The role of ALK1 and ALK5 in HUVECs and human ES cell derived endothelial cells

Jóhann Frímann Rúnarsson

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HÁSKÓLI ÍSLANDS

Hlutverk ALK1 og ALK5 í HUVEC frumum og hES ættuðum æðaþelsfrumum

Jóhann Frímann Rúnarsson

Ritgerð til meistaragráðu í Læknisfræði

Umsjónarkennari: Eiríkur Steingrímsson

Leiðbeinandi: Guðrún Valdimarsdóttir

Aðrir í meistaranámsnefnd: Magnús Karl Magnússon

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Advisor: Eiríkur Steingrímsson

Supervisor: Guðrún Valdimarsdóttir

Other members in masters committee: Magnús Karl Magnússon

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Ágrip

Síðan stofnfrumur úr fósturvísum manna (hES frumur) voru uppgötvaðar hafa þær verið vinsælt rannsóknarefni og reynst vera ómetanlegar til skilnings á fyrstu stigum fósturþroska mannsins. Ennfremur eru miklar vonir bundnar við notkun hES fruma til lækninga á hinum ýmsu sjúkdómum. Tekist hefur að sérhæfa hES frumur í frumur fósturlaganna þriggja, sem seinna í fósturþroska mynda mannslíkamann.

Í fósturþroska mannsins er fóstrið háð blóðflæði til að vaxa og dafna. Einnig er blóðflæði þroskuðum mannslíkamanum nauðsynlegt, þar sem nánast allir vefir eru háðir blóði. Þar sem blóðæðar sjá um flutning blóðs er myndun blóðæða lífsnauðsynlegt ferli, bæði í fósturþroska og þroskuðum mannslíkamanum. Blóðflæði er ekki einungis nauðsynlegt venjulegum vefjum, heldur eru krabbameinsæxli einnig háð blóði svo þau geti vaxið og meinvarpast. Æðaþelsfrumur þekja blóðæðar að innanverðu og gegna lykilhlutverki í blóðæðamyndun, þar sem þær fjölga sér og ferðast og mynda þannig blóðæðakerfi um allan líkamann. Sýnt hefur verið fram á að meðlimir TGFβ stórfjölskyldunnar hafi áhrif á blóðæðamyndun. Í flestum frumugerðum sendir TGFβ boð í gegnum TGFβ viðtaka af gerð I sem nefnist activin viðtakalíkur kínasi 5 (ALK5), en æðaþelsfrumur tjá æðaþelssértækan TGFβ viðtaka af gerð I, sem er kallaður ALK1. TGFβ getur því sent boð í gegnum ALK1 og ALK5 í æðaþelsfrumum, ólíkt öðrum frumugerðum.

Markmið verkefnisins var að skoða hlutverk ALK1 og ALK5 í blóðæðamyndun, bæði í þroskuðum mannslíkamanum og í fósturþroska mannsins. Til þess að líkja eftir æðamyndun í þroskuðum mannslíkamanum voru notaðar æðaþelsfrumur úr naflastreng (HUVECs) og blóðæðamyndun í fósturþroska mannsins var rannsökuð með æðaþelsfrumum af hES frumuuppruna. Sívirkir (ca) ALK1 og caALK5 viðtakar voru yfirtjáðir í HUVECs og líffræðileg áhrif þeirra á myndun pípulaga strúktúra og íferð metin, en þessir líffræðilegu ferlar eru einkennismerki blóðæðamyndunar. Reynt var að finna óþekkt gen virkjuð af ALK1 og ALK5. Sýnt var fram á að með því að valda aukinni tjáningu á Id1 ýtir ALK1 undir myndun pípulaga strúktúra og íferð fruma. Aftur á móti hefur ALK5 neikvæð áhrif á blóðæðamyndun, líklega með því að valda aukinni tjáningu á TSP-1. Til að meta hlutverk ALK1 og ALK5 í blóðæðamyndun æðaþelsfruma af hES frumuuppruna, var sett upp aðferð til að sérhæfa hES frumur í æðaþelsfrumur. Metið var hvort frumurnar hefðu æðaþelsfrumueiginleika með því að skoða próteintjáningu og með því að framkvæma líffræðileg próf sem líkja eftir blóðæðamyndun *in vivo*. Til að ákvarða mikilvægi ALK1 í blóðæðamyndun í fósturþroska var notað mótefni sértækt gegn ALK1. Sérhæfingaraðferðin skilaði sér í frumum með eiginleika æðaþelsfruma. Niðurstöður úr tilraunum með ALK1 mótefninu gefa til kynna mikilvægi ALK1 í blóðæðamyndun æðaþelsfruma af hES frumuuppruna, en hafa skal í huga að þetta eru bráðabirgðaniðurstöður. Á heildina litið varpa niðurstöður rannsóknarinnar frekara ljósi á hlutverk ALK1 og ALK5 í blóðæðamyndun, bæði í fósturþroska og í þroskuðum mannslíkamanum.

Abstract

Since human embryonic stem cells (hES cells) were discovered they have been studied intensively, revealing hES cells as a powerful research tool for understanding early embryonic development in humans. Furthermore, hES cells are promising candidates in therapeutic treatments and stem cell therapies. hES cells have been differentiated into cells of all the three germ layers that at later stages of development form the human body.

During human embryonic development, the embryo is dependent on blood supply for nurture and growth. Blood supply is also vital for the adult human body, as almost all tissues depend on blood flow. Since blood is supplied via blood vessels, formation of blood vessels is an essential process both during human embryonic development and during postnatal stages of life. Blood vessel formation is also crucial for tumors in cancer, as tumors are dependent on nutrition via blood supply for them to be able to grow and metastasize. Endothelial cells line the interior surface of blood vessels and they are necessary in formation of blood vessels, as they proliferate, migrate and invade the extracellular matrix (ECM) to form vascular structures throughout the human body. Members of the TGFβ superfamily have been shown to play a role in blood vessel formation. In most cell types, TGFβ signals through the TGFβ type I receptor activin receptor-like kinase 5 (ALK5) but endothelial cells express the endothelial specific TGFβ type I receptor ALK1. TGFβ can therefore signal via the ALK1 and the ALK5 receptor in endothelial cells.

The aim of this project was to study the role of ALK1 and ALK5 in blood vessel formation, both in the adult human body and during human embryonic development, using human umbilical vein endothelial cells (HUVECs) and hES cell derived endothelial cells, respectively. Constitutively active (ca) ALK1 and caALK5 were overexpressed in HUVECs before their biological effects on tube-like formation and invasion were evaluated. Efforts were made to identify novel genes activated by ALK1 and ALK5. It was shown that ALK1 promotes tube-like formation and invasion by upregulating Id1, while ALK5 has a negative effect on blood vessel formation, likely through upregulation of thrombospondin-1 (TSP-1). To study the role of ALK1 and ALK5 in vascular development of hES cell derived cells, a method was set up to differentiate hES cells into endothelial cells. The endothelial properties of the differentiated cells were determined by evaluating protein expression and by performing biological assays recapitulating angiogenesis *in vivo.* A neutralizing antibody specifically raised against ALK1 was used to determine the importance of ALK1 in vascular embryonic development. The differentiation method resulted in a cell population with endothelial properties. Results indicate the importance of ALK1 in hES cell derived vascular development, although the findings are preliminary. Taken together, this study provides further insight into the role of ALK1 and ALK5 in blood vessel formation, both during human embryonic development and in the adult human body.

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Abbreviations

Ac-LDL	Acetylated low-density lipoprotein
ALK	Activin receptor-like kinase
BMP	Bone morphogenetic protein
bFGF	Basic fibroblast growth factor
BRE	BMP responsive element
caALK	Constitutively active ALK receptor
CM	Conditioned medium
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl Sulfoxide
dnALK	Dominant negative ALK receptor
EB	Embryoid body
ECM	Extracellular matrix
EMT	Epithelial-Mesenchymal transition
EDTA	Ethylene diamine tetracetic acid
FBS	Fetal bovine serum
hES cell	Human embryonic stem cell
HRP	Horseradish peroxidase
HUVEC	Human umbilical vein endothelial cell
Id	Inhibitor of differentiation, inhibitor of DNA binding
MEEC	Mouse embryonic endothelial cell
MEF	Mouse embryonic fibroblasts
mES cell	Mouse embryonic stem cell
Microarray	Gene expression array
MMP	Matrix metalloprotease
PAI	Plasminogen activator inhibitor
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
R-Smad	Receptor-regulated Smad
α -SMA	α-smooth muscle actin
TGFβ	Transforming growth factor beta
TSP	Thrombospondin

1 Introduction

1.1 Stem cells

Stem cells can be found in all organisms. They are defined as cells that are not terminally differentiated and can divide indefinitely. Upon cell division the daughter cells can either remain stem cells or differentiate into a certain cell type, for example cells of the heart, kidney and muscle. There are two types of stem cells, tissue specific stem cells and embryonic stem cells. Tissue specific stem cells can be found in many adult tissues and are crucial in tissue repair. The cells can give rise to cell types present in the tissue where they are located. For example, hematopoietic stem cells can form blood cells and epidermal stem cells give rise to cells of the epidermis. Since tissue specific stem cells can only differentiate into a few cell types, they are termed multipotent. Embryonic stem cells originate from embryos and they can differentiate into all cell types of the body. Therefore they are termed pluripotent (Alberts et al., 2008; Gilbert, 2006). A totipotent cell, for example the zygote, can then give rise to all cell types of the embryo and adult, including germ cells and extra-embryonic tissues such as the placenta (Gilbert, 2006).

1.2 Embryonic stem cells

In early human embryonic development, fertilization occurs 12-24 hours after ovulation. 4-5 days after fertilization the blastocyst has formed. The blastocyst consists of an outer layer, which is termed trophoectoderm, and the inner cell mass. The trophoectoderm later forms the placenta and the inner cell mass gives rise to all the fetal tissues (Gilbert, 2006). An embryonic stem cell line can be created by isolating the cells of the inner cell mass from the blastocyst and culture them on a plate, (Figure 1) either previously coated with feeder cells or in a specific medium, the cells can divide indefinitely while maintaining their pluripotency and stem cell characteristics (Mummery, 2011; Smith, 2001).

The discovery of embryonic stem cells can be traced back to the mid-1970s, where developmental biologists studied teratocarcinomas, a tumour of the testis found both in mice and humans. The tumours consist of a diverse mixture of tissues such as muscle, bone, hair and even teeth. The researchers were able to induce teratocarcinomas by grafting mouse embryos under the skin of mice, and showed that the tumours turned out to contain some undifferentiated stem cells. These stem cells could then be isolated and cultured while maintaining their ability to differentiate. Following this discovery, scientists wondered if isolation of stem cells directly from mouse embryos was possible, that is, without the formation of a teratocarcinoma, as reviewed in Passier & Mummery (2003). In 1981 the first mouse embryonic stem cell line was generated, when the inner cell mass of a mouse embryo was isolated and transferred onto a culture plate previously coated with mouse embryonic fibroblasts (Evans & Kaufman, 1981). Almost 20 years later, in 1998, Thomson and colleagues generated the first human embryonic stem cell line (Thomson et al., 1998).

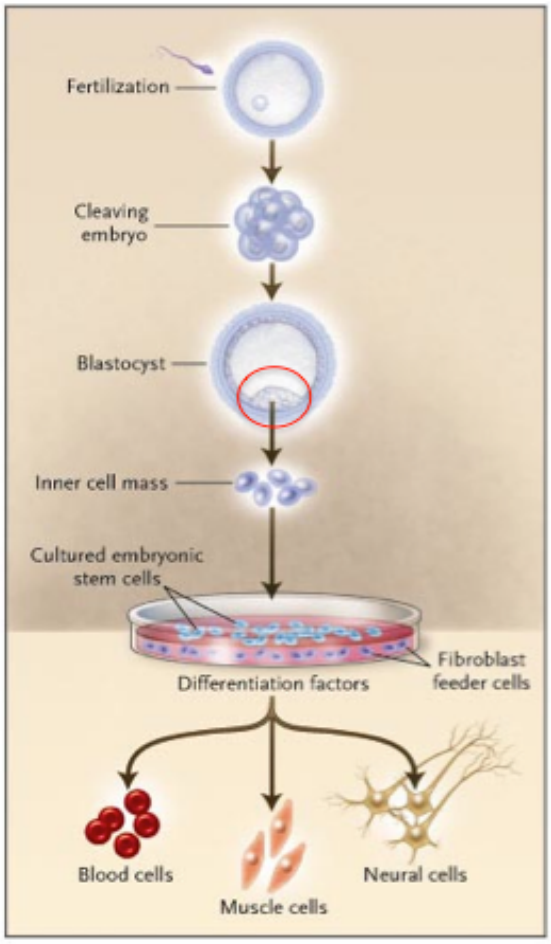


Figure 1. Origin of human embryonic stem cells. The inner cell mass of the blastocyst (red circle) is isolated and placed on a culture plate previously coated with feeder cells. hES cells can then be kept in their pluripotent state or differentiated into all cell types of the body (Gearhart, 2004).

1.3 Culturing of human embryonic stem cells

Embryonic stem cells must be cultured under specific conditions in order to maintain their pluripotency and viability. However, the culturing conditions of mouse and human embryonic stem cells are not the same, as reviewed in Pera & Trounson (2004). Mouse embryonic stem cells (mES cells) are cultured in a medium containing leukemia inhibitory factor (LIF), which maintains their pluripotency (Williams et al., 1988). Human embryonic stem cells (hES cells) on the other hand, are usually cultured on a layer of feeder cells and with a medium supplemented with basic fibroblast growth factor (bFGF), but it has been demonstrated that these conditions sustain the pluripotent state of the hES cells (Thomson et al., 1998; Xu et al., 2005). The feeder cells used in hES cell culture are mouse embryonic fibroblasts (MEFs), which both provide an attachment for the hES cells and secrete growth factors necessary for their survival and maintenance of pluripotency (Smith, 2001). Prior to using MEFs as feeder cells it is essential to prevent their division by irradiating them or treating them with mitomycin C (Terskikh et al., 2006)

hES cells in culture have a distinct morphology as they form tightly packed colonies in between the feeder cells (Figure 2). The colonies are routinely passaged in order to maintain their self-renewal abilities, expand them or to use them for experiments. Colonies are dissociated with enzymatic solutions during the passaging procedure and seeded on new tissue culture plates (Thomson et al., 1998)

Even though hES cells cultured on MEF feeder cells are adequate for some research applications, depletion of feeder cells is necessary in certain hES cell experiments. For example hES cells can not be cultured with MEFs for transplantation applications due to possible pathogen transmission and infection (Klimanskaya et al., 2005; Martin et al., 2005).

Xu and colleagues demonstrated in 2001 that hES cells could maintain their pluripotency without co-culturing them with MEFs. The hES cells were cultured on Matrigel™ (BD Biosciences) or natural laminin in conditioned medium (CM) collected from MEFs (Xu et al., 2001). Although this feeder-free culturing method was a breakthrough, it was not completely optimal due to the use of medium conditioned by MEFs. In 2006, a feeder-independent hES cell culture system was reported, where the cells were cultured on Matrigel in a defined culture medium, later marketed as mTeSR®1 (STEMCELL Technologies) (Ludwig et al., 2006).

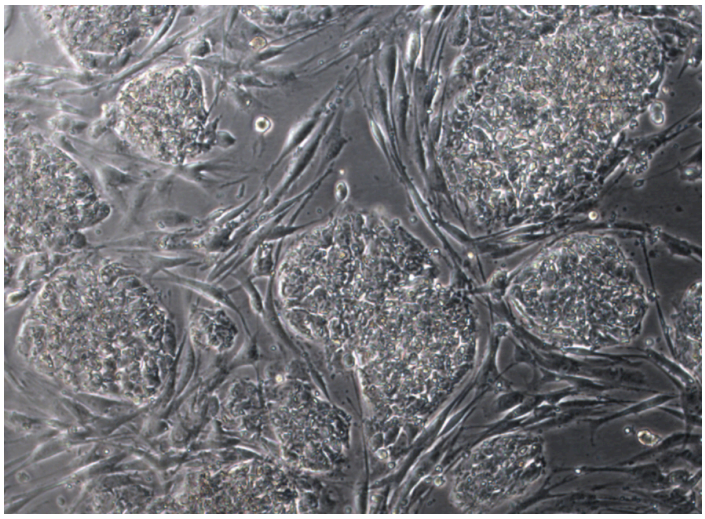


Figure 2. hES cell colonies cultured on MEF feeder cells.

Various factors contribute to sustaining pluripotency of hES cells, both external factors, such as growth factors, and intracellular factors. Among the transcription factors that play a role in maintaining hES cell pluripotency, Sox2, Oct3/4 and Nanog are the most essential ones (Botquin et al., 1998; Chambers et al., 2003; Mitsui et al., 2003; Nichols et al., 1998). These key regulatory factors can induce activation of genes that maintain self-renewal of hES cells while silencing developmental factors and therefore inhibiting differentiation (Jaenisch & Young, 2008).

1.3.1 Differentiation of human embryonic stem cells

Pluripotent hES cells can be differentiated into all cell types of the three germ layers. In order to differentiate hES cells into various cell types, they must be directed into different pathways. At present, knowledge of hES cell differentiation methods is rather limited but mainly, hES cells can be differentiated using either spontaneous differentiation or directed differentiation. During spontaneous differentiation, the cells are not differentiated into a specific lineage, so with this method one gets a mixture of various cell types. However, if the differentiation is directed, the cells are directed towards a specific lineage or cell type (Mummery et al., 2003; Passier & Mummery, 2003).

hES cells are usually differentiated by forming aggregates called embryoid bodies (EBs). Embryoid bodies are ball-like structures that consist of derivatives of all three germ layers and are thought to mimick the early stages of embryonic development (Mummery et al., 2003). When embryoid bodies are formed, the differentiation is spontaneous and the outcome is a mixture of many cell types. In order to direct the differentiation the embryoid bodies can for example be stimulated with various growth factors, resulting in a higher percentage of the desired cell type. Embryoid bodies can then be disaggregated and certain cell types isolated from them using flow cytometry (Carpenter et al., 2003). For example, endothelial cells can be isolated from hES cell-derived EBs by sorting PECAM-positive cells from the EBs. The PECAM-positive cells can then be expanded to give rise to an enriched population of endothelial cells (Levenberg et al., 2002).

Besides directing differentiation of hES cells towards a specific cell type by stimulating them with grown factors, the same can also be done by co-culturing undifferentiated hES cells with other cell types or by gene manipulation. However, these differentiation methods have only resulted in a mixture of various cell types, but not a in pure culture of a certain cell type. In co-cultures, hES cells are cultured with a cell type that is known to induce differentiation towards a desired lineage (Passier & Mummery, 2003). In 2001, Kaufman and colleagues used a co-culture method to generate a hES-derived cell population that contained some cells that showed characteristics of hematopoietic precursors. They did this by co-culturing hES cells with a yolk sac endothelial cell line or a murine bone marrow cell line, which both support some bone marrow-derived hematopoietic progenitors (Kaufman et al., 2001). In a recently published paper, hES-derived cardiac progenitors were generated using gene manipulation. As Nkx2.5 is a known cardiac marker, eGFP sequences were targeted to the NKX2.5 locus of hES cells. Isolation of NKX2.5$^{eGFP/W}$ hES cells resulted in a pure population of cardiac progenitor cells (Elliott et al., 2011).

1.4 Vascular formation

As previously described, embryonic stem cells can differentiate into all cell types of the body, meaning they can give rise to all the three germ layers; ectoderm, mesoderm and endoderm. At later stages of development, the ectoderm forms the neural system and skin, the epithelium of anus and mouth, pigment cells, parts of the eye, the pituitary gland and parts of the adrenal glands. The endoderm gives rise to the inner lining of the gastrointestinal and respiratory tracts, along with bladder, thyroid, thymus, liver and the pancreas lining. Finally, the mesoderm forms the cardiovascular system, skeletal muscle, bone, cartilage, fat, bone marrow, blood and some parts of the urogenital system (Mummery, 2011).

1.4.1 Blood vessel formation

During development of a human embryo the cardiovascular system is responsible for providing nutrition through blood supply for the embryo to be able to grow and nourish. This is also the case in the developed human body, as almost all tissues depend on blood supply. The cardiovascular system consists of the heart, blood vessels and blood cells. This organ system is the first functional unit in development of an embryo, the heart being the first functional organ (Alberts et al., 2008; Gilbert, 2006). However, the heart does not function until the blood vessels have formed their first circulatory loops. The blood vessels do not sprout from the heart, but form independently and connect to the heart soon after formation. In the developing embryo, blood vessel formation depends on two processes: vasculogenesis and angiogenesis. During vasculogenesis, formation of blood vessels occurs *de novo* and angiogenesis refers to formation of new blood vessels from pre-existing ones (Risau & Lemmon, 1988; Risau et al., 1988). Vasculogenesis only takes place during embryonic development but angiogenesis also occurs in the adult human body, in order to maintain integrity of tissues and physiological homeostasis during inflammation, wound healing and woman's menstruation (Folkman, 1995; Pardali et al., 2010). In addition, angiogenesis is a crucial process for tumors during cancer. As normal tissues, many tumors are dependent on nutrition via blood supply for them to be able to grow, survive and metastasize (Carmeliet, 2000; Valdimarsdottir, 2004).

Vasculogenesis is divided into three phases. In the first phase, cells leave the primitive streak in the posterior of the embryo and become hemangioblasts, which are precursor cells for blood vessels and blood cells. The hemangioblasts accumulate and form blood islands, where the inner cells of the islands become blood progenitor cells while the outer cells become angioblasts. These angioblasts then expand in the second phase of vasculogenesis and finally they differentiate into endothelial cells, which line the blood vessels on the inside. In the third and final phase of vasculogenesis, endothelial cells form tube-like structures which connect and form a network of capillaries, termed the primary capillary plexus (Gilbert, 2006).

Angiogenesis consists of two phases; activation phase and resolution (maturation) phase (Figure 3). The activation phase initiates when endothelial cell proliferation is induced (Figure 3A). This induction can be caused by growth factors or hypoxia. This results in increase of vascular permeability and degradation of the extracellular matrix, which enables endothelial cells to migrate and

form new tube-like structures (Figure 3B) (Valdimarsdottir, 2004). The cells pioneering this formation of capillary sprouts are called tip cells. As the name implies, the tip cell is located at the tip of the sprout and it leads the way in forming new sprouting structures. The tip cell has different characteristics than the stalk cells following behind it. The stalk cells divide and form a lumen while the tip cell does not divide but forms many long filopodia (Alberts et al., 2008). During the resolution phase, endothelial cell proliferation and migration stops (Figure 3C). This transition is followed by vessel maturation and reconstitution of the basement membrane. To stabilize the structures formed by endothelial cells and to form the rest of the vessel wall, the endothelial cells recruit pericytes and in larger vessels, smooth muscle cells (Valdimarsdottir, 2004). The endothelial cell structures are further supported by the basement membrane, which also regulates the behaviour of the endothelial cells. Large molecules, such as laminin and collagen IV, comprise the basement membrane by forming sheet-like structures. During the resolution phase in angiogenesis, the basal membrane is highly crosslinked but upon activation of the endothelial cells the components of the basal membrane are exposed and interact with the endothelial cells (Kalluri, 2003).

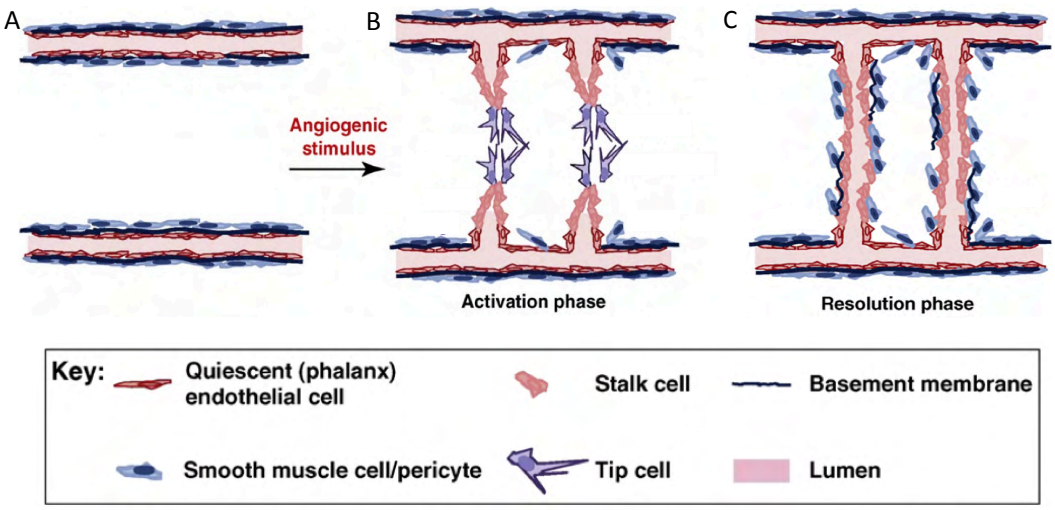


Figure 3. The two phases of angiogenesis. (A) Endothelium prior to angiogenic stimulus. (B) Activation phase caused by angiogenic stimulus. (C) Resolution phase (Pardali et al., 2010).

During blood vessel formation, there is an interplay between endothelial cells and the extracellular matrix (ECM). ECM is a complex structure of polysaccharides and proteins which the cells secrete, and in which the cells are embedded. The ECM is made and organized by the cells within it and it provides support for organization of tissues as well as control for various cellular mechanisms (Alberts et al., 2008; Kalluri, 2003). The environment of endothelial cells, which allows them to organize into tube structures, is made of extracellular factors such as the adhesive glycoproteins laminin, fibronectin and collagens. When endothelial cells invade the ECM, they secrete proteases that degrade the ECM, called matrix metalloproteases (MMPs). Finally, integrins provide adhesion of the endothelial cells to the ECM (Valdimarsdottir, 2004).

MMPs are enzymes released by endothelial cells, cleaving all components of the ECM. MMPs are both secreted and membrane-bound, the latter called the membrane-type MMPs (MT-MMPs). MT1-MMP is both involved in degradation of ECM constituents and activation of other MMPs, while MMP activation is also dependent on growth factors (Rizzino, 1988; Sternlicht & Werb, 2001). Furthermore, the growth factor TGFβ is known to have a major effect on formation and degradation of the ECM, both by increasing accumulation of numerous ECM proteins, and by increasing synthesis of protease inhibitors, such as plasminogen activator inhibitor-1 (PAI-1), while inhibiting protease production (Egeblad & Werb, 2002; Rizzino, 1988).

Integrins play a role in various features of angiogenesis, such as extension, vascular fusion and lumen formation. Additionally, regulation of activity of proteases that degrade the basement membrane is dependent on integrins (Kalluri, 2003). As previously mentioned, integrins induce adhesion of endothelial cells to the ECM, but furthermore, they modulate intracellular signalling pathways that contribute to the cytoskeletal organization (Hood & Cheresh, 2002).

1.5 The TGFβ superfamily

Many growth factors can have an effect in maintaining pluripotency of hES cells or differentiating them into various cell types. The transforming growth factor β (TGFβ) superfamily is a family of growth factors that have been shown to affect the differentiation of hES cells (Shi & Massague, 2003). Members of the superfamily play a key role in a developing embryo, especially in mesodermal differentiation and maturation. Knock-out studies in mice have demonstrated that knocking out TGFβ superfamily genes causes serious defects in heart and vessel formation in the embryo (Goumans & Mummery, 2000). Considering these reports, it is interesting to look further into the role of the TGFβ superfamily in mesodermal differentiation, especially in the two processes of blood vessel formation; vasculogenesis and angiogenesis.

Signal transduction of a TGFβ superfamily member from the cell membrane to the nucleus occurs by binding of a ligand to TGFβ type I receptors, known as activin receptor-like kinase (ALK), and type II receptors. When the binding takes place, the constitutively active type II receptor phosphorylates the type I receptor, which causes phosphorylation of receptor-regulated Smad proteins (R-Smads) (Heldin et al., 1997; Shi & Massague, 2003). Next, the phosphorylated R-Smads unite with Smad4 and the complex is translocated into the nucleus where the proteins initiate transcription of their target genes (Derynck & Feng, 1997). This cascade is shown schematically in Figure 4A.

Within the TGFβ superfamily there are a few subfamilies, the two largest ones being the TGFβ and the bone morphogenetic protein (BMP) subfamilies. Members of these two subfamilies follow different signalling pathways, resulting in different transcriptional activity (Figure 4B). A ligand from the TGFβ subfamily binds to the ALK5 receptor and phosphorylates Smad2/3. On the other hand, a ligand from the BMP subfamily binds to the ALK2/3/6 receptors, resulting in phosphorylation of Smad1/5/8 (Chen & Massague, 1999; Derynck & Zhang, 2003; Oh et al., 2000; Shi & Massague, 2003; ten Dijke et al., 1994; Yamashita et al., 1995).

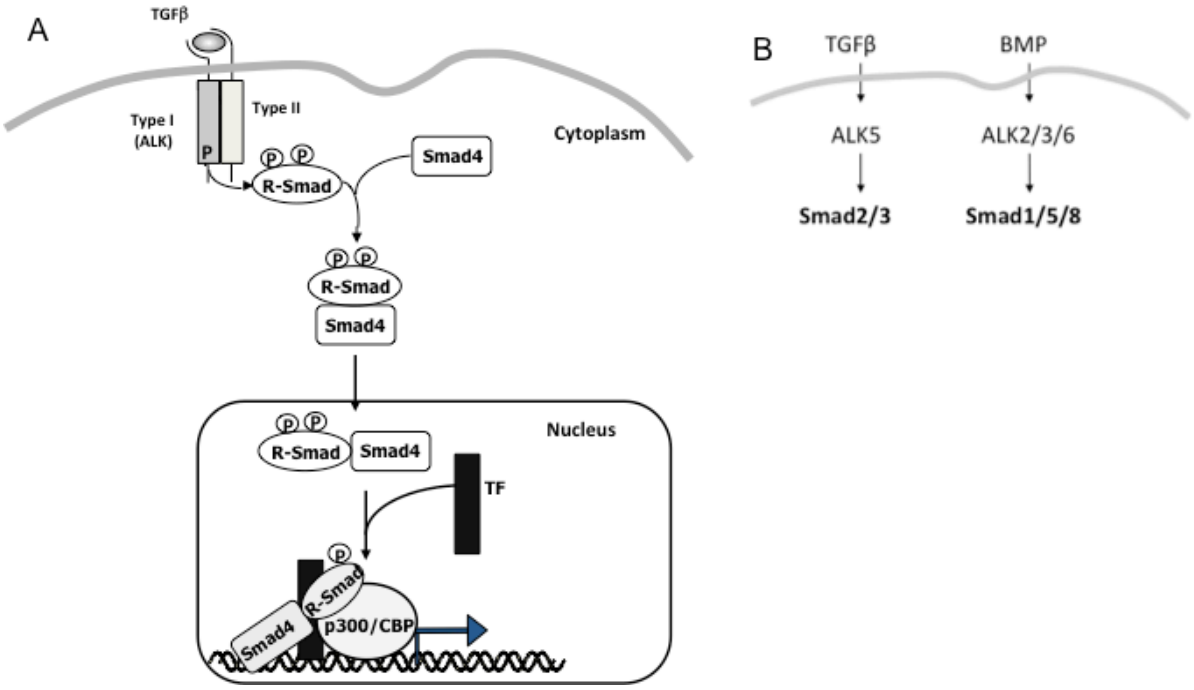


Figure 4. The TGFβ signal transduction pathway and its two main branches, the TGFβ pathway and the BMP pathway. (A) A schematic overview of the TGFβ signal transduction pathway. (B) A schematic overview of signalling pathways of the two main subfamilies of the TGFβ superfamily, TGFβ (left) and BMP (right) (Valdimarsdottir, 2004).

1.5.1 The TGFβ superfamily in blood vessel formation

Blood vessel formation is balanced by many growth factors, both activators and inhibitors. For example, basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), angiopoietins and platelet-derived growth factor (PDGF) all contribute to the complex process that is blood vessel formation (Lindahl et al., 1997; Mamou et al., 2006; Yancopoulos et al., 2000). Additionally, various members of the TGFβ superfamily have a pivotal role in the formation of blood vessels. Numerous studies have demonstrated the importance of the TGFβ and BMP pathways in both angiogenesis and vasculogenesis. However, results have been confusing, showing opposite effects of TGFβ superfamily members on angiogenesis as reviewed in Pardali et al. (2010).

Members of the BMP subfamily have been shown to play a key role in determining the fate of hES cells, with the BMP pathway inducing hES cell differentiation (James et al., 2005; Ying et al., 2003). In fact, the BMP pathway has been shown to direct hES cells into mesodermal differentiation. Furthermore, hES cells stimulated with BMP4 are prone to differentiate into $CD34^+$ $CD31^+$ vascular progenitor cells (Bai et al., 2010).

Inhibitors of differentiation (Id) proteins are known to play a role in angiogenesis. In a dominant negative manner, Id proteins inhibit the gene expression of basic Helix Loop Helix (bHLH) proteins, which tend to regulate differentiation and proliferation of various cell types. Id proteins consist solely of the HLH region but lack the basic region, which binds DNA, so when they bind to bHLH proteins they inhibit their binding to DNA (Zebedee & Hara, 2001). There are four identified members of the Id family, Id1-Id4, and Id1 is a known target gene of BMP signalling in hES cells (Hollnagel et al., 1999;

Jen et al., 1996; Xu et al., 2002). Two of the Id proteins, Id1 and Id3 seem to contribute to the process of angiogenesis. Expression of Id1 and Id3 is upregulated during blood vessel formation both in embryogenesis and tumorigenesis (Benezra et al., 2001).

TGFβ is thought to be a key regulator in blood vessel formation and has both been reported to have pro- and anti-angiogenic effects (Pepper, 1997; Roberts et al., 1986). These findings were quite surprising until it was demonstrated that in endothelial cells, TGFβ can activate two type I receptor/Smad signalling pathways, resulting in opposite cellular mechanisms. While TGFβ signals through the TGFβ type I receptor ALK5 in most cell types, endothelial cells express an endothelial specific type I receptor, termed ALK1. In endothelial cells, TGFβ can therefore both signal via the ALK5 and the ALK1 receptor. When TGFβ signals through the ALK1 receptor, it induces the activation phase in blood vessel formation, whereas TGFβ/ALK5 signalling leads to inhibition of the activation phase. Given these results, it seems that the activation phase is regulated by TGFβ through balancing these two receptors, with ALK1 signalling promoting the activation phase and ALK5 signalling inducing the resolution phase (Goumans et al., 2002). Additionally, the activation phase is also induced by ectopically expressing the ALK1 downstream target gene, Id1, while inhibiting its expression gives opposite results (Goumans et al., 2002; Valdimarsdottir et al., 2002). Even though TGFβ/ALK1 and TGFβ/ALK5 signalling appear to have opposite roles in vascular development, results from studies on ALK5 deficient mice suggest that ALK5 might be essential for sufficient TGFβ/ALK1 signalling (Goumans et al., 2003). However, this interplay of ALK1 and ALK5 has been questioned as deleting ALK5 in mouse endothelium does not have an effect on vessel morphogenesis (Park et al., 2008). Consistent with these results, Shao et al. demonstrated that suppressing ALK5 in bovine aortic endothelial cells (BAECs) does not affect phosphorylation of Smad1/5/8 induced by BMP9/ALK1 signalling (Shao et al., 2009). Considering these results, the relationship and possible interaction between ALK1 and ALK5 need to be studied further.

In addition to ALK1, there is another receptor predominantly expressed on endothelial cells, termed endoglin (CD105). Endoglin is a transmembrane glycoprotein that serves as an accessory receptor for TGFβ (Cheifetz et al., 1992). The importance of endoglin in vascular development has been demonstrated by knock-out studies in mice and by its linkage to vascular disorders in humans, which will be discussed later (Arthur et al., 2000). Furthermore, some findings have indicated that endoglin has a crucial role in balancing the TGFβ/ALK1 and TGFβ/ALK5 pathways (ten Dijke et al., 2008).

Until recently, it was believed that the only ligand that bound to ALK1 was TGFβ. However, results have demonstrated that BMP9 binds with high affinity to ALK1 and endoglin in endothelial cells (Brown et al., 2005; Scharpfenecker et al., 2007). BMP10 has actually also been shown to bind to ALK1, but with lower affinity than BMP9 (David et al., 2007). While the role of BMP10 is unkown, the growth factor is thought to be important in cardiac development, as BMP10 inhibition in mice results in cardiac defects on an embryonic level and death of the embryo (Chen et al., 2004). Both pro- and anti-angiogenic effects of BMP9 have been reported. BMP9 displays anti-angiogenic effects by inhibiting both FGF-induced and VEGF-induced angiogenesis (Scharpfenecker et al., 2007). However, proangiogenic effects of BMP9 have also been demonstrated, as BMP9 induces proliferaction of various endothelial cell types *in vitro* and *in vivo* (Suzuki et al., 2010). Furthermore, one study has

provided evidence that together BMP9 and TGFβ might have synergistic proangiogenic effects (Cunha et al., 2010).

1.5.2 The TGFβ superfamily and vascular defects

As previously mentioned, the TGFβ superfamily has been shown to be crucial in mesodermal differentiation and development in mice, demonstrated by gene ablation studies (Goumans & Mummery, 2000). Knock-out of TGFβ superfamily genes such as ALK1 and endoglin resulted in absence of vessels in a mouse yolk sac resulting in death of the embryo (Figure 5). It has been verified that the TGFβ superfamily also plays a key role in the human mesoderm, especially in vessel formation, as mutations of various TGFβ superfamily genes can result in vascular disorders in humans.

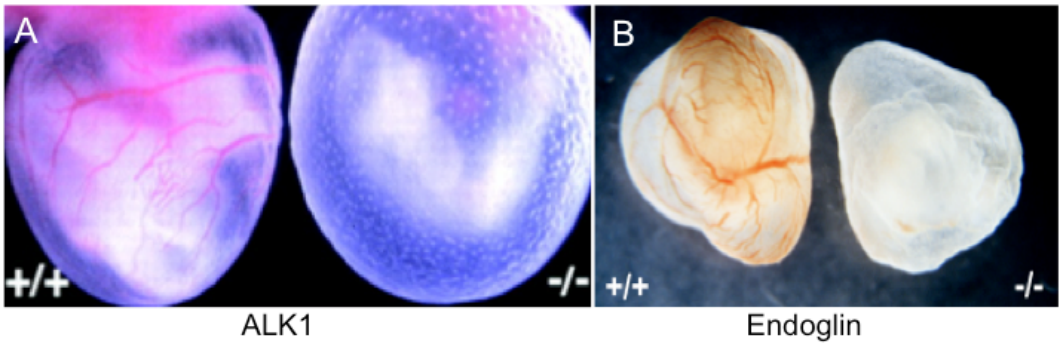


Figure 5. Demonstration of the importance of the TGFβ superfamily in vascular development in mice. (A) Wildtype mouse yolk sac (+/+) and ALK1 knock-out mouse yolk sac (-/-) (Oh et al., 2000). (B) Wildtype mouse yolk sac (+/+) and endoglin knock-out mouse yolk sac (-/-) (Arthur et al., 2000).

The vascular disorder hereditary haemorrhagic telangiectasia (HHT), also known as Osler-Weber-Rendu syndrome (Govani & Shovlin, 2009; Guttmacher et al., 1995; Shovlin et al., 2000), is one of the most common autosomal dominant disorders, affecting 1 in 5-8000 individuals (Bideau et al., 1992; Kjeldsen et al., 1999). The disorder is characterized by development of abnormal vascular structures, which can be ranging from dilated microvessels to larger arteriovenous malformations (AVM) (Govani & Shovlin, 2009). These malformations can occur in various circulations of the body, such as the hepatic, pancreatic, pulmonary and spinal circulations. Other symptoms are severe chronic gastrointestinal and nasal bleeding, along with anaemia and visible dilated blood vessels on finger tips and lips (Guttmacher et al., 1995; Lacout et al., 2010; Shovlin et al., 2000). There are a few types of HHT, but HHT type 1 (HHT1) and HHT type 2 (HHT2) are caused by mutations in TGFβ superfamily genes (Govani & Shovlin, 2009). If an individual is hetereozygous for mutations in the endoglin gene he/she develops HHT1 (McAllister et al., 1994), while mutations in the ALK1 gene lead to HHT2 (Johnson et al., 1996). This is interesting considering that both the endoglin receptor and the ALK1 receptor are thought to play a role in vascular development (Arthur et al., 2000; Goumans et al., 2002)

Pulmonary arterial hypertension (PAH) refers to a group of vascular diseases defined by an increase in mean pulmonary artery pressure, over 25 mm Hg at rest and over 30 mm Hg during exercise (Tuder et al., 1998). PAH is often associated with known diseases, such as human immunodeficiency virus (HIV) and connective tissue disorders (Rich, 1998). A subset of PAH is primary pulmonary hypertension (PPH), which has the same characteristics as PAH but seems to arise from an obscure cause, displaying proliferation of endothelial and smooth muscle cells along with vascular remodeling (Rich et al., 1987). PPH is a rather rare disorder, with 1-2 reported cases per million people in developed countries (Abenhaim et al., 1996). Since PPH seems to occur spontaneously, scientists have searched for gene mutations that could be responsible for causing the disorder. Mutations of BMP receptor type II gene (BMPR2), a TGFβ superfamily member, have been linked to PPH. The effect of these mutations highlight the importance of TGFβ superfamily members in maintaining vascular integrity (Massague & Chen, 2000).

Besides being crucial in biological processes such as development, reproduction and wound healing, angiogenesis is also critical in tumour growth, with new blood vessels forming in order to enable the tumour to grow and metastasize to other organs (Carmeliet, 2000; Valdimarsdottir, 2004). The overwhelming evidence for the importance of ALK1 in vascular development has generated interest in the endothelial specific receptor as a target in anti-angiogenic therapies against cancer. Recently, Hu-Lowe et al. underlined the significance of ALK1 in angiogenesis and tumour growth by using a neutralizing antibody specifically targeting ALK1 (Hu-Lowe et al., 2011). The novelty of this antibody is its specificity against human ALK1 (van Meeteren et al., 2012). This antibody inhibited xenograft tumour growth in mice by diminishing angiogenesis of both blood and lymphatic vessels. Accordingly, in a human melanoma model, the antibody perturbed vascular development and had a negative effect on tumour growth. Given these results, ALK1 might be a promising target for therapeutic use to inhibit tumour growth in cancers (Hu-Lowe et al., 2011).

1.5.3 Thrombospondin-1 in angiogenesis

Various proteins have been reported to either promote or inhibit angiogenesis, both *in vitro* and *in vivo* (Carmeliet, 2000). However, it is interesting that some of the inhibitors originate in the ECM and their active form can vary. Matrix-derived anti-angiogenic factors, such as thrombospondins (TSPs) 1 and 2, are active as full-length proteins, while some are solely effective as fragments derived from larger matrix molecules such as fibronectin and type XV collagen (restin) (Armstrong & Bornstein, 2003). Due to the importance of angiogenesis in tumour growth, some of the factors inhibiting angiogenesis have become of interest as potential candidates for therapeutic use against cancer (Cao, 2001). In addition to clinically testing these candidates, their molecular mechanisms have been investigated and there have been advances in determining the intracellular signalling pathways activated by the inhibitors (Armstrong & Bornstein, 2003).

TSP was among the first anti-angiogenic proteins discovered, termed TSP-1 when other TSP proteins were discovered. TSPs are defined as large multimeric ECM proteins that play a role in migration, proliferation, adhesion, survival and differentiation of numerous cell types (Adams, 2001). Bouck et al. first discovered the anti-angiogenic potency of TSP-1, when a factor secreted by baby

hamster kidney cells (BHK cells) was isolated. This factor was shown to inhibit both migration of bovine adrenal capillary endothelial cells and corneal neovascularization. The factor was identified as TSP-1, which had previously been known to be secreted by fibroblasts, platelets, aortic endothelial cells and smooth muscle cells (Good et al., 1990). Later on, TSP-1 was shown have inhibiting effect on tube formation, adhesion and proliferation of various endothelial cell types *in vitro,* as it was also demonstrated to inhibit neovascularization in a chick chorioallantoic membrane, a highly vascular membrane necessary for chick development (Bornstein & Sage, 1994; Gilbert, 2006; Iruela-Arispe et al., 1999). It has also been demonstrated that the sister protein TSP-2 inhibits proliferation and migration of endothelial cells in culture (Panetti et al., 1997; Volpert et al., 1995).

The ALK1 receptor is thought to promote the activation phase in angiogenesis, while the ALK5 receptor seems to be active in the resolution phase (Goumans et al., 2002). The interplay between TSP-1 and TGFβ superfamily members in angiogenesis is not fully understood. Based on my data, I suggest that there is possible linkage between TSP-1 and ALK5. However, it is not known if there is a direct connection between TSP-1 and ALK5 and through which pathway TSP-1 is being upregulated.

Project aim

Blood vessel formation is a vital process both during embryonic development and during postnatal stages of life, such as in wound healing and inflammation. Furthermore, blood vessel formation plays an essential role in cancer, as tumors are dependent on the formation of new blood vessels for their growth and metastasis. Various members of the TGFβ superfamily have been shown to be important in blood vessel formation and even though the TGFβ receptors ALK1 and ALK5 seem to play a key role in vascular development, a lot remains to be studied. Therefore, the aim of this project was to evaluate the role of ALK1 and ALK5, both in angiogenesis of mature endothelial cells and in vascular development of hES cell derived endothelial cells.

The thesis is divided into two main parts:

1. The role of ALK1 and ALK5 in endothelial cells

   The effect of TGFβ superfamily members on gene expression in endothelial cells was evaluated. Biological effects of the ALK1 and ALK5 receptors on tube-like formation and invasion were determined. Furthermore, efforts were made to identify novel genes activated by ALK1 and ALK5 during angiogenesis.

2. The role of ALK1 and ALK5 in vascular development of hES cell derived endothelial cells

   In order to obtain hES cell derived endothelial cells, a method was set up to differentiate hES cells into endothelial cells. The endothelial properties of the differentiated cells were evaluated by examination of protein expression and by performing biological assays recapitulating angiogenesis *in vivo*. The importance of ALK1 in human vascular embryonic development was determined using a neutralizing antibody specifically raised against ALK1.

2 Materials and methods

2.1 Cell culture and maintenance of hES cells

Three human embryonic stem cell lines, HUES9, HES2 and HES3, were used in these experiments. HUES9 was obtained from Douglas A. Melton at Howard Hughes Medical Institute, Harvard University (Cowan et al., 2004). HES2 and HES3 were obtained from ES Cell international (ESI) (Reubinoff et al., 2000). hES cells were routinely cultured on a monolayer of irradiated primary mouse embryonic fibroblasts (MEF cells). MEF cells were obtained from bioproduct distributor ATCC-LGC Standards. The cells are used as a feeder layer to support the growth and maintenance of hES cells in the undifferentiated state. MEF cells were seeded onto 6-well culture plates in MEF medium (Table 1), 5 x 10^5 cells per well.

Pluripotent hES cells were passaged every three days and hES cell growth medium (Table 2) changed daily. The growth medium was supplemented with 10 ng/ml bFGF (PeproTech) and either 10% conditioned medium or 25% mTeSR medium (STEMCELL technologies). Conditioned medium was collected from MEF cells, but the cells secrete factors that enrich both cell growth and pluripotency. 4 ml of hES cell growth medium (Table 2) was applied to wells that only contained MEF cells. The medium was collected and replaced with fresh hES medium every 24 hours. Before use, the conditioned medium was filter-sterilized using 0.22 µm filters.

Table 1. MEF medium.

MEF medium	Stock concentration	Final concentration
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 nM	2 mM
Non Essential Amino Acids (Invitrogen)		100x dilution

Table 2. hES cell growth medium.

hES cell growth medium	Stock concentration	Final concentration
DMEM/F-12 + GlutaMAX (31331-028) (Invitrogen)		
Conditioned medium (CM)		10%
KO Serum Replacement (Invitrogen)		20%
Non Essential Amino Acids (Invitrogen)		100x dilution
GlutaMAX (Invitrogen)	200 nM	2 mM
Penicillin (Invitrogen)	5000 U/ml	50 U/ml
Streptomycin (Invitrogen)	5000 µg/ml	50 µg/ml
2-Mercaptoethanol (Invitrogen)	55 mM	77 µmol/ml

2.1.1 Passage of hES cells

In order to culture hES cells, hES cell growth medium (Table 2) was prewarmed at 37°C. Culture medium was removed from wells of a 6 well culture plate and discarded before washing cells once with 1 ml 1 x PBS. Cells were dissociated with 0.5 ml TrypLE (Invitrogen) and incubated at 37°C for 5 minutes. TrypLE was inactivated using 2.5 ml hES medium, transferred to a 15 ml tube and centrifuged at 1000 rpm for 5 minutes. Supernatant was removed, pellet resuspended in hES medium and pipetted 6-10 times to get a single cell suspension. Cells were finally split in 1:10 ratio on 6 well culture plates.

2.2 Differentiation of hES cells

Two methods were used to differentiate hES cells, embryoid bodies (EBs) in suspension and culture of hES cells on a matrigel layer. In both methods growth factors that affect the TGFβ and BMP pathways were used.

2.2.1 Embryoid bodies in suspension

This differentiation protocol was merged from two published papers (Leschik et al., 2008; Yang et al., 2008).

In order to generate EBs in suspension, hES cells were cultured on MEFs in 6 well culture plates. Cells were dissociated into clumps using 1 mg/ml dispase (Invitrogen) diluted in DMEM/F12 medium and incubated for 15 minutes at 37°C with mild shaking. Dispase was inactivated with MEF medium, cells scraped and harvested into 15 ml tubes. Cells were centrifuged at 800 rpm for 4 minutes, supernatant removed and cells carefully resuspended in hES cell differentiation medium (Table 3). Cell clumps were transferred to 12 well low attachment plates (BD-Falcon) in hES cell differentiation medium (Table 3) without growth factors or containing 10 ng/ml BMP4 (Peprotech). Embryoid bodies formed in 2-3 days. After 10-11 days in suspension, EBs were harvested for MACS.

Table 3. hES cell differentiation medium.

hES cell differentiation medium	Stock concentration	Final concentration
hES cell growth medium (Table 2)		
α–Monothioglycerol (α–MTG)	1.3%	400 μM

2.2.2 Differentiation on a matrigel layer

In this differentiation method, hES cells were cultured in monolayer on a matrigel layer. The cells were cultured in hES cell growth medium (Table 2), containing 25% CM for four days, untreated or treated with TGFβ (5 ng/ml) (Peprotech) or BMP4 (10 ng/ml).

2.3 Cell sorting and analysis of hES cells

Cells obtained from the embryoid bodies in suspension differentiation method were sorted for further analysis. Since the goal was to differentiate hES cells into pure endothelial cell population, cells positive for the endothelial marker CD31 were sorted with magnetic activated cell sorting (MACS). The CD31 positive cells were then analysed with immunofluorescent staining and acetylated low-density lipoprotein (Ac-LDL) assay.

2.3.1 Magnetic activated cell sorting (MACS)

This protocol was adapted from a recently published cell sorting protocol (Levenberg et al., 2010).

10-11 days old embryoid bodies were transferred to a 15 ml tube and centrifuged at 800 rpm for 4 minutes. Medium was removed, cells washed with 5 ml 1 x PBS and centrifuged at 300 g for 3 minutes. PBS was removed, EBs dissociated using 2 ml TrypLE and incubated at 37°C for a few minutes, flicking the tube regularly. Cells were pipetted up and down using a 1000 μl pipette, 2 ml 5% FBS (in PBS) added and cells passed through a 40 mm cell strainer. Cells were centrifuged at 300 g for 5 min at RT, supernatant removed, cells resuspended in 10 ml 5% FBS (in PBS) and cells counted. Cells were centrifuged at 300 g for 5 minutes at 4°C, supernatant removed and cells resuspended in 300 μl 5% FBS (in PBS). Cells were labeled by adding 100 μl FcR blocking solution (Miltenyi Biotec) and 100 μl anti-CD31 microbeads (Miltenyi Biotec). Cell suspension was stored on ice for 30 minutes. Cells were rinsed with 10 ml 5% FBS (in PBS), centrifuged at 300 g for 5 minutes at 4°C, supernatant removed and repeated. Cells were resuspended in 500 μl MACS buffer (Table 4) by pipetting thoroughly. LS columns (Miltenyi Biotec) were placed in a magnetic field, rinsed with 3 ml MACS buffer and cell suspension added to columns. Unlabeled cells which passed through the column were collected and column washed 3 times with 3 ml MACS buffer. Column was placed over a new collection tube, 5 ml MACS buffer added and plunger applied firmly to flush out magnetically labeled cells.

Table 4. MACS buffer.

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

2.3.2 Immunofluorescent staining

Cells were fixed with 4% paraformaldehyde in 1 x PBS for 30 minutes, followed by 8 minute incubation in Triton X-100 in 1 x PBS to permeabilize the cell membrane. Both steps were performed at room temperature. Next, samples were preincubated in PBS and 4% goat serum (GS). Primary antibody (Table 5) was mixed with 4% GS and incubated over night at 4°C. Samples were washed in PBS/0.05% Tween and incubated with fluorescently activated secondary antibody for 1 hour at room temperature. Samples were washed again with PBS/0.05% Tween and finally washed shortly with dH_2O containing Topro-3 for nuclear staining (Table 5) before mounting with coverslips using Fluoromount (Sigma-Aldrich) for preservation of the fluorescent signal. Immunofluorescence was visualized using a confocal Zeiss LSM 5 Pascal laser scanning microscope.

Table 5. Antibodies used for immunofluorescent staining.

Antibody against	Cat. No.	Origin	Type	Isotype	Dilution	Supplier
Phospho-Smad1	S463/465	Rabbit	Primary	IgG	1:100	Cell Signalling
Phospho-Smad2	S465/467	Rabbit	Primary	IgG	1:100	Cell Signalling
Endoglin (CD105)	555690	Mouse	Primary	IgG1	1:250	BD Biosciences
VE-cadherin (CD144)	555661	Mouse	Primary	IgG1	1:250	BD Biosciences
Id1	sc-488	Rabbit	Primary	IgG	1:50	Santa Cruz Biotechnology
α-SMA	ab7817	Mouse	Primary	IgG2a	1:200	Abcam
Topro3	T3605	-	Nucleic	-	1:500	Invitrogen
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**

2.3.3 Acetylated low density lipoprotein (Ac-LDL) assay

Acetylated low density lipoproteins conjugated to Alexa 488 anti-rabbit were diluted (10 ng/ml) in the growth medium and added to the cells grown in gelatin coated Falcon 8-well chamber slides. Cells were incubated overnight at 37°C and finally fixed with 4% paraformaldehyde before mounting with coverslips using Fluoromount (Sigma-Aldrich) for preservation of the fluorescent signal. Immunofluorescence was visualized using a confocal Zeiss LSM 5 Pascal laser scanning microscope. HUVECs served as a positive control.

2.4 Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation (ChIP) is a method used to examine the possible binding of a transcription factor of interest to the promoter region of a target gene. For a detailed protocol, see Appendix: Chromatin immunoprecipitation. In the ChIP method, antibodies are used to precipitate DNA bound by transcription factors to which the antibodies are raised against. To determine whether Smad1 signalling proteins interact with unknown target genes in endothelial differentiation of hES cells

and in angiogenesis of mature HUVECs, anti-p-Smad1 antibodies were used to immunoprecipitate the protein-DNA complex (Termen et al., 2008; Xu et al., 2008). Briefly, hES cells or HUVECs were stimulated or infected with adenoviruses. After culture on matrigel for four days, cells were harvested with TrypLE, crosslinked with 1% formaldehyde and nuclei extracted. Chromatin was sheared with sonication and DNA/protein complexes were precipitated with the antibody of interest, anti-p-Smad1. After the immunoprecipitation, samples were cleaned and PCR used (Tables 9 and 11) to screen the isolated DNA for binding sites identification.

2.5 Cell culture and maintenance of HUVECs

HUVECs were seeded onto T25 or T75 culture flasks and cultured in EGM-2 endothelial cell growth medium (Table 6). The cells were passaged every three days.

Table 6. EGM-2 endothelial cell growth medium.

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

Final concentration of supplements not available at Lonza

2.5.1 Passage of HUVECs

In order to culture HUVECs, EGM-2 endothelial cell growth medium (Table 6) was prewarmed at 37°C. Culture medium was removed from cells and discarded before washing cells once with 1 ml 1 x PBS. Cells were dissociated with TrypLE (Invitrogen) (1.5 ml for T25, 4.5 ml for T75) and incubated at 37°C for 5 minutes. TrypLE was inactivated using MEF medium (Table 1, 6. ml for T25, 10 ml for T75), transferred to a 15 ml tube and centrifuged at 1000 rpm for 5 minutes. Supernatant was removed, pellet resuspended in EGM-2 endothelial cell growth medium and pipetted 6-10 times to get a single cell suspension. Cells were finally split in 1:5 ratio.

2.6 Adenoviral infections

hES cells or HUVECs were infected with adenoviral constructs expressing Id1, constitutively active (ca) ALK1, ALK2, ALK5 and LacZ (control) using a multiplicity of infection (MOI) of 100. Prior to the infection, optimized infection efficiency of the viruses was verified. All adenoviral constructs except Id1 were tagged with hemagglutinin protein, HA. Medium was changed 16 hours after infection and hES cells or HUVECs were starved overnight before they were lysed for western blotting (see 3.7) or used in the following biological assays.

For ChIP (see 2.4), HUVECs were cultured on a matrigel layer and infected with adenoviral constructs expressing caALK1, caALK5 and LacZ (control) using a MOI of 100. Medium was changed 16 hours after infection and HUVECs were starved overnight before they were harvested for ChIP.

2.6.1 Tube-like formation assay

Chilled 8 chamber slides were coated with matrigel and incubated at 37°C for at least 30 minutes to form a gel. Adenovirally infected HUVECs were serum-starved overnight, harvested with TrypLE and seeded in the chambers, $3x10^4$ cells in each chamber. After 24 h of incubation the HUVECs were fixed with 4% paraformaldehyde and photographed. Finally, the cells were immunostained as previously described (see 2.3.2).

2.6.2 Invasion assay

Matrigel-based invasion plates (Becton Dickinson, Oxford, UK) were incubated for 2 hours at room temperature before each chamber was rehydrated in medium without serum. After seeding $4x10^4$ endothelial cells in the upper chamber and incubation for 22 hours, cells were fixed, stained overnight with crystal violet blue and cells that had invaded to the bottom of the chamber filter were counted.

2.7 Western blotting

Cells were lysed in lysis buffer (Table 7) and samples then sonicated. 2X sample buffer (Table 7) was added and samples boiled at 95°C for 10 minutes before running them on 10% or 12.5% SDS PAGE gels, depending on the size of the protein being blotted. Next, proteins were transferred to a membrane followed by preincubation for 45 minutes in 5% milk or 5% bovine serum albumin (BSA) diluted in TBS-T at room temperature. Primary antibodies (Table 8) were mixed with TBS-T containing 3% milk or 5% BSA, depending on antibodies, and incubated on a shaker over night at 4°C. Following incubation the membranes were washed with TBS-T and then incubated with secondary antibodies (Table 8) diluted in TBS-T containing 0.01% SDS on a shaker for 1 hour at room temperature. Finally, the membranes were either treated with ECL Western Blotting Detection System (GE Healthcare) and developed in a dark room with Dürr Med Medicine 260 developing device or they were scanned in Odyssey, depending on antibodies.

Table 7. Recipies for lysis buffer and sample buffer.

Lysis buffer	2X Sample buffer	
20 mM Tris (pH 7.4)	10% SDS	40 ml
150 mM NaCl	2-Mercaptoethanol (Invitrogen)	10 ml
1% Triton X-100	Glycerol	20 ml
10 % Glycerol	Tris	1.51 g
Protease inhibitors added	Bromophenol Blue	20 mg
1 mM PMSF	H_2O	30 ml
10 µg/ml Aprotinin		

Table 8. Antibodies used for western blotting.

Antibody against	Cat. No	Origin	Type	Dilution	Supplier
Actin	MAB1501R	Mouse	Primary	1:10000	Millipore
Phospho-Smad1	S463/465	Rabbit	Primary	1:1000	Cell Signalling
Phospho-Smad2	S465/467	Rabbit	Primary	1:1000	Cell Signalling
HA	11 666 606 001	Mouse	Primary	1:1000	Roche
HRP Anti-Mouse	NA931	Sheep	Secondary	1:1000	GE Healthcare
HRP Anti-Rabbit	NA934	Donkey	Secondary	1:1000	GE Healthcare
IRDye 800CW anti-mouse	926-32210	Goat	Secondary	1:20000	LI-COR Biosciences
IRDye 680RD anti-rabbit	926-68071	Goat	Secondary	1:20000	LI-COR Biosciences

2.8 RNA Isolation

RNA was isolated from stimulated or adenovirally infected HUVECs with RNeasy Mini Kit from Qiagen, which is designed to purify RNA from small amounts of starting material.

Cells were harvested according to a normal passaging protocol (see 2.5.1), collected as a pellet and supernatant removed. Cells were disrupted by adding Buffer RLT including β-mercaptoethanol (β-ME) (10 µl β-ME per 1 ml Buffer RLT). Lysate was mixed by vortexing or pipetting and homogenized by passing it at least five times through a blunt 20-gauge needle (0.9 mm diameter) fitted to an RNase-free syringe. The isolation was continued according to RNeasy Mini Kit protocol. After isolation, RNA concentration was measured using NanoDrop spectrophotometer and samples were then stored at -80°C.

2.8.1 cDNA synthesis

cDNA was synthesized using SuperScript™ II First-Strand Synthesis System for RT-PCR (Invitrogen). This system is designed to synthesize first-strand cDNA from purified total RNA.

1-5µg of total RNA was used and mixed with 1 µl Oligo(dT)12-18 and 1 µl 10mM dNTP. Sterile, distilled water was added to bring final volume to 12 µl. Mixture was heated to 65°C for 5 minutes and chilled quickly on ice. Mixture was centrifuged briefly before adding 4 µl 5X First-Strand Buffer and 2 µl 0.1 M DTT. Mixture was mixed gently and incubated at 42°C for 2 minutes. 1 µl (200 units) of Superscript™ II RT was added and mixed by pipetting gently up and down before incubating at 42°C for 50 minutes. Finally, the reaction was inactivated by heating at 70°C for 15 minutes. After cDNA synthesis, PCR reaction was performed on samples using the recipe in Table 9 and the program in Table 11. Primers used are listed in Table 10.

2.8.2 Polymerase chain reaction (PCR)

Table 9. PCR reaction.

	Volume
10 x Dream buffer	2.5 µl
dNTP	0.5 µl
Forward primer	0.5 µl
Reverse primer	0.5 µl
H_2O	20.5 µl
DreamTaq	0.06 µl
Sample	1 µl
Total volume	25 µl

Table 10. PCR primers and reaction conditions.

PCR target	Primer	Primer sequence	Product size	Cycles	Annealing temp.
hARP	hARP F 569 hARP R 684	caccattgaaatcctgagtgatgt tgaccagcccaaaggagaag	115 bp	27	60°C
hSLUG promoter	hSLUGprom-F hSLUGprom-R	gaaactggtagatactgagatgg gaaccaccggacattctctc	202 bp	40	57°C
hID1	hId1 F 185 hId1 R 337	aaacgtgctgctctacgaca gattccgagttcagctccaa	152 bp	35	51°C
hID2	hId2 F hId2 R	acgacccgatgagcctgcta tcctggagcgctggttctg	213 bp	35	51°C
hID3	hId3 F 179 hId3 R 345	tgagcttgctggacgac ccttggcatagtttggagag	571 bp	35	51°C
hTSP-1	hTSP-1 F hTSP R	agaatgctgtcctcgctgtt tttcttgcaggctttggtct	361 bp	35	55°C
hα-SMA	hα-SMA hα-SMA	cctcccttgagaagagttacgagttg agaggagcaggaaagtgttttagaagc	447 bp	30	56°C
hVE-cadherin	hVE-CADf hVE-CADr	tggagaagtggcatcagtcaacag tctacaatcccttgcagtgtgag	118 bp	25	63°C

Table 11. Standard PCR program.

Step		Temperature (°C)	Time (min)
1	Denaturing	94	4
2	Denaturing	94	1
3	Annealing	Table 10	1
4	Elongation	72	1
5	Go to step 2	Table 10	
6	Elongation	72	5
7	Forever	4	∞

3 Results

3.1 The role of ALK1 and ALK5 in angiogenesis of endothelial cells

In order to evaluate the effects of TGFβ superfamily members on gene expression in endothelial cells, HUVECs were cultured and either infected with adenoviral constructs expressing Id1, short interference (si) RNA against Id1, constitutively active (ca) ALK1, caALK5, dominant negative (DN) ALK1, DNALK5 and LacZ (control) or stimulated with growth factors of the TGFβ superfamily namely TGFβ, BMP4 and BMP9. RNA was isolated from HUVECs and cDNA synthesized. After cDNA synthesis PCR reaction was performed using various primers. As Figure 6 demonstrates, expression of the HLH gene Id1 is increased in HUVECs adenovirally infected with caALK1 or stimulated with BMP9. Id1 has been shown to be a target of BMP-Smad1/5 signalling in mouse embryonic endothelial cells (MEECs) but its sister proteins have not been studied in detail. Our results indicate that Id2 and Id3 do not have similar expression patterns to Id1 in HUVECs. Moreover it seems as if Id2 and Id3 rather compensate the loss of Id1 expression. Furthermore, Id1 was strongly upregulated in Id1 overexpressed cells and at the same time there was no Id3 upregulation. No Id1 expression was evident when Id1 was knocked down, but instead both Id2 and Id3 were expressed.

Endothelial-mesenchymal transition (EMT) is the process in which endothelial cells change shape and migrate in the surrounding tissue. It is characterized by the loss of the endothelial cell marker VE-cadherin and the upregulation of mesenchymal cell markers such as α-SMA (Goumans et al., 2008). Reports suggest that the gene expression profile can be shifted towards the upregulation of smooth muscle genes upon TGFβ-ALK5/Smad2/3 activation (Sridurongrit et al., 2008). We therefore assessed the expression of VE-cadherin and α-SMA upon activation of different branches of the TGFβ superfamily in HUVECs. Taken together, a change in the expression of those two markers was not detectable. Most reports have shown a change in the expression profile upon TGFβ activation in bovine aortic endothelial cells (BAECs) and other endothelial cell types but not in HUVECs. That could be the reason for the difference in TGFβ responsiveness.

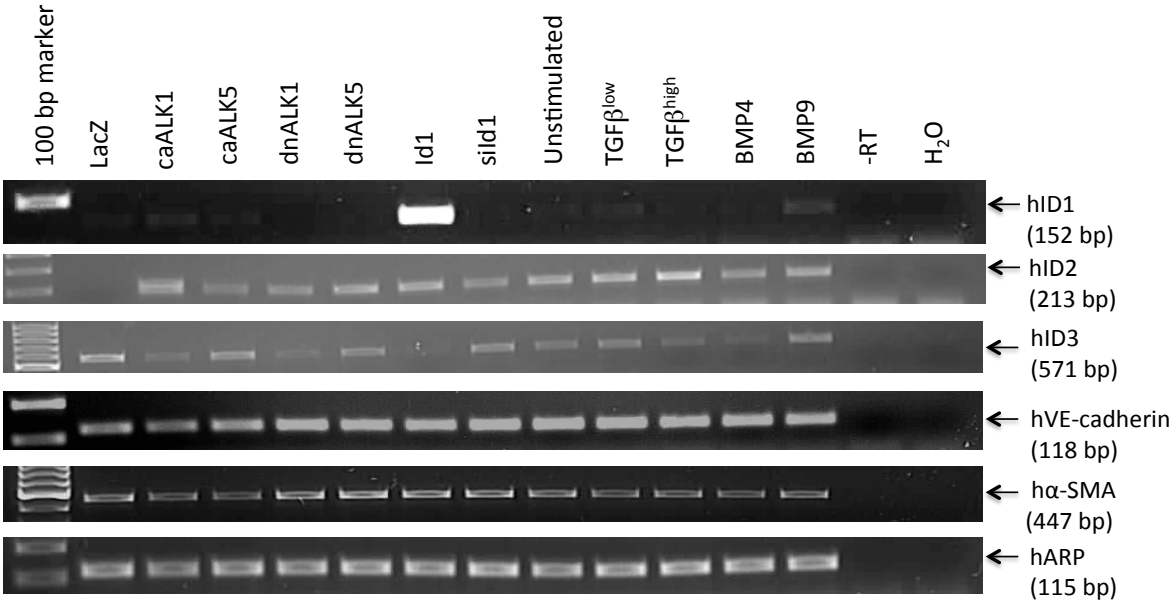


Figure 6. ALK1 induces expression of ID1 in HUVECs on an RNA level. HUVECs were either adenovirally infected or stimulated with growth factors. The adenoviral constructs used expressed constitutively active (ca) ALK1, caALK5, dominant negative (DN) ALK1, DNALK5, Id1, short intereference (si) Id1 and LacZ (control) at MOI of 100. Growth factors used were TGFβ in low (0.5 ng/ml) or high (5 ng/ml) concentration, BMP4 (10 ng/ml) and BMP9 (1 ng/ml). After starvation overnight the cells were lysed, RNA isolated and cDNA prepared. Gene expression was analysed by PCR. Amplified products and their size in basepairs are indicated on the right of the figure. The housekeeping gene hARP served as a loading control.

A striking difference between endothelial cells and other cell types is the presence of the endothelial specific TGFβ type I receptor ALK1, which has been shown to bind to TGFβ (Oh et al., 2000). ALK1 is only detected in endothelial cells whereas ALK5 is the common TGFβ type I receptor and is found in all cell types (Goumans et al., 2003; Goumans et al., 2002). Given the evidence in MEECs for the ALK1 and ALK5 receptors having opposite roles in angiogenesis (Goumans et al., 2002), their biological effect on tube-like formation of HUVECs was evaluated. In a tube-like formation assay, the abilty of endothelial cells to form capillary-like structures, or tubes, on a matrigel layer is studied (Figure 7A). During normal tube-like formation, the tubes looked thin yet well composed and the tube network was organized. Additionally, there were no big bundles of cells at the joints of tubes during normal tube-like formation. HUVECs were cultured and infected with adenoviral constructs expressing constitutively active (ca) ALK1, caALK5 and LacZ, with the last one serving as a control. Western blot results confirmed that the infection was successful (Figure 7B). After infection and serum starvation, HUVECs were seeded on chamber slides and fixed after 24 h of incubation. caALK1 infected HUVECs showed high potential to form organized tube-like structures on matrigel, while caALK5 infected HUVECs demonstrated poor ability to form tubes (Figure 7C). These results provide further evidence to the opposite effects of ALK1 and ALK5 in angiogenesis, and for the importance of ALK1 for formation of tube-like structures during the activation phase in HUVECs. Immunofluorescent stainings on these cells revealed that Id1 was highly expressed in tubes formed by caALK1 infected HUVECs, confirming that ALK1 induces expression of Id1 on a protein level as well as on an RNA level shown before (Figure 7D). The smooth muscle marker α-SMA was not expressed in ALK1 or

ALK5 infected HUVECs. This is in contrast to our results on RNA level where we observed expression of α-SMA in all conditions tested (Figure 6). Hence, it seems as if there might be a post-transcriptional regulation of α-SMA in HUVECs.

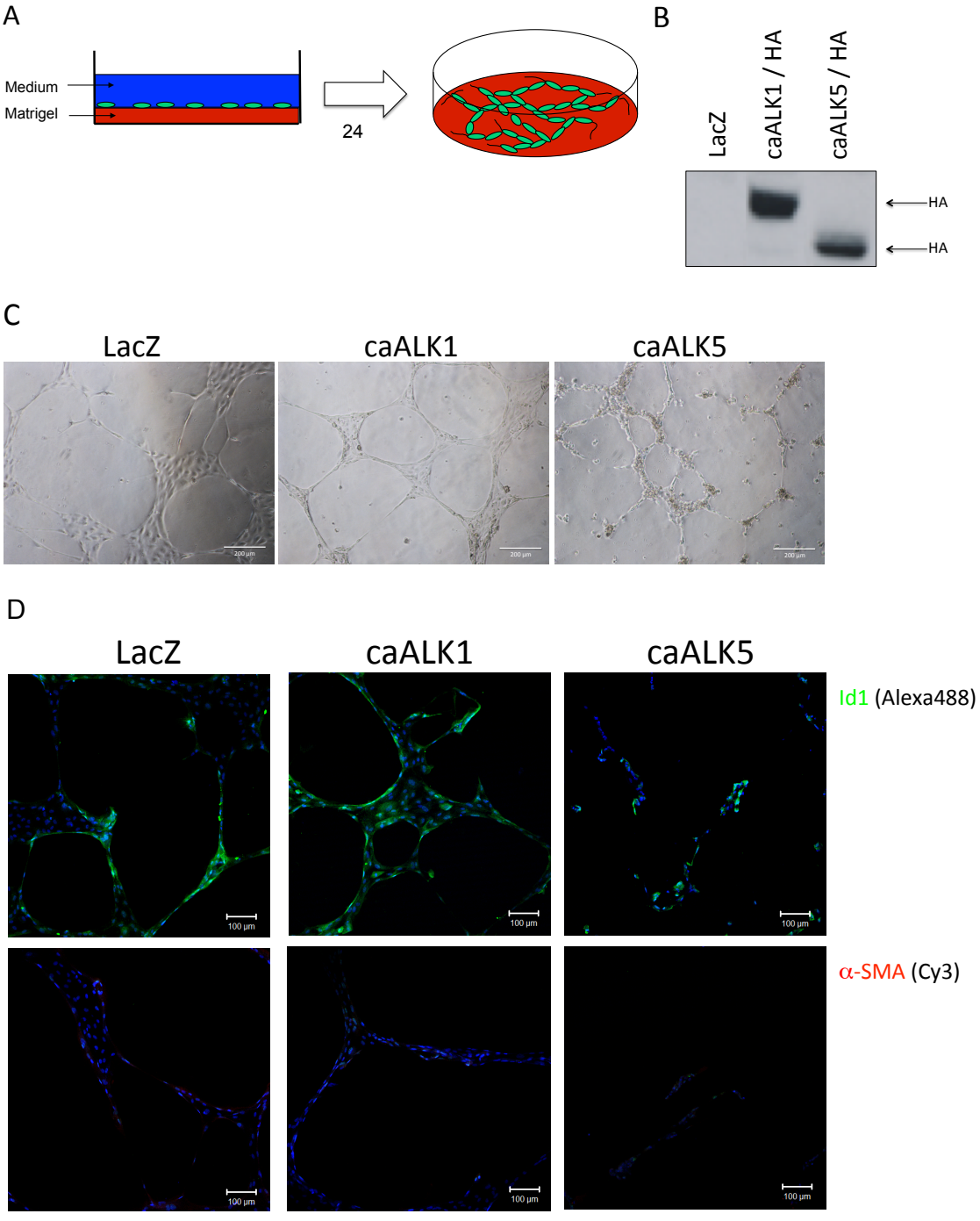


Figure 7. ALK1 promotes tube-like formation of HUVECs and induces expression of Id1 on a protein level. (A) Schematic figure showing tube-like formation assay. (B) Western blot analysis on lysates from infected HUVECs in A, using mouse anti-HA primary antibody and HRP-sheep anti-mouse secondary antibody. (C) HUVECs were infected with adenoviral constructs expressing caALK1/HA, caALK5/HA and LacZ (control). After starvation overnight the cells were harvested and a tube-like formation assay was performed, with cells being fixed 24 hours after seeding. (D) Immunofluorescent staining on fixed cells from A. Primary antibodies used were rabbit anti-Id1 and mouse anti-α-SMA. Secondary antibodies used were anti-rabbit Alexa 488 (green) and anti-mouse Cy3 (red), respectively.

During the activation phase of angiogenesis, besides migrating and forming tubes, endothelial cells must be able to degrade and invade the extracellular matrix (Valdimarsdottir, 2004). To study the opposite roles of ALK1 and ALK5 during angiogenesis even further in HUVECs, an invasion assay was performed (Figure 8A). HUVECs were infected with adenoviral constructs expressing caALK1, caALK5, LacZ (control) and Id1. The infected cells were seeded in the upper chambers of matrigel-based invasion plates and after incubation for 22 hours, the cells were fixed and finally stained overnight with crystal violet blue. Counting of cells that had invaded to the bottom of the chamber filter revealed that ALK1 signalling promoted invasiveness of endothelial cells, compared to the LacZ control (Figure 8B). Overexpression of Id1 gave similar results, which was expected since Id1 has been shown to be a downstream target of ALK1, with ALK1 inducing expression of Id1 in HUVECs, both on RNA and protein level (Figure 8B). In contrast, caALK5 inhibited invasiveness of HUVECs, causing opposite effects to ALK1. Western blot results revealed phosphorylation of Smad1/5 in caALK1 overexpressed HUVECs, demonstrating that the ALK1/Smad1/5 signalling cascade was active in these cells (Figure 8C). On the other hand, caALK5 expressing HUVECs resulted in inactivated Smad1/5 signalling.

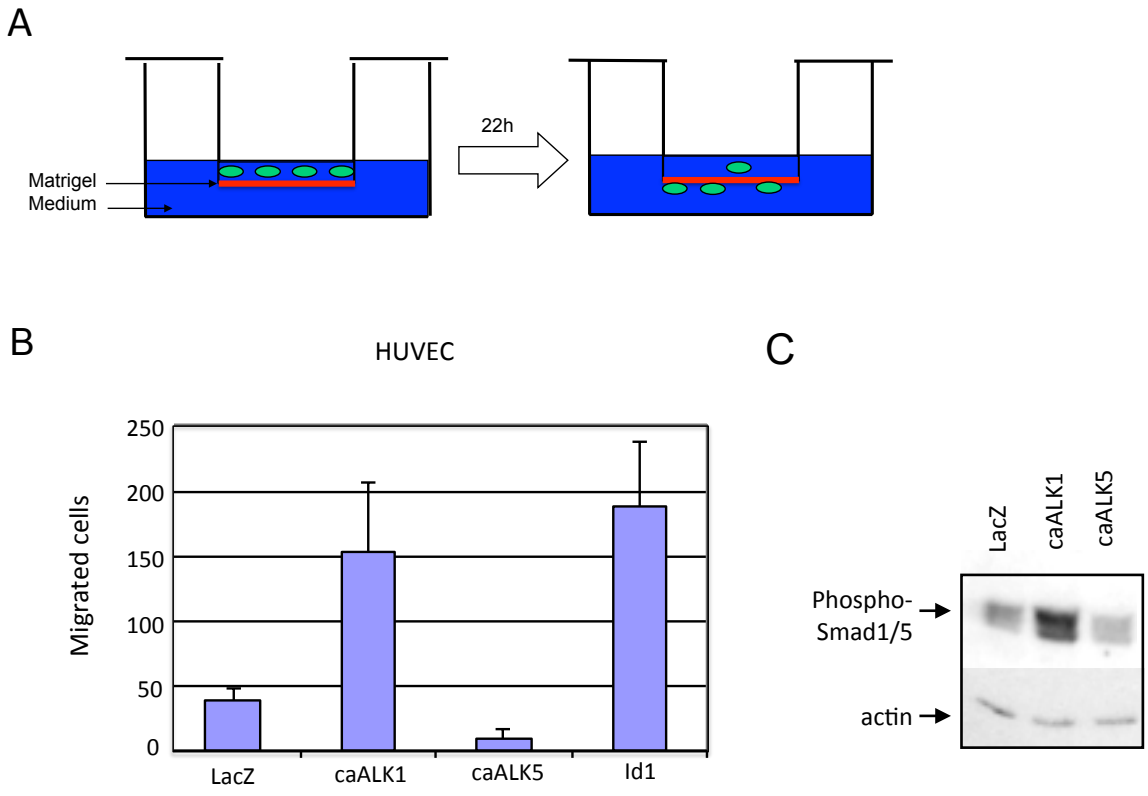


Figure 8. ALK1 promotes invasiveness of HUVECs. (A) A schematic figure of invasion assay. (B) HUVECs were infected with adenoviral constructs expressing caALK1, caALK1, Id1 and LacZ (control) at MOI of 100. After starvation overnight the cells were harvested and an invasion assay was performed. Number of migrated cells is displayed in figure. (C) Western blot analysis on lysates from LacZ, caALK1 and caALK5 infected HUVECs in A. Primary antibodies used were rabbit anti-phospho-Smad1/5 and mouse anti-actin, the latter serving as a loading control. Secondary antibodies used were HRP-donkey anti-rabbit and HRP-sheep anti-mouse, respectively.

According to results from the tube-like formation and invasion assays, activation of the endothelial specific receptor ALK1 resulted in the activation phase of angiogenesis, while activation of ALK5 had the opposite effect and resulted in the resolution phase. We were therefore interested in identifying novel genes through which the opposing TGFβ cascades were acting. ALK1/Smad1/5 has been shown to act via Id1 to promote the activation phase but ALK5/Smad2/3 had been observed to act via PAI-1 in MEECs (Goumans et al., 2003). In order to find possible new candidates of target genes especially downstream of ALK5, we infected MEECs with caALK5, the Smad7 inhibitor and LacZ (control) and studied differentiated gene expression. As figure 9A shows, several genes were upregulated in caALK5 infected MEECs compared to LacZ infected MEECs, such as plasminogen activator inhibitor-1 (PAI-1), thrombospondin-1 (TSP-1) and tissue inhibitor of MMPs (TIMP1), all three known inhibitors of MMPs (Egeblad & Werb, 2002). Given that overexpression of ALK5 induced upregulation of TSP-1, a known anti-angiogenic factor (Adams, 2001), we decided to elucidate the possible linkage between ALK5 and TSP1. As figure 9B demonstrates, we observed upregulation of TSP-1 in caALK5 overexpressed MEECs, compared to LacZ control and Smad7, an inhibitor of TGFβ signalling. Furthermore, PCR results from adenovirally infected MEECs revealed upregulation of TSP-1 in caALK5 overexpressed MEECs at the RNA level. TSP-1 was not upregulated in MEECs adenovirally infected with caALK1, dominant negative (DN) ALK5, Id1 or LacZ (control) (Figure 9C).

According to western blot results from the adenovirally infected MEECs, TSP-1 was also upregulated at the protein level only in caALK5 overexpressed cells (Figure 9D). Furthermore, Smad2 was phosphorylated in caALK5 overexpressed cells, which demonstrates that activation of ALK5 induces phosphorylation of Smad2, which results in upregulation of TSP-1.

A

GeneBank no.	Gene name	Fold change
NM_000602	PAI-1	4,67 up
NM_003246	TSP-1	2,68 up
NM_001613	ACTA2	2,60 up
NM_004368	Calponin	2,89 up
NM_003254	TIMP1	1,54 up
NM_005107	ENGL	1,14 up
NM_001795	VE-Cadherin	1,48 up
NM_002165	Id1	1,50 down
NM_002019	VEGFR1	7,23 down
NM_002253	VEGFR2	1,23 down

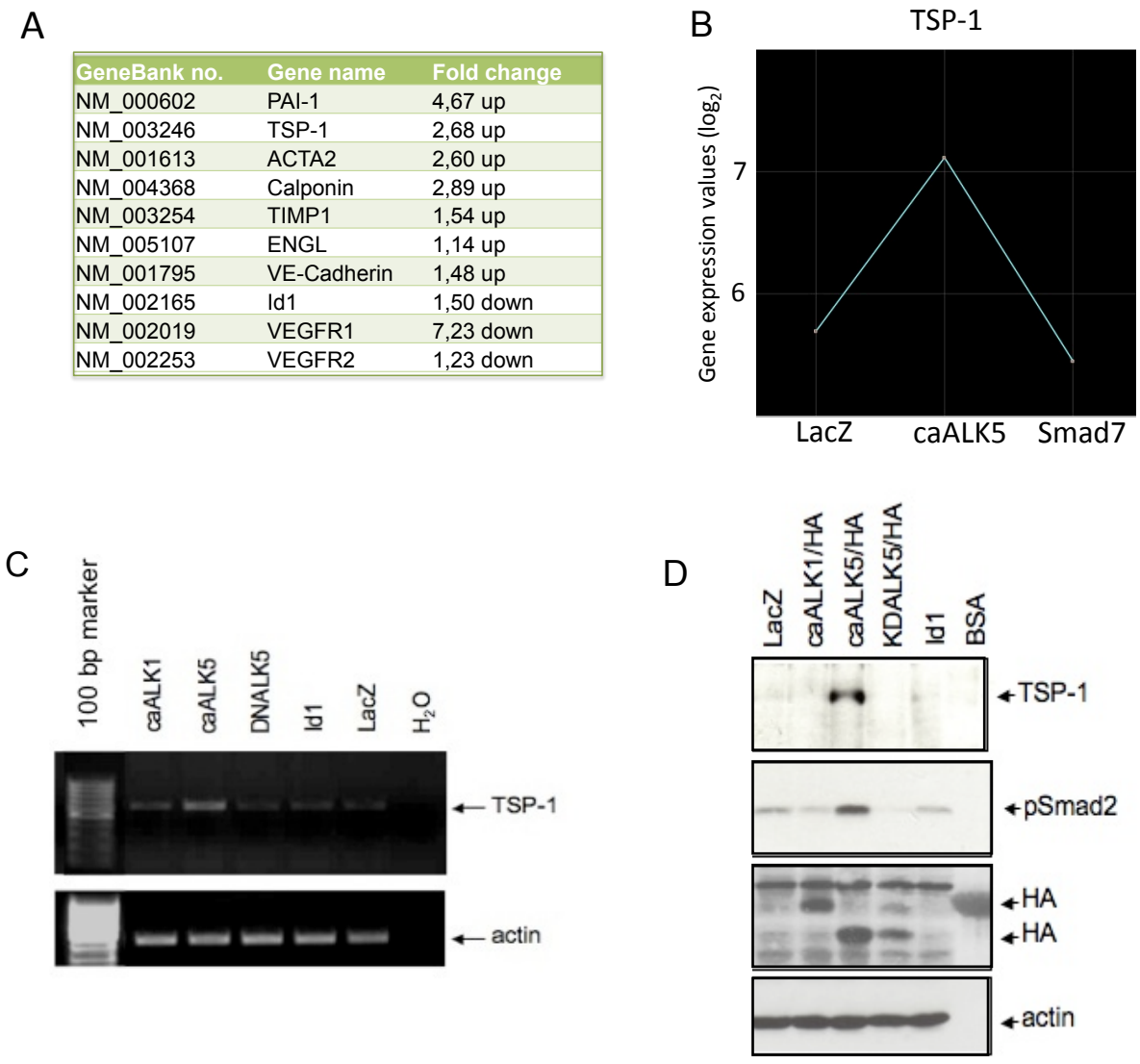


Figure 9. TSP-1 is upregulated in caALK5 overexpressed MEECs. (A) Whole-genome microarray analysis on total RNA from adenovirally infected MEECs expressing caALK5 and LacZ (control). Table shows gene expression in LacZ infected MEECs versus caALK5 infected MEECs. (B) Gene expression profiles for TSP-1. MEECs were infected with adenoviral constructs expressing caALK5, Smad7 (Smad inhibitor) and LacZ (control) (X-axis) and gene expression values were measured (Y-axis). (C) MEECs were infected with adenoviral constructs expressing caALK1, caALK5, DNALK5, Id1 and LacZ (control). After starvation overnight the cells were lysed, RNA isolated and cDNA prepared. TSP-1 expression was analysed by PCR. Actin served as a loading control. (D) Western blot analysis on lysates from infected MEECs in C. Primary antibodies used were rabbit anti-TSP-1, rabbit anti-phospho-Smad2, mouse anti-HA and mouse anti-actin, with the last one serving as a loading control. Secondary antibodies used were HRP-donkey anti-rabbit and HRP-sheep anti-mouse.

Since previous results demonstrate that ALK5 signalling induces upregulation of TSP-1 in MEECs, I wanted to investigate if this linkage between ALK5 and TSP-1 is restricted to MEECs or could be in other endothelial cell types. PCR results from adenovirally infected HUVECs revealed that TSP-1 was upregulated in caALK5 overexpressed HUVECs, confirming that ALK5 upregulates TSP-1 in HUVECs as well as in MEECs (Figure 10). Taken together, we believe that the resolution phase in endothelial cells is promoted by the ALK5/Smad2/3 pathway acting via TSP-1.

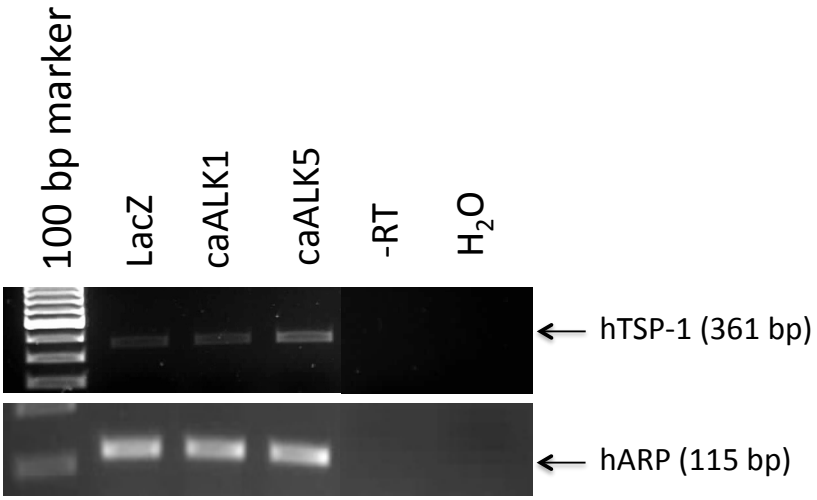


Figure 10. TSP-1 is upregulated in caALK5 overexpressed HUVECs. HUVECs were infected with adenoviral constructs expressing caALK1, caALK5 and LacZ (control). After starvation overnight the cells were lysed, RNA isolated and cDNA prepared, with –RT sample serving as a control (sample without reverse transcriptase). TSP-1 expression was analysed by PCR. hARP served as a loading control.

3.2 The role of ALK1 and ALK5 in vascular development of hES cell derived endothelial cells

After studying the role of ALK1 and ALK5 in endothelial cells we wanted to get insight into their role in human embryonic vascular development. With the isolation of hES cells it has become easier to study the lineage commitment in human development.

We set up a method to differentiate hES cells into endothelial cells (Figure 11). Embryoid bodies were formed from undifferentiated HES3 cells and they were either unstimulated or stimulated with BMP4 (10 ng/ml), since BMP4 is a known mesodermal inducer. After 10 days of incubation the EBs were dissociated and CD31 positive ($CD31^+$) cells in the population were sorted with MACS (Figure 11). $CD31^+$ cells were expanded before they were seeded on matrigel coated chamber slides and finally fixed after 24 hours of incubation. To examine the characteristics of $CD31^+$ cells and elucidate if they were in fact endothelial cells, an acetylated (ac) LDL assay was performed (Figure 12). HUVECs served as a positive control in the assay as these mature endothelial cells do take up ac-LDL. $CD31^+$ cells demonstrated uptake of ac-LDL after incubation overnight, providing evidence that we had succeeded in isolating hES derived vascular cells with endothelial properties.

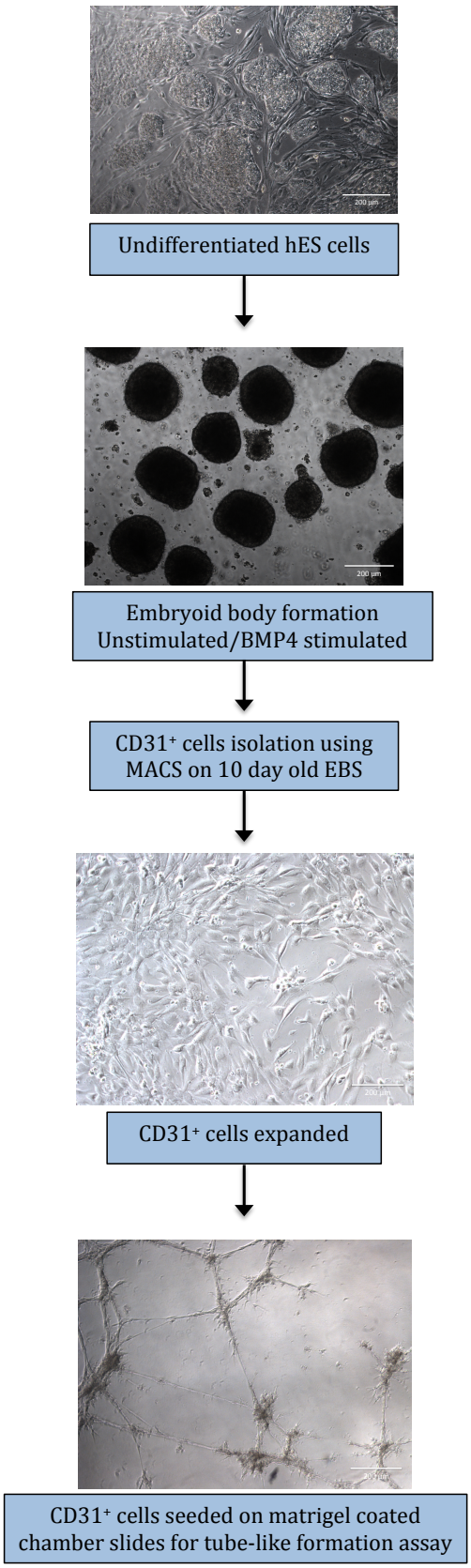


Figure 11. A flowchart of method for differentiation of hES cells into endothelial cells.

To examine protein expression of the $CD31^+$ cells and evaluate their endothelial characteristics, an immunofluorescent procedure was carried out. Unexpectedly, the smooth muscle marker α-SMA was expressed in $CD31^+$ cells on a gelatin layer (Figure 13A). This suggests that the $CD31^+$ cells have not aquired a terminal vascular fate and that they might in fact be precursor cells that can either differentiate into endothelial cells or smooth muscle cells. As figure 13B demonstrates, when a tube-like formation assay was performed on the $CD31^+$ cells, BMP4 stimulated cells formed more organized tube-like structures than unstimulated cells. Furthermore, $CD31^+$ cells showed phosphorylation of Smad1 and Smad2. Smad1 phosphorylation would be expected, especially in BMP4 stimulated cells as BMP is known to induce phosphorylation of Smad1/5/8 by signalling via ALK2/3/6 in endothelial cells (Valdimarsdottir et al., 2002). $CD31^+$ cells expressed the endothelial markers VE-cadherin and endoglin, which provides further evidence for the population having endothelial characteristics. Id1 expression was robust in tube-like structures formed by $CD31^+$ cells, which was expected especially in BMP4 stimulated cells as Id1 is a known target gene of BMP signalling (Hollnagel et al., 1999; Valdimarsdottir et al., 2002; Xu et al., 2002).

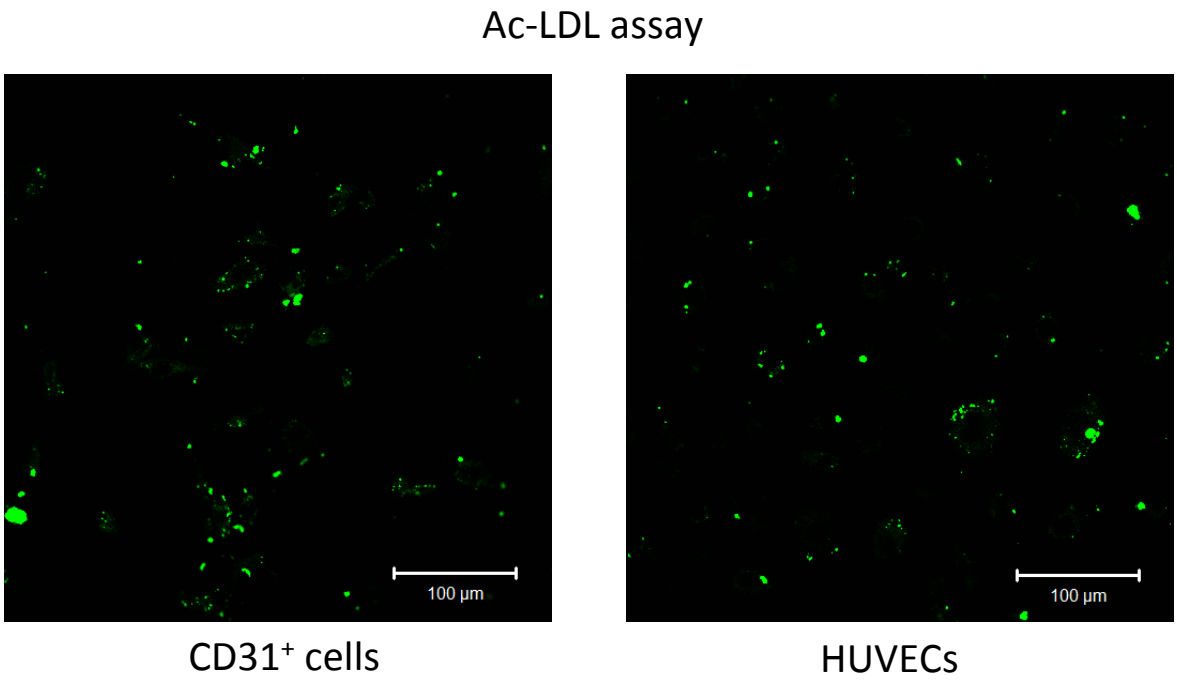
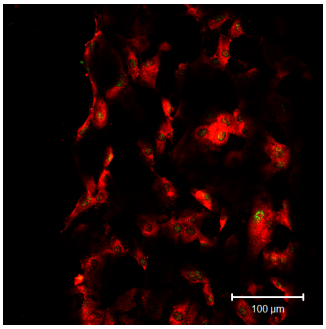


Figure 12. $CD31^+$ cells take up acetylated LDL. $CD31^+$ cells were harvested and seeded on gelatin-coated chamber slides. Acetylated LDL assay was performed on $CD31^+$ cells, with HUVECs serving as a positive control. Cells were fixed after incubation with ac-LDL overnight.

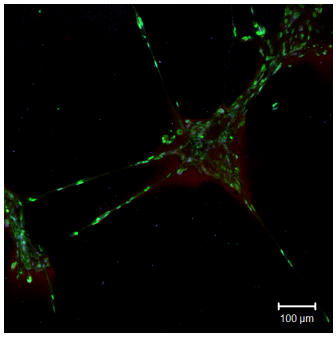
A

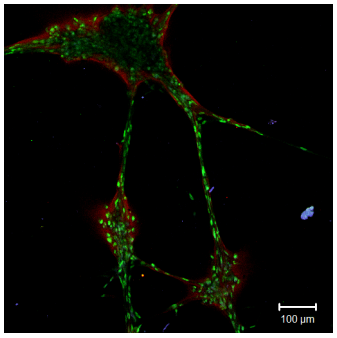


α-SMA (Cy3)
PS2 (Alexa488)

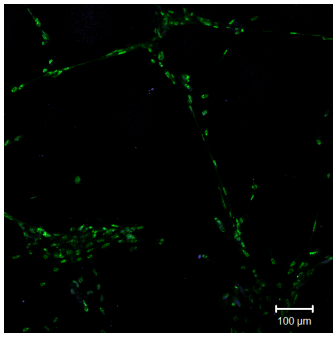
B

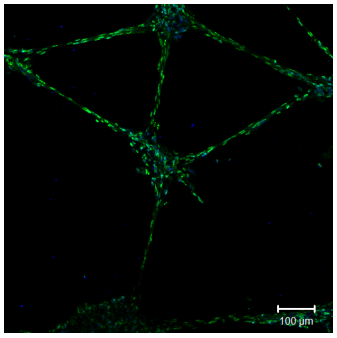
Unstimulated BMP4



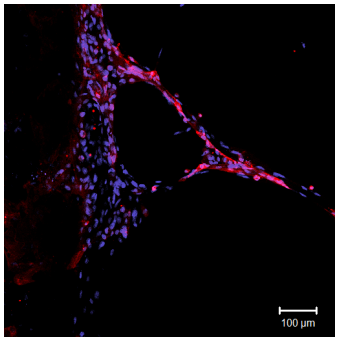


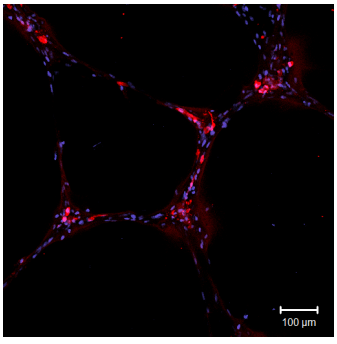
VE-Cadherin (Cy3)
p-Smad1 (Alexa488)



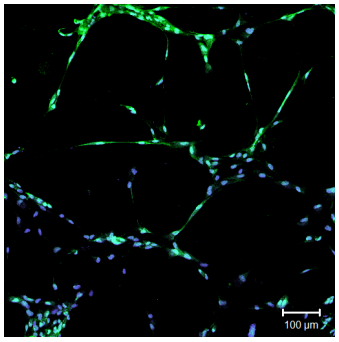


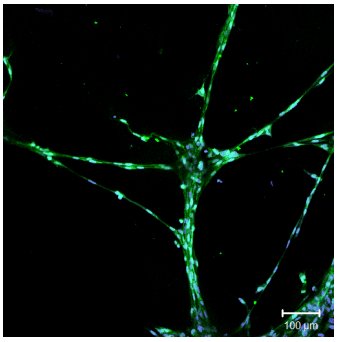
p-Smad2 (Alexa488)





Endoglin (Cy3)





Id1 (Alexa488)

Figure 13. CD31$^+$ cells form tubes, express smooth muscle marker and endothelial markers. (A) Unstimulated CD31$^+$ cells were harvested and seeded on gelatin-coated chamber slides, with cells being fixed 24 hours after seeding. Immunofluorescent staining was performed on cells. Primary antibodies used were mouse anti- α-SMA and rabbit anti-phospho-Smad2. Secondary antibodies used were anti-mouse Cy3 (red) and anti-rabbit Alexa 488 (green), respectively. (B) Unstimulated and BMP4 stimulated (10 ng/ml) CD31+ cells were harvested and a tube-like formation assay was performed, with cells being fixed 24 hours after seeding. Immunofluorescent staining was performed on cells. Primary antibodies used were anti-VE-cadherin, rabbit anti-phospho-Smad1, rabbit anti-phospho-Smad2, mouse anti-Endoglin, rabbit anti-Id1. Secondary antibodies used were anti-rabbit Alexa 488 (green) and anti-mouse Cy3 (red).

My previous findings as well as results from multiple studies underline the importance of ALK1 in angiogenesis. To evaluate the necessity of ALK1 in angiogenesis of hES cell derived endothelial cells, I performed a tube-like formation assay on CD31$^+$ cells using a neutralizing antibody specifically raised against human ALK1 (PF-03446962) (Hu-Lowe et al., 2011; van Meeteren et al., 2012). CD31$^+$ cells were cultured, harvested and incubated with or without the human ALK1 antibody (4 µl/ml) before they were seeded on matrigel-coated chamber slides, unstimulated or stimulated with TGFβ (1 ng/ml). The cells were fixed after 24 hours of incubation. Immunofluorescent stainings of the tube-like structures were performed on the cells in order to assess protein expression (Figure 14).

The results indicate that inhibiting ALK1 with the neutralizing ALK1 antibody did not have great effects on Id1 expression. Tube formation in unstimulated and TGFβ treated cells as well as Smad1/5 phosphorylation seem to be inhibited upon ALK1 inhibiton using the ALK1 antibody. Smad2 phosphorylation was not affected by the ALK1 antibody preincubation. This data can be interpreted as ALK1 being necessary for hES cell derived vascular development. Of note, these results are very preliminary and need to be repeated to obtain a clear cut answer on the importance of ALK1 in hES cell derived vascular development.

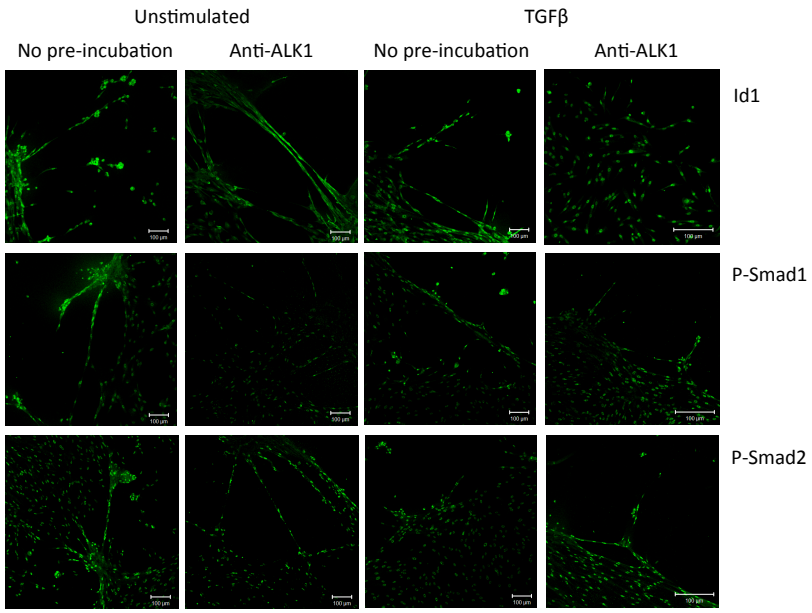


Figure 14. ALK1 might be necessary for hES cell derived vascular development. CD31$^+$ cells were harvested and incubated with or without anti-human ALK1 antibody (anti-hALK1) before performing a tube-like formation assay where cells were unstimulated or stimulated with TGFβ (1 ng/ml), with cells being fixed 24 hours after seeding. Immunofluorescent staining was performed on cells. Primary antibodies used were rabbit anti-Id1, rabbit anti-phospho-Smad1 and rabbit anti-phospho-Smad2. Secondary antibody used was anti-rabbit Alexa 488.

One aim of my study was to find novel genes directly activated by the TGFβ superfamily type I receptor during vascular differentiation of hES cells. From our previous microarray analysis we observed several vascular genes being upregulated during hES cell differentiation (data not shown). To examine that, a chromatin immunoprecipitation (ChIP) was performed on hES cells during differentiation using an antibody specifically raised against phosphorylated Smad1, a transcription factor downstream of the BMP type I receptors ALK1, ALK2, ALK3 and ALK6 (Figure 15A). IgG antibody was used as a negative control to visualize any unspecific binding. Follwing the ChIP, a PCR was performed on samples using various primers to determine which genes the phosphorylated Smad1 had bound to. In order to undergo early differentiation, hES cells are thought to upregulate EMT transcription factors. Since SLUG was one of the genes highly upregulated according to the microarray data I tested if Smad1 was able to bind directly to the SLUG promoter. Results from PCR where hSLUG promoter region primers were used, show that a band could be visualized in phosphorylated Smad1 IP samples while there was no unspecific binding in IgG IP samples (Figure 15B). These findings provide evidence for the binding of phosphorylated Smad1 to the hSLUG promoter region in hES cells, that in turn initiate transcription of the SLUG gene.

In addition to examining gene activation during early differentiation of hES cells, I was interested in finding novel genes which were directly activated by the TGFβ superfamily type I receptors ALK1 and ALK5 during vascular development of hES cells and angiogenesis of endothelial cells. To study this, I infected HUVECs with adenoviral constructs expressing constitutively active (ca) ALK1, caALK5 and LacZ (control). A ChIP was performed on the infected HUVECs using an antibody specifically raised against either phosphorylated Smad1 or phosphorylated Smad2. PCR was performed on ChIP samples in order to screen for binding site identification. Primers for various promoter regions were used, such as for VE-cadherin, PAI-1, TSP-1 and the BMP responsive element (BRE), which is the promoter region for Id1. The same was done in hES cells. Due to performance problems with the ChIP, no further results could be obtained using this method, despite spending much time on trying to troubleshoot the problems. Efforts were made to adjust every parameter and step of the ChIP protocol. The sonication step seemed to be a possible cause of the problem, but in spite of troubleshooting every step, it did not show favourable results.

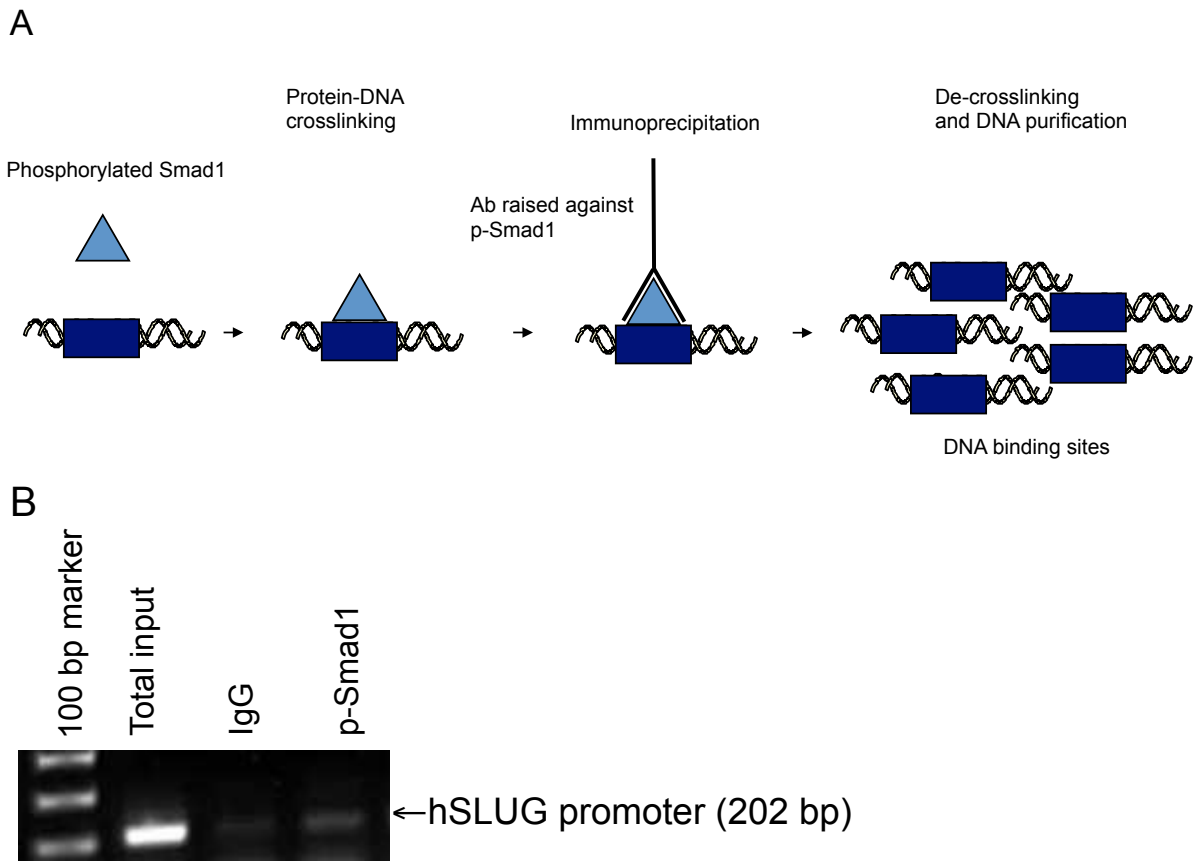


Figure 15. Phosphorylated Smad1 binds to the hSLUG promoter region, initiating transcription of the SLUG gene. (A) A ChIP was performed on unstimulated hES cells according to the schematic figure, using anti-phospho-Smad1 antibody. (B) Possible binding of p-Smad1 to the hSLUG promoter was evaluated by performing PCR on ChIP samples from A. Total input represented total DNA and IgG served as an antibody specificity control.

4 Discussion

Many factors contribute to the vital biological process blood vessel formation. In the present study, I examined the roles of the TGFβ type I receptors ALK1 and ALK5 in adult angiogenesis and embryonic vascular development. To assess the role of the two receptors in adult angiogenesis *in vitro*, mature endothelial cells (HUVECs) were used. ALK1 and ALK5 exhibited opposite effects in angiogenesis. Activation of the endothelial specific receptor ALK1 turned out to promote tube-like formation and invasion, hallmarks of the activation phase. Furthermore, ALK1 induced expression of Id1, both at the protein and RNA levels. Activation of ALK5 on the other hand gave the opposite effect, resulting in poor tube-like formation and invasion. These results underline the opposing effects of ALK1 and ALK5 in adult angiogenesis, with ALK1 promoting the activation phase and ALK5 inducing the resolution phase. Furthermore, activation of ALK5 induced upregulation of the anti-angiogenic factor TSP-1 both in MEECs and HUVECs, providing further evidence for ALK5 inducing the resolution phase.

The effect of ALK1 and ALK5 during human embryonic vascular development, was evaluated using hES cell derived endothelial cells. A method was set up to differentiate hES cells into endothelial cells. Results demonstrate that using the method, isolation of hES derived vascular cells with endothelial properties was successful, as the cells formed tube-like structures, expressed endothelial markers and exhibited uptake of acetylated LDL. Efforts were made to find novel genes directly activated by the TGFβ superfamily type I receptor during vascular differentiation of hES cells. Results suggest that when hES cells undergo differentiation, phosphorylated Smad1 binds to the hSLUG promoter region, initiating transcription of the SLUG gene. The importance of ALK1 during hES cell vascular differentiation was studied using a human ALK1 neutralizing antibody. Preliminary data suggest that ALK1 is necessary for tube-like formation.

4.1 The role of ALK1 and ALK5 in angiogenesis of endothelial cells

Results from adenovirally infected HUVECs confirm Id1 as a target of ALK1 effect. Ectopic expression of caALK1 promoted tube-like formation and invasion, with overexpression of its downstream target, Id1, also resulting in increased invasion. Id2 and Id3 have different expression patterns from Id1 and they even seem to compensate the loss of Id1 expression. The effect of the Id proteins on blood vessel formation has been studied to some extent and in a study by Lyden and colleagues, the importance of Id1 proteins in vascularization was evaluated by knocking out Id genes in mice. Interestingly, knocking out Id1 alone did not result in a detectable phenotype. Only Id1-Id3 double knockout mice displayed vascular malformation, confirming that both Id1 and Id3 need to be absent in order to cause poor vascularization (Lyden et al., 1999). These findings coincide with our results which suggest that once Id1 expression is absent, Id3 compensates for its loss. Considering this information, it is likely that the Id1-3 proteins contribute to blood vessel formation.

Microarray results from MEECs reveal that ectopic expression of caALK5 induces upregulation of the anti-angiogenic factor TSP-1. Invasiveness is one of the hallmarks of the activation phase of

angiogenesis, as endothelial cells invade the ECM when they form vascular structures. Key factors in this process are matrix metalloproteases (MMPs), which degrade the ECM and therefore enable endothelial cells to penetrate the ECM (Sternlicht & Werb, 2001). Given the anti-angiogenic effect of TSP-1, it would be interesting to evaluate the interaction of the protein with ECM proteins such as MMPs. There are a few types of MMPs. They are generated as inactive zymogens and are converted to the active form, normally either by other MMPs or serine proteases (Sternlicht & Werb, 2001). For example, MMP3 has been shown to activate MMP9 *in vitro* by cleaving the pro-MMP9 protein (Okada et al., 1992; Ramos-DeSimone et al., 1999). It has been demonstrated that in the presence of TSP-1, MMP3-dependant activation of pro-MMP9 is inhibited in a dose-dependent manner *in vitro*. In addition, the presence of TSP-1 has been shown to inhibit activation of MMP2 in vitro (Bein & Simons, 2000). Consistent with these findings, endothelial cells from TSP-1 null mice exhibit higher levels of active MMP2 than wild type mice (Lawler et al., 1993). Taken together, these findings show that TSP-1 can inihibit the activation of MMP2 and MMP9, therefore inhibiting angiogenesis.

Given this apparent connection between TSP-1 and MMPs and the finding that ALK1 induces Id1 expression, resulting in activation phase, it is plausible that during the activation phase, Id1 could be inhibiting TSP-1 expression or hindering the effects of TSP-1 on MMPs or other ECM proteins. To examine this would be a worthy goal. That could be done by infecting endothelial cells with shTSP-1 and assessing gene expression upon the knockdown of TSP-1. In this project, efforts were made to infect HUVECs with shTSP-1 in order to evaluate its effect on tube-like formation and gene expression. The infection was not technically successful and needs to be repeated in order to obtain valuable results. Such experiments may provide further knowledge for targeting TSP-1 for anti-angiogenic treatments against cancer.

4.2 The role of ALK1 and ALK5 in vascular development of hES cell derived endothelial cells

The effect of the TGFβ/ALK1 signalling on angiogenesis has been elucidated in various endothelial cell types, such as MEECs and BAECs. However, in order to gather insight into the TGFβ/ALK1 pathway in embryonic vascular development and which genes were being activated by the pathway, we generated hES cell derived endothelial cells. Next we wanted to evaluate if there was a direct binding of downstream activators of the TGFβ/ALK1 pathway to various promoter regions, such as the BMP responsive element (BRE), which is the promoter region for Id1. To study this we performed a chromatin immunoprecipitation (ChIP) assay on hES derived endothelial cells using an antibody raised against phosphorylated Smad1. However, the ChIP was not successful, despite extensive efforts in troubleshooting the method. Therefore, the binding of phosphorylated Smad1 to BRE and other promoter regions must be determined once the ChIP has been optimized.

In addition to determining which genes are activated by the TGFβ/ALK1 pathway, we wanted to elucidate the specific effect of ALK1 on embryonic vascular development. We used a neutralizing antibody specifically raised against human ALK1 (anti-hALK1). This antibody has previously been used to determine the specific effect of ALK1 in angiogenesis of mature endothelial cells (HUVECs)

(van Meeteren et al., 2012) but the novelty of our study is in examining the effect of ALK1 in embryonic vascular development by using the antibody on hES-derived endothelial cells. This antibody is currently in clinical trials and its use could shed light on ALK1 as a valuable target in anti-angiogenesis therapies. In HUVECs, TGFβ was clearly shown to bind to ALK1, resulting in increased tube-like formation and phosphorylation of Smad1. However, the anti-hALK1 antibody seemed to block the binding of TGFβ to ALK1, reducing tube-like formation and phosphorylation of Smad1 (Hu-Lowe et al., 2011). These findings indicate the necessity of the TGFβ/ALK1 pathway in hES cell derived vascular development. However, these results are preliminary and the experiments must be repeated in order to obtain further insights into the importance of ALK1 in hES cell derived vascular development.

4.3 Concluding remarks

In this study, I have confirmed the opposite effects of ALK1 and ALK5 in HUVECs, with ALK1 being proangiogenic. Different expression patterns of Id3 compared to the ALK1 target Id1 provide further evidence for Id3 being able to compensate for the loss of Id1 during angiogenesis. Furthermore, the ALK5-induced upregulation of TSP-1 underlines the importance of ALK5 during the resolution phase. These findings provide insights into the molecular mechanisms behind adult angiogenesis and could be valuable to improve anti-antiogenic treatments against tumor growth and metastasis.

Bibliography

Abenhaim, L., Moride, Y., Brenot, F., Rich, S., Benichou, J., Kurz, X., Higenbottam, T., Oakley, C., Wouters, E., Aubier, M., Simonneau, G., & Begaud, B. (1996). Appetite-suppressant drugs and the risk of primary pulmonary hypertension. International Primary Pulmonary Hypertension Study Group. *N Engl J Med, 335*(9), 609-616.

Adams, J. C. (2001). Thrombospondins: multifunctional regulators of cell interactions. *Annu Rev Cell Dev Biol, 17*, 25-51.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., & Walter, P. (2008). *Molecular biology of the cell* (Fifth ed.). New York: Garland Science, Taylor & Francis Group.

Armstrong, L. C., & Bornstein, P. (2003). Thrombospondins 1 and 2 function as inhibitors of angiogenesis. *Matrix Biol, 22*(1), 63-71.

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. *Developmental Biology, 217*(1), 42-53.

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.

Bein, K., & Simons, M. (2000). Thrombospondin type 1 repeats interact with matrix metalloproteinase 2. Regulation of metalloproteinase activity. *J Biol Chem, 275*(41), 32167-32173.

Benezra, R., Rafii, S., & Lyden, D. (2001). The Id proteins and angiogenesis. *Oncogene, 20*(58), 8334-8341.

Bideau, A., Brunet, G., Heyer, E., Plauchu, H., & Robert, J. M. (1992). An abnormal concentration of cases of Rendu-Osler disease in the Valserine valley of the French Jura: a genealogical and demographic study. *Ann Hum Biol, 19*(3), 233-247.

Bornstein, P., & Sage, E. H. (1994). Thrombospondins. *Methods Enzymol, 245*, 62-85.

Botquin, V., Hess, H., Fuhrmann, G., Anastassiadis, C., Gross, M. K., Vriend, G., & Scholer, H. R. (1998). New POU dimer configuration mediates antagonistic control of an osteopontin preimplantation enhancer by Oct-4 and Sox-2. *Genes Dev, 12*(13), 2073-2090.

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.

Cao, Y. (2001). Endogenous angiogenesis inhibitors and their therapeutic implications. *Int J Biochem Cell Biol, 33*(4), 357-369.

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

Carpenter, M. K., Rosler, E., & Rao, M. S. (2003). Characterization and differentiation of human embryonic stem cells. *Cloning Stem Cells, 5*(1), 79-88.

Chambers, I., Colby, D., Robertson, M., Nichols, J., Lee, S., Tweedie, S., & Smith, A. (2003). Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell, 113*(5), 643-655.

Cheifetz, S., Bellon, T., Cales, C., Vera, S., Bernabeu, C., Massague, J., & Letarte, M. (1992). Endoglin is a component of the transforming growth factor-beta receptor system in human endothelial cells. *J Biol Chem, 267*(27), 19027-19030.

Chen, H., Shi, S., Acosta, L., Li, W., Lu, J., Bao, S., Chen, Z., Yang, Z., Schneider, M. D., Chien, K. R., Conway, S. J., Yoder, M. C., Haneline, L. S., Franco, D., & Shou, W. (2004). BMP10 is essential for maintaining cardiac growth during murine cardiogenesis. *Development, 131*(9), 2219-2231.

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.

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., Pardali, E., Thorikay, M., Anderberg, C., Hawinkels, L., Goumans, M. J., Seehra, J., Heldin, C. H., ten Dijke, P., & Pietras, K. (2010). Genetic and pharmacological targeting of activin receptor-like kinase 1 impairs tumor growth and angiogenesis. *J Exp Med, 207*(1), 85-100.

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.

Derynck, R., & Feng, X. H. (1997). TGF-beta receptor signaling. *Biochim Biophys Acta, 1333*(2), F105-150.

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

Egeblad, M., & Werb, Z. (2002). New functions for the matrix metalloproteinases in cancer progression. *Nat Rev Cancer, 2*(3), 161-174.

Elliott, D. A., Braam, S. R., Koutsis, K., Ng, E. S., Jenny, R., Lagerqvist, E. L., Biben, C., Hatzistavrou, T., Hirst, C. E., Yu, Q. C., Skelton, R. J., Ward-van Oostwaard, D., Lim, S. M., Khammy, O., Li, X., Hawes, S. M., Davis, R. P., Goulburn, A. L., Passier, R., Prall, O. W., Haynes, J. M., Pouton, C. W., Kaye, D. M., Mummery, C. L., Elefanty, A. G., & Stanley, E. G. (2011). NKX2-5(eGFP/w) hESCs for isolation of human cardiac progenitors and cardiomyocytes. *Nat Methods, 8*(12), 1037-1040.

Evans, M. J., & Kaufman, M. H. (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature, 292*(5819), 154-156.

Folkman, J. (1995). Seminars in Medicine of the Beth Israel Hospital, Boston. Clinical applications of research on angiogenesis. *N Engl J Med, 333*(26), 1757-1763.

Gearhart, J. (2004). New human embryonic stem-cell lines--more is better. *N Engl J Med, 350*(13), 1275-1276.

Gilbert, S. F. (2006). *Developmental biology* (6 ed.). Sunderland, Massachusetts: Sinauer Associates.

Good, D. J., Polverini, P. J., Rastinejad, F., Le Beau, M. M., Lemons, R. S., Frazier, W. A., & Bouck, N. P. (1990). A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin. *Proc Natl Acad Sci U S A, 87*(17), 6624-6628.

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

Goumans, M. J., Valdimarsdottir, G., Itoh, S., Lebrin, F., Larsson, J., Mummery, C., Karlsson, S., & ten Dijke, P. (2003). Activin receptor-like kinase (ALK)1 is an antagonistic mediator of lateral TGFbeta/ALK5 signaling. *Mol Cell, 12*(4), 817-828.

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.

Goumans, M. J., van Zonneveld, A. J., & ten Dijke, P. (2008). Transforming growth factor beta-induced endothelial-to-mesenchymal transition: a switch to cardiac fibrosis? *Trends Cardiovasc Med, 18*(8), 293-298.

Govani, F. S., & Shovlin, C. L. (2009). Hereditary haemorrhagic telangiectasia: a clinical and scientific review. *Eur J Hum Genet, 17*(7), 860-871.

Guttmacher, A. E., Marchuk, D. A., & White, R. I., Jr. (1995). Hereditary hemorrhagic telangiectasia. *N Engl J Med, 333*(14), 918-924.

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.

Hollnagel, A., Oehlmann, V., Heymer, J., Ruther, U., & Nordheim, A. (1999). Id genes are direct targets of bone morphogenetic protein induction in embryonic stem cells. *J Biol Chem, 274*(28), 19838-19845.

Hood, J. D., & Cheresh, D. A. (2002). Role of integrins in cell invasion and migration. *Nat Rev Cancer, 2*(2), 91-100.

Hu-Lowe, D. D., Chen, E., Zhang, L., Watson, K. D., Mancuso, P., Lappin, P., Wickman, G., Chen, J. H., Wang, J., Jiang, X., Amundson, K., Simon, R., Erbersdobler, A., Bergqvist, S., Feng, Z., Swanson, T. A., Simmons, B. H., Lippincott, J., Casperson, G. F., Levin, W. J., Stampino, C. G., Shalinsky, D. R., Ferrara, K. W., Fiedler, W., & Bertolini, F. (2011). Targeting activin receptor-like kinase 1 inhibits angiogenesis and tumorigenesis through a mechanism of action complementary to anti-VEGF therapies. *Cancer Res, 71*(4), 1362-1373.

Iruela-Arispe, M. L., Lombardo, M., Krutzsch, H. C., Lawler, J., & Roberts, D. D. (1999). Inhibition of angiogenesis by thrombospondin-1 is mediated by 2 independent regions within the type 1 repeats. *Circulation, 100*(13), 1423-1431.

Jaenisch, R., & Young, R. (2008). Stem cells, the molecular circuitry of pluripotency and nuclear reprogramming. *Cell, 132*(4), 567-582.

James, D., Levine, A. J., Besser, D., & Hemmati-Brivanlou, A. (2005). TGFbeta/activin/nodal signaling is necessary for the maintenance of pluripotency in human embryonic stem cells. *Development, 132*(6), 1273-1282.

Jen, Y., Manova, K., & Benezra, R. (1996). Expression patterns of Id1, Id2, and Id3 are highly related but distinct from that of Id4 during mouse embryogenesis. *Dev Dyn, 207*(3), 235-252.

Johnson, D. W., Berg, J. N., Baldwin, M. A., Gallione, C. J., Marondel, I., Yoon, S. J., Stenzel, T. T., Speer, M., Pericak-Vance, M. A., Diamond, A., Guttmacher, A. E., Jackson, C. E., Attisano, L., Kucherlapati, R., Porteous, M. E., & Marchuk, D. A. (1996). Mutations in the activin receptor-like kinase 1 gene in hereditary haemorrhagic telangiectasia type 2. *Nat Genet, 13*(2), 189-195.

Kalluri, R. (2003). Basement membranes: structure, assembly and role in tumour angiogenesis. *Nat Rev Cancer, 3*(6), 422-433.

Kaufman, D. S., Hanson, E. T., Lewis, R. L., Auerbach, R., & Thomson, J. A. (2001). Hematopoietic colony-forming cells derived from human embryonic stem cells. *Proc Natl Acad Sci U S A, 98*(19), 10716-10721.

Kjeldsen, A. D., Vase, P., & Green, A. (1999). Hereditary haemorrhagic telangiectasia: a population-based study of prevalence and mortality in Danish patients. *J Intern Med, 245*(1), 31-39.

Klimanskaya, I., Chung, Y., Meisner, L., Johnson, J., West, M. D., & Lanza, R. (2005). Human embryonic stem cells derived without feeder cells. *Lancet, 365*(9471), 1636-1641.

Lacout, A., Pelage, J. P., Lesur, G., Chinet, T., Beauchet, A., Roume, J., & Lacombe, P. (2010). Pancreatic involvement in hereditary hemorrhagic telangiectasia: assessment with multidetector helical CT. *Radiology, 254*(2), 479-484.

Lawler, J., Duquette, M., Urry, L., McHenry, K., & Smith, T. F. (1993). The evolution of the thrombospondin gene family. *J Mol Evol, 36*(6), 509-516.

Leschik, J., Stefanovic, S., Brinon, B., & Puceat, M. (2008). Cardiac commitment of primate embryonic stem cells. *Nat Protoc, 3*(9), 1381-1387.

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.

Lindahl, P., Johansson, B. R., Leveen, P., & Betsholtz, C. (1997). Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. *Science, 277*(5323), 242-245.

Ludwig, T. E., Levenstein, M. E., Jones, J. M., Berggren, W. T., Mitchen, E. R., Frane, J. L., Crandall, L. J., Daigh, C. A., Conard, K. R., Piekarczyk, M. S., Llanas, R. A., & Thomson, J. A. (2006). Derivation of human embryonic stem cells in defined conditions. *Nat Biotechnol, 24*(2), 185-187.

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.

Mamou, F., May, K. S., Schipper, M. J., Gill, N., Kariapper, M. S., Nair, B. M., Brewer, G., Normolle, D., & Khan, M. K. (2006). Tetrathiomolybdate blocks bFGF- but not VEGF-induced incipient angiogenesis in vitro. *Anticancer Res, 26*(3A), 1753-1758.

Martin, M. J., Muotri, A., Gage, F., & Varki, A. (2005). Human embryonic stem cells express an immunogenic nonhuman sialic acid. *Nat Med, 11*(2), 228-232.

Massague, J., & Chen, Y. G. (2000). Controlling TGF-beta signaling. *Genes Dev, 14*(6), 627-644.

McAllister, K. A., Grogg, K. M., Johnson, D. W., Gallione, C. J., Baldwin, M. A., Jackson, C. E., Helmbold, E. A., Markel, D. S., McKinnon, W. C., Murrell, J., & et al. (1994). Endoglin, a TGF-beta binding protein of endothelial cells, is the gene for hereditary haemorrhagic telangiectasia type 1. *Nat Genet, 8*(4), 345-351.

Mitsui, K., Tokuzawa, Y., Itoh, H., Segawa, K., Murakami, M., Takahashi, K., Maruyama, M., Maeda, M., & Yamanaka, S. (2003). The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell, 113*(5), 631-642.

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.

Nichols, J., Zevnik, B., Anastassiadis, K., Niwa, H., Klewe-Nebenius, D., Chambers, I., Scholer, H., & Smith, A. (1998). Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell, 95*(3), 379-391.

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.

Okada, Y., Gonoji, Y., Naka, K., Tomita, K., Nakanishi, I., Iwata, K., Yamashita, K., & Hayakawa, T. (1992). Matrix metalloproteinase 9 (92-kDa gelatinase/type IV collagenase) from HT 1080 human fibrosarcoma cells. Purification and activation of the precursor and enzymic properties. *J Biol Chem, 267*(30), 21712-21719.

Panetti, T. S., Chen, H., Misenheimer, T. M., Getzler, S. B., & Mosher, D. F. (1997). Endothelial cell mitogenesis induced by LPA: inhibition by thrombospondin-1 and thrombospondin-2. *J Lab Clin Med, 129*(2), 208-216.

Pardali, E., Goumans, M. J., & ten Dijke, P. (2010). Signaling by members of the TGF-beta family in vascular morphogenesis and disease. *Trends in Cell Biology, 20*(9), 556-567.

Park, S. O., Lee, Y. J., Seki, T., Hong, K. H., Fliess, N., Jiang, Z., Park, A., Wu, X., Kaartinen, V., Roman, B. L., & Oh, S. P. (2008). ALK5- and TGFBR2-independent role of ALK1 in the pathogenesis of hereditary hemorrhagic telangiectasia type 2. *Blood, 111*(2), 633-642.

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.

Pera, M. F., & Trounson, A. O. (2004). Human embryonic stem cells: prospects for development. *Development, 131*(22), 5515-5525.

Ramos-DeSimone, N., Hahn-Dantona, E., Sipley, J., Nagase, H., French, D. L., & Quigley, J. P. (1999). Activation of matrix metalloproteinase-9 (MMP-9) via a converging plasmin/stromelysin-1 cascade enhances tumor cell invasion. *J Biol Chem, 274*(19), 13066-13076.

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.

Rich, S. (1998). Clinical insights into the pathogenesis of primary pulmonary hypertension. *Chest, 114*(3 Suppl), 237S-241S.

Rich, S., Dantzker, D. R., Ayres, S. M., Bergofsky, E. H., Brundage, B. H., Detre, K. M., Fishman, A. P., Goldring, R. M., Groves, B. M., Koerner, S. K., & et al. (1987). Primary pulmonary hypertension. A national prospective study. *Ann Intern Med, 107*(2), 216-223.

Risau, W., & Lemmon, V. (1988). Changes in the vascular extracellular matrix during embryonic vasculogenesis and angiogenesis. *Developmental Biology, 125*(2), 441-450.

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.

Rizzino, A. (1988). Transforming growth factor-beta: multiple effects on cell differentiation and extracellular matrices. *Developmental Biology, 130*(2), 411-422.

Roberts, A. B., Sporn, M. B., Assoian, R. K., Smith, J. M., Roche, N. S., Wakefield, L. M., Heine, U. I., Liotta, L. A., Falanga, V., Kehrl, J. H., & et al. (1986). Transforming growth factor type beta: rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. *Proc Natl Acad Sci U S A, 83*(12), 4167-4171.

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.

Shao, E. S., Lin, L., Yao, Y., & Bostrom, K. I. (2009). Expression of vascular endothelial growth factor is coordinately regulated by the activin-like kinase receptors 1 and 5 in endothelial cells. *Blood, 114*(10), 2197-2206.

Shi, Y., & Massague, J. (2003). Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell, 113*(6), 685-700.

Shovlin, C. L., Guttmacher, A. E., Buscarini, E., Faughnan, M. E., Hyland, R. H., Westermann, C. J., Kjeldsen, A. D., & Plauchu, H. (2000). Diagnostic criteria for hereditary hemorrhagic telangiectasia (Rendu-Osler-Weber syndrome). *Am J Med Genet, 91*(1), 66-67.

Smith, A. G. (2001). Embryo-derived stem cells: Of mice and men. *Annual Review of Cell and Developmental Biology, 17*, 435-462.

Sridurongrit, S., Larsson, J., Schwartz, R., Ruiz-Lozano, P., & Kaartinen, V. (2008). Signaling via the Tgf-beta type I receptor Alk5 in heart development. *Developmental Biology, 322*(1), 208-218.

Sternlicht, M. D., & Werb, Z. (2001). How matrix metalloproteinases regulate cell behavior. *Annu Rev Cell Dev Biol, 17*, 463-516.

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.

ten Dijke, P., Goumans, M. J., & Pardali, E. (2008). Endoglin in angiogenesis and vascular diseases. *Angiogenesis, 11*(1), 79-89.

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.

Termen, S., Tollin, M., Rodriguez, E., Sveinsdottir, S. H., Johannesson, B., Cederlund, A., Sjovall, J., Agerberth, B., & Gudmundsson, G. H. (2008). PU.1 and bacterial metabolites regulate the human gene CAMP encoding antimicrobial peptide LL-37 in colon epithelial cells. *Mol Immunol, 45*(15), 3947-3955.

Terskikh, A. V., Bryant, P. J., & Schwartz, P. H. (2006). Mammalian stem cells. *Pediatr Res, 59*(4 Pt 2), 13R-20R.

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.

Tuder, R. M., Lee, S. D., & Cool, C. C. (1998). Histopathology of pulmonary hypertension. *Chest, 114*(1 Suppl), 1S-6S.

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

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.

van Meeteren, L. A., Thorikay, M., Bergqvist, S., Pardali, E., Stampino, C. G., Hu-Lowe, D., Goumans, M. J., & ten Dijke, P. (2012). Anti-human activin receptor-like kinase 1 (ALK1) antibody attenuates bone morphogenetic protein 9 (BMP9)-induced ALK1 signaling and interferes with endothelial cell sprouting. *J Biol Chem, 287*(22), 18551-18561.

Volpert, O. V., Tolsma, S. S., Pellerin, S., Feige, J. J., Chen, H., Mosher, D. F., & Bouck, N. (1995). Inhibition of angiogenesis by thrombospondin-2. *Biochem Biophys Res Commun, 217*(1), 326-332.

Williams, R. L., Hilton, D. J., Pease, S., Willson, T. A., Stewart, C. L., Gearing, D. P., Wagner, E. F., Metcalf, D., Nicola, N. A., & Gough, N. M. (1988). Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature, 336*(6200), 684-687.

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, C., Rosler, E., Jiang, J., Lebkowski, J. S., Gold, J. D., O'Sullivan, C., Delavan-Boorsma, K., Mok, M., Bronstein, A., & Carpenter, M. K. (2005). Basic fibroblast growth factor supports undifferentiated human embryonic stem cell growth without conditioned medium. *Stem Cells, 23*(3), 315-323.

Xu, R. H., Chen, X., Li, D. S., Li, R., Addicks, G. C., Glennon, C., Zwaka, T. P., & Thomson, J. A. (2002). BMP4 initiates human embryonic stem cell differentiation to trophoblast. *Nat Biotechnol, 20*(12), 1261-1264.

Xu, R. H., Sampsell-Barron, T. L., Gu, F., Root, S., Peck, R. M., Pan, G., Yu, J., Antosiewicz-Bourget, J., Tian, S., Stewart, R., & Thomson, J. A. (2008). NANOG is a direct target of TGFbeta/activin-mediated SMAD signaling in human ESCs. *Cell Stem Cell, 3*(2), 196-206.

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.

Yancopoulos, G. D., Davis, S., Gale, N. W., Rudge, J. S., Wiegand, S. J., & Holash, J. (2000). Vascular-specific growth factors and blood vessel formation. *Nature, 407*(6801), 242-248.

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.

Ying, Q. L., Nichols, J., Chambers, I., & Smith, A. (2003). BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. *Cell, 115*(3), 281-292.

Zebedee, Z., & Hara, E. (2001). Id proteins in cell cycle control and cellular senescence. *Oncogene, 20*(58), 8317-8325.

Appendix: Chromatin immunoprecipitation (ChIP)

The chromatin immunoprecipitation was performed in the following way:

Day 1:

1. Grow cells on CellStart on 2 wells of 6 well plates, 1 large culture plate or a culture flask. Remove and discard culture media and wash cells with PBS. Use 0.05% Trypsin/EDTA or TrypLE (0.3 ml for 6 well plate, 2.5-3 ml for large culture plate) and incubate at 37°C for 5 minutes.
2. Inactivate the Trypsin/TrypLE with 2,5 ml differentiation medium and transfer the supernatant to a 15 ml tube.
3. Centrifuge at 1000 rpm for 5 minutes. Wash the cell pellet once with 1 ml PBS in an eppendorf tube.
4. Resuspend the cells in 1ml 1xPBS containing 1% formaldehyde (0.27ml of 37% formaldehyde to 10ml PBS) in an eppendorf tube to cross-link and incubate at RT with gentle agitation for 10 minutes.
5. Centrifuge the cells at 5000 rpm for 2 minutes (eppendorf centrifuge) and resuspend the cells in 1ml PBS containing 0.125M Glycine (0.625ml of 2M Glycine to 10ml PBS) and incubate for 5 minutes at RT with gentle shaking to stop the cross-linking reaction and centrifuge as above.
6. Resuspend the pellet with 1ml PBS and centrifuge the cells at 5000 rpm for 2 minutes.
7. Gently resuspend the cell pellet in 400µl of cold cell lysis buffer (CLB) and incubate for 10 minutes on ice. Centrifuge at 3000 rpm for 5 minutes at 4ºC to collect nuclei.
8. Remove the supernatant and resuspend nuclei in 1ml RIPA buffer with protease inhibitors and store on ice for 10 min. Split samples into vials so there isn't more than 300 µl in each vial (for sonication) ≈ each sample into 3-4 vials
9. Sonicate samples for 15 minutes. Using these conditions, the DNA is sheared to approximately 500-2000 bp fragments.
10. After the sonication, centrifuge at 13000 rpm for 5 minutes at 4ºC.
11. Remove the supernatant to a 1.5 ml eppendorf tube and add 0.5ml of RIPA buffer with protease inhibitors, the final volume is 1.5 ml.
12. For immunoprecipitation reaction, preclear chromatin by adding 40µl protein G-separose (GE

Healthcare) and incubate for 1-2 hour at 4ºC with rotation (DynaBeads® Protein G (Invitrogen) were also used, then the following centrifugation steps were substituted for a magnetic field).

13. Centrifuge the beads at 3000 rpm for 1 minute at 4ºC. The samples are ready for immunoprecipitation reactions. Use the supernatant, 200 µl chromatin per Ti reaction and 400 µl chromatin per serum and antibody reaction as follows: total input (Ti) sample with no antibody, 3µl of Fetal Bovine Serum sample with no antibody, and finally 5µg of antibody of interest.
14. Incubate over night at 4ºC with rotation.

Day 2:

15. Add 60 µl of the homogenous protein G-Sepharose suspension per reaction (except for Ti reaction). Incubate for 1 hour at 4°C with rotation.
16. Centrifuge the protein G-Sepharose beads at 13000 rpm for 1 min at 4ºC. Save the supernatant from the Ti sample with no antibody, on ice.
17. Remove the supernatant with antibodies and serum and wash the pellet four times with 1ml of RIPA buffer. For each wash, vortex briefly and centrifuge at 13000 rpm for 1 minute at 4ºC. Leave the tubes undisturbed for a minute before removing the supernatant.
18. Wash the pellet similarly, once with 1ml of IP wash buffer 2 (IPWB2) and once with 1ml of TE pH 8.0.
19. After the last wash, centrifuge as above and remove the last traces of buffer. Use the Ti sample as well from here on. Elute antibody/protein/DNA complexes by adding 150µl of IP elution buffer and rotate for at least 15 minutes. Centrifuge at 14000 rpm for 1 min and remove the supernatants to clean tubes. Repeat and combine both elutions in the same tube (total volume = 300 µl). Elute Ti samples with 100 µl of IP elution buffer and rotate. Do not repeat.
20. Add 1.5µl of RNase A (10mg/ml stock) and 18µl of 5M NaCl (final concentration of 0.3M) to each sample with or without antibodies. Incubate the samples at 65ºC for 6 hours.
21. Add 9µl of proteinase K (10mg/ml stock) and incubate overnight at 45ºC.

Day 3:

22. Samples removed from heat block and 1 volume (same amount as in eppys) of Phenol Chloroform Isoamylalcohol with Tris alkaline added to samples (54 µl of Tris alkaline into 1 ml of Ph:Chl:Iso).
23. Vortex thoroughly and centrifuge at 13.000 rpm for 5 minutes.
24. The samples have formed two phases. Remove the upper phase to a new eppendorf tube (tilt the tube while removing the phase).
25. To precipitate nucleic acids add the following substances to each sample:

    5 µg of glycogen (0.5 µl of 20 µg/µl stock to sample)

    1/9 volume of 3M sodium acetate (pH 5.2)

    1 µl of tRNA (5 mg/ml stock)
26. Vortex, add 2 volumes of 100% ethanol to samples and precipitate at -20°C over night.

Day 4:

27. Centrifuge at 13200 rpm for at least 20 minutes at 4ºC and wash the pellet with 500µl of ice-cold 70% ethanol and centrifuge at 13200 rpm for 10 min at 4°C.
28. Remove the supernatant carefully and airs dry the pellets for 15 minutes.
29. Resuspend the pellets in 60 µl of water for the total input (Ti) and 30 µl of water for the other samples.
30. The samples are ready for further analysis by Reverse Transcription polymerase chain reaction (RT-PCR).

ChIP buffers: Chromatin Immunoprecipitation (ChIP) assay

Stock:

CLB: Cell Lysis Buffer, RIPA: RIPA Lysis Buffer and IPWB2: IP wash Buffer 2, IPEB: IP Elution Buffer.

Tris HCl (pH8)

Tris HCl (pH 7.0-7.2)

5M NaCl: 1 liter

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

1M NaCl:

100ml of 1M NaCl = 20ml of 5M NaCl and adjust the volume to 100ml with H_2O. Store at RT. (RIPA)

10% NP-40:

10ml NP-40 and adjust the volume to 100ml with H_2O.

CLB: use only 2ml to make buffer. RIPA: Use 10ml for 1L buffer. IPWB2: use only 5ml to make buffer. Store at RT.

Na butyarate 1M: 50ml

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

0.5 M EDTA:

pH = 8.0 1 liter

186.12 g disodium ethylenediaminetetraacetate – $2H_2O$ ($Na_2EDTA – 2H_20$, MW: 372.24) in 800ml H_2O stir vigorously on a magnetic stirrer. Adjust to pH 8.0 with NaOH (~ 20g NaOH pellets) and adjust volume to 1 liter H_2O. Divide into aliquots and sterilize by autoclaving. Store at RT.

Note: the disodium salt of EDTA will only solute when pH of the solution is adjusted to 8.0 by the addition of NaOH.

5M LiCl: 100ml

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

1M LiCl: 100ml

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

NaHCO3:

Elution buffer: Dissolve 1,68g of $NaHCO_3$ in 150ml H_2O stir vigorously on a magnetic stirrer. 20ml of 10% SDS mixed to the solution. Adjust volume to 200ml with H_2O. Store at RT.

10% SDS: 1 liter

Dissolve 100g sodium dodecyl sulfate crystals (SDS) in 900ml H_2O. Heat to 68°C to solute the crystals. Adjust to pH to 7.2 with HCl (-50µl). Adjust volume to 1 liter with H_2O. Dispense into aliquots and store at room temperature.

Note: the fine crystals of SDS disperse easily, wear a mask when weighing SDS and clean the weighing area and balance after use.

If SDS crystals precipitate (e.g. due to cold temperature), it can be resolved by warming the solution at 37°C.

Cell Lysis Buffer: 100ml

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

RIPA Lysis Buffer: 1 liter

150mM NaCl

0.1% SDS

0.5% deoxycholate

0.5% Triton X-100

50mM Tris (pH8)

Adjust volume to 1 liter with H_2O. Dispense into aliquots; in 15ml tubes and 50ml tubes.

For storage:

15ml tubes with RIPA Lysis Buffer and protease inhibitors. Store the solution at 4°C.

50ml tubes with RIPA Lysis Buffer. Store at RT.

IP wash Buffer 2 (IPWB2): 50ml

Adjust volume to 15ml and add 500µl of Tris HCl (pH 8.1)

12.5ml of 1M LiCl

500µl of 0.1M EDTA

5ml of 10% NP-40

10ml of 5% deoxycholic acid = DOC

Adjust volume to 50ml H_2O.

IP Elution Buffer (IPEB):

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