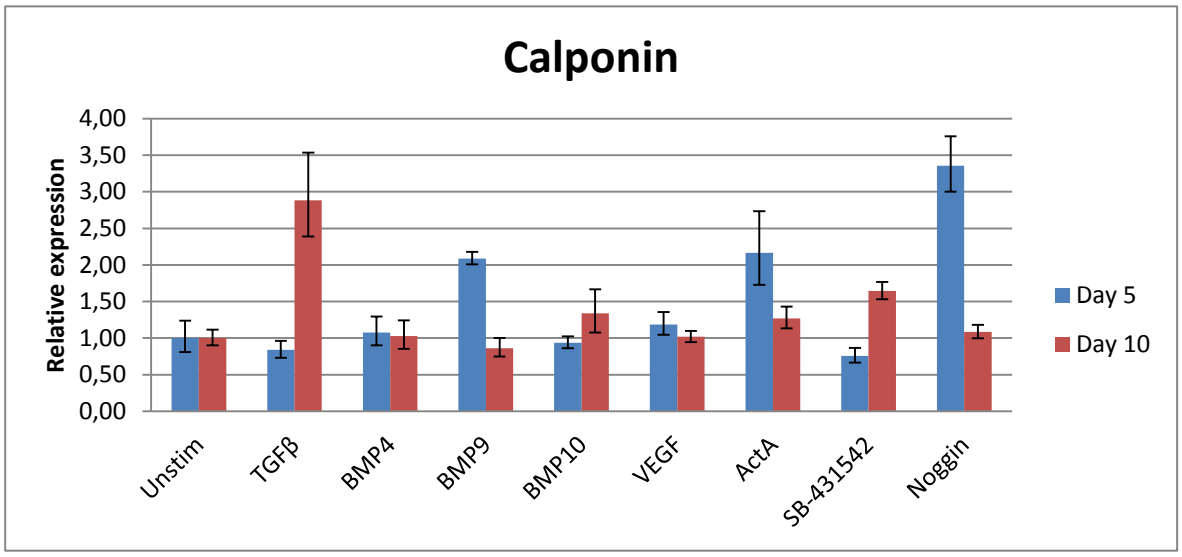


Figure 23. TGFβs components, TGFβ, ActA and Noggin upregulate smooth muscle marker Calponin during hES cell differentiation.
EBs were treated with various stimulants and inhibitors for 5 and 10 days, and unstimulated cells used as a control. Calponin expression was assessed with qRT-PCR. $P < 0.05$. Error bars, s.d. of experimental values performed in triplicate.

In order to see if the TGFβ superfamily has any effect on SMC development during EB formation, I studied Calponin expression. Calponin is a late marker for smooth muscle cells, being a calcium binding protein. Calponin is responsible for binding many actin binding proteins, phospholipids and regulating the actin/myosin interaction. Calponin was upregulated almost 3.5 fold when cells were stimulated with Noggin for the first 5 days. The TGFβ seems to play the biggest part in inducing Calponin expression, along with ActivinA, resulting in more than 2 to 3.5 fold upregulation. Other ligands tested did not have much effect on Calponin expression (Figure 23).

Recent reports have shown that the BMP-Smad1 pathway plays an important role in angiogenesis and therefore we expected that branch to be important during vascular enrichment. To our surprise TGFβ was by far the most effective inducer of all the markers we assessed in the embryoid bodies differentiation model. To get more insight into the role of the two main branches in the TGFβ pathway, we decided to overexpress hES cells with 3 different constitutively active (ca) type I receptors as well as Id1. hES cells were infected with an adenoviral construct of caALK1, caALK2, caALK5 and Id1, using a multiplicity of infection (MOI) of 100. LacZ was used as a negative control. To assess gene expression, cells were harvested after 20 hours, RNA extracted and cDNA made and amplified by PCR. The results showed that Calponin and CD31 was most upregulated in caALK5 overexpessed cells and the late endothelial cell marker VE-Cadherin showed highest level of expression in caALK1 overexpressed cells (Figure 24).

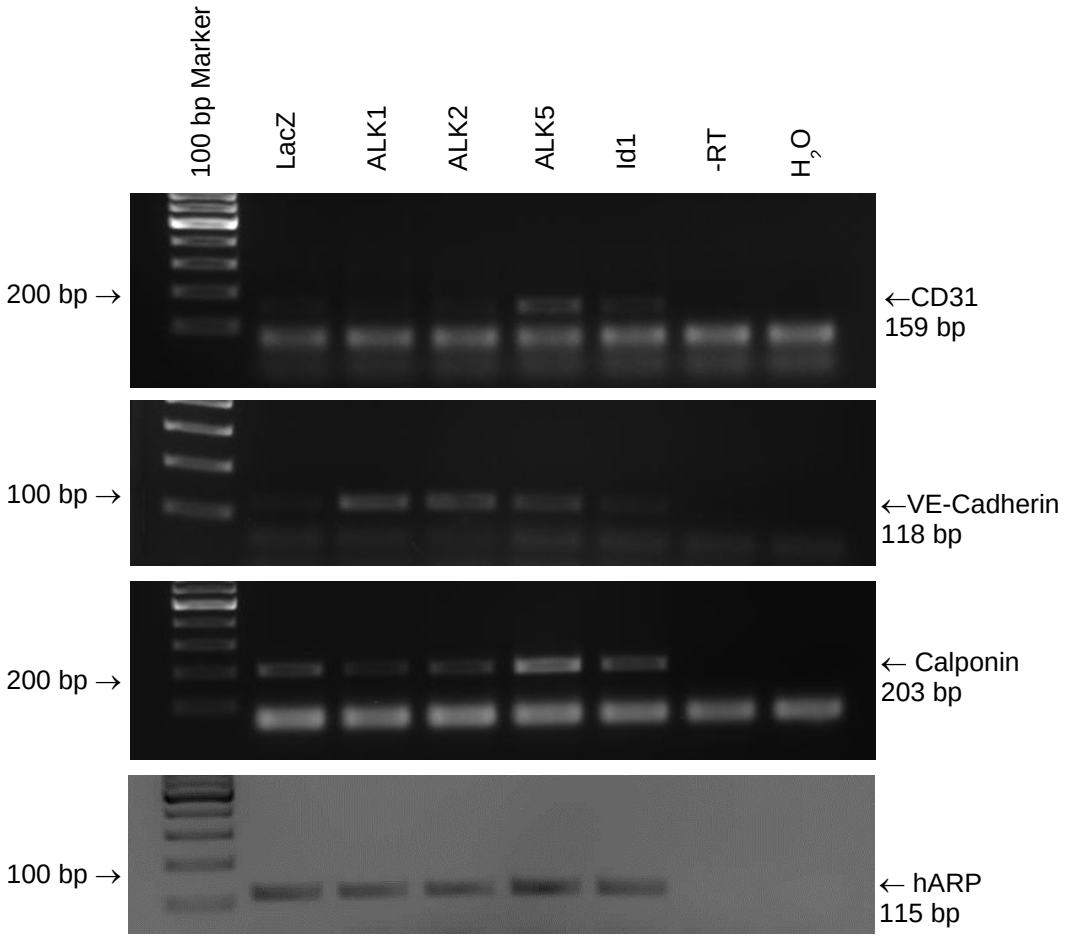


Figure 24. hES cells infected by adenoviral constructs.
hES cells overexpressing caALK5 upregulate the smooth muscle marker Calponin as well as early vascular marker CD31, whereas caALK1 and caALK2 upregulate the late endothelial marker VE-Cadherin.

This is consistent with my qRT-PCR data in that TGFβ results in upregulation of early vascular marker CD31. VE-Cadherin is a late endothelial marker and is more expressed in caALK1 overexpressed cells, possibly by BMP9 or the TGFβ/ALK1 pathway. We therefore suggest that TGFβ is necessary in upregulating TGFβ vascular specific genes, such as ALK1 and Id1 and the early vascular marker CD31. After upregulation of ALK1, BMP9 is able to induce the endothelial marker VE-Cadherin via ALK1 with the help of the co-receptor Endoglin.

4.3 Cell sorting of vascular progenitors

One aim of the project was to address whether sprouting takes place during vascular expansion by stimulating hES-derived CD31+ cells with different TGFβ ligands and assess tube formation. Since previous reports have shown BMP signalling and inhibition of the TGFβ signalling cascade to induce endothelial proliferation and maintenance (Bai et al., 2010; Boyd et al., 2007; James et al., 2010), that

became the main focus of this project. It is very important to take notice of growth factor concentration and time and duration of stimulation. Poon and colleagues reported that TGFβ stimulation for the first 3-8 days decreases EB vessel formation (Poon et al., 2006). TGFβ and Notch signalling are more likely to mediate hES cell differentiation towards smooth muscle cells (SMC) (Kurpinski et al., 2010). On the other hand, James et al., reported that early activation of this pathway, before day 7, is required for mesodermal precursor differentiation, and inhibition after day 7 to maintain endothelial identity (James et al., 2010). In 2010 Bai et al., reported that high concentration of BMP can also induce hES cell to differentiate towards mesoderm (Bai et al., 2010).

A part of this project was to develop a cell sorting technique using the MACS method. Our previous work on EB formation and immunostaining of the whole EBs, was not fully convincing. By sorting CD31+ cells, (vascular progenitor cells), we hoped for more concrete results. We wanted to improve the enrichment of hES cell derived endothelial cells and to study their potential to make tube-like structures by using different members of the TGFβ superfamily.

To induce spontaneous differentiation, undifferentiated hES colonies were dissociated into clumps and embryoid bodies formed in suspension (Materials and methods 3.4.1.). They were cultured for 10 days in serum-free differentiation medium on non-adherent culture plates. Previous reports were based on cultures in the presence of serum, which contains many unknown factors. In this study, we used serum replacement in order to avoid any disturbance of growth factors or inhibitors from the serum. The embryoid bodies were cultured in differentiation medium alone or the medium was supplemented with 10 ng/ml BMP4. The morphology differed slightly between culture conditions. On days 3-5 the EBs looked compact and round in both conditions. On day 10 unstimulated EBs still looked compact and round, however and to our surprise the BMP4 stimulated EBs had attached to the culture plate in some of the wells (Figure 25).

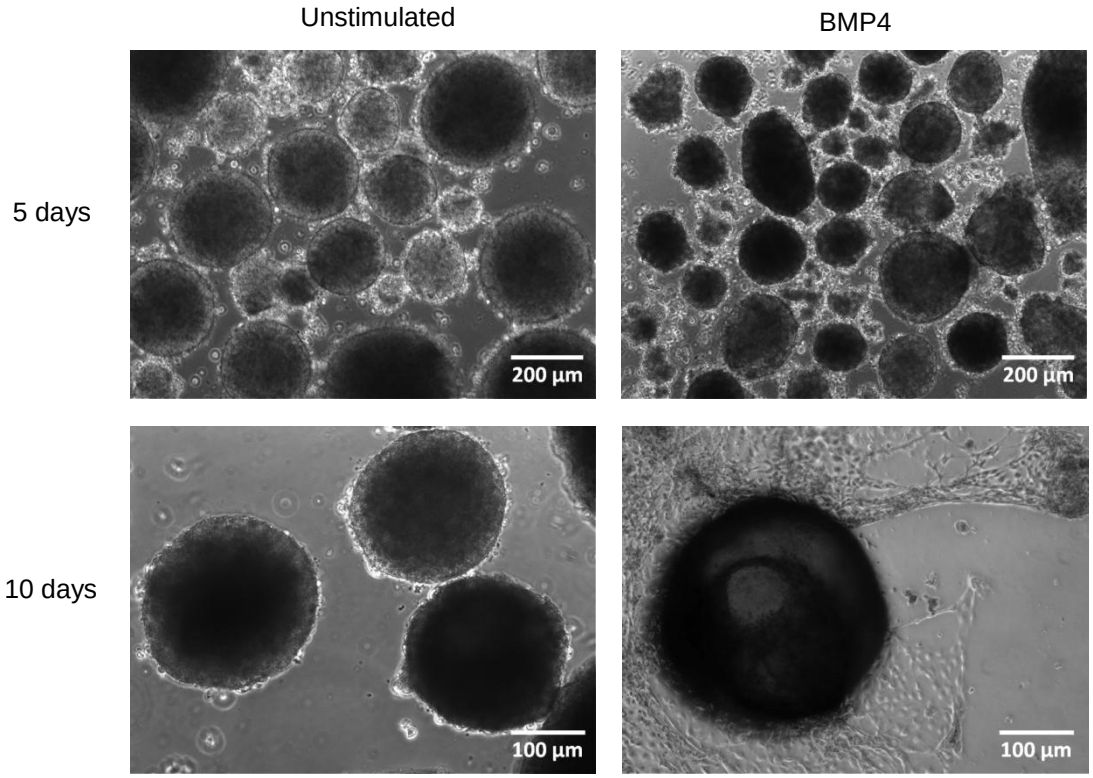


Figure 25. Embryoid body formation in suspension.
Embryoid bodies were cultured for 10 days in differentiation medium alone, or the medium was supplemented with 10 ng/ml BMP4.

By day 10, cell separation was performed using the MACS method (Materials and methods 3.5.1.). EBs were harvested from suspension culture and CD31+ cells purified by cell sorting using anti-CD31 antibody. Those cells are believed to be vascular cells (Levenberg et al., 2010). The sorted cells were plated onto gelatin coated culture plates and expanded (Figure 26). CD31+ cells were then plated onto matrigel coated chamber slides for tube like formation assay (Figure 29). Figure 26 shows proliferation time of vascular cells cultured on gelatin. By day 5 there were few cells in both conditions, however cells stimulated with BMP4 induced endothelial cell proliferation faster than unstimulated cells. By day 13 both conditions showed similar proliferation rate (data not shown).

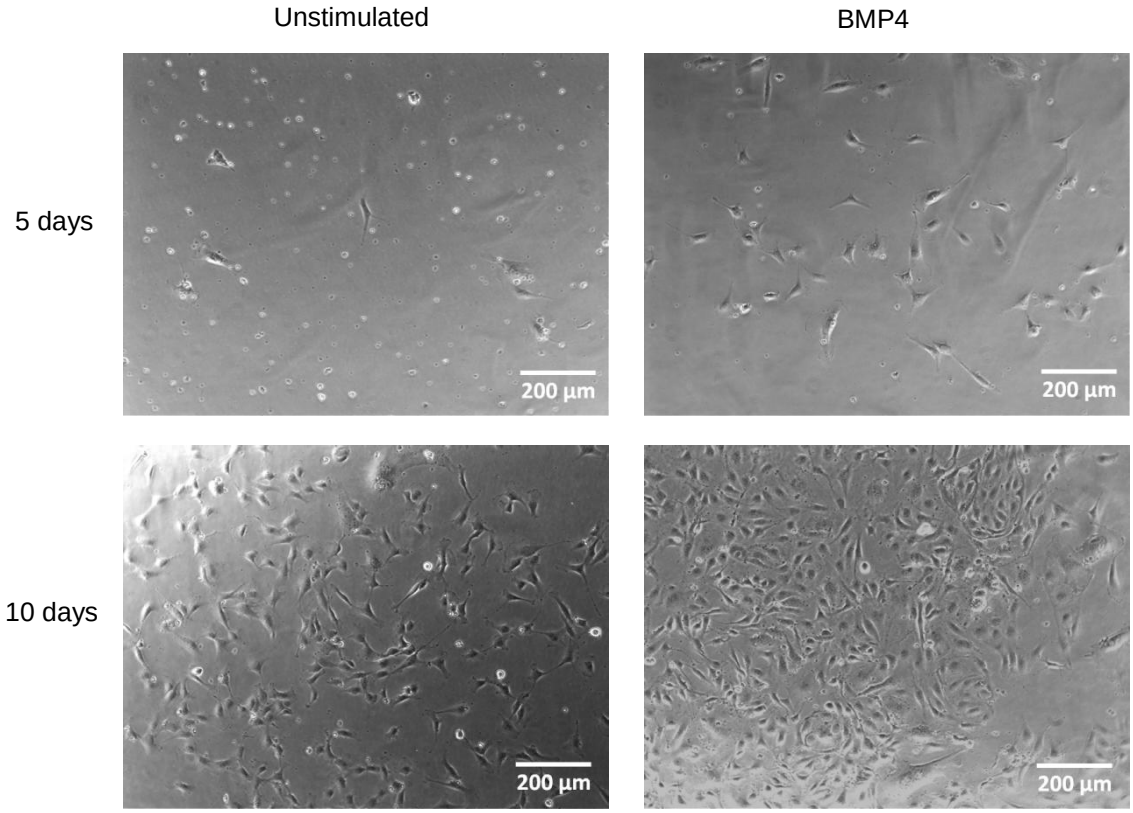
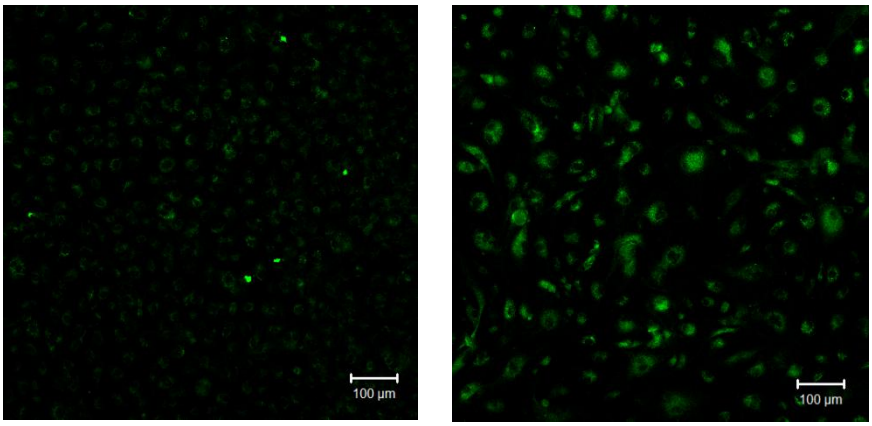


Figure 26. BMP4 stimulated cells induce more CD31+ cells.
Stimulation with 10 ng/ml BMP4 induced more proliferation of CD31+ sorted cells on gelatin.

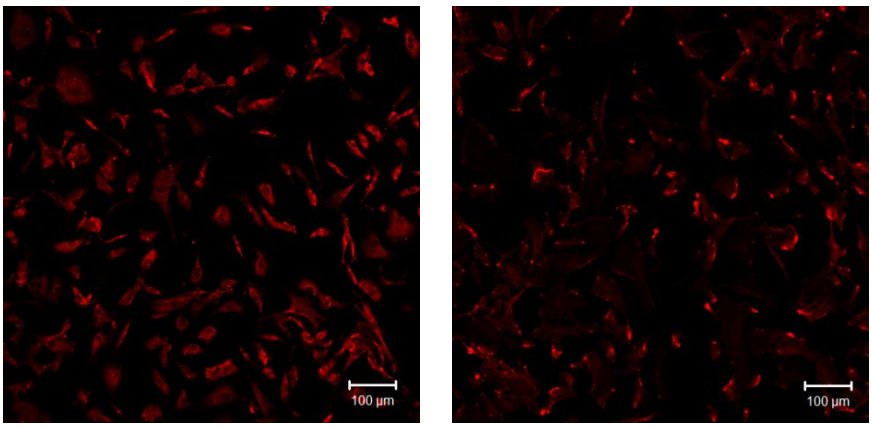
We wanted to confirm that the proliferated CD31+ sorted cells were indeed vascular cell that had endothelial potential. One way to characterize ECs is by Acetyleted-LDL (ac-LDL) uptake (Li et al., 2011). Fluorescently labeled ac-LDL was added to the CD31+ sorted cell culture to confirm if the sorted cells were indeed mature ECs. Human umbilical vein endothelial cells (HUVECs) were used as a positive control as they are true mature ECs and take up ac-LDL. As seen in Figure 27, HUVECs were able to take up ac-LDL and so did the CD31+ sorted cell, confirming that this cell population consists of endothelial cells.



HUVEC CD31^{+} cells

Figure 27. Acetylated low density lipoprotein assay.
To confirm that the CD31+ sorted cell population had indeed endothelial potential, fluorescently labeled ac-LDL was added to CD31+ cell culture to observe their ability to take up ac-LDL. HUVEC cells were used as a positive control, they are mature endothelial cells that take up ac-LDL.

Besides showing endothelial potential we wanted to know if the CD31+ sorted cell population also had smooth muscle potential. We therefore seeded the CD31+ sorted cells onto gelatin and immunofluorecently stained with antibody against smooth muscle marker αSMA. We observed that the cells expressed αSMA as well as the endothelial marker Endoglin (Figure 28).



αSMA Endoglin

Figure 28. CD31+ cell population is able to differentiated into both endothelial and smooth muscle cells.
CD31+ cells were plated on to gelatin for immunofluorescent staining. The cells were fixed and stained with primary antibodies anti-αSMA and anti-Endoglin. Secondary antibody used was anti-mouse Cy3 (red).

To study the effects of TGFβ growth factors on the CD31+ sorted cell population, several biological assays were performed that mimic angiogenesis *in vivo*. The assay we used was tube-like formation assay and tubes were assessed using immunofluorescent stainings.

Figure 29 shows morphology images of CD31+ cells on matrigel, where both unstimulated and BMP4 stimulated cells formed tubes.

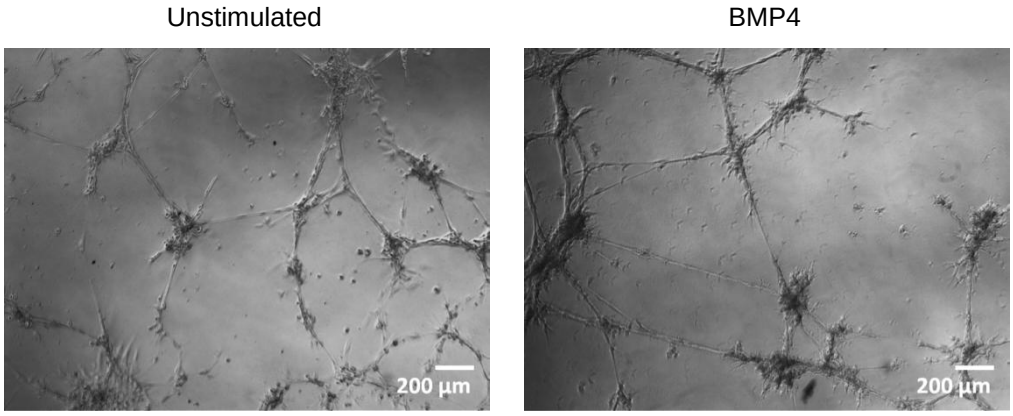


Figure 29. hES derived CD31 + cells form tubes on matrigel.
The biological assay of tube-like formation was performed on matrigel to see both unstimulated and BMP4 stimulated cells potential to form tubes.

Both untreated and BMP4 stimulated cells were able to form tubes. As seen in Figure 30 BMP4 promotes more organized tube like formation. Untreated and BMP4 stimulated cells induce similar expression of endothelial markers. BMP4 stimulated cells induced slightly more phosphorylation of BMP downstream transcription factor Smad1 than unstimulated cells and show more VE-Cadherin expression (Figure 30). Surprisingly, CD31+ cells showed phosphorylation of TGFβ downstream transcription factor Smad2 in both conditions. Both conditions showed similar expression of transmembrane co-receptor Endoglin, but BMP4 treated cells expressed Id1 at modest level. Interestingly, αSMA expression was also seen in BMP4 stimulated cells. Our results are consistent with previous findings by Valdimarsdottir and colleagues, were they report that BMP receptor/Smad activation stimulate endothelial cell migration and tube-like formation. This indicates that BMP has a role in regulating differentiation and organization of newly formed endothelial cells (Valdimarsdottir et al., 2002), rather than in later developmental stages.

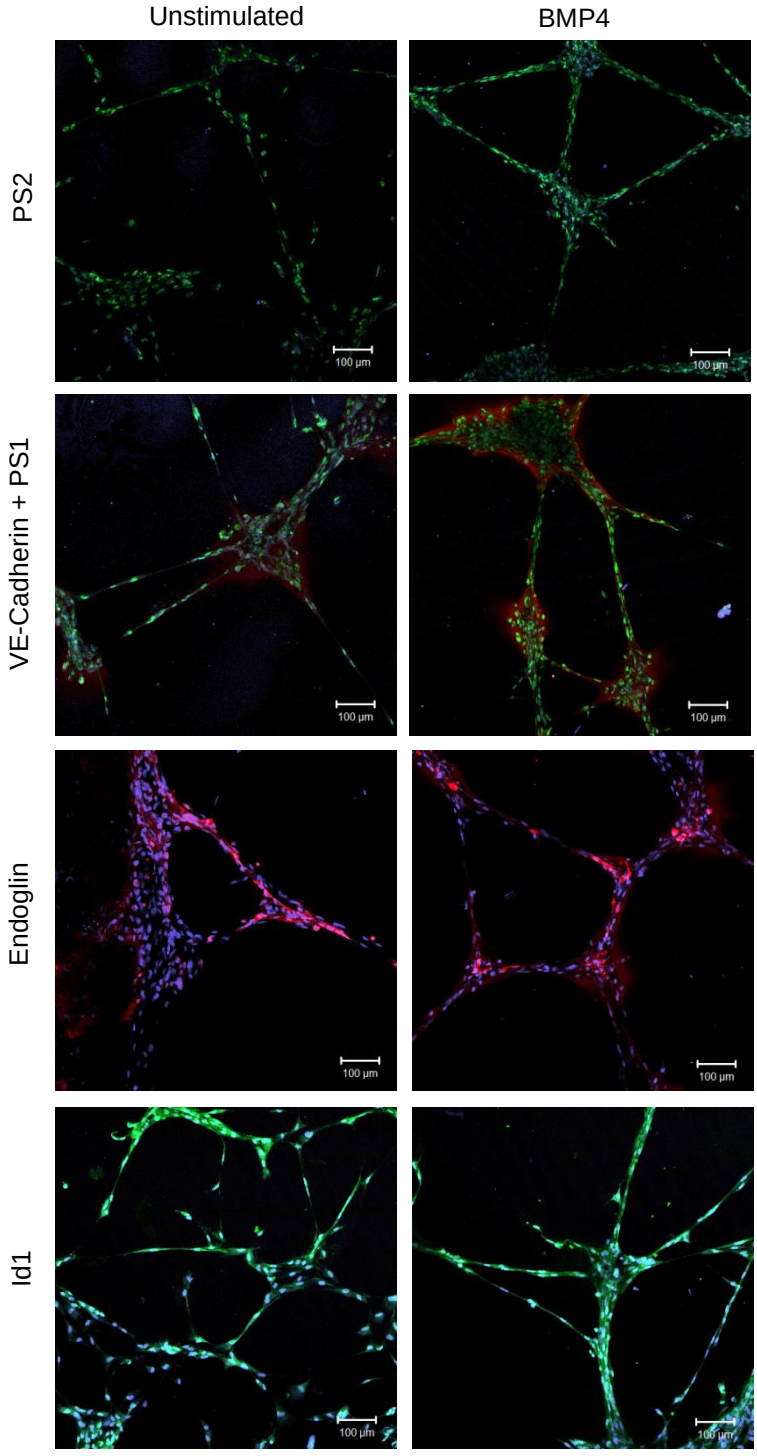


Figure 30. BMP4 induces hES cell mesodermal differentiation.

BMP4 stimulated cells upregulate more vascular markers and form more organized tubes compared to untreated cells. For nuclei staining, all conditions were stained with Topro3.

To broaden our view on the effect of TGFβ superfamily components, hES-derived endothelial cells were stimulated with either stimulants or inhibitors. Endothelial cells have an endothelial specific type I receptor, ALK1. TGFβ has been known to signal both through ALK5 and ALK1 with opposite affects. Activation of the ALK1-Smad1/5/8 pathway showed EC proliferation and migration. Upon binding to ALK5 it causes phosphorylation of Smad2/3, leading to inhibition or activation (Goumans et al., 2002). The SB-431542 inhibitor is known to inhibit the TGFβ pathway.

Recently, another member of the TGFβ superfamily, BMP9 was shown to bind with high affinity to ALK1 in endothelial cells, but its effect on endothelial cells is controversial. Reports have suggested that the BMP9/ALK1 cascade inhibits proliferation and migration of endothelial cells (David et al., 2007; Scharpfenecker et al., 2007). Three years later, Suzuki et al., reported BMP9 to induce proliferation and migration of endothelial cells (Suzuki et al., 2010). Hence, the role of BMP9 on ECs is still unclear. Moreover, to our knowledge BMP9 has not been studied in hES derived endothelial cells. Therefore, we wanted to get more insight into the effect of BMP9 on hES derived vascular cells.

Endothelial cell properties of hES derived CD31+ cells were evaluated in cord like formation. BMP9 treatment of CD31+ cells resulted in induced tube-like formation much faster than the other ligands tested. Consistent with increased tube formation upon BMP9 treatment, these cells also had the highest expression of the endothelial marker VE-Cadherin. BMP9 induces endothelial cell differentiation and they have acquired an endothelial fate, since no αSMA expression could be detected. Upon BMP9 treatment phosphorylation of Smad1 was observed as well as expression of Id1. BMP10 stimulated cells show some tendency to form tubes and show expression of Endoglin, as well as BMP9 stimulated cells. We observed that TGFβ treated CD31+ cells showed tendency to form tubes expressing both VE-Cadherin and Endoglin. Phosphorylation of Smad2 was observed with most intensity in TGFβ stimulated cells, as expected but to our surprise, p-Smad2 could also be detected in BMP9 treated cells. No tubes were formed when the TGFβ branch was inhibited with SB-431542 inhibitor (Figure 31). Kinase insert domain receptor (KDR), also known as vascular endothelial growth factor receptor 2 (VEGFR-2) is an early mesodermal marker. Since CD31+ cell population has assessed endothelial fate, not KDR expression could be detected (data not shown).

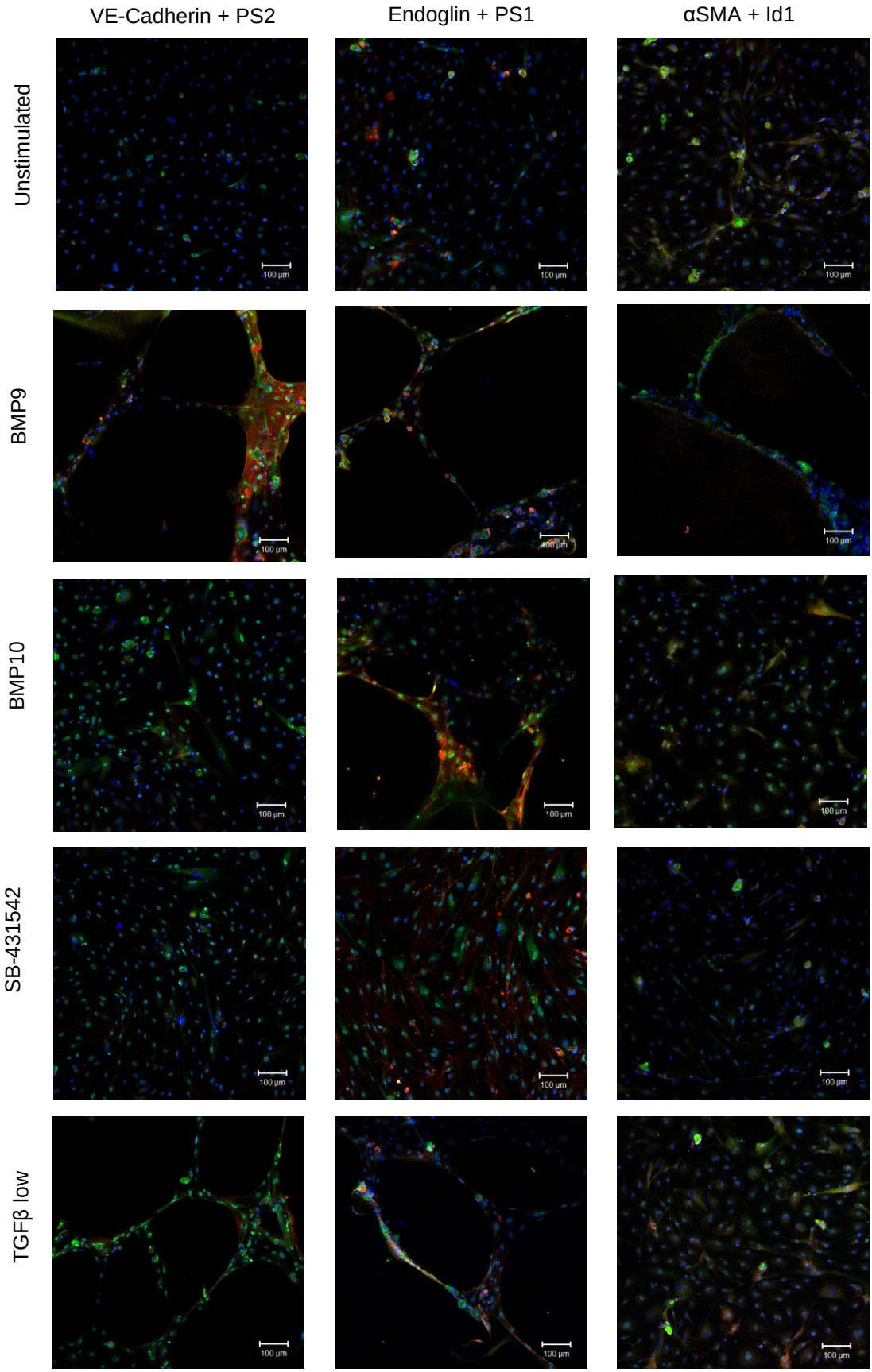


Figure 31. BMP9 induces endothelial cell differentiation.
BMP9 induces tube formation and endothelial cell marker expression much earlier that other ligands.

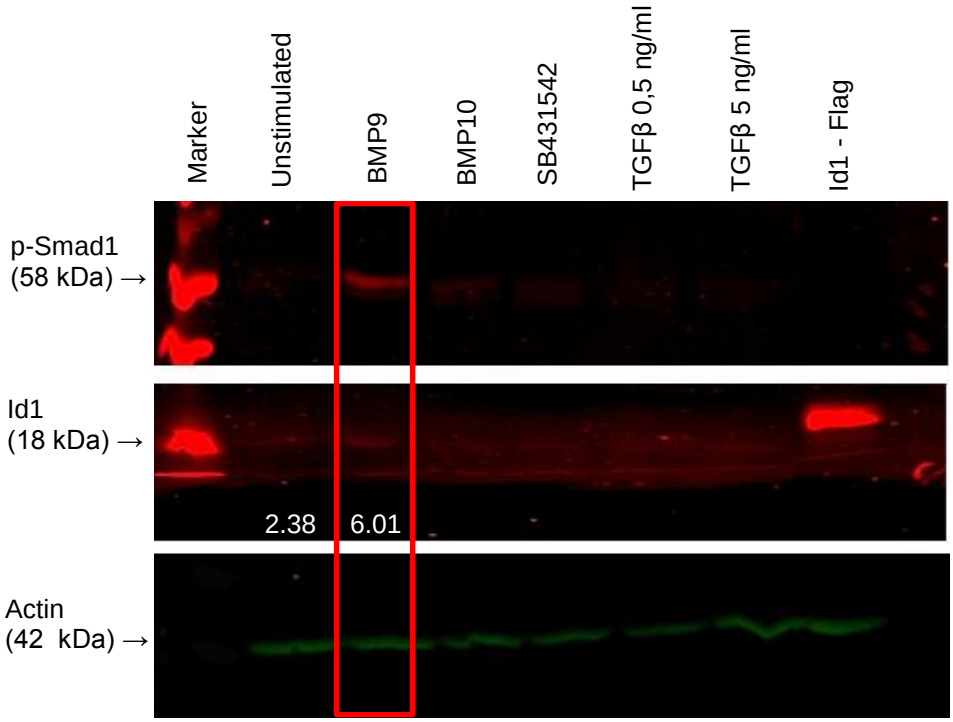
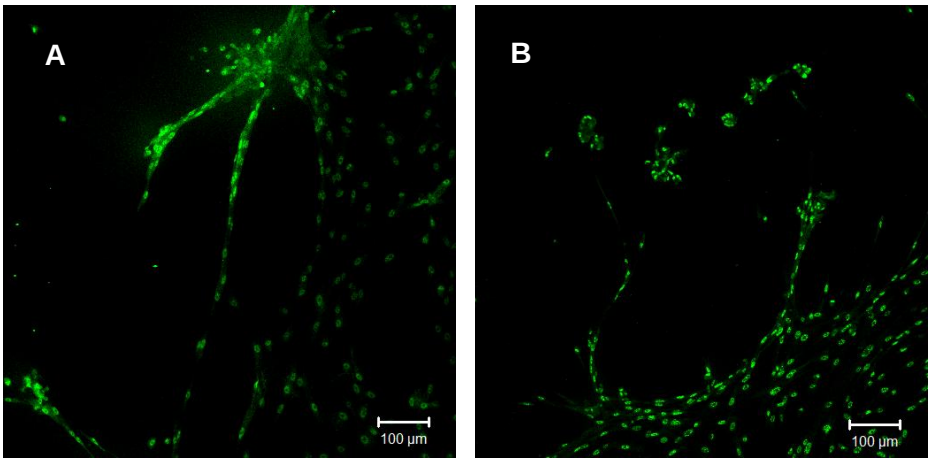


Figure 32. BMP9 induces phosphorylation of transcription factor Smad1 and expression of Id1. When hES cells are stimulated with BMP9 it caused phosphorylation of Smad1 and expression of Id1. Showing, that BMP9/ALK1/Smad1 pathway is active, leading to almost 3 fold translation of Id1 compared to unstimulated cells.

It is important to identify key signalling pathways, how growth factors and inhibitors act on hES cell fate. How these factors interact and activate transcription factors downstream of pathways of interest is equally important. We were interested in knowing if BMP9 is activating the Smad1/5/8 branch in hES derived cells. Cell lysates were analysed on western blot. Figure 32 shows that stimulation with BMP9 induces phosphorylation of Smad1 and expression of Id1 protein.

4.3.1 Anti-BMP9

Given the involvement of BMP9 signalling in hES derived vascular cell sprouting and the fact that serum in the EGM medium might contain bioactive BMP9 (David et al., 2007) we made an attempt to pre-incubate CD31+ cells with or without anti-BMP9 neutralizing antibody. Figure 33 shows p-Smad1 activation in untreated cells. BMP9 pre-incubated cells did show less organized tube formation and less activation of p-Smad1 than cells not pre-incubated with BMP9 antibody. The experiment was only done once and needs to be repeated to give reliable results.



Pre-incubation with IgG antibody Pre-incubation with p-Smad1 antibody

Figure 33. Anti-BMP9 neutralizing antibody leads to less organized tube formation and decreases Id1 expression.
(A) CD31+ cells were pre-incubated for 30 minutes with IgG antibody that served as a negative control. (B) CD31+ cells were pre-incubated with anti-BMP9 neutralizing antibody (0.1 µg/ml) for 30 minutes. To assess p-Smad1 activation, cells were fixed and stained with primary anti-p-Smad1 antibody. Secondary antibody used was anti-Alexa Fluor 488 (green).

For all fluorescent stainings, cells were stained with IgG antibody and secondary antibodies, anti-mouse Cy3 (red) and anti-rabbit Alexa Fluor 488 (green). This was done to exclude any unspecific binding of IgG or secondary antibodies. In Figure 34 negative controls can be seen. Since all negative controls were successful, Figure 34 applies to all previous immunostaining experiments.

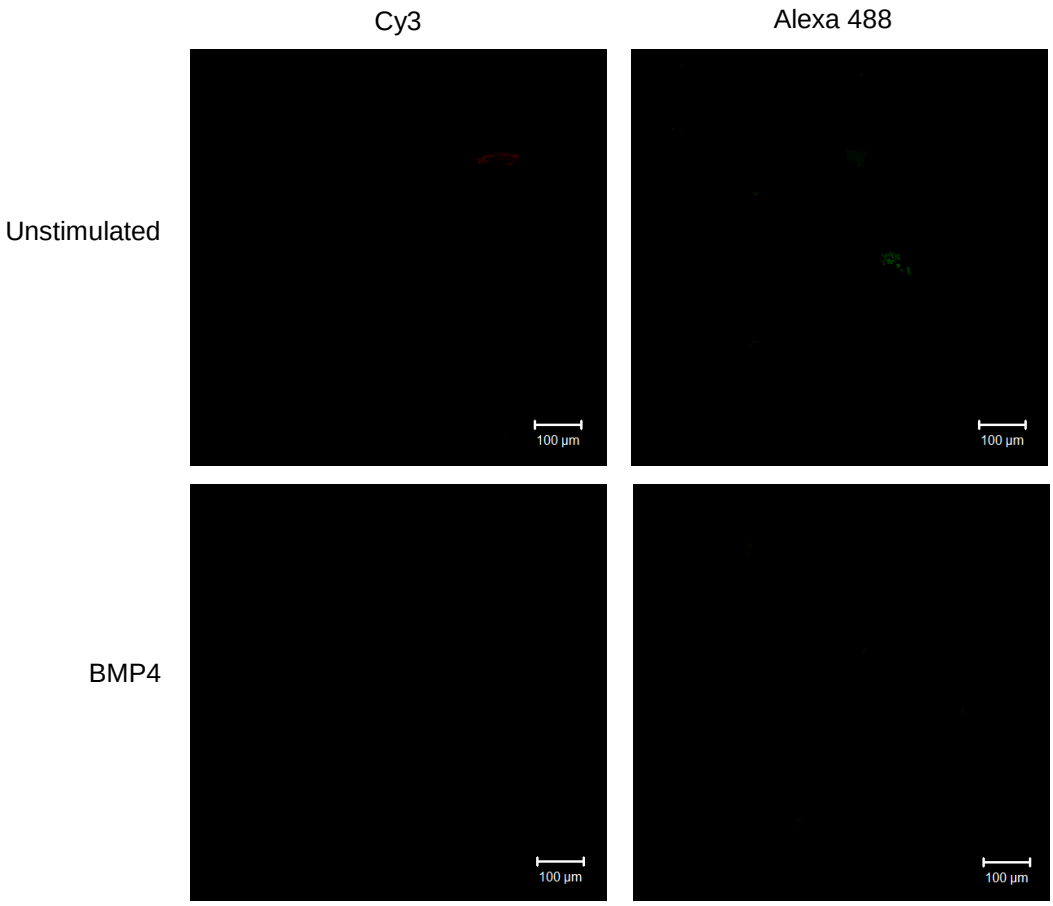


Figure 34. Negative control for immunofluorescent staining of hES cells.

5 Discussion

It is of great importance to elucidate the mechanisms behind early human vascular development. From knockout studies in mice we have learned that the TGFβ superfamily may play an important role in determining hES cell fate towards the vascular lineage. In this study we used hES cells as an *in vitro* model to analyze human vascular development and to further improve currently used techniques to make vascular cell derivation more efficient for future clinical applications. I was able to establish a differentiation method to isolate hES derived vascular cells (CD31 positive cells). This vascular cell population expressed both endothelial and smooth muscle cell markers, suggesting that these cells are vascular progenitors, with the ability to further mature either into endothelial cells or smooth muscle cells. The most interesting results indicate that BMP9 is by far the strongest inducer of tube formation in hES derived CD31+ cells. The BMP9 stimulated cells did not express αSMA anymore and we can therefore decuce that BMP9 promotes the differentiation of these vascular precursors towards mature endothelial cells.

5.1 BMP involvement in vascular outgrowths

What directs pluripotent stem cell differentiation into lineage-specific cells still remains partially unknown. Many papers suggest what molecular signals control differentiation and vessel formation. Boyd et al proposed BMP4 to be one of those growth factors, that are required for mesodermal formation and vascular- and heamatopoietic differentiation. Their findings indicate that BMP4 enhances the formation and outgrowth of an immature vascular system in hES cells (Boyd et al., 2007). Bai and colleagues demonstrated that BMP2/4/7 but not BMP9, promotes human embryonic stem cell differentiation into CD34+CD31+ vascular progenitor cells (Bai et al., 2010). Boyd et al used endothelial growth medium containing serum supplemented with 100 ng/ml BMP4. Serum contains various known and unknown growth factors and inhibitors. This high BMP dose is above the bioactive concentration. Hence, we were interested in recapitulating these vascular outgrowth experiments without serum and using ten times lower dose of BMP4 to see the real effect of BMP4 during hES cell vascular commitment.

EBs grown in differentiation medium without the addition of BMP4 did not show any outgrowth. Outgrowths could only be detected in EBs grown in differentiation medium supplemented with BMP4, consistent with the above mentioned studies. The EGM-2 medium induced vascular outgrowth of EBs, consistent with Yu and colleagues (Yu et al., 2009). Addition of BMP4 in EGM-2 medium resulted in abnormal hyper outgrowth, possibly because the EGM-2 medium contains various growth factors including VEGF and possibly BMP4. To get more insight into the BMP effect on vascular outgrowth, we decided to overexpress a constitutively active (ca) form of the BMP type I receptor, ALK2 and the BMP downstream target Id1. hES cells infected with adenoviral constructs of caALK2 and Id1, confirmed BMP4 to signal through ALK2 receptor and Id1 as the cells showed similar effects on EB outgrowth, respectively. To confirm that the EB outgrowths were indeed vascular outgrowths, immunostainings for vascular marker expression were done. Unfortunately, we did not succeed in

staining whole embryoid bodies despite great efforts in optimizing the protocol and therefore the results were not as clear cut as we hoped for.

5.2 TGFβ induces vascular marker expression in embryoid bodies

Since vasculogenesis in human ES cells has not been studied thoroughly, current understanding of early human embryonic development is for the most part based on mouse experiments. Because of the discrepancy in regard to which TGFβ superfamily members induce mesodermal differentiation and the proliferation and migration of vascular cells, we were interested in studying which vascular genes were expressed upon different treatment with members of the TGFβ superfamily during hES differentiation. To elucidate the effects of specific growth factors on hES cells, we performed qRT-PCR for several genes of interest. Again, to avoid any noise from the serum we now used chemically defined medium (Ng et al., 2008).

Our findings suggest that upon TGFβ stimulation, CD31 expression was elevated. No upregulation could be detected for the first 5 days, but by day 10 the expression had increased almost 5 fold. In 2006, Poon and colleagues published a paper highlighting the difference between early human and mouse embryonic development. Both Poon and other laboratories have shown that TGFβ and its signalling components are crucial for early vascular development in mice. On the other hand, Poon et al reported that TGFβ treatment was inhibiting the expression of CD31 in human EB vessel formation. According to James et al, stimulation with TGFβ for the first 7 days and followed by inhibition thereafter is essential to achieve a large number of stable and proliferative endothelial cells (James et al., 2010). TGFβ might affect differentiation of ES cells into the emergence and maintenance of endothelial precursors (Poon et al., 2006).

Our results showed robust Endoglin expression upon BMP9 stimulation for the first 5 days (and inhibition with Noggin). These results are consistent with Suzuki's results, were VEGFR2+ derived hES cells cultured in serum-free medium supplemented with 30 ng/ml VEGF and 1 ng/ml BMP9 showed upregulation of Endoglin.

Activin receptor-like kinase 1, ALK1, is only expressed in endothelial cells. Both TGFβ and BMP9 have been shown to bind to the ALK1 receptor activating a different branch of the TGFβ superfamily leading to different effects (Ricard et al., 2012; Suzuki et al., 2010). Our results demonstrated that ActivinA and TGFβ induce ALK1 expression, ActivinA for the first 5 days and TGFβ later on in the differentiation process. In 2002, Goumans and colleagues reported that the activation state of the endothelium being able to signal via two distinct TGFβ type-I receptors i.e. ALK5 and ALK1. There they display TGFβ/ALK1 pathway to induce endothelial cell migration and proliferation of mouse embryonic endothelial cells (MEEC). Increased ALK1 signalling resulted in increased Id1 expression (Goumans et al., 2002). According to our results TGFβ stimulation resulted in almost 5 fold expression of Id1. Id1 has been thought to be the target of the BMP branch. We believe that the signal is going through TGFβ/ALK1, causing phosphorylation of Smad1/5/8 and in turn activating transcription of Id1.

In a recent article, TGFβ (and Notch) signalling is believed to mediate hES cell differentiation into smooth muscle cells (Kurpinski et al., 2010). According to our results TGFβ stimulation during later

stages of hES cell differentiation does induce Calponin expression. From our previous results on the possible role of BMP4 on vascular outgrowths we had expected BMP4 to be a potent inducer of vascular markers during hES cell differentiation observed by qRT-PCR. To our surprise it had the opposite effect. Moreover, Noggin subjected hEBs had increased vascular marker expression along with TGFβ, which was the strongest inducer. Possibly, the activation state of the endothelium might be dependent on the balance of ALK1 versus ALK5 activation that is induced by TGFβ (Goumans et al., 2002), BMP4 and inhibition with Noggin.

5.3 BMP9 induces tube like formation of hES derived vascular cells

One part of my project was to establish a cell sorting technique for isolating hES derived vascular cells using the MACS method and use this method to address sprouting during vascular expansion by stimulating hES-derived CD31+ cells with different TGFβ ligands. Among other growth factors, BMP9, a relatively new member of the superfamily was of special interest to us. The effect of BMP9 on endothelial cells is debated in the literature and to our knowledge it has not been studied in hES derived vascular cells. Therefore we attempted to elucidate the effect of BMP9 and other TGFβ superfamily members on vascular expansion in hES derived vascular cells.

When hES cells were either stimulated or inhibited with a member of the TGFβ superfamily, BMP9 was by far the fastest to induce tube-like formation and to upregulate vascular markers compared to other growth factors used. hES cells were harvested for western blot protein detection to confirm BMP9/Smad1/5/8 activation.

To date much more is known about the effect of the TGFβ superfamily on mature endothelial cells than during early development. Human embryonic stem cells can come in great use to study early vasculogenesis and to characterize various human vascular diseases. Several research groups around the world are studying the effects of the TGFβ superfamily on early vascular development. The biological effects of BMP9 stimulation on ES cells has caused a discrepancy among the research community. Suzuki and colleagues reported BMP9 to induce proliferation and migration of endothelial cells in mice (Suzuki et al., 2010). Meanwhile, other groups have reported that BMP9 binds with high affinity to ALK1 in endothelial cells, but that the BMP9/ALK1 cascade inhibits proliferation and migration of mouse endothelial cells (David et al., 2007; Scharpfenecker et al., 2007). We wanted to elucidate the effects of BMP9 in human embryonic stem cell derived-vascular cells. Our experiments are novel in two ways. The effect has never been studied in human embryonic stem cells or in early human development. From our results, as said before, BMP9 was much more potent in inducing tube-like formation and to upregulate vascular markers compared to other growth factors used. This indicates that BMP9 indeed induces endothelial properties, in line with Suzuki's results. Surprisingly, phosphorylation of Smad2 was continuously present, even though Smad2 is a known TGFβ target. In the sprouting assays we used EGM medium including serum which may explain the effects of Smad2 activation. In a paper from 2009, Upton and colleagues also demonstrated that BMP9 was able to induce stimulation of Smad2 and phosphorylation of Smad2 (Upton et al., 2009). Another ligand, BMP10 is thought to be able to compensate for the absence of BMP9.

Defects in retinal vascularization were not observed in *BMP9-KO* in mice. However when the extracellular domain of ALK1 was injected intraperitoneally, retinal vascularization was diminished. These findings suggested another ligand for ALK1. When BMP9-KO mice were injected with neutralizing BMP10 antibody, vascularization was induced, indicating that BMP9 and BMP10 are able to substitute each other (Ricard et al., 2012).

We made an attempt to use previously mentioned neutralizing BMP9 antibody. Unfortunately this attempt was not successful. hES cells were pre-incubated with or without anti-BMP9. Both these conditions were stimulated with various growth factors from the TGFβ superfamily. The difference between those two conditions was not clear, indicating that the pre-incubation might not have been successful. No reliable results can be concluded from this experiment and can only be predicted as preliminary. When culture without pre-incubation was compared to pre-incubated cells, based on endothelial maker expression and tube formation, hardly any difference could be detected. In regard to both BMP9 and BMP10 stimulated cells, they even seemed to induce tube formation and endothelial expression when pre-incubated. To examine the specific effect of BMP9 on human embryonic derived vascular cells it is very important to repeat this experiment.

6 Conclusions

Based on previous results, hES cell differentiation towards endothelial cells is fairly complex. Previous work has suggested the importance of BMP4 to direct hES cells into mesodermal differentiation, and possibly towards vascular cell differentiation. When studying both vasculo- and angiogenesis during this project, BMP4 treated EBs showed the ability to form outgrowths. However immunofluorescent stainings were not able to confirm that the tube formation expressed endothelial markers. Hence, we used a different approach for differentiating EBs towards the vascular linage.

Using chemically defined serum-free medium to form EBs, we obtained different results. qRT-PCR results strongly indicated that TGFβ have a vital role in vasculogenesis by directing hES cells towards vascular cell differentiation. By using a chemically defined medium, we can be quite confident that the effects we see are caused by externally added growth factors.

The CD31+ sorted cell population ("vascular cells") had both endothelial and smooth muscle cell properties. "Vascular" cells treated with BMP9 induced tube-like formation much faster than the other ligands tested, and differentiated the cells towards the endothelial linage, expressing endothelial markers VE-Cadherin and Endoglin. Whereas, if these "vascular" cells were TGFβ treated they showed differentiation tendency towards smooth muscle cells, expressing Calponin and αSMA (Figure 35).

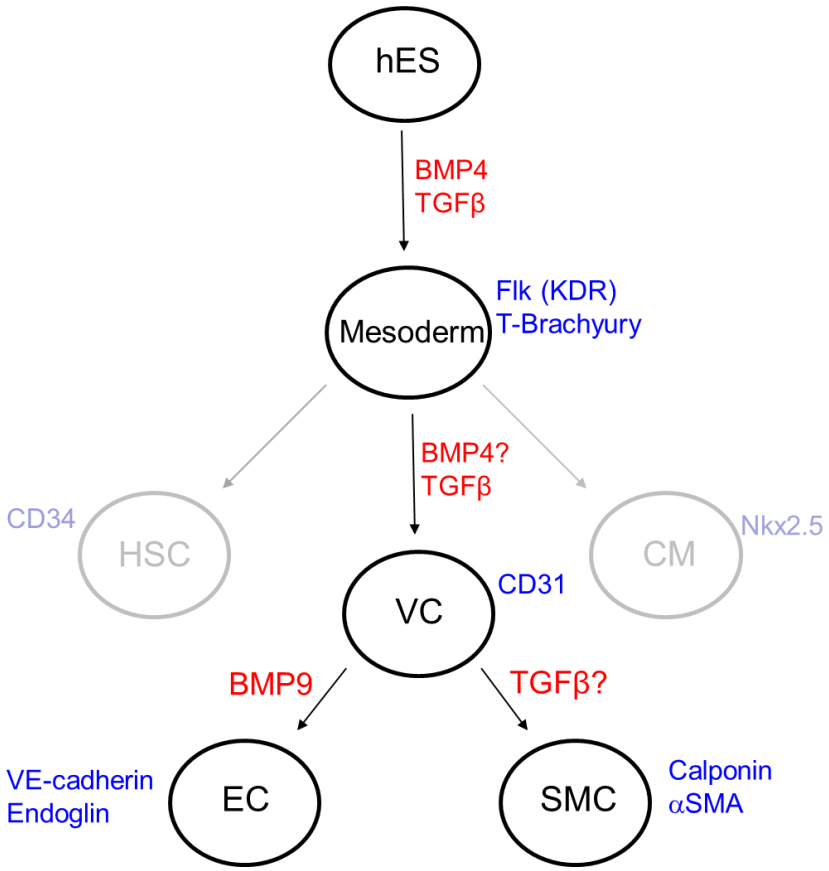


Figure 35. hES derived endothelial cell differentiation model.
A proposal of hES cell differentiation towards endothelial cells and possibly smooth muscle cells.

7 References

Arthur, H. M., Ure, J., Smith, A. J., Renforth, G., Wilson, D. I., Torsney, E., Charlton, R., Parums, D. V., Jowett, T., Marchuk, D. A., Burn, J., & Diamond, A. G. (2000). Endoglin, an ancillary TGFbeta receptor, is required for extraembryonic angiogenesis and plays a key role in heart development. *Dev Biol, 217*(1), 42-53.

Asahara, T., Murohara, T., Sullivan, A., Silver, M., van der Zee, R., Li, T., Witzenbichler, B., Schatteman, G., & Isner, J. M. (1997). Isolation of putative progenitor endothelial cells for angiogenesis. *Science, 275*(5302), 964-967.

Ausprunk, D. H., & Folkman, J. (1977). Migration and proliferation of endothelial cells in preformed and newly formed blood vessels during tumor angiogenesis. *Microvasc Res, 14*(1), 53-65.

Bai, H., Gao, Y., Arzigian, M., Wojchowski, D. M., Wu, W. S., & Wang, Z. Z. (2010). BMP4 regulates vascular progenitor development in human embryonic stem cells through a Smad-dependent pathway. *J Cell Biochem, 109*(2), 363-374.

Baron, M. H. (2001). Molecular regulation of embryonic hematopoiesis and vascular development: a novel pathway. *J Hematother Stem Cell Res, 10*(5), 587-594.

Beppu, H., Kawabata, M., Hamamoto, T., Chytil, A., Minowa, O., Noda, T., & Miyazono, K. (2000). BMP type II receptor is required for gastrulation and early development of mouse embryos. *Dev Biol, 221*(1), 249-258.

Blanco, R., & Gerhardt, H. (2012). VEGF and Notch in Tip and Stalk Cell Selection. *Cold Spring Harb Perspect Med*.

Bollerot, K., Pouget, C., & Jaffredo, T. (2005). The embryonic origins of hematopoietic stem cells: a tale of hemangioblast and hemogenic endothelium. *APMIS, 113*(11-12), 790-803.

Bourdeau, A., Dumont, D. J., & Letarte, M. (1999). A murine model of hereditary hemorrhagic telangiectasia. *J Clin Invest, 104*(10), 1343-1351.

Boyd, N. L., Dhara, S. K., Rekaya, R., Godbey, E. A., Hasneen, K., Rao, R. R., West, F. D., 3rd, Gerwe, B. A., & Stice, S. L. (2007). BMP4 promotes formation of primitive vascular networks in human embryonic stem cell-derived embryoid bodies. *Exp Biol Med (Maywood), 232*(6), 833-843.

Brown, M. A., Zhao, Q., Baker, K. A., Naik, C., Chen, C., Pukac, L., Singh, M., Tsareva, T., Parice, Y., Mahoney, A., Roschke, V., Sanyal, I., & Choe, S. (2005). Crystal structure of BMP-9 and functional interactions with pro-region and receptors. *J Biol Chem, 280*(26), 25111-25118.

Burridge, P. W., Anderson, D., Priddle, H., Barbadillo Munoz, M. D., Chamberlain, S., Allegrucci, C., Young, L. E., & Denning, C. (2007). Improved human embryonic stem cell embryoid body homogeneity and cardiomyocyte differentiation from a novel V-96 plate aggregation system highlights interline variability. *Stem Cells, 25*(4), 929-938.

Carmeliet, P. (2000). Mechanisms of angiogenesis and arteriogenesis. *Nat Med, 6*(4), 389-395.

Carmeliet, P. (2003). Angiogenesis in health and disease. *Nat Med, 9*(6), 653-660.

Cartwright, P., McLean, C., Sheppard, A., Rivett, D., Jones, K., & Dalton, S. (2005). LIF/STAT3 controls ES cell self-renewal and pluripotency by a Myc-dependent mechanism. *Development, 132*(5), 885-896.

Chambers, I., & Smith, A. (2004). Self-renewal of teratocarcinoma and embryonic stem cells. *Oncogene, 23*(43), 7150-7160.

Chang, H., Huylebroeck, D., Verschueren, K., Guo, Q., Matzuk, M. M., & Zwijsen, A. (1999). Smad5 knockout mice die at mid-gestation due to multiple embryonic and extraembryonic defects. *Development, 126*(8), 1631-1642.

Chen, Y. G., & Massague, J. (1999). Smad1 recognition and activation by the ALK1 group of transforming growth factor-beta family receptors. *J Biol Chem, 274*(6), 3672-3677.

Chinoy, M. R. (2003). Lung growth and development. *Front Biosci, 8*, d392-415.

Choi, K. D., Yu, J., Smuga-Otto, K., Salvagiotto, G., Rehrauer, W., Vodyanik, M., Thomson, J., & Slukvin, I. (2009). Hematopoietic and endothelial differentiation of human induced pluripotent stem cells. *Stem Cells, 27*(3), 559-567.

Cleaver, O., & Melton, D. A. (2003). Endothelial signaling during development. *Nat Med, 9*(6), 661-668.

Costa, M., Dottori, M., Sourris, K., Jamshidi, P., Hatzistavrou, T., Davis, R., Azzola, L., Jackson, S., Lim, S. M., Pera, M., Elefanty, A. G., & Stanley, E. G. (2007). A method for genetic modification of human embryonic stem cells using electroporation. *Nat Protoc, 2*(4), 792-796.

Cowan, C. A., Klimanskaya, I., McMahon, J., Atienza, J., Witmyer, J., Zucker, J. P., Wang, S., Morton, C. C., McMahon, A. P., Powers, D., & Melton, D. A. (2004). Derivation of embryonic stem-cell lines from human blastocysts. *N Engl J Med, 350*(13), 1353-1356.

Cunha, S. I., & Pietras, K. (2011). ALK1 as an emerging target for antiangiogenic therapy of cancer. *Blood, 117*(26), 6999-7006.

Dang, S. M., Kyba, M., Perlingeiro, R., Daley, G. Q., & Zandstra, P. W. (2002). Efficiency of embryoid body formation and hematopoietic development from embryonic stem cells in different culture systems. *Biotechnol Bioeng, 78*(4), 442-453.

David, L., Mallet, C., Mazerbourg, S., Feige, J. J., & Bailly, S. (2007). Identification of BMP9 and BMP10 as functional activators of the orphan activin receptor-like kinase 1 (ALK1) in endothelial cells. *Blood, 109*(5), 1953-1961.

Dejana, E., Tournier-Lasserve, E., & Weinstein, B. M. (2009). The control of vascular integrity by endothelial cell junctions: molecular basis and pathological implications. *Dev Cell, 16*(2), 209-221.

Derynck, R., & Zhang, Y. E. (2003). Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature, 425*(6958), 577-584.

Dickson, M. C., Martin, J. S., Cousins, F. M., Kulkarni, A. B., Karlsson, S., & Akhurst, R. J. (1995). Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice. *Development, 121*(6), 1845-1854.

Feraud, O., Cao, Y., & Vittet, D. (2001). Embryonic stem cell-derived embryoid bodies development in collagen gels recapitulates sprouting angiogenesis. *Lab Invest, 81*(12), 1669-1681.

Ferreira, L. S., Gerecht, S., Shieh, H. F., Watson, N., Rupnick, M. A., Dallabrida, S. M., Vunjak-Novakovic, G., & Langer, R. (2007). Vascular progenitor cells isolated from human embryonic stem cells give rise to endothelial and smooth muscle like cells and form vascular networks in vivo. *Circ Res, 101*(3), 286-294.

Flamme, I., Frolich, T., & Risau, W. (1997). Molecular mechanisms of vasculogenesis and embryonic angiogenesis. *J Cell Physiol, 173*(2), 206-210.

Folkman, J., & D'Amore, P. A. (1996). Blood vessel formation: what is its molecular basis? *Cell, 87*(7), 1153-1155.

Franzen, P., ten Dijke, P., Ichijo, H., Yamashita, H., Schulz, P., Heldin, C. H., & Miyazono, K. (1993). Cloning of a TGF beta type I receptor that forms a heteromeric complex with the TGF beta type II receptor. *Cell, 75*(4), 681-692.

Gerhardt, H., Golding, M., Fruttiger, M., Ruhrberg, C., Lundkvist, A., Abramsson, A., Jeltsch, M., Mitchell, C., Alitalo, K., Shima, D., & Betsholtz, C. (2003). VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *J Cell Biol, 161*(6), 1163-1177.

Geudens, I., & Gerhardt, H. (2011). Coordinating cell behaviour during blood vessel formation. *Development, 138*(21), 4569-4583.

Gilbert, S. F. (2003). The morphogenesis of evolutionary developmental biology. *Int J Dev Biol, 47*(7-8), 467-477.

Goumans, M. J., & Mummery, C. (2000). Functional analysis of the TGFbeta receptor/Smad pathway through gene ablation in mice. *Int J Dev Biol, 44*(3), 253-265.

Goumans, M. J., Valdimarsdottir, G., Itoh, S., Rosendahl, A., Sideras, P., & ten Dijke, P. (2002). Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors. *EMBO J, 21*(7), 1743-1753.

Heldin, C. H., Miyazono, K., & ten Dijke, P. (1997). TGF-beta signalling from cell membrane to nucleus through SMAD proteins. *Nature, 390*(6659), 465-471.

Herbert, S. P., & Stainier, D. Y. (2011). Molecular control of endothelial cell behaviour during blood vessel morphogenesis. *Nat Rev Mol Cell Biol, 12*(9), 551-564.

Hill, K. L., Obrtlikova, P., Alvarez, D. F., King, J. A., Keirstead, S. A., Allred, J. R., & Kaufman, D. S. (2010). Human embryonic stem cell-derived vascular progenitor cells capable of endothelial and smooth muscle cell function. *Exp Hematol, 38*(3), 246-257 e241.

Ikushima, H., & Miyazono, K. (2010). TGFbeta signalling: a complex web in cancer progression. *Nat Rev Cancer, 10*(6), 415-424.

Isogai, S., Lawson, N. D., Torrealday, S., Horiguchi, M., & Weinstein, B. M. (2003). Angiogenic network formation in the developing vertebrate trunk. *Development, 130*(21), 5281-5290.

Itoh, S., Landstrom, M., Hermansson, A., Itoh, F., Heldin, C. H., Heldin, N. E., & ten Dijke, P. (1998). Transforming growth factor beta1 induces nuclear export of inhibitory Smad7. *J Biol Chem, 273*(44), 29195-29201.

Itskovitz-Eldor, J., Schuldiner, M., Karsenti, D., Eden, A., Yanuka, O., Amit, M., Soreq, H., & Benvenisty, N. (2000). Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. *Mol Med, 6*(2), 88-95.

Jacquemin, P., Yoshitomi, H., Kashima, Y., Rousseau, G. G., Lemaigre, F. P., & Zaret, K. S. (2006). An endothelial-mesenchymal relay pathway regulates early phases of pancreas development. *Dev Biol, 290*(1), 189-199.

Jain, R. K. (2003). Molecular regulation of vessel maturation. *Nat Med, 9*(6), 685-693.

James, D., Nam, H. S., Seandel, M., Nolan, D., Janovitz, T., Tomishima, M., Studer, L., Lee, G., Lyden, D., Benezra, R., Zaninovic, N., Rosenwaks, Z., Rabbany, S. Y., & Rafii, S. (2010). Expansion and maintenance of human embryonic stem cell-derived endothelial cells by TGFbeta inhibition is Id1 dependent. *Nat Biotechnol, 28*(2), 161-166.

Johansson, B. M., & Wiles, M. V. (1995). Evidence for involvement of activin A and bone morphogenetic protein 4 in mammalian mesoderm and hematopoietic development. *Mol Cell Biol, 15*(1), 141-151.

Kane, N. M., Meloni, M., Spencer, H. L., Craig, M. A., Strehl, R., Milligan, G., Houslay, M. D., Mountford, J. C., Emanueli, C., & Baker, A. H. (2010). Derivation of endothelial cells from human embryonic stem cells by directed differentiation: analysis of microRNA and angiogenesis in vitro and in vivo. *Arterioscler Thromb Vasc Biol, 30*(7), 1389-1397.

Kane, N. M., Xiao, Q., Baker, A. H., Luo, Z., Xu, Q., & Emanueli, C. (2011). Pluripotent stem cell differentiation into vascular cells: a novel technology with promises for vascular re(generation). *Pharmacol Ther, 129*(1), 29-49.

Kirton, J. P., & Xu, Q. (2010). Endothelial precursors in vascular repair. *Microvasc Res, 79*(3), 193-199.

Kurpinski, K., Lam, H., Chu, J., Wang, A., Kim, A., Tsay, E., Agrawal, S., Schaffer, D. V., & Li, S. (2010). Transforming growth factor-beta and notch signaling mediate stem cell differentiation into smooth muscle cells. *Stem Cells, 28*(4), 734-742.

Lammert, E., Cleaver, O., & Melton, D. (2001). Induction of pancreatic differentiation by signals from blood vessels. *Science, 294*(5542), 564-567.

Lane, K. B., Machado, R. D., Pauciulo, M. W., Thomson, J. R., Phillips, J. A., 3rd, Loyd, J. E., Nichols, W. C., & Trembath, R. C. (2000). Heterozygous germline mutations in BMPR2, encoding a TGF-beta receptor, cause familial primary pulmonary hypertension. *Nat Genet, 26*(1), 81-84.

Larrivee, B., Freitas, C., Suchting, S., Brunet, I., & Eichmann, A. (2009). Guidance of vascular development: lessons from the nervous system. *Circ Res, 104*(4), 428-441.

Larrivee, B., Prahst, C., Gordon, E., del Toro, R., Mathivet, T., Duarte, A., Simons, M., & Eichmann, A. (2012). ALK1 signaling inhibits angiogenesis by cooperating with the Notch pathway. *Dev Cell, 22*(3), 489-500.

Larsson, J., Goumans, M. J., Sjostrand, L. J., van Rooijen, M. A., Ward, D., Leveen, P., Xu, X., ten Dijke, P., Mummery, C. L., & Karlsson, S. (2001). Abnormal angiogenesis but intact hematopoietic potential in TGF-beta type I receptor-deficient mice. *EMBO J, 20*(7), 1663-1673.

Lawson, K. A., Dunn, N. R., Roelen, B. A., Zeinstra, L. M., Davis, A. M., Wright, C. V., Korving, J. P., & Hogan, B. L. (1999). Bmp4 is required for the generation of primordial germ cells in the mouse embryo. *Genes Dev, 13*(4), 424-436.

Lechleider, R. J., Ryan, J. L., Garrett, L., Eng, C., Deng, C., Wynshaw-Boris, A., & Roberts, A. B. (2001). Targeted mutagenesis of Smad1 reveals an essential role in chorioallantoic fusion. *Dev Biol, 240*(1), 157-167.

Levenberg, S., Ferreira, L. S., Chen-Konak, L., Kraehenbuehl, T. P., & Langer, R. (2010). Isolation, differentiation and characterization of vascular cells derived from human embryonic stem cells. *Nat Protoc, 5*(6), 1115-1126.

Levenberg, S., Golub, J. S., Amit, M., Itskovitz-Eldor, J., & Langer, R. (2002). Endothelial cells derived from human embryonic stem cells. *Proc Natl Acad Sci U S A, 99*(7), 4391-4396.

Levenberg, S., Zoldan, J., Basevitch, Y., & Langer, R. (2007). Endothelial potential of human embryonic stem cells. *Blood, 110*(3), 806-814.

Li, D. Y., Sorensen, L. K., Brooke, B. S., Urness, L. D., Davis, E. C., Taylor, D. G., Boak, B. B., & Wendel, D. P. (1999). Defective angiogenesis in mice lacking endoglin. *Science, 284*(5419), 1534-1537.

Li, Z., Hu, S., Ghosh, Z., Han, Z., & Wu, J. C. (2011). Functional characterization and expression profiling of human induced pluripotent stem cell- and embryonic stem cell-derived endothelial cells. *Stem Cells Dev, 20*(10), 1701-1710.

Luttun, A., Carmeliet, G., & Carmeliet, P. (2002). Vascular progenitors: from biology to treatment. *Trends Cardiovasc Med, 12*(2), 88-96.

Lyden, D., Young, A. Z., Zagzag, D., Yan, W., Gerald, W., O'Reilly, R., Bader, B. L., Hynes, R. O., Zhuang, Y., Manova, K., & Benezra, R. (1999). Id1 and Id3 are required for neurogenesis, angiogenesis and vascularization of tumour xenografts. *Nature, 401*(6754), 670-677.

Macias-Silva, M., Abdollah, S., Hoodless, P. A., Pirone, R., Attisano, L., & Wrana, J. L. (1996). MADR2 is a substrate of the TGFbeta receptor and its phosphorylation is required for nuclear accumulation and signaling. *Cell, 87*(7), 1215-1224.

Maltsev, V. A., Wobus, A. M., Rohwedel, J., Bader, M., & Hescheler, J. (1994). Cardiomyocytes differentiated in vitro from embryonic stem cells developmentally express cardiac-specific genes and ionic currents. *Circ Res, 75*(2), 233-244.

Mannello, F., & Tonti, G. A. (2007). Concise review: no breakthroughs for human mesenchymal and embryonic stem cell culture: conditioned medium, feeder layer, or feeder-free; medium with fetal calf serum, human serum, or enriched plasma; serum-free, serum replacement nonconditioned medium, or ad hoc formula? All that glitters is not gold! *Stem Cells, 25*(7), 1603-1609.

Massague, J. (1990). The transforming growth factor-beta family. *Annu Rev Cell Biol, 6*, 597-641.

Massague, J. (2000). How cells read TGF-beta signals. *Nat Rev Mol Cell Biol, 1*(3), 169-178.

Mishina, Y., Suzuki, A., Ueno, N., & Behringer, R. R. (1995). Bmpr encodes a type I bone morphogenetic protein receptor that is essential for gastrulation during mouse embryogenesis. *Genes Dev, 9*(24), 3027-3037.

Miyazawa, K., Shinozaki, M., Hara, T., Furuya, T., & Miyazono, K. (2002). Two major Smad pathways in TGF-beta superfamily signalling. *Genes Cells, 7*(12), 1191-1204.

Miyazono, K., Kamiya, Y., & Morikawa, M. (2010). Bone morphogenetic protein receptors and signal transduction. *J Biochem, 147*(1), 35-51.

Miyazono, K., Kusanagi, K., & Inoue, H. (2001). Divergence and convergence of TGF-beta/BMP signaling. *J Cell Physiol, 187*(3), 265-276.

Mummery, C., Ward-van Oostwaard, D., Doevendans, P., Spijker, R., van den Brink, S., Hassink, R., van der Heyden, M., Opthof, T., Pera, M., de la Riviere, A. B., Passier, R., & Tertoolen, L. (2003). Differentiation of human embryonic stem cells to cardiomyocytes: role of coculture with visceral endoderm-like cells. *Circulation, 107*(21), 2733-2740.

Mummery, C. L. (2011). *Stem cells : scientific facts and fiction* (1st ed.). London ; Burlington, MA: Academic Press.

Nakagami, H., Nakagawa, N., Takeya, Y., Kashiwagi, K., Ishida, C., Hayashi, S., Aoki, M., Matsumoto, K., Nakamura, T., Ogihara, T., & Morishita, R. (2006). Model of vasculogenesis from embryonic stem cells for vascular research and regenerative medicine. *Hypertension, 48*(1), 112-119.

Ng, E. S., Davis, R., Stanley, E. G., & Elefanty, A. G. (2008). A protocol describing the use of a recombinant protein-based, animal product-free medium (APEL) for human embryonic stem cell differentiation as spin embryoid bodies. *Nat Protoc, 3*(5), 768-776.

Nourse, M. B., Halpin, D. E., Scatena, M., Mortisen, D. J., Tulloch, N. L., Hauch, K. D., Torok-Storb, B., Ratner, B. D., Pabon, L., & Murry, C. E. (2010). VEGF induces differentiation of functional endothelium from human embryonic stem cells: implications for tissue engineering. *Arterioscler Thromb Vasc Biol, 30*(1), 80-89.

Oh, S. P., Seki, T., Goss, K. A., Imamura, T., Yi, Y., Donahoe, P. K., Li, L., Miyazono, K., ten Dijke, P., Kim, S., & Li, E. (2000). Activin receptor-like kinase 1 modulates transforming growth factor-beta 1 signaling in the regulation of angiogenesis. *Proc Natl Acad Sci U S A, 97*(6), 2626-2631.

Pal, R., Totey, S., Mamidi, M. K., Bhat, V. S., & Totey, S. (2009). Propensity of human embryonic stem cell lines during early stage of lineage specification controls their terminal differentiation into mature cell types. *Exp Biol Med (Maywood), 234*(10), 1230-1243.

Passier, R., & Mummery, C. (2003). Origin and use of embryonic and adult stem cells in differentiation and tissue repair. *Cardiovasc Res, 58*(2), 324-335.

Pepper, M. S. (1997). Transforming growth factor-beta: vasculogenesis, angiogenesis, and vessel wall integrity. *Cytokine Growth Factor Rev, 8*(1), 21-43.

Piek, E., Heldin, C. H., & Ten Dijke, P. (1999). Specificity, diversity, and regulation in TGF-beta superfamily signaling. *FASEB J, 13*(15), 2105-2124.

Poon, E., Clermont, F., Firpo, M. T., & Akhurst, R. J. (2006). TGFbeta inhibition of yolk-sac-like differentiation of human embryonic stem-cell-derived embryoid bodies illustrates differences between early mouse and human development. *J Cell Sci, 119*(Pt 4), 759-768.

Reissmann, E., Jornvall, H., Blokzijl, A., Andersson, O., Chang, C., Minchiotti, G., Persico, M. G., Ibanez, C. F., & Brivanlou, A. H. (2001). The orphan receptor ALK7 and the Activin receptor

ALK4 mediate signaling by Nodal proteins during vertebrate development. *Genes Dev, 15*(15), 2010-2022.
Reubinoff, B. E., Pera, M. F., Fong, C. Y., Trounson, A., & Bongso, A. (2000). Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. *Nat Biotechnol, 18*(4), 399-404.
Ricard, N., Ciais, D., Levet, S., Subileau, M., Mallet, C., Zimmers, T. A., Lee, S. J., Bidart, M., Feige, J. J., & Bailly, S. (2012). BMP9 and BMP10 are critical for postnatal retinal vascular remodeling. *Blood, 119*(25), 6162-6171.
Risau, W. (1997). Mechanisms of angiogenesis. *Nature, 386*(6626), 671-674.
Risau, W., Sariola, H., Zerwes, H. G., Sasse, J., Ekblom, P., Kemler, R., & Doetschman, T. (1988). Vasculogenesis and angiogenesis in embryonic-stem-cell-derived embryoid bodies. *Development, 102*(3), 471-478.
Rufaihah, A. J., Haider, H. K., Heng, B. C., Ye, L., Tan, R. S., Toh, W. S., Tian, X. F., Sim, E. K., & Cao, T. (2010). Therapeutic angiogenesis by transplantation of human embryonic stem cell-derived CD133+ endothelial progenitor cells for cardiac repair. *Regen Med, 5*(2), 231-244.
Scharpfenecker, M., van Dinther, M., Liu, Z., van Bezooijen, R. L., Zhao, Q., Pukac, L., Lowik, C. W., & ten Dijke, P. (2007). BMP-9 signals via ALK1 and inhibits bFGF-induced endothelial cell proliferation and VEGF-stimulated angiogenesis. *J Cell Sci, 120*(Pt 6), 964-972.
Shi, Y., & Massague, J. (2003). Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell, 113*(6), 685-700.
Silva, J., Nichols, J., Theunissen, T. W., Guo, G., van Oosten, A. L., Barrandon, O., Wray, J., Yamanaka, S., Chambers, I., & Smith, A. (2009). Nanog is the gateway to the pluripotent ground state. *Cell, 138*(4), 722-737.
Smith, A. G. (2001). Embryo-derived stem cells: of mice and men. *Annu Rev Cell Dev Biol, 17*, 435-462.
Sng, J., & Lufkin, T. (2012). Emerging stem cell therapies: treatment, safety, and biology. *Stem Cells Int, 2012*, 521343.
Soldner, F., Hockemeyer, D., Beard, C., Gao, Q., Bell, G. W., Cook, E. G., Hargus, G., Blak, A., Cooper, O., Mitalipova, M., Isacson, O., & Jaenisch, R. (2009). Parkinson's disease patient-derived induced pluripotent stem cells free of viral reprogramming factors. *Cell, 136*(5), 964-977.
Solloway, M. J., & Robertson, E. J. (1999). Early embryonic lethality in Bmp5;Bmp7 double mutant mice suggests functional redundancy within the 60A subgroup. *Development, 126*(8), 1753-1768.
Souchelnytskyi, S., Nakayama, T., Nakao, A., Moren, A., Heldin, C. H., Christian, J. L., & ten Dijke, P. (1998). Physical and functional interaction of murine and Xenopus Smad7 with bone morphogenetic protein receptors and transforming growth factor-beta receptors. *J Biol Chem, 273*(39), 25364-25370.
Souchelnytskyi, S., Tamaki, K., Engstrom, U., Wernstedt, C., ten Dijke, P., & Heldin, C. H. (1997). Phosphorylation of Ser465 and Ser467 in the C terminus of Smad2 mediates interaction with Smad4 and is required for transforming growth factor-beta signaling. *J Biol Chem, 272*(44), 28107-28115.
Stadtfeld, M., & Hochedlinger, K. (2010). Induced pluripotency: history, mechanisms, and applications. *Genes Dev, 24*(20), 2239-2263.
Suzuki, Y., Ohga, N., Morishita, Y., Hida, K., Miyazono, K., & Watabe, T. (2010). BMP-9 induces proliferation of multiple types of endothelial cells in vitro and in vivo. *J Cell Sci, 123*(Pt 10), 1684-1692.
Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., & Yamanaka, S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell, 131*(5), 861-872.
ten Dijke, P., Yamashita, H., Ichijo, H., Franzen, P., Laiho, M., Miyazono, K., & Heldin, C. H. (1994). Characterization of type I receptors for transforming growth factor-beta and activin. *Science, 264*(5155), 101-104.
Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S., & Jones, J. M. (1998). Embryonic stem cell lines derived from human blastocysts. *Science, 282*(5391), 1145-1147.
Tremblay, K. D., Dunn, N. R., & Robertson, E. J. (2001). Mouse embryos lacking Smad1 signals display defects in extra-embryonic tissues and germ cell formation. *Development, 128*(18), 3609-3621.

Ungrin, M. D., Joshi, C., Nica, A., Bauwens, C., & Zandstra, P. W. (2008). Reproducible, ultra high-throughput formation of multicellular organization from single cell suspension-derived human embryonic stem cell aggregates. *PLoS One, 3*(2), e1565.

Upton, P. D., Davies, R. J., Trembath, R. C., & Morrell, N. W. (2009). Bone morphogenetic protein (BMP) and activin type II receptors balance BMP9 signals mediated by activin receptor-like kinase-1 in human pulmonary artery endothelial cells. *J Biol Chem, 284*(23), 15794-15804.

Urness, L. D., Sorensen, L. K., & Li, D. Y. (2000). Arteriovenous malformations in mice lacking activin receptor-like kinase-1. *Nat Genet, 26*(3), 328-331.

Valdimarsdottir, G. (2004). *TGFbeta signal transduction in endothelial cells*. Uppsala: Uppsala Universitet.

Valdimarsdottir, G., Goumans, M. J., Rosendahl, A., Brugman, M., Itoh, S., Lebrin, F., Sideras, P., & ten Dijke, P. (2002). Stimulation of Id1 expression by bone morphogenetic protein is sufficient and necessary for bone morphogenetic protein-induced activation of endothelial cells. *Circulation, 106*(17), 2263-2270.

Valdimarsdottir, G., & Mummery, C. (2005). Functions of the TGFbeta superfamily in human embryonic stem cells. *APMIS, 113*(11-12), 773-789.

Vallier, L., Touboul, T., Chng, Z., Brimpari, M., Hannan, N., Millan, E., Smithers, L. E., Trotter, M., Rugg-Gunn, P., Weber, A., & Pedersen, R. A. (2009). Early cell fate decisions of human embryonic stem cells and mouse epiblast stem cells are controlled by the same signalling pathways. *PLoS One, 4*(6), e6082.

Waite, K. A., & Eng, C. (2003). From developmental disorder to heritable cancer: it's all in the BMP/TGF-beta family. *Nat Rev Genet, 4*(10), 763-773.

Wang, L., Li, L., Shojaei, F., Levac, K., Cerdan, C., Menendez, P., Martin, T., Rouleau, A., & Bhatia, M. (2004). Endothelial and hematopoietic cell fate of human embryonic stem cells originates from primitive endothelium with hemangioblastic properties. *Immunity, 21*(1), 31-41.

Wang, Z. Z., Au, P., Chen, T., Shao, Y., Daheron, L. M., Bai, H., Arzigian, M., Fukumura, D., Jain, R. K., & Scadden, D. T. (2007). Endothelial cells derived from human embryonic stem cells form durable blood vessels in vivo. *Nat Biotechnol, 25*(3), 317-318.

Watabe, T., & Miyazono, K. (2009). Roles of TGF-beta family signaling in stem cell renewal and differentiation. *Cell Res, 19*(1), 103-115.

Winnier, G., Blessing, M., Labosky, P. A., & Hogan, B. L. (1995). Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse. *Genes Dev, 9*(17), 2105-2116.

Winslow, T. (2001). Stem cells: Scientific progress and future research directions [Electronic Version], from http://stemcells.nih.gov/staticresources/info/scireport/PDFs/fullrptstem.pdf

Wozney, J. M., Rosen, V., Celeste, A. J., Mitsock, L. M., Whitters, M. J., Kriz, R. W., Hewick, R. M., & Wang, E. A. (1988). Novel regulators of bone formation: molecular clones and activities. *Science, 242*(4885), 1528-1534.

Wrana, J. L., Attisano, L., Wieser, R., Ventura, F., & Massague, J. (1994). Mechanism of activation of the TGF-beta receptor. *Nature, 370*(6488), 341-347.

Xu, C., Inokuma, M. S., Denham, J., Golds, K., Kundu, P., Gold, J. D., & Carpenter, M. K. (2001). Feeder-free growth of undifferentiated human embryonic stem cells. *Nat Biotechnol, 19*(10), 971-974.

Xu, R. H., Peck, R. M., Li, D. S., Feng, X., Ludwig, T., & Thomson, J. A. (2005). Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. *Nat Methods, 2*(3), 185-190.

Yamanaka, S. (2008a). Induction of pluripotent stem cells from mouse fibroblasts by four transcription factors. *Cell Prolif, 41 Suppl 1*, 51-56.

Yamanaka, S. (2008b). Pluripotency and nuclear reprogramming. *Philos Trans R Soc Lond B Biol Sci, 363*(1500), 2079-2087.

Yamashita, H., ten Dijke, P., Huylebroeck, D., Sampath, T. K., Andries, M., Smith, J. C., Heldin, C. H., & Miyazono, K. (1995). Osteogenic protein-1 binds to activin type II receptors and induces certain activin-like effects. *J Cell Biol, 130*(1), 217-226.

Yang, L., Soonpaa, M. H., Adler, E. D., Roepke, T. K., Kattman, S. J., Kennedy, M., Henckaerts, E., Bonham, K., Abbott, G. W., Linden, R. M., Field, L. J., & Keller, G. M. (2008). Human cardiovascular progenitor cells develop from a KDR+ embryonic-stem-cell-derived population. *Nature, 453*(7194), 524-528.

Yang, X., Castilla, L. H., Xu, X., Li, C., Gotay, J., Weinstein, M., Liu, P. P., & Deng, C. X. (1999). Angiogenesis defects and mesenchymal apoptosis in mice lacking SMAD5. *Development, 126*(8), 1571-1580.

Ye, L., Chang, J. C., Lin, C., Sun, X., Yu, J., & Kan, Y. W. (2009). Induced pluripotent stem cells offer new approach to therapy in thalassemia and sickle cell anemia and option in prenatal diagnosis in genetic diseases. *Proc Natl Acad Sci U S A, 106*(24), 9826-9830.

Yoder, M. C. (2009). Defining human endothelial progenitor cells. *J Thromb Haemost, 7 Suppl 1*, 49-52.

Yoshimoto, M., & Yoder, M. C. (2009). Developmental biology: Birth of the blood cell. *Nature, 457*(7231), 801-803.

Yu, J., Huang, N. F., Wilson, K. D., Velotta, J. B., Huang, M., Li, Z., Lee, A., Robbins, R. C., Cooke, J. P., & Wu, J. C. (2009). nAChRs mediate human embryonic stem cell-derived endothelial cells: proliferation, apoptosis, and angiogenesis. *PLoS One, 4*(9), e7040.

Yu, J., Vodyanik, M. A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J. L., Tian, S., Nie, J., Jonsdottir, G. A., Ruotti, V., Stewart, R., Slukvin, II, & Thomson, J. A. (2007). Induced pluripotent stem cell lines derived from human somatic cells. *Science, 318*(5858), 1917-1920.

Zambidis, E. T., Peault, B., Park, T. S., Bunz, F., & Civin, C. I. (2005). Hematopoietic differentiation of human embryonic stem cells progresses through sequential hematoendothelial, primitive, and definitive stages resembling human yolk sac development. *Blood, 106*(3), 860-870.

Zhang, H., & Bradley, A. (1996). Mice deficient for BMP2 are nonviable and have defects in amnion/chorion and cardiac development. *Development, 122*(10), 2977-2986.

Zhang, J., Wilson, G. F., Soerens, A. G., Koonce, C. H., Yu, J., Palecek, S. P., Thomson, J. A., & Kamp, T. J. (2009). Functional cardiomyocytes derived from human induced pluripotent stem cells. *Circ Res, 104*(4), e30-41.

Appendix A - Buffers

Materials and methods, chapter 3.5.1

MACS buffer
0.5 g BSA in 50 ml PBS
0.074 EDTA g in 50 ml PBS
Mix and filter

Materials and methods, chapter 3.9.3.

Lysis buffer
20 mM Tris (pH 7.4)
150 mM NaCl
1% Triton X-100
10% Glycerol
ADD
Protease inhibitor:
• 1 mM PMSF
• 10 µg/ml Aprotinin

2X sample buffer	
SDS	10%
2-βmercaptoethanol	10%
Glycerol	20%
Tris	130mM
Bromophenol Blue	0.1%

Results chapter 4.4.1., page 49

IF buffer	Stock sol.	End sol.	Volume
Triton X -100	2%	0.2%	5 ml
Tween20	0.1%	0.05%	25 ml
BSA	1%	0.1%	5 ml
PBS	-	-	15 ml