A new role of transcription factor SOX17 as potential interaction partner of KLF4 and EGR-1 in human coronary artery smooth muscle cells and in differentiating mouse ES-cells

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1. Introduction

The blood vessel development, comprising vasculogenesis and angiogenesis, establishes the vascular network during embryogenesis for supporting the outgrowing tissues with nutrients and oxygen. The same is the case in the adult organism. An intact vascular network is therefore important for keeping a healthy state, and its genetic pathways need to be tightly regulated. This is especially important for the two cell types, mainly composing the vascular wall, the endothelial cells and the vascular smooth muscle cells (VSMCs), the latter one being important for the stability of the blood vessel and regulating the blood pressure. As proliferation of VSMCs, e.g. in response to the growth factor PDGF-BB (Platelet derived growth factor-BB) or as consequence of high LDL (Low density lipoprotein) concentrations in the blood, is associated with severe vascular diseases, like atherosclerosis, the transcriptional regulations of these processes become more and more the focus for the development of therapeutical interventions. Therefore, transcription factors which respond to PDGF-BB and LDL in VSMCs, like it is known to be the case for Egr-1 and Klf4, are preferred candidates for further examinations. During the progression of this thesis, it appeared that the transcription factor Sox17, so far known as endodermal marker, is expressed in VSMCs in human and mice, and is moreover inducible by PDGF-BB and LDL. By this, Sox17 is a new candidate for having a regulatory transcriptional function in this cell context.

1.1. The vascular network

1.1.1. The structure of blood vessels

In humans and other organisms, one can mainly recognize three different types of blood vessels: 1) arteries, which transport the high oxygen concentrated blood to the periphery; 2) veins, which are responsible for the return of the blood to the lung and the heart; and, 3) the capillaries, which are the smallest vessels, connecting arteries and veins. As the capillaries consist of one layer of endothelial cells, they are in direct contact with the surrounding tissue and are able to promote the exchange of nutrients, oxygen and carbon dioxide. All types of vessels have one layer of endothelial cells (Intima). This layer is, in the case of the bigger vessels, the arteries and veins, surrounded by multiple layers of smooth muscle cells (Media). In form of

fiber and collagen, the adventitia connects as third layer the blood vessel with the surrounding tissue.

1.1.2. Vasculogenesis

One of the earliest events arising in organogenesis is the development of the vascular system. For the support of the developing organs during embryogenesis, oxygen and nutrients are transported by *de novo* formed blood vessels. The process of forming primitive vascular networks from endothelial progenitor cells (angioblasts) is called vasculogenesis (Risau et al., 1995). Already in this state, the determination of the vessel, becoming a vein or an artery is made, indicating that this is genetically determined. Later, this first primitive vascular network differentiates by enforcement of some vessels on the one hand, and degradation of some other vessels on the other hand ("branching and pruning"). Vasculogenesis mainly occurs in the embryo, but also in the adult organism, as one often finds in case of tumor-induced angiogenesis or ischemic injury a combination of vasculogenesis (postnatal vasculogenesis) and angiogenesis (neoangiogenesis). In this case, endothelial progenitor cells are recruited from the bone marrow and differentiate in the tumor tissue into mature endothelial cells (Asahara et al., 1997; Folkman et al., 1995; Vajkoczy et al., 2003).

1.1.3. Angiogenesis

This process describes the outgrowth of new vessels from preexisting ones, a process comprising basement degradation, migration and proliferation of endothelial cells, which form a vascular tube (Risau et al., 1997). Afterwards, pericytes and vascular smooth muscle cells are recruited. These cells settle in multiple layers surrounding the endothelial cell layer. The communication between endothelial cells and vascular smooth muscle cells/pericytes is critical for the formation of a functional vasculature (Hirschi et al., 1996; Hungerford et al., 1999) as disruptions of the interaction of both cell types is associated with severe and often lethal vascular defects (Armulik et al., 2005). Angiogenesis is an invasive cellular process, which requires the activity of growth factors (e.g. VEGF, FGF), proteolytic enzymes, extracellular matrix proteins (e.g. MMP-9) and adhesion receptors (e.g. ICAM-1,

VCAM-1, Pecam-1). These processes have to be tightly regulated. An angiogenic stimulus is followed by the enlargement of the vessel and a decrease of cell-cell contacts. Proteases of the surrounding tissue degrade parts of the stroma as well as of the basement membrane. This enables the activated endothelial cells to proliferate and migrate to form a tube structure. The same is true for the vascular smooth muscle cells or the pericytes, which also proliferate and migrate in response to different stimuli (e.g., PDGF-BB = platelet derived growth factor) and complete the formation of the new vessels (Majak et al., 1990). Both cell types still have to differentiate afterwards. Physiological angiogenesis occurs in the adult organism only during the reproductive cycle, in the placenta during pregnancy and during injury repair (Goede et al., 1998). In contrast, pathological angiogenesis occurs during tumor growth and the forming of metastases, proliferative retinopathies, chronic inflammatory diseases like psoriasis and after ischemic injury (Folkman et al., 1995; Garner et al., Vasular diseases in *Pathobiology of ocular disease*, 2nd edn.; Marcel Dekker, New York 1994).

One of the signaling pathways, implicated in vasculogenesis and angiogenesis is the wnt signaling pathway (Goodwin et al., 2002). The wnt antagonists Wnt, and FRP (Frizzled Related Protein) and the receptor Fz (Frizzled) are expressed by endothelial and vascular smooth muscle cells (Wright et al., 1999; Monkley et al., 1996; Ishikawa et al., 2001). Activation of wnt signaling is associated with angiogenesis and vascular remodeling processes, whereas an inhibition of the wnt pathway leads to vessel destabilisation. Many targets of the wnt pathway are known to play a role in angiogenesis (e.g. c-myc, cyclinD1, VEGF, MMP-7, cx43, fibronectin) (Van der Heyden et al., 1998; Ai et al., 2000; Wang et al., 2002). β-catenin, a key player in wnt signaling, is stabilized in neovascular endothelium and in neointimal smooth muscle cells in a time-dependent fashion, thereby inducing the expression of wnt-responsive genes (Blankesteijn et al., 2000; Wang et al., 2002).

1.2. Angiogenic Factors

1.2.1. Vascular Endothelial Growth Factor (VEGF) and its biological functions

For angiogenesis to take place there has to be an angiogenic signal. So far, many different molecules have been shown to induce angiogenesis, like vascular

endothelial growth factor (VEGF), acidic fibroblast growth factor (FGF), basic FGF, hepatocyte growth factor (HGF), transforming growth factor (TGF) α and β, tumor necrosis factor α (TNF-α), interleukin (IL)-8, angiogenin and the angiopoietins (Folkmann et al., 1992; Yancopoulos et al., 2000; Ferrara et al., 1997).

Hypoxia is the central stimulus for induction of angiogenesis, as it induces the transcription of HIF-1α (Hypoxia induced factor-1 alpha) and thereby the expression of VEGF (Semenza et al., 2002; Tsuzuki et al., 2000; Liu et al., 1995; Forsythe et al., 1996; Shweiki et al., 1992). The latter is secreted as homodimer and provides structural similarity to platelet derived growth factor (PDGF) (Keck et al., 1989). It is responsible for the induction of vasculogenesis as well as of angiogenesis (Ferrara et al., 1989; Leung et al., 1989; Xiu et al., 1995). A disrupture of a single allele of VEGF causes abnormal blood vessel formation and embryonic lethality (Carmeliet et al., 1996; Ferrara et al., 1996). In contrast to other growth factors, like the fibroblast growth factors, VEGF is an endothelial cell specific mitogen (Leung et al., 1989; Plouet et al., 1989). The VEGF factors comprise five members, named VEGF-A to VEGF-E, of which VEGF-A is the best characterized protein, being able to activate endothelial cells, whereas VEGF-C and VEGF-D regulate lymphatic angiogenesis (Karkkainen et al., 2002; Leung et al., 1989; Plouet et al., 1989; Nagy et al., 2002). To activate endothelial cells, VEGF-A has to bind to its specific receptors, which belong to the tyrosine kinase family (Neufeld et al., 1999; Terman et al., 1992). Two VEGF receptors exist, VEGFR-1 (flt-1) and VEGFR-2 (flk-1) (Shibuya et al., 1990; Terman et al., 1991; Gerber et al., 1997; Waltenberger et al., 1994; Maru et al., 1998; Fong et al., 1995). The importance of Flk-1 becomes clear, as mice deficient for this receptor show a failure of blood island development and vasculogenesis (Shalaby et al., 1995). The receptors are only able to activate signal transduction if they build homodimers upon binding of VEGF. Thereby, kinases of each receptor phosphorylate tyrosine residues of the interacting receptor. This is the initiation of the signaling cascade in the endothelial cells (Matsumoto et al., 2001). It follows a series of different phosphorylations, involving the protein kinase C, MAPK and ERK-1/2 which leads to expression of MMP (matrix metalloproteinase), eNOS (endothelial specific NO-synthetase) and cyclin D1. Another pathway leads to activation of vinculin and by this to migration of endothelial cells. An important function of VEGF is the increase of the vessel permeability (Dvorak et al., 1995). Thereby, blood plasma

proteins can extravasade and interact with the endothelial and vascular smooth muscle cells. Moreover, VEGF is known to be the key mediator of survival of endothelial cells, preventing serum-starvation induced apoptosis via the phophatidylinositol (PI)-3 kinase -Akt pathway and inducing the expression of the anti-apoptotic proteins Bcl-2 and A1 in endothelial cells (Gerber et al., 1998; Benjamin et al., 1999; Yuan et al., 1996).

1.2.2. The Fibroblast Growth Factors (FGFs)

Another group of angiogenic factors includes the fibroblast growth factors (FGFs). They are a heparin-binding protein group of 23 members, with differentiation-promoting, growth and antiapoptotic properties (Basilico et al., 1992). As FGFs show mitogenic activity on different cell types, they are, in contrast to VEGF, not endothelial cell specific since most cell types express FGF receptors. The most potent angiogenic stimuli in this group are FGF1 and FGF2. Uncontrolled expression of FGF2 is associated with neovascularization, tumor growth, and progression of atherosclerotic plaque development, as T-lymphocytes, infiltrating in the diseased tissues, release FGF2 (Peoples et al., 1995). Moreover for FGF2 an autocrine or paracrine role in T-lymphocytes has been proposed, as these cells express FGF2 and provide in parallel heparin-binding FGF-like bioactivity (Peoples et al., 1995; Blotnick et al., 1994).

1.2.3. The angiopoietins and their receptor Tie2

The angiopoietin family consists of four members, of which angiopoietin1 (Ang1) and angiopoietin2 (Ang2) have a very important angiogenic function (Suri et al., 1996; Tian et al., 2002). About Ang3 and Ang4 not much is known. Both, Ang1 and Ang2, bind to the Tie2 receptor, a transmembrane tyrosine kinase receptor, which is expressed early in the embryo. Tie2 is endothelial cell specific and is only activated, when the angiopoietins bind to it as oligomers. Disruptions in the Tie2 receptor or its agonist ligand Ang1, are associated with severe vascular malformations, caused by a reduced or lack of vascular smooth muscle cell (VSMC) recruitment whereas an activating mutation in the *tie2* gene results in venous malformations, characterized by abnormal SMCs on the vascular wall (Vikkula et al., 1996; Sato et al., 1995; Suri et

al., 1996). Therefore the Tie2 signaling pathway is considered to be critical for endothelial cell - smooth muscle cell communication.

Ang1 and Ang2 have been reported to behave as competitive antagonists, as Ang1 stabilizes the vessel integrity after binding to Tie2, whereas Ang2 leads to destabilization, preventing the binding of Ang1 to Tie2, and thereby inhibiting the Ang1 dependent signaling cascades (Maisonpierre et al., 1997). Moreover Ang2 supports the disconnection of endothelial and vascular smooth muscle cells and the degradation of the extracellular matrix. By this, the migration of endothelial cells is initiated. Therefore, Ang1 and Ang2 show a different expression pattern, the latter primarily expressed in the growing vessels, whereas Ang1 is mainly expressed in matured vessels (Maisonpierre et al., 1997).

1.2.4. The Hepatocyte Growth Factor (HGF)

Another factor, providing angiogenic stimulatory properties is the mesenchymal-derived hepatocyte growth factor (HGF). Being expressed in different cell types, among them endothelial and vascular smooth muscle cells, this factor is implied in different cellular responses, comprising cytoskeleton reorganization, growth, and motility (Wolf et al., 1991; Torok et al., 1996). It has been reported, that HGF induces SMC migration via binding to its known receptor c-met, activating an ERK1/2 signaling cascade (Ma et al., 2003; Taher et al., 2002; Aoyagi et al., 1999). By Kobayashi et al., (2006) it has been demonstrated, that Ang1 induces HGF expression in endothelial cells (ECs), which leads to the recruitment of SMCs towards the ECs. This effect was shown to be abrogated by Ang2. This finding demonstrates a new regulatory mechanism of SMC recruitment, involving Ang1 and Ang2, as well as HGF.

1.2.5. The Platelet Derived Growth Factor (PDGF)

The group of Platelet Derived Growth Factors (PDGFs) is composed of A, B, C, and D chains, occurring in different constellations as homo- or hetero-dimers of two different chains. These dimers specifically interact with homo- or hetero-dimers of tyrosine kinase possessing receptors. Thereby, the PDGFs provide angiogenic effects, are implicated in embryogenesis, platelet activation and in pathophysiological

processes such as atherosclerosis, restenosis, fibrosis, and tumorigenesis (Heldin et al., 1999; Cao et al., 2002; Ding et al., 2000; Fang et al., 2004). PDGF-A, B, as well as PDGF-C have been found to be expressed in vascular SMCs of the intact arterial wall, providing mitogenic effects on the SMCs, significantly effecting their proliferation and migration (Dijkmans et al., 2002; Uutela et al., 2001; Heldin and Westermark, 1999). Therefore, an inactivation of PDGF-B in transgenic mice results in vascular defects with loss of pericytes and VSMCs (Lindahl et al., 1997). Important regulators of the transcription, at sites of stress and mechanical injury, of PDGF-A and PDGF-B chain are Egr-1 (Early growth response factor-1) and Sp1 (Khachigian et al., 1996; Khachigian et al., 1997).

1.2.6. The ephrin ligands and their receptors

Another group of important factors involved in angiogenesis are members of the family of ephrin ligands and ephrin receptors. They are found in many different cell types and are not restricted to endothelium. Both the ephrin ligand and the ephrin receptor are membrane-bound. Depending on how the ephrin ligands are anchored in the plasma membrane, they are divided into type A and type B. The same is the case for the receptors, which are also devided into EphA and EphB. Corresponding ligand and receptor preferentially bind to each other. The most important functions of the ligands and the receptors of the Ephrin family during angiogenesis are the mediation of cell-adhesion to extracellular matrix, the cell migration and juxtacrine cell-cell contacts (Cheng et al., 2002a). Ephrins found in the vascular cells are ephrin A1, which plays a role in the tumor necrosis factor α induced inflammatory angiogenesis, and ephrin B1 that promotes endothelial capillary-like assembly and attachment *in vivo* (Pandey et al., 1995; Stein et al., 1998). Moreover, the ephrin receptors EphB3 and EphB4 and Ephrin B2 are expressed in the vascular cell context. The fact, that an abrogation of the EphA receptor results in a specific inhibition of VEGF-induced angiogenesis, underlines the importance of this protein family in this vascular context (Cheng et al., 2002b). Another indication for the implication in pathological angiogenesis is the detection of high expression levels of ephrin A1 and EphA2 in tumor angiogenesis (Ogawa et al., 2000). Similar effects have been reported for ephrin B2 and EphB4 (Martiny-Baron et al., 2001).

1.3. Vascular processes in embryonic development

1.3.1. Vascular development along the mammalian body axis

By *de novo* aggregation of angioblasts along the anterior/posterior body axis, the dorsal aorta and cardinal vein occur in response to VEGF, secreted by the endoderm. In response to sonic hedgehog (Shh), produced by the notochord, the somites produce VEGF and thereby support angiogenic processes, building the intersomitic vessels (Vokes et al., 2004). The notochord secrets moreover bone morphogenetic protein (BMP) inhibitors, noggin and chordin, and thereby determines the patterning of the axial vasculature (Reese et al., 2004; Nimmagadda et al., 2005). By this, an avascular region around the notochord is built. Later on, the secretion of VEGF from the neural tube results in the recruitment of somite-derived angioblasts. These cells form the perineural vascular plexus, which encases the neural tube at midgestation. Further on, sonic hedgehog signaling mediates the angiogenic sprouting within the neural tube.

1.3.2. Vascular processes in the developing liver and prancreas

The liver derives from the ventral foregut endoderm. At this early time point, it appears as a multilayered epithelium, surrounded by endothelial cells (ECs). The ECs invade into this presumptive liver bud and aggregate into sinusoids, while hepatoblasts begin to migrate from the endoderm into the underlying septum transversum. These liver sinusoidal endothelial cells (LSEC) respond to VEGF and are thereby able to secrete hepatic mitogens, hepatic growth factor (HGF) and interleukin-6 (IL6) (Le Couter et al, 2003). In consequence, hepatic growth is promoted and hepatocytes are protected from toxic insult. Thereby endothelial cells are essential for the development of the liver in the embryonic state and have the capacity, after stimulation, to provide trophic and nutritional support to a damaged adult liver (Matsumoto et al., 2001).

In case of the developing pancreas, which forms in close association with the dorsal aorta and vitelline veins, endothelial cells also associate with pancreatic endocrine islet cells (e.g. insulin producing cells). These endocrinic cells can directly secrete hormones in the blood. On the other hand, ECs influence the differentiation of

pancreatic cells and support thereby the stabilization of a functional pancreas. In this respect, the dorsal aorta has been implicated as being necessary for the insulin production in endoderm tissue (Lammert et al., 2003; Yoshitomi et al., 2004).

1.3.3. Vascular processes in the developing kidney

Podocytes are specialized cells that build the support structures of the functional glomerulus. They express high levels of VEGF and attract thereby endothelial cells and promote migration and maturation of the ECs. On the other hand, ECs promote the maturation of the podocytes and mesangial-cells and thereby the formation of a functional glomerulus. Throughout life, the functioning of the glomerulus is highly dependent on endothelial function controlled by precise VEGF levels (Eremina et al., 2003; Mattot et al., 2002).

1.3.4. Vascular processes in placental development

For the interchange of oxygen, nutrients and growth factors between the maternal and fetal side, the establishment of a vascular system is necessary. From the maternal side, spiral arteries enter the placenta, where they underlie an invasion process of fetal trophoblasts. Moreover, fusion of the allantois to the chorion from the fetal side is necessary, and a branching invasion of the fetal capillaries into the chorionic trophoblast. To initiate these vascular processes, the placenta secrets in parallel with the standard angiogenic factors also some placenta specific ones, like placental lactogen-related hormones, proliferin, proliferin-related protein and placental-like growth factor (PlGF) which can exert angiogenic and anti-angiogenic actions on the placental vasculature (Antiero et al., 2003; Jackson et al., 1994). PlGF is a member of the VEGF family and is strongly expressed in the placenta where it enhances, by binding to Flt1, the VEGF signaling through Flk1 (Maglione et al., 1991; Park et al., 1994).

1.4. Pathological Angiogenesis

The vascular network has to be tightly regulated to keep the blood vessels intact. An imbalance of proliferation and differentiation of vascular cells, induced by pro- and

anti-angiogenic factors, results in vascular malformations and in vascular disease (pathological angiogenesis). So far, disorders in angiogenesis have been implicated in more than 70 diseases. Except of the cycling ovary and the placenta during pregnancy, angiogenesis normally does not occur in the adult organism, where most of the blood vessels remain quiescent. Nevertheless, endothelial cells and vascular smooth muscle cells retain their ability of fast phenotypic switching and rapid proliferation in response to environmental stimuli like hypoxia or growth factors (Carmeliet et al., 2003). During this process, angiogenesis is activated in repair processes. In case of a disruption of the balance of stimulatory and inhibitory signaling, thereby an angiogenic switch is caused, which mostly results in malignant, ocular, and inflammatory disorders. If the switch causes endothelial cell dysfunction, diseases like ischaemic heart disease or preeclampsia are the consequence (Soman et al., 2006; Semenza, 2003). Vessels are malformed or regressed and revascularization and regeneration is prevented. Moreover angiogenic processes play a decisive role in tumor progression and metastasis, supplying the growing tumor with new vessels, which transport O_2 and nutrients to the malignant tissues (tumor angiogenesis) (Kerbel, 2000). Because of this fact, the vascular cells constitute an important target for pharmaceutical interventions in tumor growth. An example for such an intervention is the recently used anti-VEGF antibody for preventing pathological angiogenesis (GENETECH). An inhibition of VEGF-induced angiogenesis has been described to suppress tumor growth *in vivo* (Kim et al., 1993).

The vascular disease with the highest mortality rate in the western world is atherosclerosis. The development of this disease is initiated by a disruption of the integrity of the intima, in most cases caused by high LDL (low density lipoprotein) concentrations in the blood, but also by a high blood pressure lasting for longer time periods, or a combination of both factors. A high LDL concentration is in some cases genetically predicted, but in most cases a consequence of high fat diet, smoking and lack of exercise. As a result of the disruption of the endothelium, it leads to an inflammatory reaction. Leukocytes, like macrophages and neutrophils adhere to the intima and extravasade in the subendothelial space. The leukocytes secrete cytokines, which stimulate the disruption of cell-cell contacts in the vascular cell layers. Furthermore, proliferation of the vascular smooth muscle cells is induced, e.g. by PDGF, causing a phenotypic switch (Heldin and Westermark, 1999). The media

gets thicker and starts to expand in the lumen of the vessel. Thereby, big plaques, mainly consisting of vascular smooth muscle cells and activated macrophages, arise and lead, in the worst case scenario, to a complete closure of the blood vessel causing severe ischemic injury to tissues downstream of the occluded vessel. During the progression of the atherosclerotic plaques, some areas calcify. It was shown that some vascular smooth muscle cells undergo transdifferentiation into chondrocytes under these pathological conditions (Bobryshev, 2005; Abedin et al., 2004). Thereby the vascular SMCs loose their myofilaments and acquire the ability to produce type II collagen. An important transcription factor, involved and strongly upregulated in this calcification process, is Sox9, which has been also implicated in chondrocyte development during embryogenesis (Wright et al., 1995; Lefebvre et al., 1998). So as one can see from the example of atherosclerosis, vascular smooth muscle cells have a high potential for being targeted for therapeutic intervention.

1.5. Vascular cells

Different cell types contribute to the composition of the vascular blood vessel wall. These are on the one hand the endothelial progenitor cells, which derive from the bone marrow and differentiate at the sites of the outgrowing vessels into mature endothelial cells, and on the other hand the vascular smooth muscle cells, which surround the endothelial cells, thereby stabilizing the blood vessel. The communication of both, the endothelial and the smooth muscle cells has to be tightly regulated to provide an intact vascular network.

1.5.1. Endothelial progenitor cells

The endothelial progenitor cells originate in many areas of the embryo, including the blood islands in the yolk sac and differentiate in the periphery of these islands. During vascularization of the embryo, embryonic endothelial progenitor cells, form primitive vascular tubes and start to differentiate into mature endothelial cells. In the adult, endothelial progenitor cells, deriving from the bone marrow, circulate in the blood and are recruited during neovascularization, which can take part in the case of tissue ischemia, vascular trauma, and tumor growth (Asahara et al., 1997; Folkman et al., 1995; Vajkoczy et al., 2003; Takahashi et al., 1999). Markers of the endothelial

progenitor cells are Tie2, c-Kit, Sca-1, CD34 and low Flk1 (Yamaguchi et al., 1993). As adult EPCs are difficult to isolate and maintain for further examinations, one might use mouse embryonic EPCs as a model system (Hatzopoulos et al., 1998).

1.5.2. Mature endothelial cells

After tube formation, the endothelial progenitor cells start differentiating into mature endothelial cells, marked by the expression of CD31 (Pecam-1), VEGFR-2 (Flk-1), VE-cadherin, Tie-1 and Tie2 (Sato et al., 1995). Pecam-1 is an adhesion molecule, which belongs to the immunoglobulin gene superfamily and is mainly expressed by endothelial cells (Newman et al., 1990; Simmons et al., 1990). VE (Vascular Endothelial)-Cadherin is an important endothelial specific cell-junction protein, whose targeted null-mutations result in abrogation of vascular structure formation (Vittet et al., 1997). After differentiation and tube formation, the mature endothelium stays in a quiescent state. Nevertheless, endothelial cells have the ability to quickly respond to environmental changes (angiogenic stimuli) by changing their phenotype from a quiescent cell to a proliferating and migrating one (Carmeliet et al., 2003). As already mentioned before, ECs communicate directly with adjacent cells or tissues, releasing different cytokines and growth factors, as for example PIGF during vascular processes in placental development. But not only the ECs signal, but also the surrounding tissue itself signals back to the ECs, resulting in a reciprocal signaling cascade. Thereby, the ECs in different tissues differ, concerning their morphological appearance, but also their expression pattern. The EC phenotype is divided in continuous, fenestrated or discontinuous (Majno et al., 1977). ECs, lining the microvessels are often fenestrated, as well as the ones in the liver, which are additionally often also discontinous. An example for a continuous endothelium is the endothelial layer of the capillaries in skeletal muscle, heart, lung and brain. Moreover, one can also find a functional heterogeneity of the ECs, playing a role in vasodilation, vasoconstriction, blood coagulation and anticoagulation, acute inflammation, wound healing, leukocyte homing and diapedesis. Other functions comprise fibrinolysis, taking part in acute inflammation, atherogenesis, antigen presentation and catabolism of lipoproteins (Gerritsen et al., 1987). Examples for different expression patterns between endothelial cells include the transcriptional differences between arteries and veins. It was found, that Ephrin B2 was expressed in arteries, whereas

Ephrin B4 was much higher expressed in veins than in arteries (Wang et al., 1998). Moreover, an artery specific expression of Notch and Gridlock genes has been shown in zebrafish embryos (Lawson et al., 2001; Zhong et al., 2000).

1.5.3. Vascular Smooth Muscle Cells (VSMCs)

The vascular smooth muscle cells are surrounding the endothelial cell layer in multiple sheets in case of the big vessels and are thereby functioning as regulators of vessel stability and blood pressure. During angiogenic processes, we find on the one hand the endothelial cell sprouting process, leading to a vascular tube formation, and on the other hand the recruitment of perivascular cells. These perivascular cells comprise the smooth muscle cells (SMCs), in case of large vessels, and the pericytes at sites of microvessels (vascular maturation). These processes need a tightly regulated communication between endothelial cells and perivascular cells. Disruptions in these interactions mostly result in severe vascular defects. The Angiopoietin/Tie2 signaling is known to play a key role in the vascular maturation process (Morisada et al., 2006). This was shown by generating mice with defects in Tie2 or its ligand angiopoietin 1. These mice die during embryogenesis because of a reduced or complete lack of recruitment of vascular SMCs (Sato et al., 1995; Suri et al., 1996). As already mentioned above, Ang1 is able to recruit SMCs and participates in vascular maturation having a stabilizing function, whereas binding of Ang2 to tie2 causes destabilization of the EC - SMC interaction and initiates new vessel sprouting.

Factors, that are known to regulate migration of vascular smooth muscle cells, are platelet-derived growth factor (PDGF), basis fibroblast growth factor-2 (bFGF), and transforming growth factor (TGF) (Majack et al., 1990). PDGF-BB has been demonstrated to induce expression of bFGF in vascular smooth muscle cells via the ERK 1/2 cascade (Pinutcci and Mignatti, 2005). Moreover, hepatocyte growth factor (HGF) has been shown to induce migration of vascular SMCs, being induced by Ang1 (Kobayashi et al., 2006). Ang2 blocks Ang1 induced HGF production and thereby vascular SMC recruitment.

Factors, involved in the differentiation of SMCs are msx2 and necdin (Brunelli et al., 2005). Moreover the serum response factor (SRF) plays a critical role in smooth myogenesis, as mice deficient for SRF show a strong reduction of differentiated SMCs. TGF-β1 has also been implicated in the differentiation of vascular SMCs, inducing the expression of a number of smooth muscle differentiation markers, like smooth muscle myosing heavy chain (SMMHC), smooth muscle alpha-actin and smoothelin (Hautmann et al., 1999; Chambers et al., 2003). The latter is a cytoskeletal protein whose distribution is restricted to smooth muscle cells (Van der Loop et al., 1996; Krämer et al., 2000). The expression of smoothelin differs from vessel to vessel (Johansson et al., 1999). Arteries, being smaller than 2mm, show a strong expression of smoothelin, whereas the expression decreases with increasing vessel size. Another factor, being important for SMC differentiation is myocardin, as mice deficient for this gene, show a strong reduction of SM cells (Du et al., 2003).

Vascular smooth muscle cells are known to occur in two different states: on the one hand the proliferating (synthetic) cell and on the other hand the differentiated (contractile) one. They are able to change between these two phenotypes in response to changes in local environment (Owens et al., 1996; Owens et al., 2004). A factor known to participate in this phenotypic switching is Notch. It has been demonstrated that Notch decreases SMC differentiation marker expression in a CBF-1/RBP-Jκ-dependent manner in human arterial SMCs (Morrow et al., 2005). Such regulations are important, e.g. after vascular injury, when the SMCs have to change their phenotype, from the differentiated and contractile state to the proliferating one, to support the growth and repair of the injured vessels (Bär et al., 2002).

To sum up the most important features, vascular SMCs play a decisive role in the stability of a blood vessel and the tight regulation of blood pressure. Therefore arteries, which sustain high blood pressure, show multiple layers of SMCs, whereas veins, which have a lower blood pressure, have fewer layers of SMCs. During angiogenesis, the SMCs assume a proliferating state with high migratory abilities (synthetic state). Afterwards, the SMCs change their appearance under the influence of the environment and acquire the differentiated state, characterized by the expression of vascular SMC markers, like smoothelin, alpha smooth muscle actin (alpha-SMA), smooth muscle myosin heavy chain (SMMHC), Calponin, and

SM22alpha (Solway et al., 1995; Zhang et al., 2001; Van der Loop et al., 1996; Van der Loop et al., 1997). This is the case in adult vessels, when the SMCs primarily occur in the quiescent form (contractile function). The SMCs change this appearance in case of vascular injury or vascular diseases like atherosclerosis (Bär et al., 2002; Yutani et al., 1993; Ross et al., 1999; Schwartz et al., 1998). Moreover, this phenotypic switch is associated with the pathogenesis of restenosis following coronary angioplasty and/or stent implantation, pulmonary hypertension, and asthma (James et al., 1989; Newby et al., 1999) One factor regulating this phenotypic modulation from differentiated to proliferating cells is Notch (Morrow et al., 2005).

Moreover, one can divide SMCs to tonic or phasic phenotypes. The vascular SMCs belong to the tonic cell type, which means that these cells have a slow rate of force activation and relaxation, lower maximum speeds of shortening and good force maintenance. The phasic SMCs are mainly found in the gastrointestinal tract and show a high rate of force activation and relaxation, high maximum speeds of shortening and poor force maintenance.

Smooth muscle cell are known to arise from different precursor lineages during embryogenesis. Coronary smooth muscle cells for example are partly derived from proepicardial cells (Landerholm et al., 1999; Mikawa et al., 1996). In contrast to this, smooth muscle cells from the thoracic aorta and of the aortic arch are mainly derived from migrating neural crest cells (Itu et al., 1993; Topouzis et al., 1996; Bergwerff et al., 1998). In the case of the peripheral vasculature, smooth muscle cells are recruited from the surrounding mesenchyme by endothelial cells (Roberts et al., 2000). Because of these different origins, it is not surprising, that multiple factors regulate the differentiation of the vascular SMC subtypes. One group of proteins, implicated in these differentiation processes is the Hox protein group. Hoxa10 for example has been shown to specifically activate the expression of telokin, which is exclusively expressed in SMCs of the uterus and the colon (El-Mounayri et al., 2005). Opposite to this, Hoxb8 represses the activity of many SMC specific genes (El-Mounayri et al., 2005).

1.6. The Sox (<u>S</u>ry b<u>ox</u>) proteins

As the transcriptional regulation of proliferation and differentiation of vascular cells plays a crucial role in the maintenance of the vascular network, transcription factors involved in these processes become more and more the focus of strategies against vascular disease. The Sox protein group comprises some proteins which are known to play a role in vasculogenesis and angiogenesis, and becomes therefore interesting for further examinations. The sox proteins build a group of transcription factors that have been implicated in many different developmental and proliferative processes in different tissues (Wegner et al., 1999; Pevny et al., 1997; Bowles et al., 2000). They are expressed during embryogenesis as well as in adulthood. The Sox proteins are highly conserved during evolution and expressed from Drosophila to man (Soullier et al., 1999). The main feature of this group is the DNA binding domain, the so-called High mobility group domain (HMG), which is highly conserved between the different members (Coriat et al., 1993; Denny et al., 1992). The HMG box is a 79 aminoacid domain, which binds to the minor groove of the DNA and bends it in a sequence-specific manner (Grosschedl et al., 1994). The name of the Sox protein group derives from the HMG-box containing sex-determining gene *sry* (SOX = sry box), which is part of this protein family (Harley et al., 1994). This gene is located on the Y chromosome and is responsible for initiating testis development during mammalian embryogenesis (Sinclair et al., 1990; Gubbay et al., 1990). SRY shares around 50% homology within its HMG domain with the SOX proteins. Besides binding to the DNA, the HMG domain provides different features.

These are the interactions with other transcription factors, like Importin, for the transport into the nucleus, and with adapter proteins, for the formation of multiprotein complexes. Depending on the homology in their HMG domain, the Soxes are divided into seven subgroups, in which the members of each subgroup share more than 80% homology. More than 30 members are known so far, building groups A-G (Bowles et al., 2000).
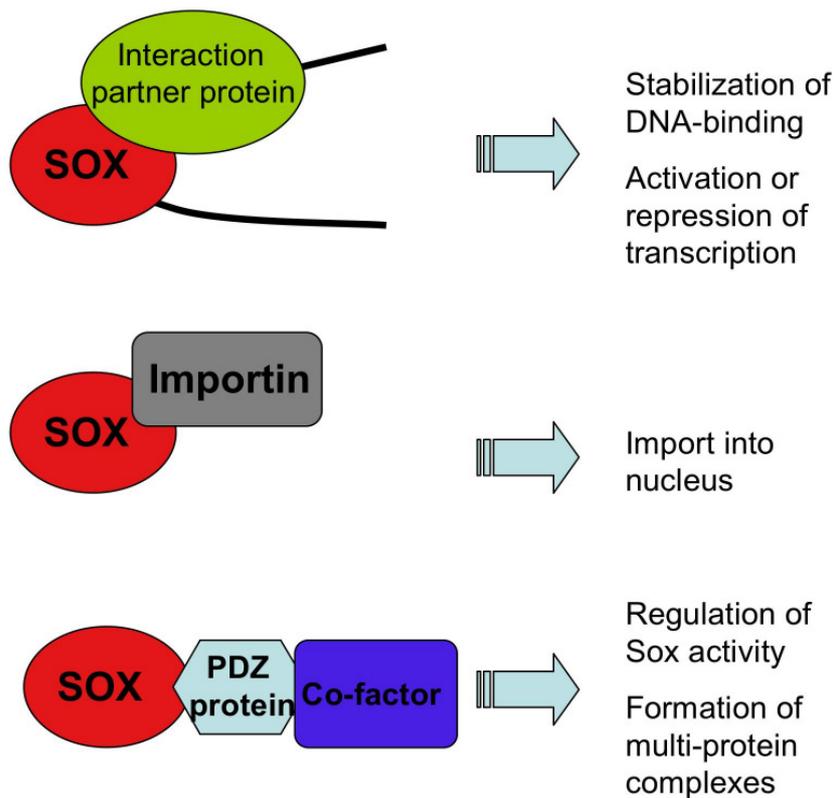
Figure 1.1 Protein-Interactions of the Sox proteins. The Sox proteins are able to bind to other transcription factors, importin (via the NLS sequence in the HMG domain), and adapter proteins for building multi-protein complexes.

The Sox proteins have been implicated in many different developmental processes. Sox9, for example, is involved in sex-determination and chondrogenesis, whereas Sox1 is a crucial factor in lens development (Wright et al., 1995; Kent et al., 1996; Morais da Silva et al., 1996). Another example is Sox4, which is taking part in cardiac development and lymphocyte differentiation (Geijsen et al., 2001; van de Wetering et al., 1993). An important feature of the Sox protein group is the fact that they have to interact with another protein to transactivate the expression of their target genes and the fact that different Sox proteins can be expressed in the same cell type at the same time (Kuhlbrodt et al., 1998; Lefebvre et al., 1998). The partner a Sox protein interacts with is cell-type specific and the biological readout is dependent on the interaction partner (Wilson and Koopman, 2002). By this, the Sox factors can interact with different proteins, depending on the context, and thereby inhibit or activate the transcription of putative target genes (Kamachi et al., 2000). For this reason, Sox proteins are considered to be bifunctional. For example, Sox2 is known to interact with Oct3/4 in embryonic stem cells, but also cooperates with Pax6 in lens cells to enhance the expression of *delta-crystallin* (Kamachi et al., 2001; Botquin et al., 1998;

17

Nishimoto et al., 1999). Another example is Sox17, which binds to β-catenin in the context of the wnt-signaling and thereby inhibits the transcription of β-catenin target genes (Takash et al., 2001). On the other hand, Sox17 is responsible for the induction of early endodermal genes, like Foxa1, Foxa2 and GATA4 (Sinner et al., 2004). Another characteristic ability of Sox family members is the fact that some of them are able to compensate for the function of each other acting in redundant fashion as in the case for Sox7 and Sox17 during early endodermal development (Kanai-Azuma et al., 2002).

1.6.1. Sox protein subgroup F – Sox 7, Sox 17 and Sox 18

As already mentioned before, some sox proteins are known to play a crucial role in vascular development, two of them are members of the subgroup F, which comprises Sox7, Sox18 and Sox17, the latter one known as endodermal factor. In cyclic AMP treated endothelial progenitor cells, all three factors are induced, leading to the assumption that all of them are decisive transcriptional proteins during vascular outgrowth (Antonis Hatzopoulos, 2002; data not published). The first member of subgroup F is Sox7, a transcription factor that has so far been shown to play a role in early endodermal development, but also in vasculogenesis during embryonic development (Takash et al., 2001). *Xenopus* Sox7 is supplied maternally (Fawcett and Klymkowsky, 2004). It is known, that Sox7 transactivates, in concert with Sox17, the expression of Laminin a1 in parietal endoderm (Niimi et al., 2004). Sox7 is co-expressed with Sox17 in the extra-embryonic endoderm and a functional compensation of both factors in this context has been supposed (Kanai-Azuma et al., 2002). Both, Sox7 and Sox17 induce the expression of the pan-endodermal marker endodermin and the expression of fibronectin (Shirai et al., 2004). Moreover, it has been demonstrated, that Sox7 is an immediate-early target of VegT in *Xenopus* (Zhang et al., 2004). VegT, a T-box transcription factor, initiates mesoendodermal differentiation (Xanthos et al., 2001; Zhang et al., 1998). As downstream target of VegT, Sox7 induces expression of the Nodal-related genes *Xnr1, 2, 4, 5* and *6* (Zhang et al., 2004). Moreover it is able to initiate the transcription of the homeodomain transcription factor *Mixer*, and the endodermal marker *SOX17β* in *Xenopus* (Zhang et al., 2004). Sox7 is, like Sox3 and Sox17, able to inhibit β-catenin/TCF (T-cell factor) signaling via direct binding to β-catenin (Takash et al.,

2001). It has been demonstrated, that Sox7 and GATA4 are competitive activators of Fgf-3 expression (Murakami et al., 2004).

Sox17 was at first described as stage-specific transcription activator during mouse spermatogenesis (Y. Kanai et al., 1996). In *Xenopous*, two forms of Sox17 have been discovered, called Sox17α and Sox17β (Hudson et al., 1997). These two forms are known to play an inhibitory role in the wnt-signaling pathway by binding to β-catenin/TCF and thereby blocking the DNA binding domain of the two factors (Zorn et al., 1999). Moreover, Sox17 has been implicated in the determination of endoderm in *Xenopus*, mouse and zebrafish (Hudson et al., 1997; Kanai-Azuma et al., 2002; Alexander et al., 1999). Mice deficient for Sox17 do not form gut endoderm (Kanai-Azuma et al., 2002). It has been observed that Sox17 is crucial for the maintenance and differentiation of the definitive endoderm of the embryonic gut. The phenotype of Sox17 knock out mice includes apoptosis of the endoderm cells in the foregut. Moreover, the endoderm of the mid- and hindgut does not expand. So Sox17 is a decisive factor for the differentiation of ES-cells to the endodermal lineage. Of the three members of subgroup F, only Sox17 is expressed in the definitive gut endoderm. Sox18 is absent in the endoderm, whereas Sox7 is co-expressed with Sox17 in the extra-embryonic visceral endoderm (Kanai-Azuma et al., 2002). This and the fact that there are no severe defects in the visceral endoderm of Sox17-/- mice might be due to the fact that Sox7 is able to compensate Sox17 function in this specific context. In the endodermal development, different target genes of Sox17 have been identified. It has been shown, that Sox17 binds to the promoter regions of Laminin a1 (Lama1) and Fibronectin during endodermal development (Niimi et al., 2004; Shirai et al., 2004). Via its C-terminal transactivation domain, Sox17 stimulates expression of its target genes, which include Foxa1 and Foxa2 (Sinner et al., 2004). In humans, Sox17 shows a wide expression pattern, being detected in heart, lung, spleen, testis, ovary, placenta, gastrointestinal tract, fetal lung, and kidney.

Sox18, the third member of subgroup F, is known to be a key player in vascular development, being involved in endothelial cell specification. It is transiently expressed in the embryonic vasculature, in the intersomitic vessels, but also in the adult organism when neovascularization takes place (Darby et al., 2001).
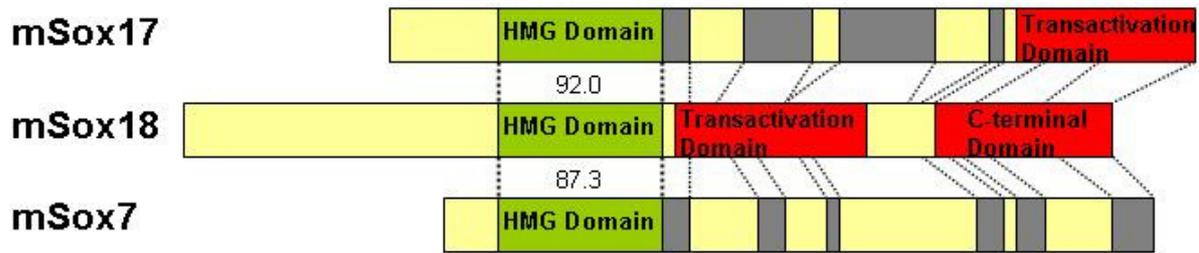
Figure 1.2 Structural comparison of murine subgroup F Sox proteins. The known functional areas are labeled in green and red. The percentages indicate the degree of similarity of the HMG domain of the three Sox proteins. The grey areas show significant similarities of murine Sox17 and Sox7 to murine Sox18. The dotted lines indicate the corresponding regions of the three soxes.

During embryogenesis, Sox18 is first detected in the allantois and in the yolk sac blood islands at 7.5 dpc and persists at these sites until 8.5 dpc (days post coitum). Subsequently, Sox18 is expressed in the paired dorsal aortae and the developing cardiovascular system (Pennisi et al., 2000b). During embryogenesis and wound repair in adults, Sox18 shows a similar expression pattern as Flk-1 (fetal liver kinase-1) and Collagen IV, two endothelial-specific genes known to be induced during pathological angiogenesis (Darby et al., 2001; Pennisi et al., 2000b). This, and the fact that Sox18 is absent in Flk1 knockout mice, underlines the importance of Sox18 in earliest stages of vascular development and during neovascularization processes in adult organisms suggesting that it might be an early target of the VEGF/VEGFR axis. Sox18 is moreover detected in the developing hair follicles (Olsson et al., 2001). In adult tissues, Sox18 is detectable in lung, heart, and skeletal muscle tissues. Four naturally occurring Sox18 allelic mutations are known, called *Ra, RaJ, Ragl* and *RaOP* which cause severe defects in hair and skin development and cardiovascular defects (Pennisi et al., 2000b). In contrast, a mouse deficient for Sox18 is viable and shows only mild phenotypic changes suggesting that the allelic mutants might have a more severe, dominant negative effect (Pennisi et al., 2000a). The Sox18-/- mice also show a small reduction in frequency and pigmentation of coat hairs (Pennisi et al., 2000a). Moreover, VCAM1 has been shown to be a direct target of Sox18 (Hosking et al., 2003).

1.7.   Krüppel-like Factor 4 (KLF4)

KLF4, also known as GKLF (gut-enriched KLF4), belongs to a family of zinc-finger transcription factors, called Krüppel-like factors (Bieker et al., 2001; Shields et al., 1996; Dang et al., 2000). The human KLF family comprises 25 members, which include both Sp1-like and KLF-like factors. Some of these proteins are expressed ubiquitously, like Sp1 and KLF6, whereas others appear to be tissue-specific, like KLF5 in the intestine and KLF4 in the gut (Conkright et al., 1999; Ohnishi et al., 2000). Recently, KLF4 has also been demonstrated to be expressed in vascular smooth muscle cells, repressing the expression of smooth muscle cell differentiation markers, like SMMHC (Smooth muscle myosin heavy chain). Therefore it is supposed to have an important regulatory role in vascular maturation processes.

KLFs regulate critical aspects of cellular development, differentiation and activation. KLF1 (EKL erythroid Krüppel-like factor) for example is involved in red blood cell maturation (Nuez et al., 1995). Another member, KLF2 (LKLF; lung Krüppel-like factor), is important for maintaining the quiescent phenotype in single-positive T-cells (Kuo et al., 1997).

KLF4 contains, like all Krüppel-like factors, three zinc-finger domains in its C-terminal region. Via their C terminus the KLFs bind to either a CACCC element or a GC-box (Shields et al., 1998). The N-Terminus is involved in transcriptional activation as well as protein-protein interactions with other transcription factors (Bieker et al., 1996; Bieker et al., 2001; Feinberg et al., 2004). Like the Sox proteins, KLFs can also act as activators or repressors of transcription, depending on the interaction partner and the cellular context (Dang et al., 2000; Dang et al., 2002; Ghaleb et al., 2005). KLF4 regulates genes that are involved in differentiation, proliferation and apoptosis. Mice deficient in KLF4 show defects in skin differentiation and a reduced number of secretory goblet cells in the colon (Katz et al., 2002; Segre et al., 1999). Moreover, the KLF4 knock out mice show defects in gastric differentiation and have precancerous changes in the stomach (Katz et al., 2005; Wei et al., 2005). Because of the severe skin defects, these mice die within 15 hours after birth. Moreover, KLF4 has been implicated in activation of macrophages at proinflammatory conditions (Feinberg et al., 2005). It is inducible by IFN-γ, LPS and TNF-α and decreased by

TGF-β1 in macrophages (Chen et al., 2000). After stimulation with IFN-γ or LPS, KLF4 initiates on one hand the activation of iNOS and on the other hand inhibits TGF-β1/Smad3 signaling by competitive binding to the transcription activator p300/CBP (Feinberg et al., 2005). Besides, KLF4 has also been shown to play a role in vascular development, being induced by PDGF-BB in vascular smooth muscle cells and thereby downregulating the expression of multiple SMC marker genes, for example smoothelin, smooth muscle (SM)-22α, and SM-actin (Dandre et al., 2004; Kawai-Kowase and Owens, 2006; Yoshida et al., 2006; Holycross et al., 1992). KLF4 is weakly expressed in differentiated vascular smooth muscle cells *in vivo*, but is strongly upregulated after vascular injury and, as mentioned above, in response to the mitogen PDGF-BB. These observations led to the proposal that KLF4 might play a key role in the phenotypic switch of vascular SMCs form a quiescent, differentiated cell, to a proliferating and migrating cell, being capable of contributing to the repair of vascular injury.

As its other name GKLF (gut krüppel like factor) implicates, KLF4 is known as an important epithelial transcription factor in the gut and skin where it regulates differentiation and cell proliferation (Segre et al., 1999; Katz et al., 2002). It can act as tumor suppressor, but also as an oncogene (Dang et al., 2003). KLF4 is able to bind β-catenin and thereby to inhibit the wnt signaling pathway and by this, uncontrolled cell proliferation (Zhang et al., 2006). This interaction has been shown to play a critical role in homeostasis of the normal intestine, as well as in tumorigenesis of colorectal cancers. Mutations of KLF4 lead to a loss of wnt signaling repression in the colon and, in consequence, to uncontrolled proliferating crypt cells, leading to colon cancer. Therefore KLF4 is, in this context, considered as a tumor suppressor gene (Shie et al., 2000; Zhao et al., 2004). The same mechanism of KLF4 action was observed in gastric cancers (Wei et al., 2005). In contrast, KLF4 seems to be an oncogene in the case of pancreatic and breast cancers, being upregulated under these conditions. This underlines the ability of KLF4 to function in a cell type specific manner (Foster et al., 2000; Pandya et al., 2004).

As mice deficient for KLF4 have normal colonocytes and enteroendocrine cells, but a decreased number of goblet cells, KLF4 is thought to regulate the differentiation of this cell type (Katz et al., 2002). This observation, and the fact that KLF4 binds β-

catenin, suggests that an inhibition of the wnt pathway contributes to goblet cell differentiation. Moreover, zebrafish KLF4 has been shown to be essential for anterior mesendoderm/pre-polster differentiation and hatching (Gardiner et al., 2005).

1.8. Early Growth Response Factor 1 (EGR-1)

As ubiquitously eypressed transcription factor, EGR-1 has also been implicated in vascular development, having a key regulatory role in vascular processes, promoting e.g. vascular smooth muscle cell proliferation. Therefore it has to be considered in examinations of regulatory processes in vascular cells.

The zinc-finger transcription factor EGR-1 (Early Growth Response Factor 1) is expressed from the beginning of embryonic development and also ubiquitously in the adult organism, being present mainly in the brain, especially in the hippocampus, in the heart, the lung and to lower levels in the kidney, spleen and liver (McMahan et al., 1995). Together with EGR2, EGR3, EGR4 and NGFI-B, EGR-1 builds the group of early growth response proteins (EGRs). It binds to DNA by a COOH-terminal binding domain, comprising three zinc finger regions (C_2H_2-type), which regulate transcription via binding to the consensus sequence CGCCC(C/G/T)CGC (Cao et al., 1993; Christy et al., 1989). EGR-1 contains two activator domains, one repressor domain and a nuclear localization signal. It is known to be a nuclear factor that functions as transcriptional regulator of differentiation and proliferation, in response to extracellular stimuli, like. PDGF, Hypoxia, physical forces (Lau et al., 1987; Hjoberg et al., 2003). EGR-1 is an immediate early gene, whose activity is partly modulated by binding of the co-repressors Nab1 (NGFI-A-binding protein 1) and Nab2, which bind via their NCD1 domain (Nab conserved domain 1) to the R1 domain of EGR-1, thereby preventing the long-term activation effects of EGR-1 (Russo et al., 1995). Nab1 is constitutively expressed in most cells, whereas Nab2 is upregulated in the same conditions as EGR-1. This repressor is a direct target gene of EGR-1, establishing a negative feedback loop of EGR-1 activity (Kumbrink et al., 2005). Regarding vascular processes, EGR-1 is known to be upregulated after vascular injury, as this is the case in atherosclerotic lesions and in neointimal regions (McCaffrey et al., 2000; Santiago et al., 1999a). Moreover it has been implicated as a key mediator of inflammation associated with the first early steps of atherosclerosis, inducing the

expression of cytokines and growth factors. In this context, two EGR-1 target genes have been identified: TECK, a CC-Chemokine attracting lymphocytes and IP-30, playing an important role in IFN-induced inflammation (Fu et al., 2003; Vicari et al., 1997; Luster et al., 1988). By contrast, CTGF and TRAIL are significantly repressed by EGR-1 in mature endothelial cells (Fu et al., 2003). CTGF and TRAIL are known to induce apoptosis of vascular smooth muscle cells (Hishikawa et al., 2000; Gochnico et al., 2000). Taken together, these data indicate that EGR-1 is part of the mechanisms that promote neointimal formation after vascular injury (Santiago et al., 1999). Besides this, EGR-1 induces the expression of many other vascular genes, like PDGF-A and B, bFGF, TGF-β, TNFα, Apolipoprotein A1, macrophage colony-stimulating factor (MCSF), tissue factor (TF), urokinase-type plasminogen activator (u-PA), Interleukin-2, Intracellular adhesion molecule-1 (ICAM-1), and Fetal liver tyrosine kinase (Flt-1) (Akuzawa et al., 2000; Guha et al., 2001; Yao et al., 1997; Siverman and Collins, 1999). As these target genes themselves also induce the expression of EGR-1, one can assume this to be an EGR-1 mediated autocrine loop mechanism within blood vessels. In mature endothelial cells (HUVECs), EGR-1 upregulates ID2 (Inhibitor of Differentiation 2), PAX2, Nab2, and p300. The latter may function as a positive cofactor, in contrast to Nab2, which acts as a negative cofactor.

Moreover, EGR-1 is expressed in monocytes and seems to play a role in the differentiation of macrophages (Nguyen et al., 1993). Like KLF4, EGR-1 can act as either a tumor suppressor or tumor promoter. In many human tumors like fibrosarcoma, glioblastoma and breast cancer, EGR-1 is described as tumor suppressor gene (Pignatelli et al., 2003). In case of prostate cancer, EGR-1 is known to play a tumor growth-promoting role (Baron et al., 2003). EGR-1 knock out mice are viable, but have reduced body size and are, as a consequence of hormonal deregulations, sterile (Lee et al., 1996).

1.9. The FunGenES project

Goal of the FunGenES (Functional Genomics of Engineered ES-cells) consortium is the identification and characterization of tissuespecific markers during embryogenesis. Therefore new cellular and molecular tools should be developed to characterize gene function and to enable the development of strategies for therapeutical interventions. A future vision is e.g. to be able to replace organ

transplantations, and to replace animal drug screenings with ES-cell derived methods. As our working group is interested in vascular cells, which mainly derive from the mesoderm, we are focused on this lineage and the genes that are involved into its differentiation.

1.9.1. ES-cell differentiation mimics embryonic development

In the early 1980s, mouse embryonic stem (ES) cell lines were established for the first time (Evans and Kaufman 1981; Martin et al., 1981). ES-cells derive from the inner cell mass of the blastocyst and have a pluripotent capacity, being able to differentiate in the three germ layers endoderm, ectoderm and mesoderm. An important feature of ES-cells is their ability for self-renewal, meaning prolonged symmetrical cell division in culture, resulting in identical pluripotent progeny. In vitro, one can maintain the pluripotency and self-renewal of the ES-cells by adding leukemia inhibitory factor (LIF) to the cell culture medium. LIF belongs to the Interleukin (IL)-6 family of cytokines. It binds to its receptor, the glycoprotein 130 (gp130) and thereby activates the Jak kinases with recruitment of STAT3 (signal transducer and activator of transcription) and Shp-2 (Burdon et al., 2002).

ES-cells show an almost unlimited proliferation capacity in cell culture and can retain their pluripotency. Mouse ES-cells are also characterized by a relatively short cell cycle time (12-15 h) with a short G1 phase. Moreover they possess enzyme activities for alkaline phosphatase (ALP) and telomerase. Markers for the undifferentiated state of ES-cells are for example the membrane-bound protein SSEA-1, and the Oct3/4 POU domain containing transcription factor, which is essential for the initial development of pluripotency in the inner cell mass of the blastocyst (Niwa et al., 2000; Pesce and Schöler, 2000). Nanog, a homeodomain protein also takes part in keeping the undifferentiated state of the ES-cells (Review: Chamers, I. 2004). In addition, Sox2 and FoxD3 are involved in these processes and BMP (bone morphogenetic protein) dependent induction of ID2 (inhibitor of differentiation) target genes is sufficient for keeping the ES-cells undifferentiated (Hanna et al., 2002). Moreover, the MEK/ERK signaling is involved in ES-cell renewal and differentiation, as inhibition of this pathway is necessary to maintain self-renewal. ERK and SHP-2 seem to counteract the STAT3 mediated proliferative effects, and thereby promote differentiation (Niwa et al., 1998). A list of other markers for the undifferentiated state

of ES-cells comprise Rex-1, Genesis, GBX2, UTF1, Pem, and L17, which are upregulated in the inner cell mass of the blastocyst and downregulated when differentiation starts (Chapman et al., 1997).

Between mouse ES-cells and human ES-cells (hES-cells), one can find some differences, e.g. human ES-cell form mainly cystic embryoid bodies (EBs), express proteoglycans like TRA-1-60, TRA-1-81 and GCTM-2 and different subtypes of stage-specific antigens like SSEA-3 and SSEA-4, which are not expressed by mouse ES-cells (Carpenter et al., 2003). Moreover, hES-cells show a longer population doubling time of around 30 to 35 hours (mES-cells: 12-15h). Another difference is the fact that hES-cells cannot be cultured in LIF to maintain the undifferentiated state, but have to be co-cultured with a feeder layer (Daheron et al., 2004). On the other hand, hES-cells and mES-cells also have some common features like the Oct3/4 expression, high telomerase activity, and the maintained proliferative potential for prolonged periods in culture. Both cell types are able to retain their normal karyotype.

1.9.2. Endoderm, Mesoderm and Ectoderm arise from the inner mass of the blastocyst

The fertilized egg is totipotent, as it is able to generate an entire organism. This state of totipotency is retained by early progeny of the zygote up to the eight-cell stage of the morula. Further differentiation leads to the formation of the blastocyst, which consists of undifferentiated inner cells (also "inner cell mass" = ICM), surrounded by outer trophoblasts. The cells of the ICM are pluripotent, meaning the ability to differentiate into all cell types of the embryo. The three different germ layers, endoderm, ectoderm and mesoderm, and the primordial germ cells, the founder cells of male and female gametes, arise from this pluripotent inner cell mass. In adults, multipotent stem and progenitor cells still exist in tissues and organs, like haematopoetic progenitor cells in the bone marrow, hair stem cells in the hair follicles, and neuronal stem cells in the brain ventricles, etc. They have the function to replace lost or injured cells.

The ectoderm gives rise to different cell types like neurons, glial cells and epithelial cells. One of the first markers of the neuroectoderm is Sox1 (Pevny et al., 1998).

Tissues arising from the mesodermal lineage are muscle (including vascular smooth muscle cells and cardiomyocytes), cartilage, bone, blood and connective tissue. Blood and endothelium in the yolk sac differentiate from a common precursor, the so-called hemangioblast, at around day 6.5.

As third germ layer, the endoderm gives rise to the gut, the pancreas, the thyroid, the thymus and the liver (Review Wells and Melton, 1999). In part, also vascular smooth muscle cells are generated by the endodermal lineage. The pancreas develops from dorsal and ventral regions of the foregut. The endodermal precursors arise from the anterior primitive streak. This corresponds to early and mid gastrula organizer (Wells and Melton, 1999; Kinder et al., 2001; Lawson et al., 1991). During mouse development, one can recognize two types of endoderm: the definitive endoderm and the visceral endoderm, which gives rise to extra-embryonic endoderm (Lu et al., 2001). The definitive endoderm forms the lining of the gastrointestinal tract and contributes tissues to the visceral organs associated with the gut (e.g. liver and pancreas) (Tam et al., 2003; Wells et al., 1999). The visceral endoderm derives directly from the inner cell mass of the blastocyst, whereas the definitive endoderm derives from the mesendoderm, a subpopulation that is giving rise to mesoderm and endoderm. A marker for endoderm in general is Sox17, which can be used in combination with goosecoid (Gsc) for dividing visceral endoderm (Sox17+/Gsc-) and definitive endoderm (Sox17+/Gsc+) (Yasunaga et al., 2005).

1.10. Goal of the thesis

The goal of this thesis was the characterization of the role of Sox transcription factors in vascular processes and vascular disease. The importance of this group in vascular biology was underlined by the knowledge of literature, which showed that Sox7 and Sox18 are implicated in angiogenesis during embryogenesis and in adults during vascular repair mechanisms. In contrast, the third member of the subgroup F, Sox17, has not been implicated in vascular processes so far, but it was known to be a decisive endodermal factor playing a crucial role of the maintenance and differentiation of the definitive endoderm of the embryonic gut.

During this thesis work, I discovered that Sox17 is strongly expressed in vascular smooth muscle cells in different mouse tissues, like kidney, spleen, lung, heart, brain, testis, and liver. Because the role of Sox17 in the vascular smooth muscle cells was unknown and as vascular SMCs are important as potential targets for therapeutic interventions, I decided to focus my work on studying the role of Sox17 in the proliferation or differentiation of the vascular SMCs and on identification of possible interaction partners in this new cell context. Moreover, as member of the FunGenES consortium, a second goal was to find an answer to the question if the subgroup F sox proteins might be involved in mesodermal lineage formation.

2. Materials

2.1. Plasmids

pGL3-Basic Vector (Promega)
pBK-CMV (Stratagene)
pBluescriptII SK (+/-) (Stratagene)

2.2. Cloning primers

Primers used for cloning of mouse Sox7, Sox17 and Sox18 sequences in various expression constructs

Primer/Gene	direction	length	Primersequence	cloning sites, usage
mSox7	5	34	tcccccgggCggccatggcctcgctgctgggcgc	full length primer, SmaI
mSox7	3	33	gctctagacctccagctctatgacacactgtag	full length primer, XbaI
mSox17	5	31	aactgcaggtctggagagccatgagcagccc	full length primer, PstI
mSox17	3	30	gctctagaccgtcaaatgtcggggtagttg	full length primer, XbaI
mSox18	5	35	cgggatcccGcccagctggaatgcagagatcgccg	full length primer, BamHI
mSox18	3	30	gctctagaggacagtgtctagcctgagatgc	full length primer, XbaI

Primers used for cloning of human SOX7, SOX17 and SOX18 sequences in various expression constructs

Primer/Gene	direction	length	Primersequence	cloning sites, usage
hSOX7	5	31	cgggatccCgcgtgcggccatggcttcgctg	full length primer, BamHI
hSOX7	3	33	cccaagcttgggcctccagctctatgacacact	full length primer, HindIII
hSOX17	5	34	cgggatcccgcctggagcgccatgagcagcccgg	full length primer, BamHI
hSOX17	3	34	cgaattccggacctgtcacacgtcaggatagttg	full length primer, EcoRI
hSOX18	5	34	cgggatcccgcgcccagctggaatgcagagatcg	full length primer, BamHI
hSOX18	3	34	cggaattcccggcggcctagccggagatgcacgc	full length primer, EcoRI

2.3. Bacteria and Cell lines

-Bacteria-
DH5α : F-. lacI- recA1, endA1, Δ(lacZY A-argF), U169, F80dlacZΔM15, supE44, thi-1, gyrA96, relA1 (Hanahan et al., 1985)

-Cell lines-
CASMCs >   Coronary Artery Smooth Muscle Cells (Cambrex)
HUVECs >   Human Umbilical Vein Endothelial Cells (Cambrex)
eEPCs (T17b) >   embryonic Endothelial Precursor Cells (isolated from day 7.5 mouse embryos)

CGR8 >   mouse embryonic stem cells isolated from 3.5 day-old
                      blastocysts (ECACC - European Collection of Cell cultures)

2.4. Cell culture media

CASMCs: SmGM®-2 - Smooth Muscle Medium-2 plus supplements (FCS
                      5%, Insulin, Gentamicin, FGF; Cambrex)
HUVECs: EGM - Endothelial Cell Medium plus supplements (FCS 5%,
                      VEGF, FGF, hGF
eEPCs Medium: DULBECCO´S MEM with 25 mM HEPES, 20% Foetal Bovine
                      Serum
                      3.5 µl β-mercaptoethanol, 1% 200 mM L-glutamine, 1%
                      Penicillin/ Streptomycin (10.000 units/ml Penicillin, 10 mg/ml
                      Streptomycin)and 1% non-essential amino acids MEM.
ES-cell Medium: GMEM with 10% FCS, 250 µl 0,1 M β-mercaptoethanol, 5 ml L-
                      Glutamine, 50 µl Leukemia Inhibitory Factor (Sigma);

2.5. Antibodies

primary antibodies
- anti-Sox17 polyclonal; goat (Santa-Cruz) native mixture
  Immunofluorescence: 1:50; Western-Blot 1:100; IP: 1 µg/ml
- anti-Klf4 polyclonal; goat (Santa-Cruz) native mixture
  Immunofluorescence: 1:50; Western-Blot 1:100; IP: 1 µg/ml
- anti-Egr-1 polyclonal; rabbit (Santa-Cruz) native mixture
  Immunofluorescence: 1:100; Western-Blot 1:200; IP: 1 µg/ml
- anti-Pecam-1 mouse (BD Biosciences)
  Immunofluorescence: 1:10
- anti-β-catenin polyclonal rabbit (Sigma-Aldrich)
  Western-Blot: 1:100; IP:1 µg/ml
- anti-Hämagglutinin monoclonal; rat; IgG1; IP: 1 µg/ml

secondary antibodies
- anti-goat Cy3-labeled (Dianova)   Immunofluorescence 1:200
- anti-goat FITC-labeled (Dianova)   Immunofluorescence 1:200
- anti-rabbit Cy3-labeled (Dianova)   Immunofluorescence 1:200
- anti-rabbit FITC-labeled (Dianova)   Immunofluorescence 1:200
- anti-mouse Cy3-labeled (Dianova)   Immunofluorescence 1:200
- anti-mouse FITC-labeled (Dianova)   Immunofluorescence 1:200

- anti-goat HRP-conjugated (Promega)   Western-Blot 1:5000
- anti-rabbit HRP-conjugated (Promega)   Western-Blot 1:2500

2.6. Chemicals and Enzymes

Company	Chemicals/Enzymes
Amersham Pharmacia	ECL-western blotting detection reagents, A-Sepharose
Chemicon	PDGF-BB
Gibco Invitrogen	EDTA, Trypsin, OPTIMEM, L-Glutamin, Penicillin-Streptomycin, Non-essential amino acids (MEM), IMDM, DMEM, Foetal Calf Serum (FCS), ultraPure agarose
Invitrogen	Lipofectamine™ 2000, Platinum®Pfx DNA Polymerase, MLV-Reverse Transcriptase
Merck Eurolab GmbH	KCL, Na_2HPO_4,, KH_2PO_4, Gylcin, Ammonium peroxide sulfate (APS), Ethanol, Isopropanol, Methanol, Sodium acetate, Magnesium sulfate, Sodium dodecyl sulfate (SDS), Tween-20, Triton X-100, Sodium-Orthovanadate
Metabion	1kB DNA Ladder, Taq-Polymerase, dNTPs, oligos
New England Biolabs	Restriction enzymes, VENT DNA Polymerase
Pierce Biotechnology	NE-PER kit
Promega	oligo(dT) 15 Primer, Taq-Polymerase, RNasin®Ribonuclease Inhibitor
Qiagen	Endo-free Maxi DNA preparation kit, PCR purification kit, RNeasy RNA isolation kit
Roche	FuGENE 6 Transfection reagent, Rapid Ligation Kit, RNase A, Canamycine, alkaline phosphatase
Sigma-Aldrich	Leukemia Inhibitory Factor (LIF), GMEM, BSA, LDL, 2-mercaptoethanol, ethidium-bromide, DMSO, TEMED, Polyacrylamide, Pre-stained SDS Molecular Weight Marker, Dithiothreitol (DTT), Bromophenol blue, ampicillin
Vector Laboratories, Burlingame	VECTASHIELD Mounting Medium, MOM detection kit

2.7. Working materials

Company	Materials
Amersham Pharmacia	Hybond-C-nitrocellulose membrane
BectonDickinson	six-well plates, 24 well plates
Costar	5, 10 and 25 ml plastic pipettes

Eppendorf	50 ml plastic pipettes
Falcon	15 and 50 ml tubes, bacteriological plates
BioRad	gel chambers for gel electrophoresis
Kimberly-Clark	Gloves, tissues
Nunc	6 and 10 cm plates
TipOne	pipette tips
SCI Science Services	PapPen

3. Methods

3.1. Cell culture

3.1.1. Cell passaging

All cell types used in this work were adherent cells, so we used trypsinization to expand cells in culture. Before splitting, the cell layer was washed once with 10 ml PBS (w/o Ca_2+ and Mg_2+). To detach the cells from the plates, they were incubated for 5 minutes with 1x Trypsin-EDTA. The enzymatic reaction was stopped by adding 5 ml of culture medium. The cell suspension was centrifuged at 1200 rpm for 5 minutes. Afterwards the supernatant was removed, and the cells were resuspended in fresh medium and plated on new gelatin-coated plates (0.1%). In case of the Coronary Artery Smooth Muscle Cells (CASMCs), no gelatin coating was necessary.

3.1.2. Differentiation of CGR8 ES-cells

The ES-cells have been differentiated by two different methods: by the "hanging drop" method or by generating cell suspensions in bacterial Petri dishes. In both cases, the protocol started with the trypsinisation of the cells for 5 min at 37°C. To stop the enzymatic reaction, 5 ml medium were added, and the cells were centrifuged at 1200 rpm for 5 min. The cell pellet was resuspended in differentiation medium, with a final concentration of $2,5x10^4$/ml. In case of the "hanging drop" method, 20 µl-drops were generated at the inside surface of a 10 cm plate lid. Each drop contained around 500 cells. In this hanging state, the cells started accumulating at the bottom of the drop and started thereby differentiating, building embryoid bodies (EBs). After two days, the generated EBs were transferred in bacterial 10 cm plates, containing 10 ml differentiation medium. In case of the cell suspension method, the resuspended undifferentiated cells were directly plated in a 10 cm bacteriological plate, containing 10 ml differentiation medium. The reason for using bacteriological plates is, that the cells do not adhere at this type of plastic, but stay in suspension and start, by accumulating with other cells, to build EBs. The advantage of the "hanging drop" method is the comparable size of the generated EBs, whereas the ones from the cell suspension often vary in their size.

Differentiation medium: IMDM (500 ml), 20% FCS, 1% Non-essential amino acids (MEM), 1% L-glutamine, 500 µl 0,1 M β-Mercaptoethanol

3.1.3. Transient Cell Transfection

For transient expression of the genes of interest, expression plasmids were transfected with Lipofectamine™ 2000 (Invitrogen) in the used cell lines. In this protocol, the cells were pre-incubated in serum-free medium, like OPTIMEM for 30 min to 1h. During this time, the DNA-Lipofectamine mix was prepared. For this, 16 µg expression plasmid and 32 µl Lipofectamine™ 2000 (in case of a 10 cm plate) were diluted separately in 1 ml OPTIMEM each. After 5 min of incubation, both solutions were added to each other and incubated together for 25 min at room temperature. Afterwards, the OPTIMEM of the pre-incubation step was removed from the cells and replaced by the DNA/Lipofectamine™ 2000 mixture. This complex was left for 4-6 hours on the cells and was finally replaced by fresh medium. Lysates for various assays have been taken 24-48 hours afterwards.

3.2. Molecular biology techniques

3.2.1. Cloning strategy

Expression plasmids of mouse and human Sox7, 17 and 18 sequences have been generated using the pBK-CMV, pBluescriptII SK (+/-) and pIRES2-eGFP vector systems from Stratagene. In case of cloning the mouse constructs, we used a modified pBluescriptII SK (+/-) vector (Stratagene), which carries an additional PGK promoter for expression in embryonic mouse cell lines. The cloned constructs were sequenced (Sequiserve, Vaterstetten) and used for transient transfection assays in different cell types.

3.2.2. Ligation

Ligations were done using the Rapid Ligation Kit (Roche) and following the instructions in the manual. The insert was used in a 10-fold excess in comparison to the vector amount. The incubation time was 10 min at room temperature.

3.2.3. Transformation of DNA in bacteria

Half of the ligated DNA from the previous step was used for the transformation in chemically-induced competent DH5α cells. The prepared bacterial cells have been stored at -80°C. For the transformation, the cells were thawed on ice for 5 minutes. The DNA (1 µg) was added, and incubated with the bacteria for 20 minutes on ice. Afterwards, the sample was heat-shocked for 2 min at 42°C. 1 ml of LB-Medium was added, and the tube was incubated for 1 h at 37°C on an Eppendorf Thermomixer, shaking at 1000 rpm. Finally, the bacteria were pelleted by centrifugation (2 min, 4000 rpm), and plated on LB plates, containing the appropriate antibiotic (ampicillin/canamycine). The plates were incubated inverted overnight at 37°C.

3.2.4. Mini-preparation of Plasmid DNA

Colonies were picked from the plates and incubated, shaking overnight at 37°C in 5 ml LB-medium, containing the appropriate antibiotic. Next day, 1.5 ml of the suspension were transferred to a 2 ml Eppendorf tube and centrifuged for 5 min at 14000 rpm. The pellet was resuspended in lysis buffer (buffer 1) and incubated for 5 minutes. Afterwards, a second lysis buffer (buffer 2) was added and the samples were incubated for 5 more minutes. Last, buffer 3, a neutralizing buffer, was added to stop the lysis reaction. The whole mixture was centrifuged for 5 minutes at full speed, and the supernatant was transferred to a new tube. To extract the DNA, 1 volume of Isopropanol and 1/10 volume of Sodium acetate (3 M) was added, and the sample was centrifuged at 14000 rpm for 10 min. After an additional washing step using 80% Ethanol, the pellet was air dried and finally resuspended in 30 µl TE-buffer.

Buffer 1:	50 mM Tris-HCl, 10 mM EDTA; pH 8.0
Buffer 2:	200 mM Sodium hydroxide, 1% SDS (w/v)
Buffer 3:	3.2 M KAc/HAc; pH 5.5

3.2.5. Maxi-Preparation of Plasmid DNA from bacteria

The Maxi-preparation of plasmid DNA was carried out using the Endotoxin-Free Maxi-Plasmid Kit from Qiagen, following the manifacturer's instructions.

3.2.6. RNA-Isolation

The RNA isolation procedure was done by using the QIAGEN RNeasy® Kit. The cells were lysed in 300 – 500 µl RLT Lysis buffer. DNA digestion (with RNase-Free DNase Set, QIAGEN GmbH) was performed to avoid contamination with genomic DNA. RNA was eluted in 50 µl of RNase-free water and stored at –80°C. RNA concentrations were measured using an Eppendorf Spectrophotometer.

3.2.7. Reverse Transcription (RT) PCR

RT-PCR is a technique to first synthesize cDNA using RNA as template; the single-stranded cDNA subsequently serves as template for amplification by PCR (Polymerase Chain Reaction) to monitor levels of gene expression. The steps were carried out in an Eppendorf Thermomixer in 1.5 ml tubes. First, 3 µg of RNA were added to autoclaved ddH$_2$O to a final volume of 15 µl. Oligo-dT primer (3.75 µl) were added to the mixture, and the tubes were incubated at 65°C for 5 minutes to denature secondary RNA structures and allow the primers to bind to the polyA tails of the mRNA molecules. Afterwards, the tubes were immediately put on ice to avoid refolding of the RNA. Further on, 11.25 µl of RT-Mix were added to the samples.

The Reverse Transcription (RT)-Mix contained:
4.5 µl NX buffer (2 M KCl, 1 M Tris-Cl pH 8.4, 1 M MgCl$_2$, 3% Tween20)

1.5 µl dNTPs (20 mM)

3.0 µl β-mercaptoethanol

0.75 µl RNasin

1.5 µl Mo-MLV Reverse Transcriptase (200 U/µl)

The entire mixture was incubated at 37°C for 55 minutes. To stop the enzymatic reaction, the tubes were heated at 95°C for 5 minutes. Finally, 270 µl autoclaved ddH$_2$0 were added to each sample to obtain a final volume of 300 µl cDNA with a concentration of ca.10 ng/µl.

3.2.8. The Polymerase Chain Reaction (PCR)

The generated cDNA was tested for different markers by conventional and quantitative PCR. Amplification protocol was as follows:

e.g.   95℃ 5 min

        95℃ 1 min

        65℃ 1 - 1.30 min

        72℃ (per 1000 bp 1 min)

        steps 2 - 4 were repeated for 24 - 35 times

        72℃ 10 min

         4℃ stored till analysis

3.2.9. Agarose gel electrophoresis

PCR amplified DNA products were separated in 1.0% agarose gels run in horizontal gel chambers in 1x TBE electrophoresis buffer. Therefore, each sample was diluted with a DNA-Loading dye (6x). The size of the DNA fragments was determined using a 1kb DNA ladder as marker. After electrophoresis, gels were stained in a 0.02% ethidium bromide solution for 10 minutes. Gels were photographed under UV light using a Polaroid camera.

TBE (10x):   108 g Tris-HCl, 55 g Borate, 400 ml EDTA (20 mM) add 600 ml H_2O; pH 8,3

DNA Loading Dye: 40% Glycerin, 60% H_2O, 0.001% Bromo-phenol-blue

3.3. Immunofluorescence

Immunofluorescence is a histological method to detect proteins in tissue sections, based on the binding of primary monoclonal or polyclonal antibodies to their respective antigens. Secondary antibodies, which are conjugated with a fluorescence dye like FITC (green) or Cy3 (red), recognize and bind to the primary antibodies leading to a complex that is detectable under a fluorescence microscope.

Therefore, frozen sections (thickness of 10 µm) were fixed for 5 min at 4℃ in a 1:1 mixture of acetone and methanol. The sections were surrounded with a PapPen to provide a hydrophobic barrier around the sections and keep antibody solutions

directly on top of the section. After fixation, sections were washed five times with 1x PBS for 10 min each and subsequently blocked for 1 hour with blocking buffer (5% bovine serum albumin in 1x PBS) to prevent unspecific antibody binding. Primary antibodies, diluted in blocking buffer were added to the sections and incubated for one hour at room temperature or O/N at 4°C. After this step, the sections were washed five times with 1x PBS and subsequently incubated with the secondary antibody for 1 hour at room temperature in blocking buffer. The secondary antibody was used in a final concentration of 1:200, diluted in 1x PBS. Finally, the sections were washed again for five times with 1x PBS, air-dried for a short time and mounted with VECTASHIELD Mounting Medium. The sections were photographed using a Zeiss Axiovert 200M digital camera (Carl Zeiss Microscope, Göttingen, Germany).

Blocking buffer: 1x PBS and 5% Bovine Serum Albumin

3.4. Cell stimulation assays

To stimulate the cells with Low density lipoprotein (LDL), Platelet-Derived Growth Factor-BB (PDGF-BB) or Transforming Growth Factor β 1 (TGF-β 1), the cells were kept in low-serum conditions changing medium 32 hours before growth factor treatment to 0,1 % FCS medium. Afterwards, the cells were stimulated with 50 ng/ml PDGF, 10 ng/ml TGF-β 1, or 100 µg/ml LDL. The substances were directly added to the 0.1% FCS medium. After mixing for even distribution, cells were incubated for the indicated time periods at 37°C. For each time point, untreated plates were used as a control. Afterwards, the lysates were taken and RNA was isolated, using the QIAGEN RNeasy® Kit. The RNA was transcribed in cDNA and the samples were tested via RT-PCR for different markers.

3.5. Luciferase-Assays

For the Luciferase - Promoter assays, 1 µg of promoter construct, cloned in the pGL3 Basic Vector, which carries the luciferase gene without its own promoter, and 1µg of the expression plasmid (SOX17-CMV) or the empty vector (pBK-CMV) were co-transfected in HeLa cells by Lipofectamine™ 2000. 24 hours later, the lysates were prepared using the RLB lysis buffer (5x) of the luciferase kit from Promega, which is diluted 1:5 with ddH$_2$O directly before usage. The cells were incubated with this

buffer, shaking for 15 min at room temperature. Afterwards, the cells were scraped and transferred in Eppendorf tubes. The lysates were frozen for 30 min at 80°C and then centrifuged at 14000 rpm for 10 min. The supernatants were transferred in a new tube and afterwards stored at -20°C or directly measured, assaying the luciferase activity by using a Chemiluminescence Photometer.

3.6.   SDS-gel protein electrophoresis

For detection of proteins after size separation, protein samples were loaded on a 12.5% polyacrylamide gel. The gel consists of two parts, a "stacking" gel and a separation gel. The first one is shorter and contains the wells for loading the samples. The samples run vertically in a BioRad gel chamber at 120 Volt separated by their molecular size. To ensure separation according to molecular size, the proteins were denatured at 95°C for 10 min, before loading on the gel. To determine the size of the detected proteins, a pre-stained molecular protein ladder was used. The gels run for approximately 1 - 2 hours and were afterwards transferred on a nitrocellulose-membrane via "wet-blotting" at 4°C for 1 h at 400 mA. The blots were then blocked with 1X PBS, containing 5 % non-fat milk powder, for 30 min. The primary antibody was diluted 1:100 in a PBS/milk mixture, and the blot was incubated in this suspension rolling over night at 4°C. Next day, the blot was washed five times with 1X PBS and afterwards incubated for 3 h at room temperature with the secondary antibody. This one was diluted at a range from 1:2500 - 5000 in PBS/milk. The blot was washed five more times with 1X PBS, dried with tissue paper, and, lastly incubated with ECL solution for 1 min. Afterwards, the blots were exposed to X-ray films, which were subsequently developed.

Transfer buffer (10x):	30 g Tris, 95 g Glycine; add ddH2O to 1l
TBS buffer:	20 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA; pH 7.5
12.5% SDS-gel:	40.4 ml H_2O, 16.5 ml Tris-HCl (pH 8,8). 825 µl 0,5M EDTA, 41.25 ml Acrylamide (30%) Bis-acrylamide (0.8%) solution; pro 10 ml SDS gel: 150 µl APS, 70 µl TEMED (=separation gel)
Stacking gel:	7.6 ml H_2O, 625 µl 2 M Tris-HCl (pH 8.8), 100 µl SDS, 1,5 ml APS, 40 µl TEMED
SDS-buffer (10x):	30 g Tris, 144 g Glycine, 100 ml SDS (10%) add ddH$_2$O to 1 l; pH 8.9

3.7. Protein immunoprecipitation

For immunoprecipitation of protein complexes, the cells were washed twice with 1 X PBS, scraped and lysed in 500 µl Lysis buffer. The lysates were incubated on ice for 30 min and afterwards sonified for around 15 times at 50 ms time constant. The lysates were then centrifuged for 30 min at 14000 rpm. During these steps, the primary antibodies were set up in 100 µl 1 X PBS each, and incubated with 30 µl pre-washed A-Sepharose beads, in case of goat and rabbit primary antibodies, and G-Protein in case of mouse and rat primary antibodies. The incubation took place in a rotator at 4°C for 1 h. During this time, the A-Sepharose/G-Protein was coupled to the primary antibody. After this incubation step, the lysate was added to the primary antibody/Sepharose mix and incubated overnight at 4°C. At the next day, the protein/antibody/Sepharose complex was washed five times with Lysis buffer (w/o protease-inhibitors) at 4°C, and spun at 5000 rpm, 5 min after each wash. The supernatant was carefully removed. Further on, the pellet was resuspended in 50 µl 1x Lämmli-buffer, containing 0.1 M DTT (Dithiotreitol), and incubated at 95°C for 10 min for elution and denaturation of the protein complexes. Afterwards, the suspension was centrifuged for 10 min at 14000 rpm to pellet and remove the A-/G-Sepharose. Western-Blots were then performed with the protein eluates. As isotype control 3F10 > anti-HA-tag was used.

Lysis buffer: 50 mM Tris-HCl, pH 8.0; 1% NP40, 150 mM Sodium chloride;
                    directly added before use: cocktail of Protease inhibitors
                    (Complete; Roche)

3.8. Co-Immunoprecipitations

To check for protein-protein interactions between different transcription factors, the immunoprecipitated proteins were loaded once again on a 12.5% SDS-gel and analysed by Western-Blot analysis. In this case, next to the precipitating antibody, we used antibodies against putative interaction partners. Also, an IgG control and moreover, a control for a non-binding partner protein, was used.

4. Results

4.1. A new role for SOX17 as potential interaction partner of EGR-1 and KLF4 in human coronary artery smooth muscle cells

Large blood vessels consist of three different layers: the intima, consisting of a single layer of endothelial cells, the media, mainly built up by multiple layers of smooth muscle cells, and, last, the adventitia, mostly consisting of connective tissue like fiber and collagenous material. Disruptions of the vessel wall are associated with severe vascular diseases, like atherosclerosis, a chronic inflammatory disease at the vascular wall (Ross et al., 1999; Libby et al., 2002). Moreover, vasculogenesis and angiogenesis, the vascular network forming processes, play a decisive role in the outgrowth of metastases, supporting the tumor tissue with oxygen and nutrients, transported by the blood. Thereby the transcriptional level of such vascular processes becomes interesting, especially for therapeutic interventions. Affymetrix data of cyclic AMP treated endothelial progenitor cells showed an increase of the members of the subgroup F of the Sox proteins (Antonis Hatzopoulos, 2002, data not published). These are Sox7, 17 and 18, whereby Sox7 and Sox18 have already been implicated in vasculogenesis during embryogenesis and angiogenesis in adult organisms (Pennisi et al., 2000b; Darby et al., 2001). Sox17 is so far mainly associated with endodermal development (Kanai-Azuma et al., 2002). Recently, Sox17 has been demonstrated to be expressed in endothelial cells, showing a redundant function with Sox18 during angiogenesis (Matsui et al., 2006). Thereby this protein group seems to be a promising target for further examinations in vascular cells.

4.1.1. Expression of Sox7, Sox17 and Sox18 in different mouse and human tissues *in vivo*

Sox proteins are widely expressed in embryogenesis as well as in adult tissues. Often many different Sox proteins are expressed at the same time in the same cell (Kuhlbrodt et al., 1998; Lefebvre et al., 1998). The biological read out depends on the interaction partner in the different cellular contexts (Kamachi et al., 2000). To get a better idea about the relative expression patterns of Sox7, Sox17 and Sox18, the distribution of these factors was examined on different mouse and human tissues.

Figure 4.1 shows the expression of Sox7, 17 and 18 in mouse tissues, like adipose, brain, colon, embryo, heart, kidney, liver, lung, muscle, spleen and testis (A). In (B) SOX7 and SOX18 were tested in the same tissues, but from human origin. Additionally bone marrow (lane 3) was tested. In (A) one can see that all three factors are expressed nearly in each tested mouse tissue, indicating a wide expression. In general, the expression of Sox17 is weaker, the strongest expression detectable in liver samples. This is consistent with Sox17 expression in endodermal cells, as the liver is one of the tissues deriving from the endoderm (Kanai-Azuma et al., 2002).
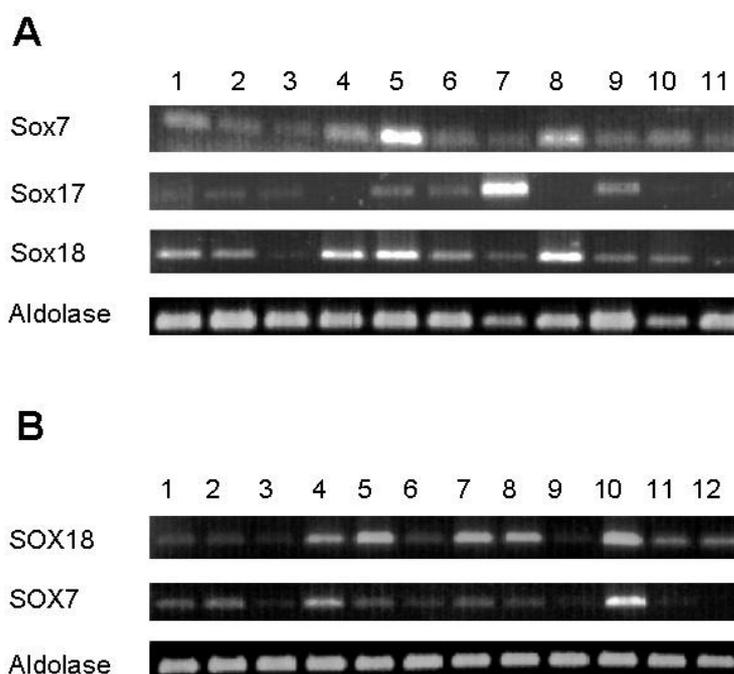
Figure 4.1 Expression of Sox7, Sox17 and Sox18 in different mouse and human tissues. The expression pattern of Sox7, Sox17 and Sox18 in mice is shown by RT-PCR (A) checking different tissues (1 = adipose, 2 = brain, 3 = colon, 4 = embryo, 5 = heart, 6 = kidney, 7 = liver, 8 = lung, 9 = muscle, 10 = spleen, 11 = testis). Part (B) shows the expression of SOX18 and SOX7 in tissues of human origin (1 = adipose, 2 = aorta, 3 = bone marrow, 4 = brain, 5 = breast, 6 = colon, 7 = heart, 8 = kidney, 9 = liver, 10 = lung, 11 = spleen, 12 = testis), examined by RT-PCR. The human cDNA samples were purchased commercially (BioCat GmbH, Germany).

Sox7 and Sox18 show a very similar expression pattern, which underscores the idea that both factors might have a compensatory function for each other, which has been suggested, as Sox18 deficient mice display only mild defects in skin and heart development, but no severe defects in the vasculature (Pennisi et al., 2000). Thereby, Sox7 seems to compensate the function of Sox18 in vascular cells. The highest expression is detectable in the heart and the lung. In the human tissues (B), SOX7 and SOX18 are mainly detectable in the brain and the lung, SOX18 is

additionally strongly expressed in breast (lane 5), liver and kidney (lane 7 and 8). Also the expression of SOX17 was examined which was so low under normal PCR conditions that it is not shown here. At least only a weak expression in the lung was detectable.

4.1.2. Expression profile of Sox7, 17 and 18 in different vascular cells *in vitro*

In a next step, we assayed the expression of the subgroup F Sox proteins ("vascular Soxes"), in cultured vascular cells. These were embryonic endothelial progenitor cells (T17b), isolated from 7.5 day-old mouse embryos, Human Umbilical Vein Endothelial Cells (HUVECs) as mature endothelial cells, and human Coronary Artery Smooth Muscle Cells (CASMCs). As the coronary artery is one of the most important arteries in the organism, and defects in its vascular cells are associated with severe diseases, this subpopulation of vascular SMCs was chosen for further examinations.
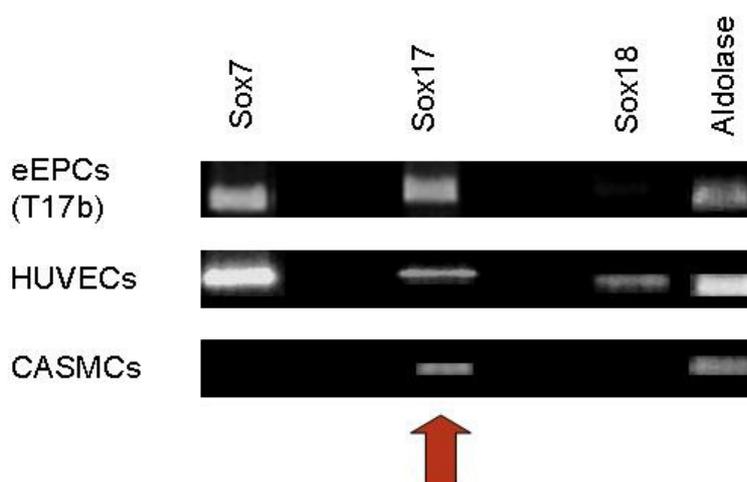
Figure 4.2 Expression profiles of subgroup F Sox proteins in vascular cells *in vitro*. Embryonic endothelial progenitor cells (eEPCs), Human Umbilical Vein Endothelial Cells (HUVECs) and Vascular Smooth Muscle Cells from the coronary artery (CASMCs) were checked via RT-PCR for expression of Sox7, 17 and 18. Sox17 is expressed in vitro in all three cell types, but the only Subgroup F member expressed in vascular smooth muscle cells (indicated by arrow).

The results showed that SOX17 is the only factor, of the three subgroup F sox proteins, which is expressed in vascular smooth muscle cells, whereas SOX7 and SOX18 are restricted to endothelial cells (figure 4.2). The expression of Sox17 in

smooth muscle cells has not been reported previously, so we decided to study its role in this cell type.

4.1.3. Expression of Subgroup F Sox proteins in different mouse tissues *in vivo*

To expand the in vitro results, indicating an expression of Sox7 and Sox18 in endothelial cells and an additional expression of Sox17 in vascular SMCs, immunofluorescence stainings on different normal mouse tissues have been performed. As we were interested in the expression in vascular cells, the three Sox proteins have been co-stained with an antibody directed against Pecam-1 (Platelet endothelial cell adhesion molecule), an endothelial cell specific marker (Simmons et al., 1990). Pecam-1 (CD31) is a receptor, expressed at the cell surface of mature endothelial cells. It belongs to the immunoglobulin superfamily and is able to induce the expression of other adhesion molecules on naive T-cells (Newman et al., 1990). Pecam-1 is mainly capable of mediating cell-matrix and cell-cell adhesion.

Using a Pecam-1 specific antibody for double immunofluorescence stainings, we detected an endothelial cell specific expression of Sox7 and Sox18 in different mouse tissues, like spleen, kidney, brain, heart, lung, and testis. In figure 3 (A) - (C), one can see a heart section showing a big blood vessel stained positive for Sox7 (A) and Pecam-1 (B) in the endothelium. The overlay of both sections (C) shows the co-expression of both factors. Moreover, Sox7 is detectable, via a biotinylated antibody in small vessels, invading in metastases of a lung tumor (E) and is moreover localized in the endothelium of the big vessels of the surrounding lung tissue (D). As angiogenesis takes place during tumor growth, the detection of Sox7 in this malignant tissue suggests an important role of Sox7 in these processes. The same observation was made for Sox18 (figure 4.3, (F)), which was also co-localized with Pecam-1 on a normal heart tissue section. Figure 4 (D) shows a middle size vessel, which is positively stained for Sox18, using a FITC-labeled secondary antibody. Pecam-1 co-localization is visualized via a Cy-3 labeled secondary antibody. Therefore, the vascular in vitro expression pattern of Sox7 and Sox18, showing a restricted expression to endothelial cells (4.1.2.), could be confirmed *in vivo*.
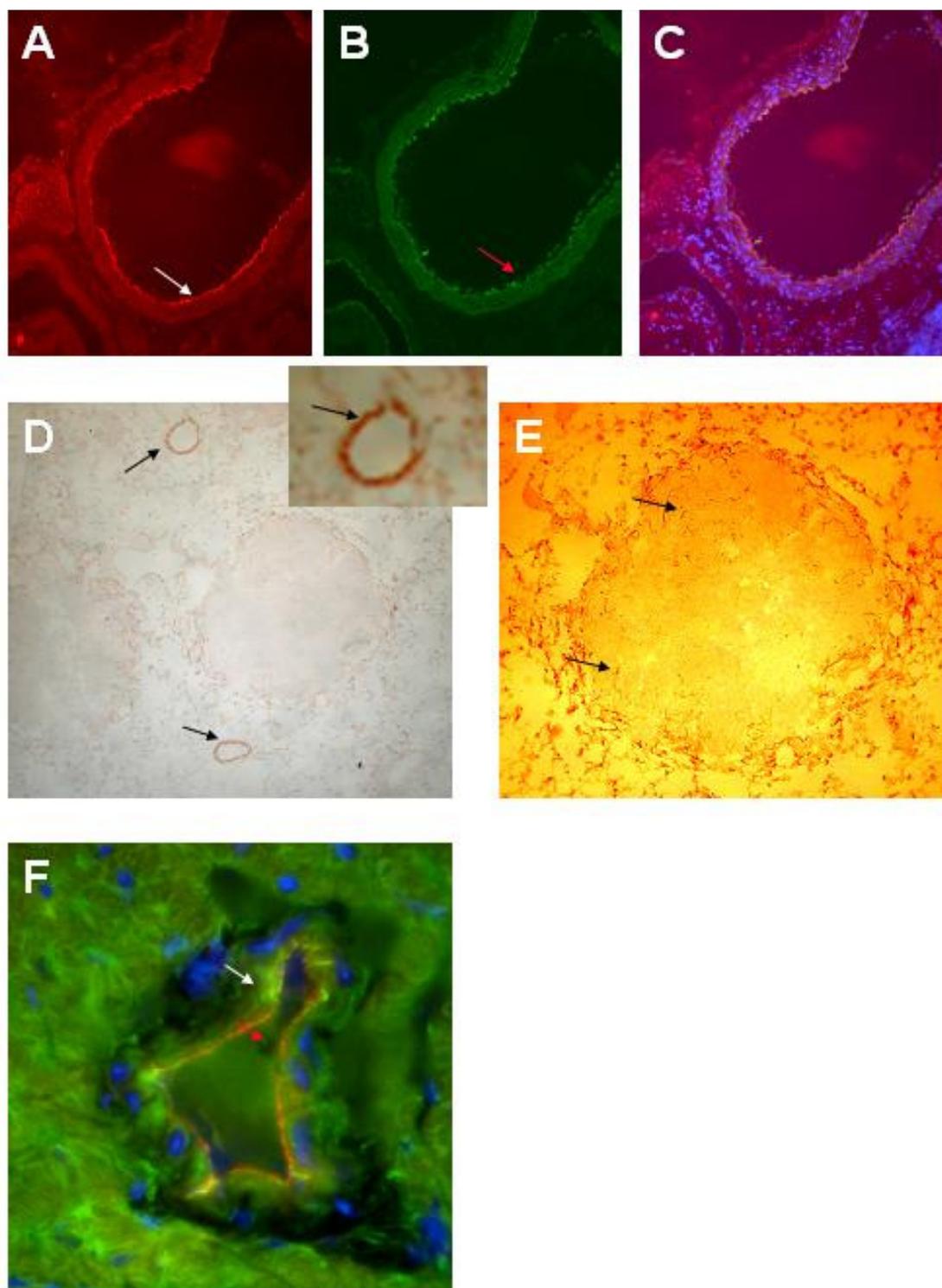
Figure 4.3 Expression of Subgroup F Sox proteins Sox7 and Sox18 in the endothelium *in vivo.*
(A) A large blood vessel in the heart was stained positive for Sox7 in the endothelium, detected by a Cy3-coupled (red color) anti-goat secondary antibody. (B) The same blood vessel was stained with Pecam1, an endothelial cell specific marker, using a FITC-labeled (green) secondary antibody. C) The overlay of (A) and (B) is showing the co-expression of both proteins in the endothelium. The nuclei were stained with DAPI (blue). (D) A lung tumor section was stained with an anti-Sox7 specific biotinylated antibody. The arrows indicate two large vessels stained positive for Sox7 (see also the magnification in the insert). Figure (E) shows a higher magnification of a metastasis growing in the lung. The arrows indicate vessels, which invade in the tumor and were positively stained for Sox7. The tumor nodules were generated by injecting LM8 osteosarcoma cells through the tail vein (Wei et al., 2004). (F) Sox18, stained with a FITC-labeled secondary antibody, is colocalized with Pecam1 (Cy3 labeled) in the endothelium of a normal heart section (shown by arrows).

45

In comparison to the observations made for Sox7 and Sox18, the third member of this subgroup, Sox17, is not expressed in the endothelium, but in the vascular smooth muscle cells *in vivo* (figure 4.4).
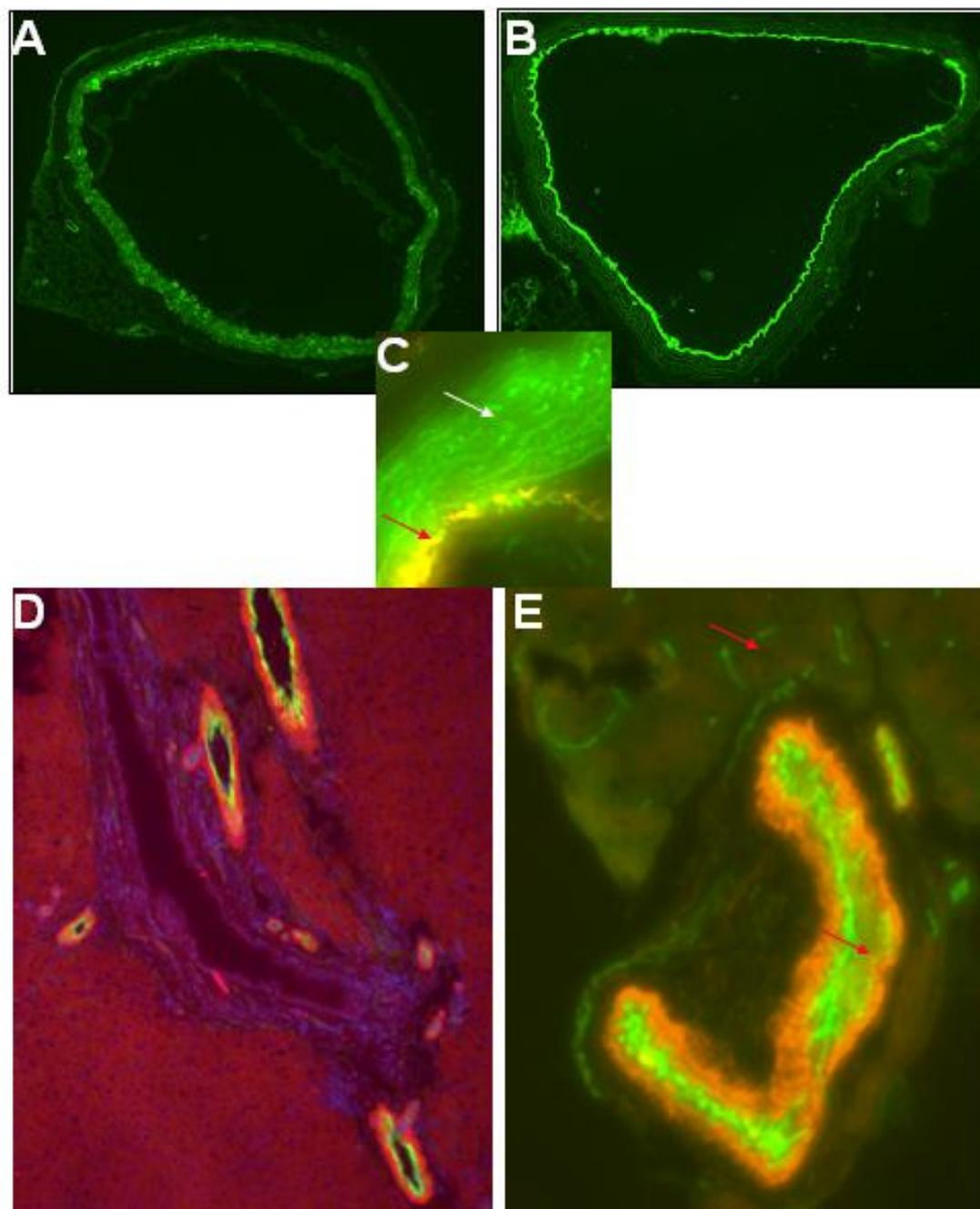
Figure 4.4 Subgroup F Sox protein Sox17 is expressed in vascular smooth muscle cells. Sox17 is expressed in the vascular smooth muscle cells of large blood vessels, detected with a FITC-labeled secondary antibody (A). In comparison, (B) shows Pecam-1 staining of the endothelium of an adjacent aortic section. Figure 4 (C) shows a high magnification of a small section of the aortic vascular wall, stained with a FITC-labeled anti-goat antibody against Sox17 (white arrow) and a Cy3-labeled ant-rat antibody directed against Pecam-1 (red arrow). (D) and (E) show a co-staining of Sox17 and Pecam-1 in a liver wildtype section, Sox17 detected with a Cy3 conjugated antibody and Pecam1 with a FITC labeled one (indicated by arrows).

This is visible in panels (A) and (B) of figure 4. Both sections show a cross section of a normal dorsal aorta. In (B), one can see an anti-Pecam-1 staining, using a FITC labeled secondary antibody, detecting Pecam-1 in the inner endothelial cell layer of the vessel. In contrast to this, Sox17 is detectable in the surrounding multiple layers, consisting of the aortic smooth muscle cells ((A) FITC stained). Figure 4.4 (C) shows the different expression of Sox17 (FITC, green) and Pecam-1 (Cy3, red) in a higher magnification in another aorta section. The location of Sox17 in vascular smooth muscle cells was moreover visible in different tissues, like spleen, kidney, brain, heart, testis, lung (data not shown), and in the liver (see figure 4 (D) and (E)).

The fact that Sox17 is expressed in vascular smooth muscle cells is a new and interesting finding, as this cell type becomes the focus of strategies to manage vascular diseases, because of the fact that vascular SMCs are critical for keeping the stability of the blood vessels and regulate the blood pressure. Thereby the question, regarding the genetic pathways regulated by Sox17 in SMCs and the conditions in which Sox17 gets activated in this cell-context, arises.

4.1.4. Response of Subgroup F Sox proteins to different stimuli in vascular cells

To begin to understand the context in which Sox7 and Sox18 might play a role in endothelial cells, human umbilical vein endothelial cells (HUVECs) were stimulated with different substances. Additionally, also the response of Sox17 was examined, as it was detected in endothelial progenitor and mature endothelial cells *in vitro* (see Chapter 4.1.2). As stimulatory agents, we used Lipopolysaccharide (LPS), Tumor growth factor beta 1 (TGF-β1), and Tumor necrosis factor alpha (TNF-α). Moreover, the cells were subjected to hypoxic conditions. LPS, as part of the cell wall of Gram-negative bacteria, simulates a cellular response to bacterial infection and causes activation of immune response intracellular pathways. *In vivo*, bacteria, which are absorbed by phagocytes, like macrophages or dendritic cells, activate via their LPS the absorbing cells to secrete cytokines, like IL (Interleukin)-1, IL-8, IL-6, IL-12 and TNF-α. Moreover, endothelial cells can be also directly activated by LPS via the Toll-like receptor 4 (TLR-4), which is highly expressed on their cell surface (Hijiya et al., 2002). TNF-α, which is also secreted by T-cells, activates the endothelial vessel wall

and increases its permeability. As consequence, endothelial cells are able to induce macrophages and other leukocytes, like neutrophils, to bind to the vessel wall and to invade in the surrounding inflamed tissues. Both signaling pathways, mediated by TLR4 and TNF-α induce NF-κB. Thereby, LPS and TNF-α cause very similar expression changes in endothelial cells (Magder et al., 2006). Factors, which are mainly induced, are chemokines, cell integrity mediating molecules, procoagulant factors, and adhesion molecules (Magder et al., 2006). TNF-α and LPS are thereby pro-inflammatory, whereas the third stimulus, TGF-β 1, is an anti-inflammatory factor. It has been shown, that a low level of TGF-β 1 expression is associated with the generation of an excessive inflammatory milieu, accelerating atherosclerosis (Grainger et al., 1995). This is accompanied by increased amounts of macrophages and T-cells, and a decrease of collagen content (Lutgens et al., 2002; Mallat et al., 2001). Moreover, TGF-β 1 is on the one hand able to induce apoptosis of endothelial cells via activation of the mitogen-activated protein kinase (MAPK) (Hyman et al., 2002), and on the other hand to initiate differentiation via the SMAD pathway.

Additionally to the treatment of the cells with different reagents, the HUVECs were cultured in a hypoxic environment (5% CO_2, 2% O_2) to imitate the conditions in hypoxic areas in disease, like tissue areas after myocardial infarction, stroke areas and tumor tissues. It is known that a hypoxic environment is created inside growing tumors inducing angiogenesis (Thomlinson et al., 1955) by an array of angiogenic factors like VEGF (Folkman et al., 1971), which is activated via the HIF1 (hypoxia-inducible factor 1)- α pathway (Liu et al., 1995; Forsythe et al., 1996; Shweiki et al., 1992).

Figure 4.5 shows a panel of differently stimulated mature endothelial cells. HUVECs were treated with LPS (100 ng/ml) and TGF-β1 (10 ng/ml) for 1 h, 2 h and 4 h (A). As one could see by RT-PCR, assaying the SOX7 mRNA expression, no changes were visible. SOX7 is not regulated by LPS, neither by TGF-β1, so it might not play a role in immunological or inflammatory reactions at the vessel wall.

A

B
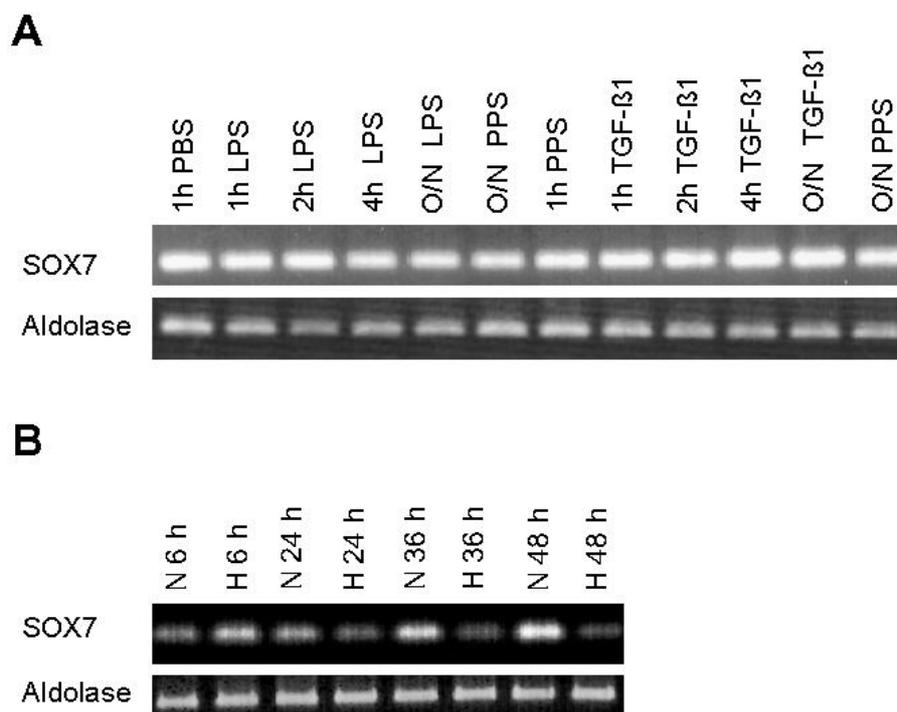
Figure 4.5 Regulation of SOX7 by different stimuli in endothelial cells. (A) SOX7 expression was examined by RT-PCR after stimulation of mature endothelial cells (HUVECs) with LPS (100 ng/ml) and TGF-β1 (10 ng/ml). Before treatment, the cells were kept quiescent for 24 h with 0,1% FCS containing medium. LPS and TGF-β1 were directly added to the medium. As control, 1X PBS was added to the cells with the same volume (100 µl) as LPS and TGF-β1. The cells were harvested after the indicated time points. (B) Quiescent HUVECs were incubated in hypoxic conditions (2% O_2, 5%CO_2), whereas the control was incubated at normal culture conditions (Normoxia = 21% O2, 5% CO2) for the indicated time points. The lysates were tested by RT-PCR for SOX7 expression (N = Normoxia, H = Hypoxia).

In contrast to this, figure 4.5 (B), shows that SOX7 is induced as cells reach confluency under normal conditions, but this induction is suppressed under Hypoxia after 36 h and 48 h. It has been demonstrated, that SOX7 is able to inhibit the transcription of wnt target genes, like c-myc, cyclin D1, MMP-7, and VEGF, by binding to β-catenin/TCF (T-cell specific factor), which has been shown *in vitro* by luciferase-assays (Takash et al., 2001). In the vasculature, the canonical wnt-signaling is mainly activated during proliferation of endothelial cells and inactive in the mature vasculature (Reviewed by Goodwin and D´Amore 2002). This means an accumulation of β-catenin in the cytoplasm, moving to the nucleus and initiating the transcription of wnt-responsive genes during proliferation, and a rare β-catenin expression in the normal, quiescent state of the adult vasculature (Blankesteijn et al., 2000; Yano et al., 2000a; Yano et al., 2000b). This knowledge leads to the hypothesis that SOX7 plays a key role in keeping the cells quiescent in the healthy

condition, whereas if it is downregulated by hypoxia, the cells can start to proliferate and differentiate.

In figure 4.6 one can see, that the same, as for SOX7, is the case for SOX17. This transcription factor is also downregulated after 24 h, 36 h and 48 h in response to Hypoxia.

Figure 4.6 SOX17 is downregulated in response to Hypoxia in mature endothelial cells. HUVECs, kept quiescent for 24 h, were cultured in hypoxic and normoxic conditions for 6 h, 24 h, 36 h, and 48 h. RNA was isolated, cDNA was prepared and checked for SOX17 expression by RT-PCR (N = Normoxia, H = Hypoxia).

For this observation one can take the same explanation like for SOX7, as it has also been shown, that Sox17 binds to β-catenin/TCF, thereby inhibiting the wnt-signaling pathway (Zorn et al., 1999). It seems that SOX7, as well as SOX17 might be important for keeping the non-proliferative state of mature endothelial cells and thereby conserving the intact vessel wall. Sox7 and Sox17 might have compensatory function for each other in this context. Some compensatory function of both factors has already been supposed, as both are co-expressed in the extra-embryonic endoderm (Kanai-Azuma et al., 2002; Taniguchi et al., 1999; Takash et al., 2001). Another indication is the fact, that both factors are known to cooperatively activate the mouse laminin alpha 1 enhancer in undifferentiated F9 cells (Niimi et al., 2004). On the other hand, we could detect Sox17 only in vitro in the endothelial cells, but not in vivo. Therefore, the response of Sox17 in hypoxic conditions might just give us a hint for its function in vascular smooth muscle cells, which will have to be examined in further experiments.

As third member of the Subgroup F Sox protein family, SOX18 has been tested for its expression in response to TNF-α, LPS and TGF-β1. The cells were stimulated for 2 h and 4 h, in case of TNF-α and LPS, and for 1 h, 2 h and 4 h in the case of TGF-β1. It

appeared that SOX18 expression is strongly reduced after TNF-α and LPS treatment, whereas it is weakly induced by TGF-β1 (figure 4.7).
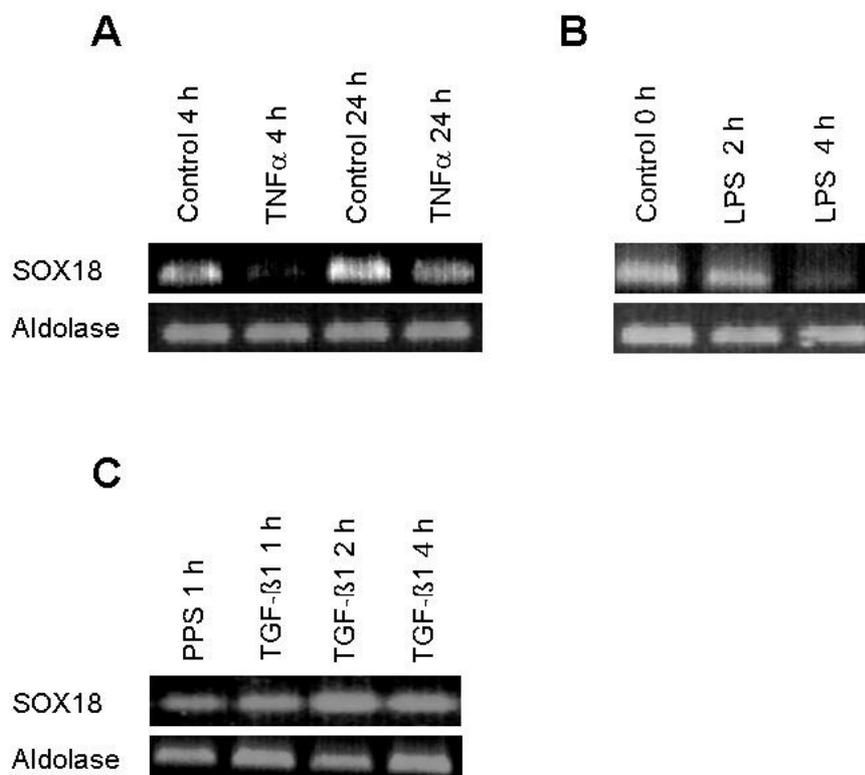
Figure 4.7 Regulation of SOX18 by different stimuli in mature endothelial cells. (A) Quiescent HUVECs were treated with TNF-α (100 ng/ml) for 4 h and 24 h. Non-treated cells were used as control. (B) Cells were stimulated with 100 ng/ml LPS for the indicated time points (2 h and 4 h). The control cells were harvested at the same time points as the stimulated cells. (C) Quiescent HUVECs were treated with TGF-β1 (10 ng/ml) for 1 h, 2 h and 4 h. The samples of all three assays were tested for SOX18 expression by RT-PCR. As control for equal cDNA amounts, the samples were tested for aldolase expression.

The fact that SOX18 responds to the cytokines in this manner, leads to the assumption, that SOX18 might be a regulator of some target genes, which are involved in early inflammatory processes in endothelial cells. But this seems to happen in a time-dependent manner, as Sox18 has been shown to be highly upregulated after 5 days and 21 days during angiogenesis in wounded tissues (Darby et al., 2001). Thereby, Sox18 might regulate both early target genes as well as late-target genes during inflammation, like VCAM-1, which has been demonstrated to be a target gene of Sox18 (Hosking et al., 2003). The fact that SOX18 is downregulated or repressed at such an early time point, but strongly expressed or induced at later time points, supposes a bifunctional behavior for

SOX18, which has already been demonstrated for many other sox proteins (Yuan et al., 1995; Nishimoto et al., 1999)

4.1.5. SOX17 is upregulated in proliferative conditions in human coronary artery smooth muscle cells

Since SOX17 is strongly expressed in vascular smooth muscle cells *in vitro* and *in vivo*, it was interesting to test if SOX17 plays a role in this cell type under proliferative conditions. Vascular smooth muscle cells are known to exist in two main states: on the one hand the proliferative, and on the other hand the differentiating one. In response to environmental changes the appearance of cells can change (Owens et al., 1996; Owens et al., 2004). The proliferation of vascular SMCs is a process, which follows tube formation from endothelial cells during angiogenesis, leading to the maturation of the newly formed blood vessels. Moreover, the proliferation of vascular SMCs is also associated with pathological angiogenesis. One main factor, implicated in these processes, in the physiological, as well as in the pathological condition, is PDGF-BB (Platelet Derived Growth Factor-BB), a growth factor, which stimulates proliferation of vascular smooth muscle cells and attracts them to migrate to the new outgrowing endothelial tube, being induced at sites of stress (Ross et al., 1993). Thereby, PDGF-BB is part of the angiogenic stimuli and by that a first choice to induce the proliferation of vascular SMCs.

To examine the effect of PDGF-BB on Sox17 expression in SMCs, the cells were kept in serum-free conditions for 32 h and afterwards treated with 50 ng/ml PDGF-BB. The control cells stayed untreated and were lysed at the same time points as the stimulated ones (2 h, 24 h, and 48 h). As shown in figure 8, SOX17 is downregulated in culture as time proceeds, but its expression is maintained after 48 hours of PDGF-BB treatment in vascular SMCs.

As a control for a successful induction, we chose EGR-1, which is known to be induced by PDGF-BB at very early time points (15 min – 1 h) after treatment (Kamimura et al., 2004; Mundschau et al., 1994). It appears that indeed EGR-1 is strongly upregulated at the early time points, indicating a successful stimulation (figure 4.8). This result suggests that SOX17 might be activated in vascular SMCs by

proliferative signals like. PDGF-BB, but that in contrast to EGR-1, SOX17 is not an early-induced gene.
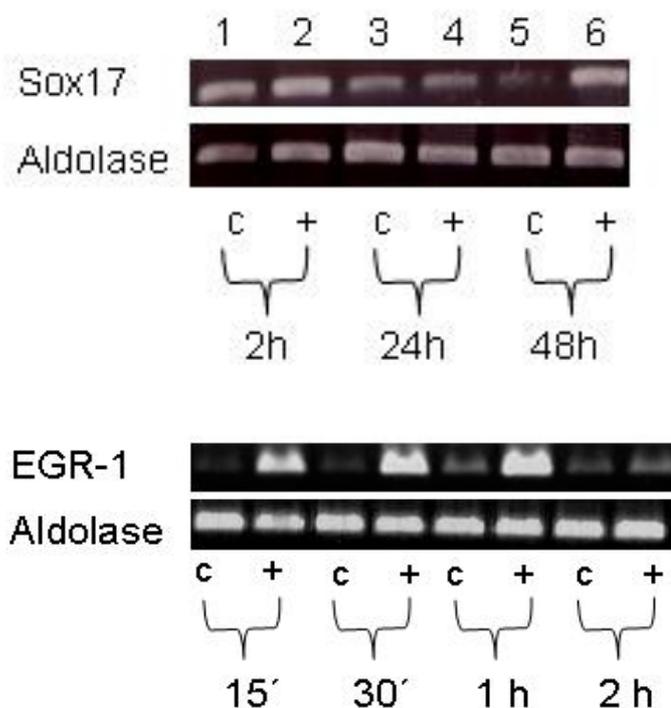
Figure 4.8 SOX17 expression is maintained after PDGF-BB stimulation in human coronary artery smooth muscle cells. Coronary artery smooth muscle cells, kept quiescent for 32 hours with 0.1% FCS containing medium, were treated with 10 ng/ml of PDGF-BB for the indicated time periods, and RNA samples were analysed by RT-PCR for SOX17 expression. The comparison of lane 5 and 6 shows that SOX17 expression is maintained at high levels after 48 h PDGF-BB stimulation, whereas it is downregulated in control cells (c = control, untreated cells; + = PDGF-BB stimulated). PCRs, using an EGR-1 specific primer, were done on shortly PDGF-BB induced samples to verify that PDGF-BB is functional (15 min, 30 min, 1 h, and 2 h). All samples were tested for aldolase to check for equal cDNA amounts.

To confirm these results, we chose as another proliferation stimulus low density lipoprotein (LDL). By binding to the low density receptor related protein 5/6 (LRP5/6), LDL activates the wnt-signaling pathway and thereby the proliferation of vascular SMCs (Wang et al., 2004). This stimulation assay should also give us a hint for possible implications of SOX17 in pathological conditions, as a high level of LDL in the circulating blood leads to the initiation of inflammatory processes at the vessel wall, a process resulting in vascular defects and disease, like atherosclerosis (Steinberg et al., 1989; Witztum et al., 1993; Ross et al., 1999; Libby et al., 2002).

As one can see in figure 9, LDL leads to an induction of SOX17 after 6 h (lane 7) and 24 h (lane 9) of treatment. Also under these experimental conditions, we used EGR-1 as control for a successful induction. It is known, that LDL upregulates EGR-1 in monocytes via the MEK-ERK1/2 pathway (Harja et al., 2004). Consistent with these previous findings, EGR-1 is upregulated very early (30 min) after LDL treatment (figure 9).
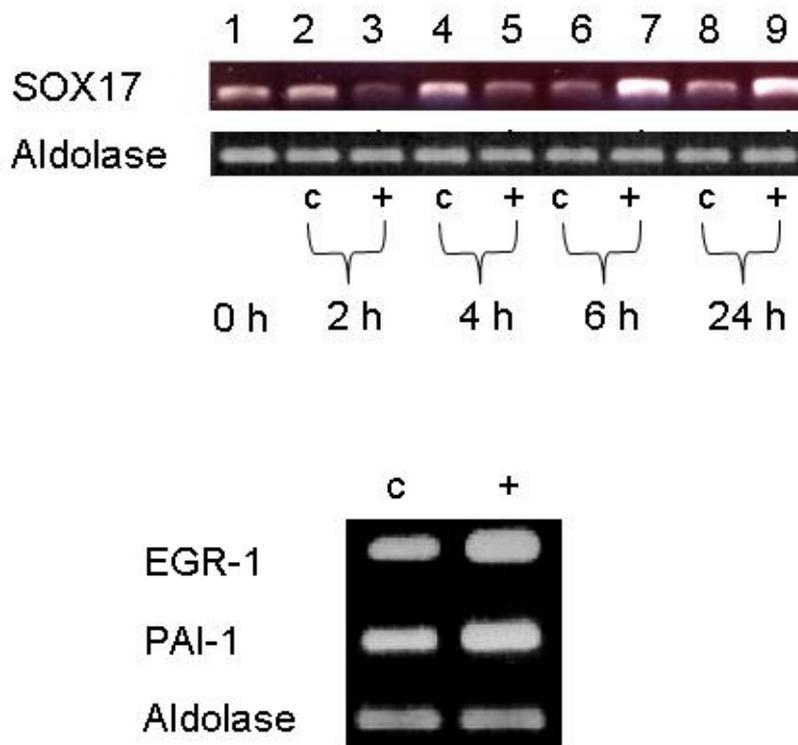
Figure 4.9 Low density lipoprotein regulates SOX17 expression in coronary artery smooth muscle cells. Quiescent (for 32 h) coronary artery smooth muscle cells were treated with aggregated LDL (50 ng/ml) for 2 h, 4 h, 6 h and 24 h. RNA samples were prepared and checked by RT-PCR for SOX17 expression. To have comparable amounts of cDNA, the samples were tested for aldolase expression. The expression of SOX17 is reduced after 2 h and 4 h of treatment and increased after 6 h and 24 h of LDL treatment. As control for a successful stimulation, SMCs, treated for 30 min with LDL (50 ng/ml) were checked for PAI-1 (Plasminogen Activation Inhibitor-1) and EGR-1.

In summary, we found that both stimuli, PDGF-BB and LDL are able to maintain or increase SOX17 expression at later time points after treatment (48 h and 24 h). These preliminary results lead to the hypothesis, that SOX17 might be a late response gene, raising the possibility that it might be involved in vascular disease in response to high LDL levels and in angiogenesis in response to PDGF-BB during vessel maturation. After these first results, the question arose about the signaling cascade in which SOX17 might be involved on the transcriptional level.

4.1.6. EGR-1 induces SOX17 expression in human coronary artery smooth muscle cells

To answer the question, in which signaling context SOX17 could participate in human vascular smooth muscle cells, we tested if EGR-1, a known transcriptional regulator of VSMC proliferation, mostly acting upstream of other transcription factors (Santiago et al., 1998), is also able to induce SOX17. Another indication to choose EGR-1 in this context is the observation in this work that EGR-1 is, like SOX17 upregulated in response to PDGF-BB and LDL but at earlier time points. This has also been demonstrated in literature (Kamimura et al., 2004). EGR-1 is for example known to induce the angiogenic factor CCN-1 and the expression of PPARgamma1 in vascular smooth muscle cells (Grote et al., 2004; Fu et al., 2002). Therefore, we postulated that EGR-1 might act upstream of a signaling cascade, in which SOX17 might also be involved in the proliferation of vascular SMCs. To test this model, EGR-1 was transiently overexpressed in human coronary artery smooth muscle cells and the expression level of SOX17 was examined (figure 4.10).
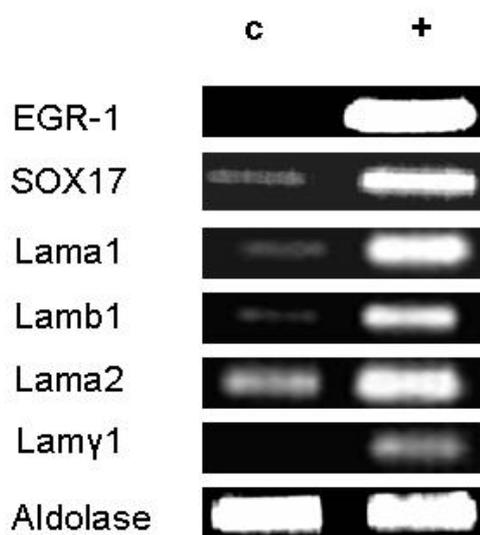
Figure 4.10 EGR-1 induces the expression of SOX17 and Lama1, Lamb1, Lama2 and Lamγ1 in human coronary artery smooth muscle cells. EGR-1, cloned in the IRES-eGFP expression plasmid was transiently overexpressed for 24 h in coronary artery smooth muscle cells, using Lipofectamine™ 2000-assisted transfection. After lysis, RNA was isolated via the Qiagen RNA isolation kit and a reverse transcription was done. The cDNA samples were checked by RT-PCR for SOX17, Lama1, Lamb1, Lama2 and Lamγ1 expression. SOX17 is strongly upregulated after EGR-1 overexpression. The same is true for Lama1, Lamb1 and Lamγ1, whereas Lama2 is only slightly upregulated. As a control for comparablel cDNA amounts, the samples were tested for aldolase expression. Empty vector (IRES-eGFP) transfected cells were used as control (c = control; + = EGR-1 transfected).

Thereby it appeared that SOX17 is strongly upregulated after 24 h overexpression of EGR-1 (figure 4.10). This observation indicates that SOX17 is downstream of EGR-1 in a genetic pathway that might control activation and/or proliferation of SMCs.

Another indication for an interaction of SOX17 and EGR-1 to regulate common target genes in coronary artery SMCs is the fact that EGR-1 overexpression in vascular SMCs leads to a strong induction of different members of the laminin family, like Lama1, Lamb1, Lama2 and Lamγ1 (figure 4.10) of which Laminin α 1 (Lama1) is a known target gene of Sox17 (Niimi et al., 2004).

4.1.7. TGF-β 1 reduces SOX17 expression in human coronary artery smooth muscle cells 48 hours after stimulation

As it was shown above, SOX17 is inducible by growth stimuli, like PDGF-BB and LDL, in vascular SMCs (4.1.5.; Figure 4.8 and 4.9). In contrast, differentiation of vascular SMCs is characterized by the expression of smooth muscle marker genes like Smooth Muscle (SM)-22alpha, SM-actin, Smoothelin, and Calponin (Ross et al., 1993; Aikawa et al., 1993; Shanahan et al., 1993). PDGF-BB inhibits differentiation (Owens et al., 2004), whereas Tumor growth factor-beta 1 (TGF-β 1) induces the differentiation of vascular smooth muscle cells, by upregulating smooth muscle contractile marker genes via a TGF-β control element (TCE) in the promoter region of these genes (Hautmann et al., 1997). Moreover, TGF-β 1 shows a concentration-dependent influence on proliferation *in vitro*, and stimulates the expression of extracellular matrix proteins (Ross et al., 1993; Reddy et al., 1993; Saltis et al., 1992; Owens et al., 1988). It is secreted by vascular SMCs as well as by macrophages. An important transcriptional regulator of the differentiation process in vascular SMCs is Notch, promoting phenotypic changes via activation of CBF-1/RBP-Jκ-dependent pathways (Morrow et al., 2005). Therefore, TGF-β 1 is important for the maintenance of vessel stability and plays a crucial role in the prevention of the development of atherosclerosis antagonizing the function of PDGF-BB (Schönherr et al., 1993).

Therefore, we examined if TGF-β 1 regulates SOX17 expression in an opposite manner to PDGF-BB. To this end, cultured human coronary artery SMCs were treated with 10 ng/ml TGF-β 1 for 30 min, 1 h, 24 h, and 48 h. The control cells were

left untreated. Using RT-PCR, SOX17 expression was examined (figure 4.11). The comparison between lanes 7 and 8 shows a strong downregulation of SOX17 after 48 h TGF-β 1 treatment (lane 8) in comparison to the untreated cells (lane 7). Interestingly, the suppression takes place at late time points, when induction was seen by PDGF-BB.
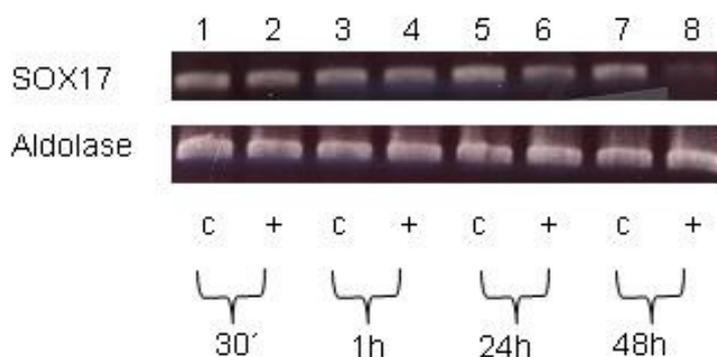
Figure 4.11 TGF-β 1 reduces SOX17 expression in human coronary artery smooth muscle cells after 48 hours. In FCS-containing medium cultured human vascular smooth muscle cells were treated with 10 ng/ml of TGF-β 1 for 30 min, 1 h, 24 h and 48 h. Therefore TGF-β 1 was directly added to the medium-containing plate. RNA was isolated at the indicated time points and tested by RT-PCR for SOX17, and as control for aldolase expression. Comparing lanes 7 and 8, a strong reduction of SOX17 expression is visible after 48 h of induction (c = control; + = TGF-β 1 treatment).

Taking into account the results from the PDGF-BB and LDL stimulation assays (4.1.5.), and the induction of SOX17 by EGR-1 (4.1.6.), we propose that SOX17 is involved in the proliferation of vascular SMCs and might be suppressed when they are induced to differentiate. As SOX17 is known to be a transcription factor that can act in a bifunctional manner, on one hand as activator and on the other hand as repressor of transcription, in conjunction with cell-type specific interaction partners, we further investigated the SOX17 and EGR-1 interaction.

4.1.8. Sox17 and Egr-1 are co-expressed in murine vascular smooth muscle cells in wildtype conditions

To expand the in vitro results, assuming a connection of EGR-1 and SOX17 in human vascular smooth muscle cells, the in vivo expression of both factors in normal aortic sections was examined. The assay was done by immunofluorescence stainings, using a Cy3-labeled secondary antibody in the case of Sox17 and a FITC-labeled one for the detection of Egr-1 on normal mouse aorta sections. It appeared

that Sox17 and Egr-1 are strongly expressed and moreover co-expressed in the multiple aortic smooth muscle cell layers (figure 4.12).
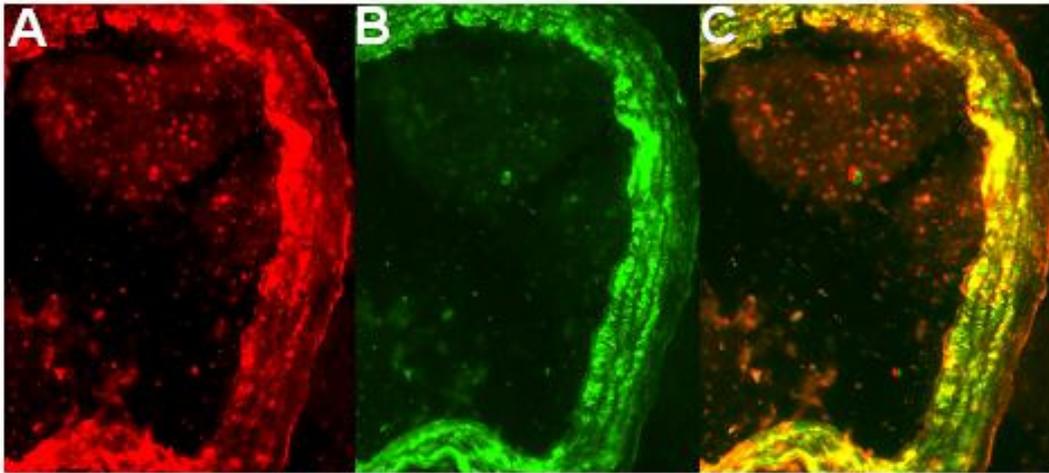
Figure 4.12 Sox17 and Egr-1 are co-expressed in vascular smooth muscle cells of normal aortas. The figure shows a part of a cross-section of a mouse coronary aorta. (A) Positive staining of the aortic smooth muscle cells for Sox17, detected via a Cy3-labeled secondary antibody. (B) shows an Egr-1 staining (FITC-labeled secondary antibody) on the same section. The overlap of both sections (C) demonstrates the co-expression of both factors (yellow areas).

These results demonstrate that the two transcription factors are co-expressed in SMCs *in vivo*, further supporting an interaction between these two proteins.

4.1.9. Sox17 is strongly expressed in atherosclerotic plaques of ApoE deficient mice

The fact that SOX17 is upregulated in proliferative conditions in vascular SMCs implies a role of this transcription factor in pathological conditions. The proliferation of the SMCs is one of the first steps in vascular abnormalities, like atherosclerosis. Therefore, Sox17 expression was examined on a section of an atherosclerotic plaque of an ApoE deficient mouse. ApoE is an important mediator of cholesterol uptake in the cell and thus keeping the cholesterol concentration in the blood low. If ApoE deficient mice are fed with a cholesterol rich diet, in this case for 12 weeks, they start to form atherosclerotic plaques as a consequence of high LDL concentrations in the blood (Nakashima et al., 1994; Breslow et al., 1996). A disruption of the vessel wall (Intima) is followed by local inflammatory processes. As a consequence of high cholesterol concentrations in the circulating blood, the endothelial cells get activated and start expressing adhesion molecules like ICAM-1 or VCAM-1 at their cell surface.

Moreover, selectins, like the endothelial cell specific E-Selectin, are upregulated and enable, in conjunction with the adhesion molecules, the binding of leukocytes (mainly monocytes and neutrophils), to the endothelial cell surface. The leukocytes roll along the endothelium, bind tightly to the wall of the intima and extravasade in the subendothelial space, where the monocytes differentiate into macrophages. The extravasation is facilitated, as activated ECs show a loss or destabilization of cell-cell interactions. In the subendothelial space, activated macrophages start to express cytokines and growth factors like PDGF-BB, which activates the proliferation and migration of vascular SMCs. As a consequence of these molecular processes, the media gets thicker and expands into the lumen of the vessel and leads finally to the formation of an atherosclerotic plaque. Thereby the plaques consist mainly of vascular SMCs and macrophages as well as extracellular matrix proteins (Gown et al., 1986).

As shown in figure 4.13, SOX17 is, additionally to its expression in the aortic smooth muscle cells, also strongly expressed in the vascular SMCs of the atherosclerotic plaque (indicated by arrows). To clarify the difference to the endothelium, anti-Pecam-1 co-staining was done, marking the endothelial cells (FITC).
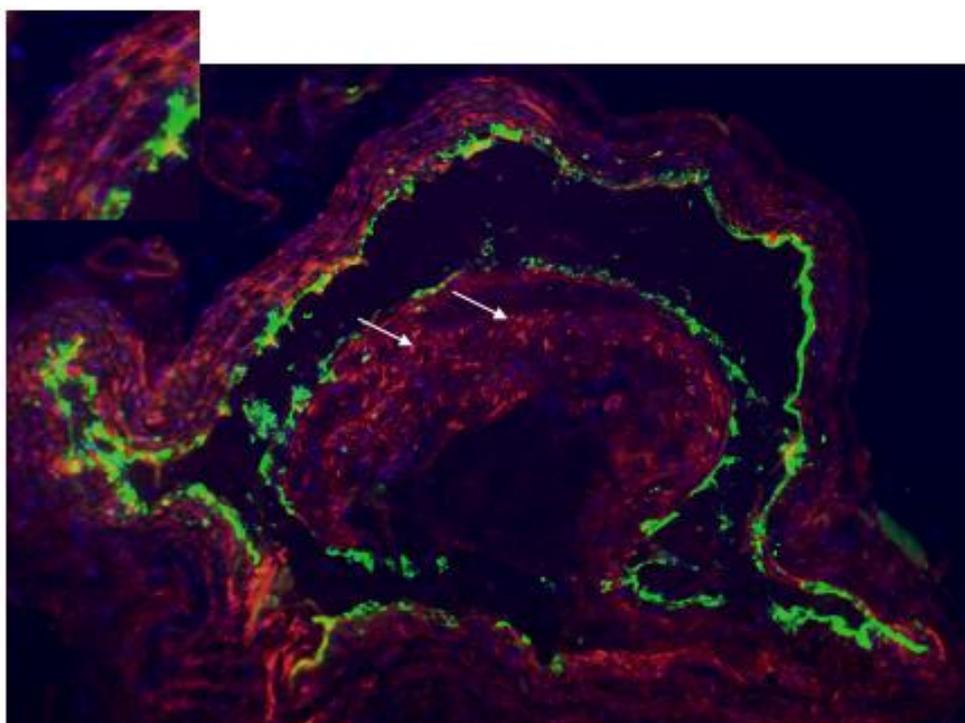
Figure 4.13 Sox17 is strongly expressed in atherosclerotic plaques of ApoE deficient mice. An aortic atherosclerotic plaque of an ApoE deficient mouse was stained for Sox17 with a Cy3-labeled antibody (indicated by arrows) and a FITC-conjugated Pecam-1 antibody (green). The figure shows an overlay of both images. Additionally, the nuclei were stained with DAPI (blue).

This observation supports the hypothesis that SOX17 might be involved in the proliferation SMCs, as most of the vascular SMCs in a plaque of that early stage (12 weeks) are still proliferating.

4.1.10. Sox17 and Egr-1 show the same expression pattern in aortic smooth muscle cells in pathological conditions
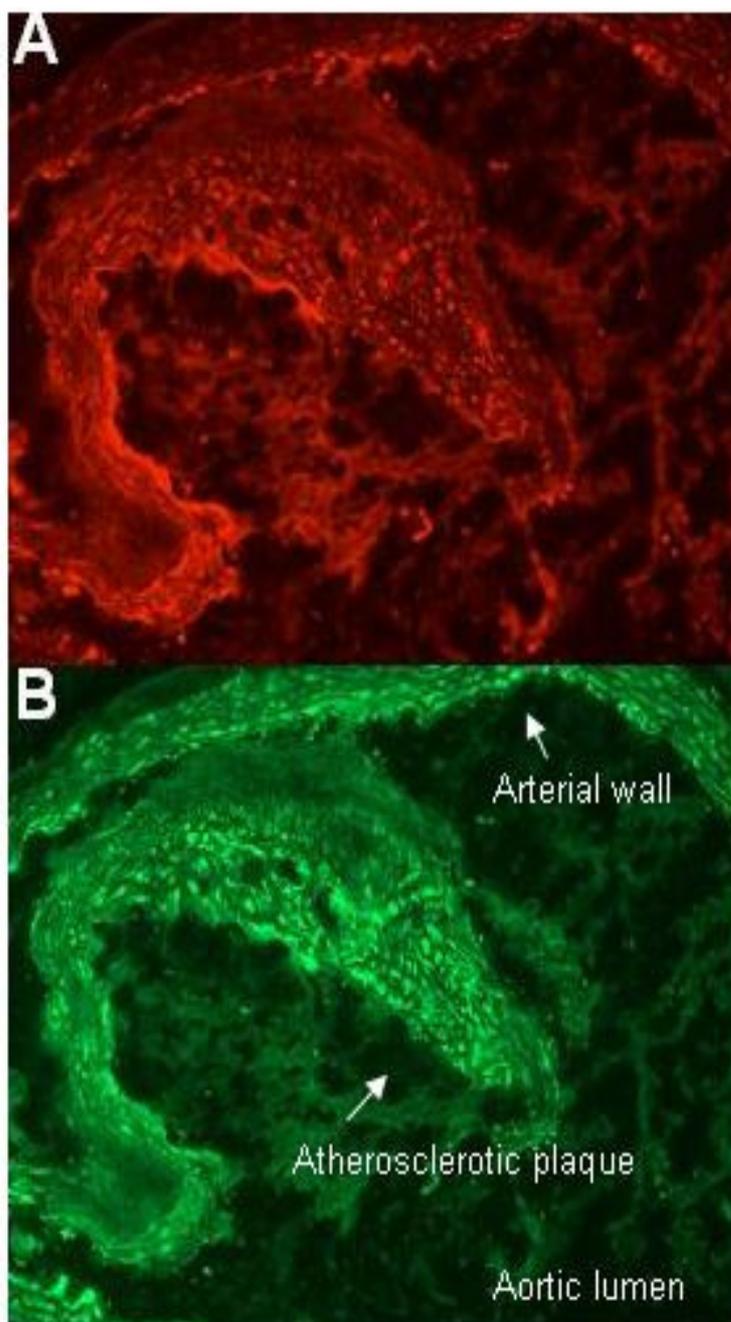
Following the observation that Sox17 is expressed in the SMCs of an atherosclerotic plaque, we examined if it is co-expressed with Egr-1, which is also known to be expressed in atherosclerotic plaques (McCaffrey et al., 2000). Therefore both transcription factors were co-stained on a section of an atherosclerotic plaque of an ApoE deficient mouse, as it was done for Sox17 in the chapter before. For this, fluorescence-labeled antibodies were used, a FITC-labeled one for Egr-1, and a Cy3-labeled one for Sox17. As shown in figure 4.14, Sox17 and Egr-1 are co-expressed in the smooth muscle cells of the atherosclerotic plaque additionally to the ones of the arterial wall itself, as already seen before.

Figure 4.14 Sox17 and Egr-1 show a similar expression pattern in aortic smooth muscle cells in pathological conditions. Sox17 (A) and Egr-1 (B) were examined by immuno-fluorescence on atherosclerotic plaque sections of an ApoE deficient mouse. For Sox17, a Cy3-conjugated secondary antibody against goat was used and for Egr-1 an anti-rabbit FITC-conjugated one.

This observation, in addition to the ones made in the stimulation with PDGF-BB and LDL assays, supports the notion that Sox17 might play a role in atherosclerosis as a target gene or an interaction partner of Egr-1 in vascular SMCs.

4.1.11.   Overexpression of EGR-1 in human coronary artery smooth muscle cells causes a strong induction of KLF4

To find other proteins that might be part of this signaling cascade, involving EGR-1 and SOX17, I further analyzed the RNA samples after overexpression of EGR-1, using RT-PCR for identifying possible target genes. Proteins, which are known to play a role in the proliferation of vascular smooth muscle cells, were tested as first candidates. One of these factors is KLF4 (Krüppel-like Factor 4), also known as GKLF (Gut-like KLF), a zinc-finger transcription factor that has been shown to be induced by PDGF-BB and thereby downregulating expression of differentiation marker genes in vascular smooth muscle cells (Liu et al., 2005). KLF4 is considered to be a key player in the phenotypic switch of vascular smooth muscle cells from the proliferating to the differentiating cell type and is thereby a predicted candidate for our signaling model (King et al., 2003). It is moreover known, that KLF4 also needs to bind to a partner protein to be transcriptionally active. As in the case of the Sox proteins, such interaction partner(s) are cell context dependent. Another parallel between KLF4 and SOX17 is their expression in the endoderm in the developing gut and the fact that both factors bind to β-catenin (Zhang et al., 2005; Shie et al., 2000; Shields et al., 1996; Stone et al., 2002). Moreover, both proteins are able to act as activators or repressors of transcriptional activation (Ghaleb et al., 2005).
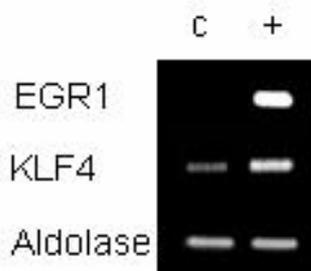
Figure 4.15 Overexpression of EGR-1 in human coronary artery smooth muscle cells causes a strong induction of KLF4. EGR-1 was transiently overexpressed in coronary artery smooth muscle cells via lipofection (Lipofectamine™2000) transfection of the EGR-1-IRES-eGFP expression vector. The transfected cells were incubated for 24 h. RNA samples were generated and tested by RT-PCR for expression of KLF4 (c = control; + = EGR-1 transfected).

Figure 4.15 shows that following transient overexpression of EGR-1 for 24 h in human coronary artery smooth muscle cells, KLF4 is strongly induced. By this, it seems that KLF4 is, like SOX17, a putative target of EGR-1. In conjunction with the previous results, one could conclude that PDGF-BB initiates EGR-1, which thereby induces SOX17 and KLF4 in vascular SMCs. This is suspected to happen during proliferation and might lead to a suppression of smooth muscle differentiation, as this is known to be the case for KLF4. Thereby it is reasonable to assume that the concerted action of EGR-1, SOX17 and KLF4 is required to maintain SMCs in the proliferative state. This will have to be confirmed by promoter analysis and in vivo via co-immunoprecipitations.

4.1.12. The human KLF4 promoter contains a putative SOX17 binding site via which SOX17 induces KLF4 expression

As we suspect SOX17, KLF4 and EGR-1 to act in concert in one signaling cascade, the question for the order in which the three factors get activated, comes up. As EGR-1 is known to be a transcriptional key player, mediating proliferation of vascular SMCs, and as the overexpression of EGR-1 induces the expression of SOX17 and KLF4, we suppose it to act at the top of the hypothesized cascade. This prediction leaves the possibility of an induction of SOX17 directly via EGR-1, or with KLF4 as intermediate, being activated via EGR-1. Moreover it might be that SOX17 as well as KLF4 get activated in parallel. To bring an order in this signaling pathway, we blasted the ENSEMBL sequence of the human KLF4 promoter against the putative binding sites of SOX17 (GACAAT) and EGR-1 (CGCCCCGC/CGCCCGCGC/CTCCCCCG C).
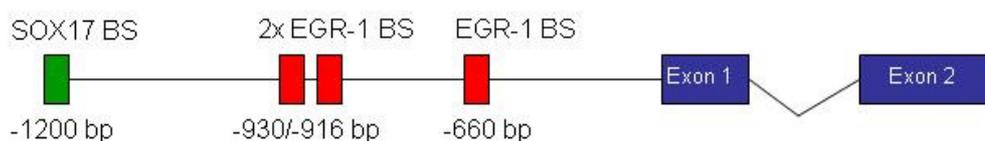
Figure 4.16 The structure of the human KLF4 promoter. Shown is a part of the human KLF4 coding sequence and 1200 bp upstream of ATG, comprising the promoter region. Blasting the human KLF4 sequence, three putative EGR-1 binding sites were identified at position -930, -916, and -660 bp (indicated by red boxes) and one putative SOX17 binding site, located 1200 bp upstream of the transcriptional initiation site (indicated by the green box). The gene comprises 4 Exons from which only 2 Exons are shown here (blue boxes).

Thereby it appeared that the promoter region of human KLF4 contains one putative SOX17 binding site at around 1200 basepairs upstream of the transcriptional initiation site (figure 4.16) and three putative EGR-1 binding sites, located 930, 916 and 660 bases upstream. Sox17 and Sox7 are known to interact with the sequence AACAAT/GACAAT, as they have been shown to bind specifically to this sequence in the promoter region of mouse Laminin alpha 1 (Lama1) (Niimi et al., 2004). At least, the detection of the putative binding sites raises the possibility that SOX17 transactivates the human KLF4 promoter. To test if this is at least the case, promoter analysis on the human KLF4 promoter were performed. Therefore three promoter constructs were cloned in a pGL3 luciferase expression plasmid.
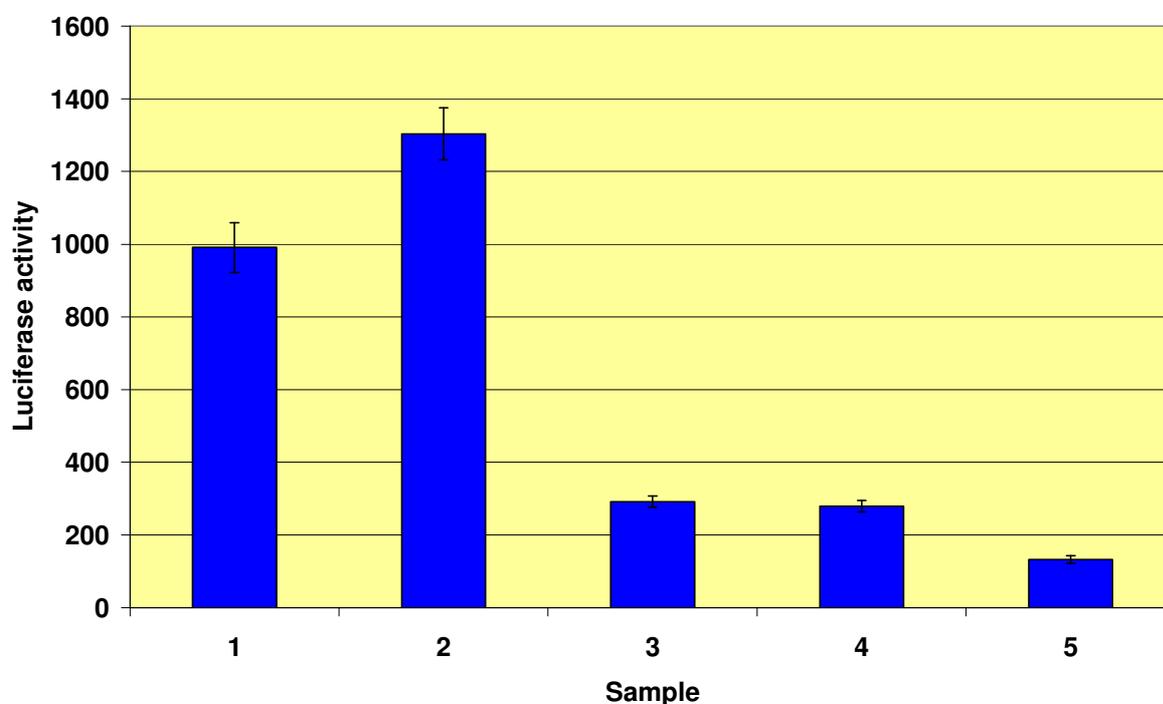
Figure 4.17 The expression of human KLF4 is induced by SOX17 via a putative SOX17 binding site, located 1200 bp upstream of ATG. To perform luciferase activity assays, three KLF4 promoter constructs were cloned in the pGL3 luciferase vector (Promega). One Construct contained the intact putative SOX17 binding site and extended 1400 bp upstream of the ATG codon of human KLF4. The second construct had point mutations in the putative SOX17 binding site (GACAAT>GGTCCT), whereas the third construct was deleted for this site. The cloned constructs were transiently transfected by Lipofectamine™ 2000 in HeLa cells. After 24 h the lysates were made and assayed for luciferase activity using the luciferase kit of promega. The graphic shows the relative luciferase activity measured in a chemiluminometer. The first sample shows the unmutated construct, co-transfected with the CMV-vector. Sample 2 was also transfected with construct 1, but moreover co-transfected with SOX17-CMV. The third and fourth samples contain the mutated construct, first co-transfected with CMV (sample 3), and in sample 4 co-transfected with SOX17-CMV. The last sample contains the deleted construct, co-transfected with the CMV vector. The measurements were three times repeated.

One construct comprises the intact putative SOX17 binding site. In the second construct the putative site was mutated (GACAAT>GGTCCT) and in the third construct the binding site was deleted. In the latter construct, only the putative SOX17 binding site was excluded from the cloned construct. All three constructs were transiently transfected in HeLa cells and co-transfected, in case of the control, with an "empty" CMV vector, and with the human SOX17-CMV expression plasmid, to test if SOX17 transiently induces KLF4 expression, at least in case of the intact and the mutated construct. To exclude any different effects of the used promoter on the induction of the luciferase activity, the CMV vector was chosen as control, as the human SOX17 sequence was also cloned into this plasmid. Therefore the measured values should be comparable.

Lane 1 and 2 show the transfection of the non-mutated construct, co-transfected with the empty CMV vector (lane 1) and with the SOX17 expression plasmid (lane 2), where it appears, that SOX17 is able to induce KLF4 expression. In comparison to the CMV-co-transfection, the induction is 30 % higher, as the activity of the transfected construct shows an activity of around 990, whereas the sample with the co-transfected SOX17-CMV plasmid has a mean value of 1300. This induction was abrogated, using the mutated and deleted constructs (figure 4.17 A, 2 and 3). In lane 3 and 4, the construct with the mutated binding site (GACAAT>GGTCCT) is co-transfected with the CMV-vector (lane 3) and with the SOX17-CMV expression plasmid (lane 4). Comparing sample 2 and 4 it appears that the luciferase activity is reduced from 1300 to 279. Moreover, if one compares sample 2 with sample 5, which shows the co-transfection of the promoter construct, deleted for the putative Sox17 binding site, with the empty CMV vector, it appears that the activity of luciferase is reduced from 1300 to 132. It is obvious, that the measured values are relatively low. A possilbe explanation for this observation might be the fact, that all samples were co-transfected with a CMV-promoter containing construct. This might due to a reduction of the luciferase activity. Nevertheless, the results were repeatable and indicate that the human SOX17-CMV construct is able to induce human KLF4 expression. A putative SOX17 binding site (GACAAT), located 1200 basepairs upstream of the start-codon, seems to be essential for this transactivation.

4.1.13. SOX17, EGR-1 and KLF4 are supposed to bind to each other in human coronary artery smooth muscle cells *in vivo*.

All previous results we had so far led us to the hypothesis that all three factors might interact and therefore bind to each other in human vascular smooth muscle cells. To prove this, immuno- and co-immunoprecipitations were performed. Therefore cultured and proliferating coronary artery smooth muscle cells were used. The cells were scraped, washed twice with 1X PBS and afterwards resuspended in lysis buffer, containing 1% NP-40. After incubation on ice (20 min), the cells were sonicated and the resulting cell fragments were pelleted by centrifugation. The clear supernatant was incubated overnight at 4°C with the primary antibody and A-Sepharose. Next day, the lysates were washed five times with lysis buffer, the pellet was resuspended in loading dye (containing 0.1M DTT) and boiled at 95°C for 10 min. At least Western-Blots were done.

All three proteins could be immunoprecipitated from the human CASMCs, as well as c-myc, which was chosen as control for a non-binding protein. As isotype control, 3F10, an antibody, directed against a Hemaglutinin tag, was used. All samples were checked on one gel for SOX17 expression using a SOX17 specific primary antibody from SantaCruz. As one can see in figure 4.18, SOX17 protein is detectable in the SOX17, EGR-1 and KLF4 precipitated samples. In contrast to this the controls are negative for SOX17.
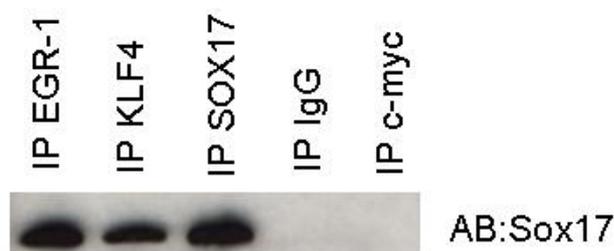
Figure 4.18 SOX17 is detectable in EGR-1 and KLF4 precipitated samples of human coronary artery smooth muscle cells *in vivo*. The lysates of the immunoprecipitations of EGR-1, KLF4, SOX17, 3F10 and c-myc, as control for a non-SOX17-binding protein, were loaded on a 12.5 % SDS gel. The blot was analysed with a SOX17 specific primary antibody (goat, polyclonal; SantaCruz). Except of the two controls (IgG and c-myc), SOX17 is detectable in all three samples (lane 1 - 3).

For the precipitation of the proteins, the same concentration of antibody was used (1 µg/ml). Therefore it seems surprising that the amount of SOX17 detected in the EGR-1 precipitated sample is comparable to the amount of protein in the SOX17 precipitated sample. As it is unreasonable that the whole pool of SOX17 protein in the cell is bound specifically to EGR-1, this observation might be due to different precipitation capacities of the antibodies. At least there might also be differences in the amount of whole protein for the reason of a loss of protein during the precipitation protocol.

Nevertheless, the observations made in this co-immunoprecipitation lead us to the conclusion that all three transcription factors might specifically bind to each other in human CASMCs, building a stable protein complex. This complex could act as activator of proliferation associated genes and repress the expression of smooth muscle differentiation marker genes, as it was already shown for KLF4 (Dandre et al., 2004; Kawai-Kowase and Owens, 2006; Yoshida et al., 2006; Holycross et al., 1992). Of course these results would still have to be confirmed in further analysis, using different controls, checking for all antibody subtypes, as the used antibodies for EGR-1, KLF4 and SOX17 are polyclonal. Moreover, one could perform the co-immunoprecipitations also the other way round, meaning a western-blot of EGR-1 on SOX17 and KLF4 precipitated lysates and a western-blot of KLF4 on SOX17 and EGR-1 precipitated samples. If these Western-Blots would show the same result, one could at least conclude that all three factors bind specifcally to each other in vascular SMCs, and as this complex is precipitated under proliferative conditions, indicate an involvement of the three interacting factors in promoting angiogenic processes, e.g. in pathological conditions, like atherosclerosis.

4.2. The FunGenES project

FunGenES (Functional Genomics in Engineered ES-cells) is a consortium whose goal is the identification and characterization of organospecific markers during embryonic development. Another objective is the development of new cellular and molecular tools to characterize gene function and to develop new ES-cell derived methods for high throughput screening of the toxicity of small molecules that are candidates for therapeutic interventions. The results could enable future therapeutic strategies for repair or regeneration of damaged or diseased organs, which might be

an alternative to organ transplantations. Moreover, the use of ES-cell derived screens could replace the use of animals in drug screening.

As endothelial cells and vascular smooth muscle cells derive mainly from the mesoderm, our goal was the identification and characterization of transcription factors involved in the differentiation of this lineage.

4.2.1. Differentiation of CGR8 ES-cells

The cultured mouse embryonic stem (ES) cells originate from the inner cell mass of the blastocyst of a day 3.5 old embryo. These cells are pluripotent, being able to differentiate in all three germ layers, comprising endoderm, ectoderm and mesoderm. As we are interested in vascular cells, like endothelial cells and vascular smooth muscle cells, we concentrated on the mesodermal lineage and the factors which are specific for mesoderm development, like e.g. Flk-1 (Shalaby et al., 1995). The differentiation of ES-cells mimics the embryonic development and it was shown that the *in vivo* expression of different cell lineage markers follows a comparable pattern to the *in vitro* differentiation. Thereby the ES-cell differentiation assay is a good model to examine the factors that are responsible for the cell lineage specification.

Figure 4.19 shows an overview of the differentiation protocol that was used in this study. The trypsinized cells were centrifuged and resuspended in differentiation medium, containing 20% FCS (Foetal Calf Serum), to a final concentration of 2.4 x 10^4. Afterwards the cells were placed in a drop-wise manner on the inner side of the lid of the plate (A). One drop of 20 µl contained around 500 cells. The cells stay like this for two days, accumulating at the bottom of the drop and starting differentiation (B). Afterwards the resulting embryoid bodies (EBs) were spilled in 10 cm bacteriological plates, containing 10 ml differentiation medium (C). The differentiating EBs stayed in suspension and grew for two more days. For further assays, in which a calculated number of cells/EBs is necessary, the EBs were then plated on e.g. 24-well plates (D). Here, the cells differentiated for 7 - 10 days and were analyzed afterwards, e.g. by immunofluorescence stainings or RT-PCR.
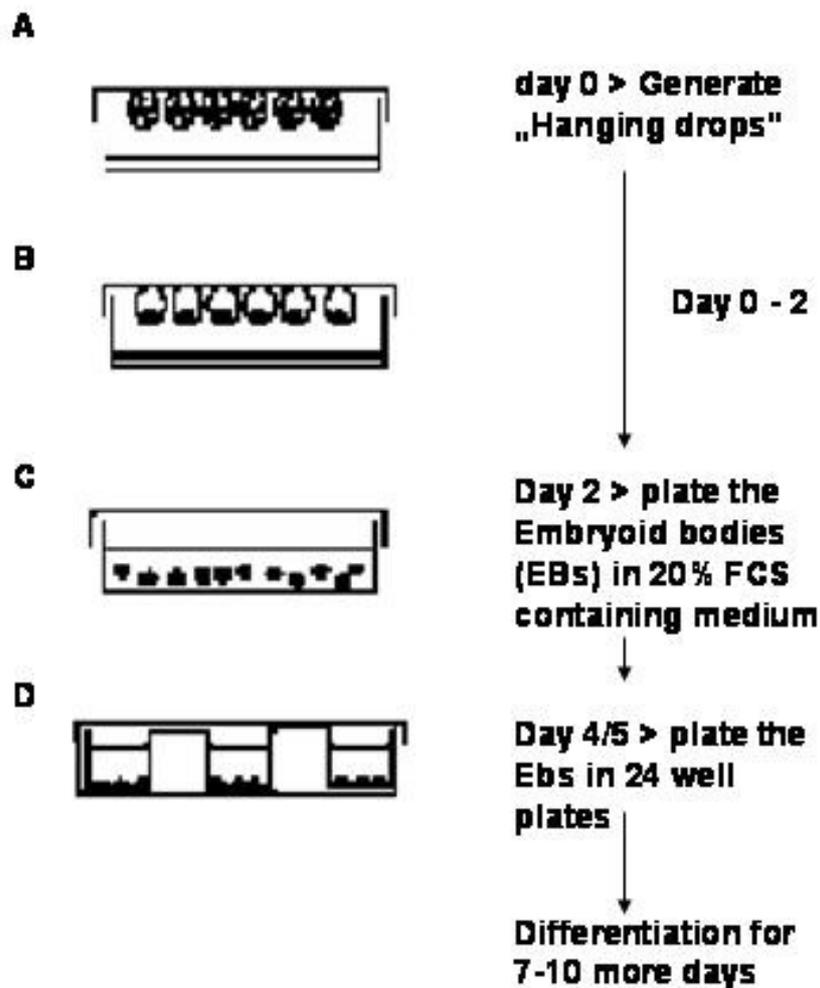
Figure 4.19 Differentiation of ES-cells (CGR8) using the hanging drop method. (A) Undifferentiated CGR8 embryonic stem cells (of a 3.5 day old embryo) were, up to a confluence of 70 - 80%, cultured on gelatin-coated plates. The cells were trypsinized, washed with 1X PBS and resuspended in 20% FCS containing differentiation medium to a final concentration of 2.4×10^4. The cells were placed in drops on the top of a 10 cm plate, each drop containing around 500 cells. Two days later the resulting embryoid bodies (B) were transferred in 10 cm bacteriological plates, containing 10 ml differentiation medium, where the EBs grow for around 5 - 7 days. The EBs were then plated in 24 well plates, growing for a few more days (3 - 6 days). The generated EBs differentiated in all three germ layers, mesoderm, endoderm and ectoderm.

Figure 4.20 (A) shows undifferentiated ES-cells, growing on a gelatin-coated plate. For the differentiation, the cells have to reach a confluence of around 70 - 80%. Figure 4.20 (B) shows 4 day old EBs cultured in differentiation medium. (C) and (D) are immunofluorescence stainings of Pecam-1 (C) and smooth muscle actin (D).
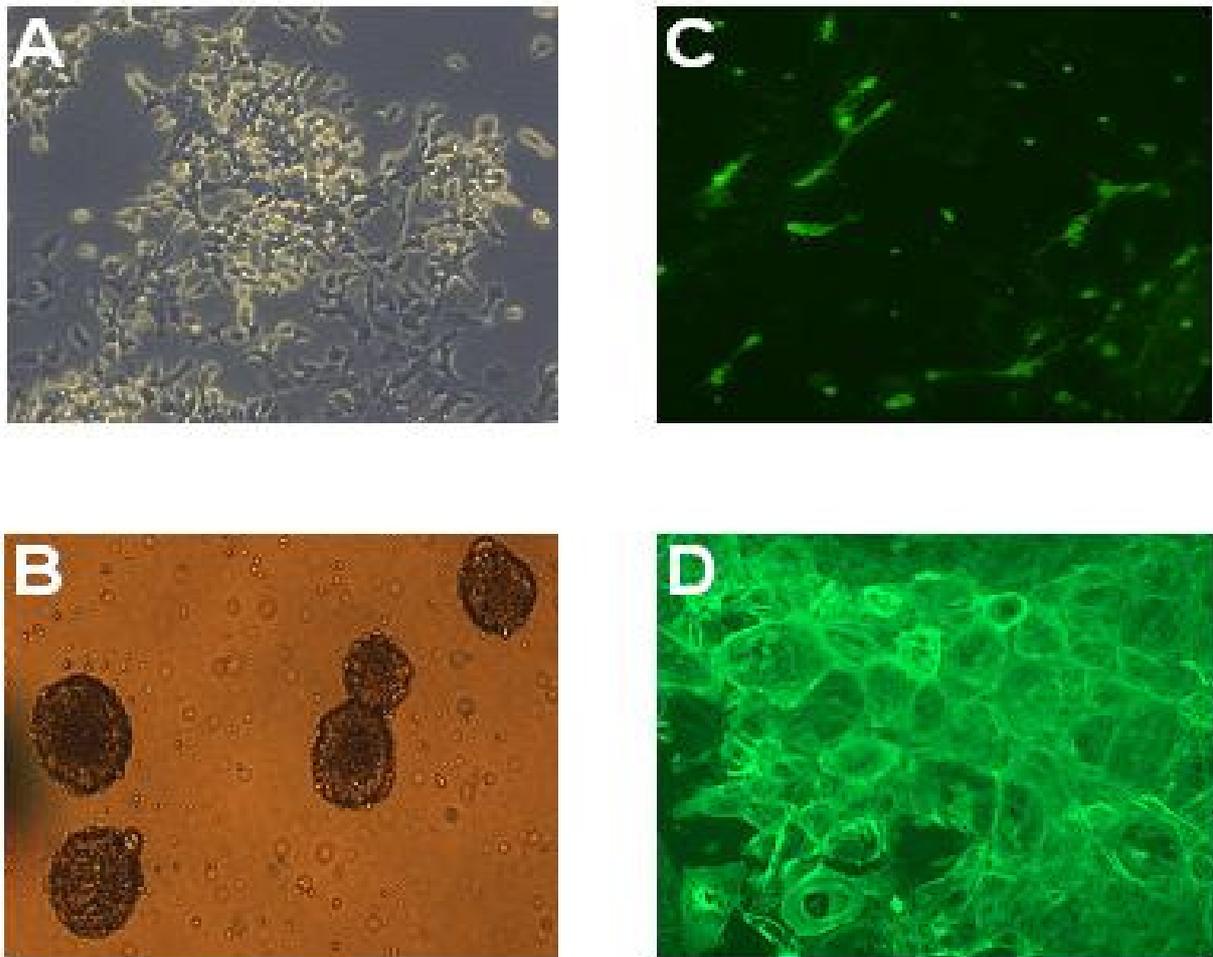
Figure 4.20 Differentiation of ES-cells (CGR8) by the hanging drop method. (A) Undifferentiated CGR8 embryonic stem cells with a confluence of 60 - 70%, cultured on gelatin-coated plates. (B) 4 day old EBs, generated by cell-suspensions, cultured in 10 cm bacteriological plates, containing 10 ml differentiation medium. At day 14 of embryonic development, the EBs, plated in 24 well plates, were stained by immunofluorescence with Pecam-1, detecting endothelial cells (C) and with smooth muscle actin, as cell-specific marker for vascular SMCs (D).

In this differentiation protocol, no cell lineage specific supplements were used so all various cell types can grow. At least it could be proven that the generation of mesoderm-deriving cell-lineages, in this case meaning endothelial and vascular smooth mucle cells, using the "hanging-drop" method, is possible. Using specific growth factors for the cultivation of the differentiating ES-cells, like VEGF or FGF, one could further on determine the differentiation of the ES-cells into endothelial specific lineages.

4.2.2. Expression profile of different lineage specific markers

To evaluate the in vitro expression pattern of the differentiating ES-cells, we tested for different lineage specific marker genes, like Flk-1 as an early endothelial marker, and VE-Cadherin, Ang2, and Tie2 as markers for mature endothelial cells (Sato et al., 1995). Moreover, Sox7, 17 and 18 were tested. Therefore every second day, lysates were taken and analysed via RT-PCR (figure 4.21).

In case of the vascular cells, one can take as example Flk-1, which comes up at day 4, which is not surprising as it is known to be an early mesodermal marker, in comparison to VE-Cadherin, which appears at day 6 as a marker for mature endothelial cells. Sox7 and Sox18 are co-expressed with VE-Cadherin, Angiopoietin2, and Tie2, which are all endothelial-cell specific markers. This underlines the endothelial cell specific expression of Sox7 and Sox18, and the knowledge that both factors functionally compensate each other. As VCAM-1 has been demonstrated to be a direct target of Sox18, it is not surprising that both factors come up in parallel at day 6.

In contrast to Sox7 and Sox18, Sox17 as third member of subgroup F comes up earlier, in parallel with Flk-1 at day 4. This is not unexpected, as Sox17 is known to be an early endodermal marker, arising around that time point during embryonic development (Kanai-Azuma et al., 2002).

As early mesodermal cells (Flk-1+) are not only differentiating in endothelial, but also in some subtypes of vascular smooth muscle cells, this parallel expression pattern of Sox17 and Flk-1 could also already give an implication for an involvement of Sox17 in such differentiation processes, especially after the finding that this transcription factor is expressed in mature VSMCs in adult human, but also in adult mice (see the results of the first part of this thesis; 4.1.2; 4.1.9).
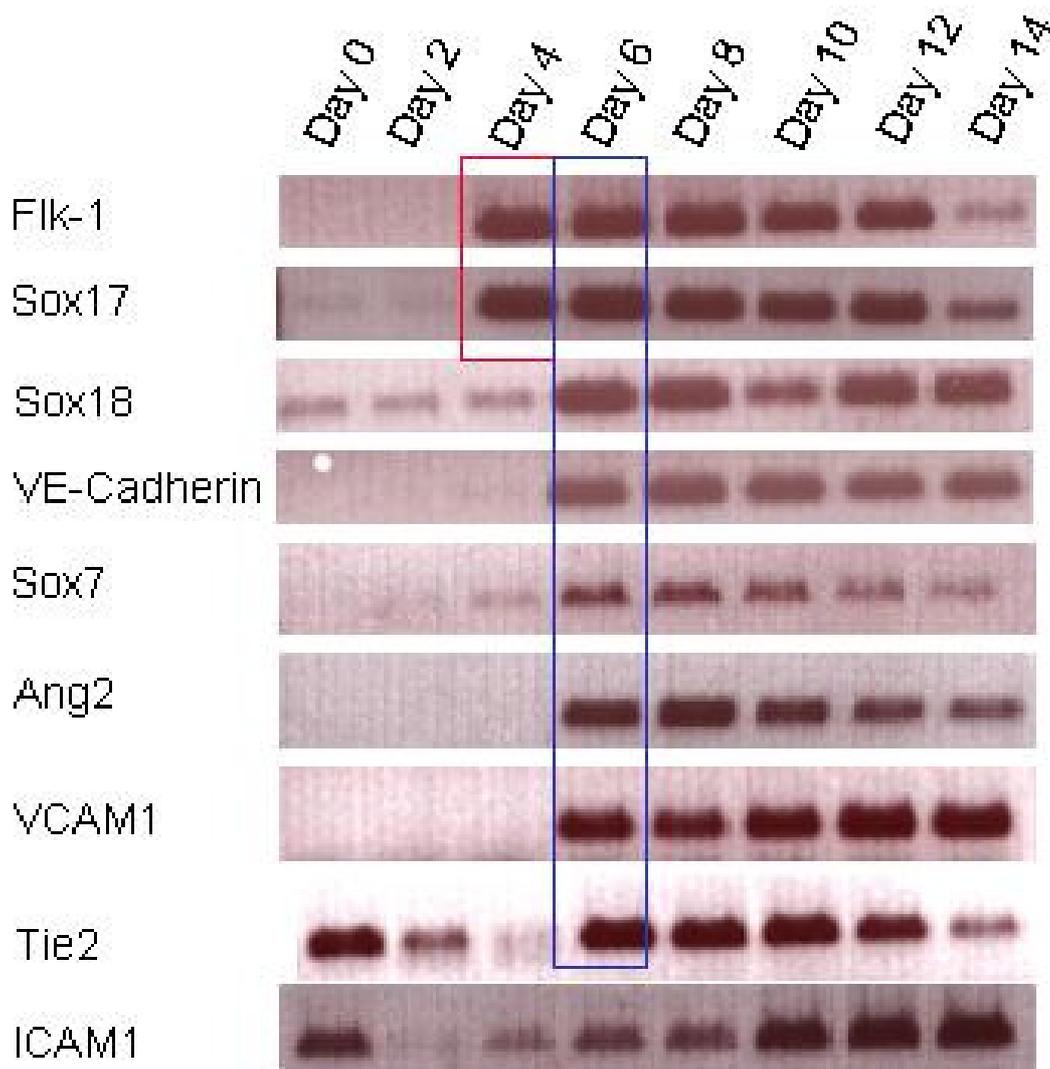
Figure 4.21 Expression pattern of CGR8 ES-cells during differentiation. The CGR8 cells were differentiated via the hanging drop method. Lysates were taken of the differentiating ES-cells each second day until day 14. Day 0 indicates the undifferentiated ES-cells. RNA was isolated and cDNA generated, which was then checked for different lineage markers via RT-PCR. Sox17 comes up at day 4 in parallel with Flk-1, an early endothelial marker, indicated by the red box. In comparison to this, mature endothelial markers, to which also Sox7 and Sox18 belong, come up at day 6 of ES-cell differentiation (blue box).

4.2.3. Klf4 binds to Sox17 in 4 day old embryoid bodies

Sox17 and Klf4 are known to play a crucial role in endoderm development, but they have not been shown to interact with each other in this context (Kanai-Azuma et al., 2002; Gardiner et al., 2005). Because of the observation that both factors seem to bind to each other in vascular smooth muscle cells, an interaction between the two factors might also take place during embryonic development. To test this idea, Sox17

and Klf4 were immunoprecipitated in parallel from 4 day old embryoid bodies (EBs). Assaying these samples for their counterparts, it appeared that Klf4 is detectable in the Sox17 immunoprecipitation (figure 4.22). By this it seems that both factors could bind to each other at day 4 of EB differentiation.

Figure 4.22 Klf4 is detectable in a Sox17 precipitated sample of 4 day old embryoid bodies *in vivo.* 3F10 (IgG/Isotype control), Sox17 and Klf4 were precipitated from 4 day old EBs and analyzed via Western-Blot, using an antibody raised against Klf4 (polyclonal, SantaCruz). Klf4 is detected at a size of 55kDa.

Taking into account the results in the smooth muscle cells described above, where SOX17, KLF4 and EGR-1 seem to build a protein complex, this might be also the case during embryonic development, even for Sox17 and Klf4. One would still have to examine, if both factors are detectable in an Egr-1 precipitated sample, and the other way round, and test for more controls, like different isotype controls as the used antibodies for the three transcription factors are polyclonal. These preliminary observations lead to the assumption that this protein complex could be an important transcriptional regulator in different cell contexts and might be involved in the differentiaton of vascular SMCs, as we detect the same interaction pattern in this cell type.

4.2.4. β-catenin is supposed to enter a protein complex comprising Sox17 and Klf4 in differentiating mouse ES-cells

It has been previously shown that Sox17 can bind to β-catenin during wnt-pathway signaling pathway and thereby inhibit the transcription of wnt-responsive genes (Zorn et al., 1999). On the other hand it has also been demonstrated that Sox17 binds to β-catenin for cooperatively activating the transcription of endodermal target genes

(Sinner et al., 2004). Also for KLF4 it is known that it binds to β-catenin (Zhang et al., 2006). For both factors the binding to β-catenin has so far been associated with endodermal development. With this knowledge it appeared to be interesting to test if the factors interact with each other, bind to β-catenin and initiate or inhibit thereby the transcription of downstream targets in the context of endodermal or mesodermal development.
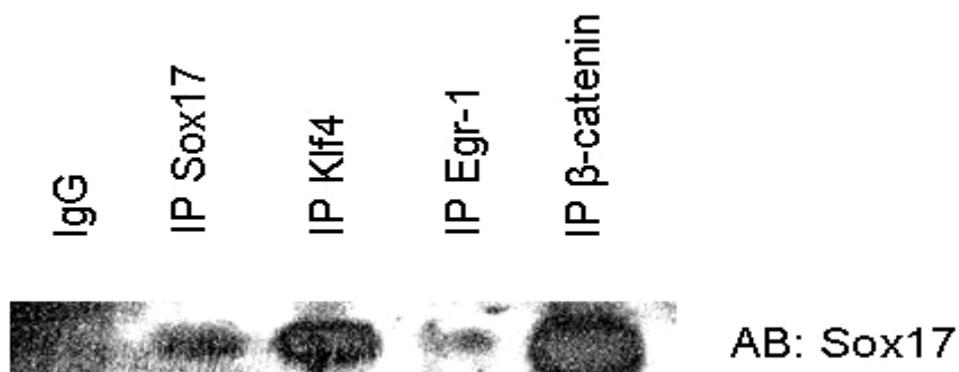
Figure 4.23 β-catenin builds a complex with Sox17 at day 4 of embryonic body differentiation. The immunoprecipitated 3F10 (isotype control), Sox17, Klf4, Egr-1 and β-catenin fractions were loaded on a 12.5 % SDS-gel and analyzed via a Sox17 specific antibody (polyclonal, SantaCruz).

As one can see in figure 4.23, Sox17 is detectable in high amounts in the β-catenin precipitated sample. The fact that the amount of Sox17 protein is much higher in this sample and the one showing the precipitated Klf4 fraction, might be due to the fact, that the antibodies have different precipitation capacities. It seems that the Sox17 antibody has a relatively low capacity, which is consistent with the result from the immunoprecipitations done in CASMCs, whereas the Klf4 antibody has a good binding capacity, as well as the one of β-catenin. Moreover one could assume that Klf4 and β-catenin strongly and with high efficiency bind to Sox17 in differentiating ES-cells. At least, in the β-catenin immunoprecipitation, using a rabbit polyclonal antibody, it occurred that this antibody has a high efficiency, as huge amounts of protein were detectable in the β-catenin specific western-blot. Nevertheless, to exclude any artefacts, one would have to equalize the protein amounts of the different samples to make conclusions about the amount of precipitated protein. At least, Sox17 seems to bind to β-catenin, and moreover also to Egr-1 in differentiating ES-cells. The co-immunoprecipitation of SOX17 and β-catenin was also tried for CASMCs, where the β-catenin precipitated sample was negative for SOX17. Therefore a complex, consisting of Sox17, Klf4, and Egr-1 might be involved in

signaling processes in different cell types, whereas β-catenin might cell-type specifically bind to this complex at day 4 of EB differentiation and the resulting complex could be involved in differentiation processes e.g. in direction of endodermal lineages.

I

5. Discussion

5.1. A new role for SOX17 as potential interaction partner of EGR-1 and KLF4 in human coronary artery smooth muscle cells

The vessel wall of arteries is composed of the intima, a single layer of endothelial cells, which is surrounded by multiple layers of vascular SMCs, building the media. A third layer forms the adventitia, in form of fiber and collagen extracellular matrix, embedding the blood vessels within the surrounding tissue. The main function of the vascular SMCs is to provide the artery with elastic properties for maintaining the vascular tone in response to environmental stimuli. An important feature of the vascular SMCs is their ability to occur in two different states, the proliferative and migrating state during vascular remodeling processes, and the differentiated one in the quiescent periods.

Atherosclerosis is a chronic inflammatory disease causing plaque formation, in whose progression the proliferation of vascular SMCs plays a major role (Ross et al., 1999; Hansson et al., 2005). As one of the fist steps in atherosclerosis, endothelial cells get activated, the endothelial-endothelial cell contacts get disrupted and monocytes infiltrate into the subendothelial space, where they differentiate into macrophages. By secretion of cytokines and growth factors, like Platelet Derived Growth Factor BB (PDGF-BB) from the endothelial cells and the macrophages, but also from the vascular SMCs themselves, the quiescent, differentiated vascular SMCs become activated to transform into migrating, proliferative SMCs (Schönherr et al., 1993). Therefore, vascular SMCs and the examination of the signaling cascades leading to their activation might be an alternative strategy to find molecular targets for therapeutic intervention in atherosclerosis.

At the beginning of this thesis I discovered, that Sox17, belonging to subgroup F of the Sox proteins, is expressed in vascular SMCs in various normal tissues. Therefore, this factor and its possible implications or its interaction with other proteins, in vascular processes went into the focus of this work.

5.1.1. Expression pattern of the subgroup F Sox proteins in vitro and in vivo in different vascular cells

To identify possible signaling pathways/conditions in which Sox7, 17 and 18 might be involved in mature endothelial cells (HUVECs), the cells were stimulated with LPS and TNF-α, as pro-inflammatory stimuli, and with TGF-β1, as an anti-inflammatory substance. Moreover the cells were cultured in hypoxic conditions, to mimic angiogenic processes, e. g. during tumor vascularization. The observation that Sox7 and Sox17 are downregulated in response to hypoxia (4.1.4.), might be connected to the fact, that both factors are able to inhibit wnt-signaling by binding to β-catenin (Zorn et al., 1999). It is known, that β-catenin is upregulated in endothelial cells after myocardial infarction, being involved in neovascularization processes in the infarcted area, where it induces the expression of the vascular growth factor VEGF (Blankesteijn et al., 2000). This knowledge leads to the hypothesis, that both factors need to be downregulated in endothelial cells in pathological conditions in order for neovascularization mediated in part by β-catenin, to take place.

The in vitro expression analysis showed moreover a response of Sox18 to LPS, TNF-α and TGF-β1. After LPS and TNF-α treatment, Sox18 was downregulated after 4 hours, whereas it was slightly upregulated after TGF-β1 stimulation. As LPS and TNF-α are pro-inflammatory substances, it might be that Sox18 represses in the normal state of the mature endothelial cell the expression of early inflammatory response genes. When this state gets disrupted, Sox18 might have to be downregulated to enable the transcription of such inflammation mediating genes. In contrast, TGF-β1 is an anti-inflammatory stimulus. The observation that Sox18 is slightly upregulated by TGF-β1 leads to the possibility, that Sox18 might be involved in anti-inflammatory processes, activating genes, which are implicated in theses processes, or repressing pro-inflammatory genes. It might be important for the conservation of the quiescent state of endothelial cells. As the time point (4 hours) of Sox18 response to TGF-β1, correlates with the ones from the previous assay, using TNF-α and LPS, the latter explanation seems to be more plausible.

Examining the expression profile of Sox7, Sox17 and Sox18, it appeared that Sox7 and Sox18 are exclusively expressed in endothelial cells in different mouse tissues

(brain, spleen, kidney, testis, heart, liver, and lung), *in vitro* and *in vivo* (see results chapter 4.1.3.). This is not surprising, as it has been shown that both factors are expressed in blood vessels during embryogenesis and in adult organisms during angiogenic processes, e.g. in vascular repair (Darby et al., 2001; Pennisi et al., 2000; Downes et al., 2001).

In contrast to this, the observation that Sox17 is expressed in vascular smooth muscle cells, which build the media of the big blood vessels, is a new finding. So far, Sox17 has been demonstrated to be critical for the development of the definitive endoderm and to be expressed during mouse spermatogenesis (Kanai-Azuma et al., 2002; Kanai et al., 1996). Moreover, an expression in endothelial cells has recently been shown (Matsui et al., 2006).

The in vitro expression profile of Sox7, 17 and 18 in different vascular cells indicates that Sox17 is exclusively expressed in vascular smooth muscle cells, in comparison to Sox7 and Sox18, which are present in endothelial cells.

5.1.2. SOX17 expression is increased in proliferative and decreased in differentiating conditions in human coronary artery smooth muscle cells

In Chapter 4.1.5., it could be demonstrated, that Platelet Derived Growth Factor (PDGF-BB) and Low Density Lipoprotein (LDL) maintain/induce high SOX17 expression in coronary artery smooth muscle cells, whereas in the untreated control cells, expression of SOX17 is downregulated as consequence of a low serum concentration in the culture medium (0.1%). Already this observation implies that SOX17 expression and its maintenance are dependent on proliferation-promoting extracellular stimuli, like in this case the FCS in the culture medium. PDGF-BB and LDL are substances, which are known to induce proliferation of vascular SMCs (VSMCs), and Sox17 seems to be involved in these processes (Libby et al., 1985; Scott-Burden et al., 1989). The proliferation and migration of the VSMCs plays an important role in the outgrowth of new blood vessels, during vasculogenesis and angiogenesis, but also in pathological conditions, as it is the case during the formation of atherosclerotic plaques. In this case, Sox17 might have an activating or repressing role for its target genes. Many Sox proteins, like Sox17, have been described as bifunctional proteins, acting as activators and/or repressors of

transcription of their target genes. Sox17 is known to induce the expression of Laminin alpha1, but represses the transcription of β-catenin responsive genes (Niimi et al., 2004; Zorn et al., 1999). The biological readout, mediated by most of the Sox proteins, depends on the cell context and the interaction partner/s (Kamachi et al., 2000). This would also be the case for the induction of Sox17 by LDL after 24 hours. Via binding to LRP5/6, LDL is known to induce the wnt-signaling pathway, which causes the proliferation of vascular smooth muscle cells (Wang et al., 2004). In a time-dependent manner, an upregulation of Sox17 after 24 hours might cause an inhibition of the wnt-signaling pathway. On the other hand, it could also be the other way round, as Sox17 has also been described to interact with β-catenin to activate the transcription of endodermal genes, like Foxa1, Foxa2, and Gata4-6 (Clements et al., 1999; Weber et al., 2000; Sinner et al., 2004). Taking into account the following results, and the ones made in the PDGF-BB stimulation assay, it would be more reasonable to predict SOX17 in a growth promoting role in VSMCs in response to LDL. This could be supported by the notion, that LDL is known to induce, besides *c-fos* and *egr-1*, the expression of platelet-derived growth factor and platelet-derived growth factor receptors in VSMCs (Ross, 1990; Sachinidis et al., 1993). As the LDL initiated signaling cascade comprises the activation of Mitogen-activated protein kinases (MAPK), like ERK, and as the same is the case for the PDGF-BB signaling cascade, one could suppose Sox17, downstream of Egr-1, which is also induced by LDL and PDGF-BB via ERK1/2, downstream of a MAP-kinase mediated pathway, with the consequence of transcription of proliferation-promoting genes (Metzler et al., 1999; Deigner et al., 1996; Kusuhara et al., 1997; Sachinidis et al., 1997; Harja et al., 2004).

As TGF-β 1 is an antagonist of PDGF-BB, inducing the differentiation of vascular SMCs, we used this factor for stimulation assays on coronary artery smooth muscle cells (Hautman et al., 1997; Schönherr et al., 1993). Thereby, we expected a downregulation of SOX17 in response to TGF-β 1 treatment as opposing effect to maintenance/induction of SOX17 after PDGF-BB and LDL treatment. This is indeed the case after 48 hours. The fact that Sox17 is downregulated by TGF-β1 correlates with the induction of β-catenin by TGF-β 1 (Masszi et al., 2004). Sox17 is known to inhibit β-catenin mediated activation of transcription, binding to the β-catenin/TCF complex (Zorn et al., 1999). In contrast, TGF-β1 enhances the binding of β-catenin to

TCF (T-cell factor transcription factor) and a cross-talk between the TGF-β1 and wnt-signaling pathway has been shown, resulting in the transcription of wnt-responsive genes (Masszi et al., 2004; Warner et al., 2005). Thereby, the observation that TGF-β1 reduces the expression of Sox17 after 48 hours, leads to the assumption, that the wnt-signaling gets activated. This pathway is known, among others, to play a role in differentiation processes during embryogenesis, as well as in adult organisms (Huelsken, et al., 2000; Mukhopadhyay et al., 2001; Heisenberg et al., 2001; Kratochwil et al., 2002). Another possibility is of course, as Sox17 acts bifunctional, and responds to growth-promoting stimuli in VSMCs, that Sox17 transcription has to be repressed, to prevent the activation of transcription of proliferation-associated genes and to influence the phenotype of the VSMCs in direction of a differentiated and quiescent state. To get a more concrete idea about this, one could make a long-lasting time course of TGF-β1 stimulation on VSMCs and afterwards examine the expression of known Sox17 target genes like laminin alpha1 and fibronectin e.g. after 60 or 72 hours.

5.1.3. EGR-1 induces SOX17 expression in human coronary artery smooth muscle cells

The fact, that the overexpression of EGR-1 in coronary artery smooth muscle cells leads to a strong induction of SOX17 is not surprising, as EGR-1 is known to respond to PDGF-BB, and thereby mediating proliferation of vascular SMCs as a transcriptional regulator, mostly acting upstream of other transcription factors, but downstream of Src and ERK kinases (Kamimura et al., 2004). Sox17 also responds to PDGF-BB and therefore seems to be a promising candidate, acting downstream or in conjunction with EGR-1 in a signaling cascade, which leads to cell proliferation of vascular SMCs. Another fact, which underlines this hypothesis, is that both factors have similar target genes, like Laminin α1 (Lama1) and Fibronectin (Shirai et al., 2004; Baron et al., 2005). Sox17 is known to induce the expression of Lama1 in F9 cells (Niimi et al., 2004). Moreover, it appears, that an overexpression of EGR-1 in coronary artery smooth muscle cells leads to a strong induction of Lama1, Lama2, Lamb1, and Lamγ1 (4.1.6., figure 11). This observation reinforces the idea of an interaction between both factors, to regulate gene expression. As the laminins and fibronectin, both belong to an important group of extracellular matrix proteins,

initiating proliferation and mediating migration of VSMCs, this parallel already implicates the biological read out of a signaling cascade, comprising Egr-1 and Sox17 in VSMCs (Majesky et al., 1990; Hedin et al., 1988; Ruoslantiet et al., 1988).

5.1.4. Sox17 and Egr-1 are co-expressed in murine vascular smooth muscle cells in normal and pathological conditions

The in vitro data, implicating Sox17 and EGR-1 in growth conditions in coronary artery smooth muscle cells, were supported by the in vivo data, showing a co-expression of both factors in vascular smooth muscle cells in normal conditions and in atherosclerotic lesions (4.1.8., 4.1.10). Egr-1 expression has already been demonstrated to be upregulated in neointima after vascular injury and in atherosclerotic lesions (McCaffrey et al., 2000; Santiago et al., 1999a). Therefore, a knockdown of Egr-1, using siRNA technology, causes a decrease of intimal hyperplasia in balloon-injured carotid through inhibiting smooth muscle cell proliferation and migration (Santiago et al., 1999b; Fahmy and Khachigian, 2002). Thereby, Egr-1 is presumed as a key regulator of signaling processes after vascular injury, initiating proliferation. By that, the observation that Egr-1 is strongly expressed in the vascular smooth muscle cells in the atherosclerotic plaque and moreover co-expressed with Sox17, enforces the hypothesis that Sox17 is involved in the proliferation of vascular SMCs in normal and pathological conditions in cooperation with Egr-1.

5.1.5. KLF4 expression is induced by overexpression of EGR-1 in human coronary artery smooth muscle and by SOX17 in promoter studies

KLF4 was one of the first candidates to look for proteins involved in a signaling cascade that comprises EGR-1 and SOX17 in the proliferation of vascular SMCs. KLF4 and EGR-1 might also act as interaction partner/s for SOX17, which needs, like other Sox proteins, to bind to another protein to be transcriptionally active (Kuhlbrodt et al., 1998; Lefebvre et al., 1998). The reason for choosing KLF4 was that it is involved in the regulation of the phenotypic switch of vascular SMCs, downregulating SMC marker genes, like SM22α, SM-actin, SMMHC (Smooth Muscle Myosin Heavy Chain) and myocardin, and is thereby associated with proliferation of the vascular

SMCs (Liu et al., 2005). Measuring KLF4 expression after EGR-1 gain-of-function studies in SMCs, it appeared that KLF4 is strongly upregulated (4.1.11.). This result and, the fact that KLF4 is inducible by PDGF-BB (Liu et al., 2005) lead to the hypothesis that PDGF-BB induces EGR-1, which in turn induces KLF4. As we postulate the same for Sox17, the question arises if these factors regulate the expression of each other in a complex regulatory loop. The fact, that Sox17 and Klf4 have already been implicated in such an autoregulatory loop, Sox17 and Gata4 inducing each other, and Klf4, inducing its own gene expression, makes this notion reasonable (Sinner et al., 2006; Mahatan et al., 1999).

To begin to address this hypothesis, the human KLF4 promoter was screened for putative SOX17 binding sites using previously characterized Sox17 binding sequences (GACAAT/AACAAT). SOX17 has been shown to activate the expression of Laminin α 1 via this binding site, which (GACAAT) has been detected at 1400 basepairs upstream of the KLF4 start-codon (Niimi et al., 2004; Chapter 4.1.12.). Moreover, three EGR-1 binding sites were found. By promoter analysis, an induction of KLF4 by SOX17 has been shown (see results chapter 4.1.12.). An induction via the putative SOX17 binding site could be confirmed, as a mutation, as well as the deletion of this site abrogated the induction. EGR-1 could also induce KLF4 expression via its binding sites in the 5´ flanking promoter region of the *klf4* gene, which has been examined in our lab. The question if both factors act synergistically for the induction still has to be answered, for example, by co-expression of EGR-1 and SOX17 in studies of the KLF4 promoter. Thereby, one would have to examine if the co-expression of the counterpart leads to a further increase of the measured relative luciferase activity.

It might also be that KLF4 and SOX17 influence the transcription of each other, as SOX17 was found to have two putative KLF4 binding sites. An autoregulatory loop function has already been demonstrated for Sox17 during early embryonic development, where Sox17 and GATA4/6 induce each other (Sinner et al., 2006). The same is the case for Sox17 and Xnr4 and for Sox17 and Bix1/2/4 (Sinner et al., 2006). As already mentioned before, also Klf4 is involved in an autoregulatory regulation, inducing its own gene expression (Mahatan et al., 1999). To examine if SOX17 and KLF4 also participate in such an autoregulatory loop, one would have to

perform promoter analysis on the SOX17 promoter, in the same way as it was done for KLF4 and, more importantly, one would have to perform in vivo assays, like chromatinimmunoprecipitation in vascular SMCs, to examine if both transcription factors bind to the promoter region of each other. So far, we can conclude, that Sox17 and Egr-1 are able to activate the transcription of the *Klf4* gene. Some Chromatinimmunoprecipitations were already done on the promoter region of SOX17 and KLF4 in human coronary artery smooth muscle cells (CASMCs). Thereby some complications came up, as up to 1×10^9 cells were needed for one assay, and the CASMCs are difficult to expand. At least its very time consuming, as the cells grow very slowly, and very expensive, as one has to expand a hight amount of cells over a long time period and as the cells and the medium are very expensive. Therefore one could take into consideration to perform this assay in another cell type, in which all three factors are highly expressed, like in endothelial progenitor cells (EPCs). At least one can not definitely say that this possibly found interaction in EPCs or another chosen cell type, will also take place in CASMCs *in vivo*, which was my intention to show. But anyway, it could enforce the results from the promoter studies, showing an induction of KLF4 by SOX17. Therefore one would also have to take into account that this assay was done in human cells and, more important, with human constructs and the human KLF4 promoter region. As the comparison of the mouse and human KLF4 promoter shows differences in the amount and location of putative SOX17 and EGR-1 binding sites, one should perhaps better consider to take a human cell line. As the HUVECs, which show a SOX17 expression in vitro, have as primary cell line the same growing properties like the CASMCs, one could test some fast-growing cell lines, like HeLa cells for SOX17, EGR-1 and KLF4 expression, to perform Chromatinimmunoprecipitations.

5.1.6. SOX17, EGR-1 and KLF4 are supposed to build a protein complex in human coronary artery smooth muscle cells *in vivo*

Supporting the hypothesis, that all three factors might bind to each other in CASMCs, and that this complex might initiate the transcription of different target genes, is the fact that already some common target genes have been described. One of them is Laminin α 1 (Lama1). As already mentioned before, it is known that Sox17 and Egr-1 initiate its transcription (Niimi et al., 2004; Chapter 4.1.6.). Also, Klf4 has been

demonstrated to transactivate Lama1, Lama3A and Lamγ1 (Piccinni et al., 2004; Miller et al., 2001; Higani et al., 2002). Another common target gene, at least for Sox17 and Egr-1, is fibronectin (Baron et al., 2005; Shirai et al., 2004). The fact that both proteins, Lama1 and fibronectin, are involved in similar processes, meaning the formation of the extracellular matrix, can already give a hint for the type of target genes of the Sox17/Egr-1/Klf4 protein complex in coronary artery smooth muscle cells. As important extracellular matrix components, fibronectin and laminin get activated after vascular injury and are part of the proliferation-initiating signaling cascade, resulting in a phenotypic switch of the VSMCs from the contractile, quiescent to the synthetic state (Majesky et al., 1990; Hedin et al., 1988). Besides this, fibronectin has been described as a decisive mediator of the migration of VSMCs, mediating cell-cell adhesion, e.g. of leukocytes, which infiltrate the injured vascular tissue. Therefore fibronectin is considered to be a key mediator of neointimal thickening after vascular injury (Ruoslantiet et al., 1988).

Moreover, the newly identified complex might act as activator or as repressor of transcription, as Sox17 and Klf4 have already been described to behave in a bifunctional manner (Niimi et al., 2004; Zorn et al., 1999). Depending on the interaction partner and/or the promoter context, Klf4 can serve as an activator or inhibitor of transcription (Dang et al., 2000; Rowland et al., 2005; Ghaleb et al., 2005). It represses, like Sox17, β-catenin, and activates, also like Sox17, the Lama1 promoter (Zorn et al., 1999; Zhang et al., 2006). This bifunctional behaviour has also been described for Egr-1, providing growth promoting activities on the one hand, and growth inhibiting properties on the other hand, depending on the cellular context (Eid et al., 1998; Huang et al., 1997).

A common interaction partner for Klf4 and Sox17 has already been described. Sox17 is known to bind to β-catenin, thereby blocking the transcription of wnt-responsive genes or to activate the transcription of endodermal genes, like *Foxa1* and *Foxa2*, in cooperation with β-catenin (Zorn et al., 1999; Sinner et al., 2004). Also Klf4 has been shown to repress β-catenin mediated gene expression in the intestine (Zhang et al., 2006).

Another similarity between all three factors is their response to PDGF-BB. All three factors get activated by PDGF-BB (Kamimura et al., 2004; Liu et al., 2005; Chapter 4.1.5.). A hypothetical signaling cascade, in which the EGR-1/SOX17/KLF4 complex might be involved, could comprise ERK and Src family kinases upstream of the complex, as an inhibition of these kinases leads to a suppression of PDGF-BB induced EGR-1 expression in vascular SMCs (Kamimura et al., 2004).

Another parallel between SOX17 and KLF4 is the fact, that both factors are downregulated by TGF-β1. This has been shown in this work in chapter 4.1.7. for SOX17, which is downregulated 48 hours after TGF-β1 treatment. It is known that Klf4 is inhibited by TGF-β1 in macrophages (Feinberg et al., 2005). Moreover, Klf4 inhibits TGF-β1/Smad3 function by competing with Smad3 for the binding to the C-Terminus of the co-activator p300 (Feinberg et al., 2005). Besides, Sox17 as well as Klf4 have been implicated in endoderm development in mice (Kanai-Azuma et al., 2002; Shie et al., 2000).

The complex might activate genes, responsible for initiating proliferation of SMCs, or repress SMC differentiation marker genes, as this function was shown for Klf4, in a PDGF-BB induced context (Dandre et al., 2004; Kawai-Kowase and Owens, 2006; Yoshida et al., 2006; Holycross et al., 1992). Another possibility is that the complex activates the transcription of VEGF, and promotes thereby angiogenesis as PDGF-BB is known to induce VEGF expression via Erk-1/2 and AP-1 (Park et al., 2006).

In summary, the results from the first part of my thesis indicate that:

- Sox17 is expressed in vascular smooth muscle cells *in vitro* in human cells and *in vivo* in mouse cells.
- SOX17 is inducible by EGR-1 in human coronary artery smooth muscle cells, as well as Lama1, Lama2, Lamb1, and Lamγ1.
- Sox17 is co-expressed with Egr-1 in aortic SMCs in normal mouse tissues and in atherosclerotic plaques of ApoE deficient mice.
- SOX17 expression is repressed 48 hours after TGF-β1 treatment in human coronary artery smooth muscle cells.
- EGR-1 induces KLF4 expression in CASMCs.

- One putative SOX17 and three putative EGR-1 binding sites were detected in the 5´-flanking promoter region of the *klf4* gene, via the first one KLF4 gets activated by SOX17.

- PDGF-BB and LDL maintain/induce the expression of SOX17 in human coronary artery smooth muscle cells.

- SOX17, KLF4, and EGR1 are supposed to bind to each other in human coronary artery smooth muscle cells *in vivo*.

These facts lead me to the following hypothesis:

EGR-1, as immediate early gene, gets activated by PDGF-BB, e.g. in response to vascular injury or during angiogenic processes. This induction is mediated by Erk1/2, which has already been shown in literature. As consequence, EGR-1 might induce the expression of SOX17 and KLF4. The three proteins build a complex that might on the one hand repress the transcription of smooth muscle marker genes, like SMMHC, and on the other hand activate the transcription of extracellular matrix proteins, like fibronectin and laminin alpha1. Thereby, the transcriptional complex promotes the phenotypic switch of the VSMCs from the contractile to the synthetic, proliferating and migrating cell. By this, the hypothetical complex might contribute to the proliferation process of the VSMCs during angiogenesis, meaning the sprouting of new blood vessels from preexisting ones, and during vascular disease, like atherosclerosis, contributing to neointimal thickening. The fact that Sox17 and Egr-1 were shown to be co-expressed in the VSMCs in the atherosclerotic plaque of ApoE deficient mice supports this suspicion. The notion that TGF-β 1, an antagonist of PDGF-BB, downregulates Sox17, and as this has also been demonstrated to be the case for Klf4, enforces the idea of an involvement of the complex in such cellular processes, as TGF- β 1 is a known activator of VSMC differentiation. The fact that I found a protein–DNA interaction, in the case of the SOX17 protein and the KLF4 promoter, and the protein-protein interaction between all three transcription factors in the co-immunoprecipitations, could at least be explained by an autoregulatory loop, in which the complex itself might initiate the transcription of its own components, as this has already been shown to be the case for all three factors in different cellular contexts.

5.2. The FunGenES project

The goal of this European consortium is the identification and characterization of tissue-specific factors and the examination of their contribution to differentiation of the cell-specific lineages. Because of its easy handling and the prevention of examinations on animals, differentiating ES-cells were used for recapitulating embryonic development. In literature it has been shown that the expression of different lineage markers in vitro corresponds to the one in vivo (Matsuura et al., 2005; Leahy et al., 1999; Choi et al., 2005).

5.2.1. Expression profile of Sox7, Sox17 and Sox18 in differentiating ES-cells

The examination of the expression of the three subgroup F Sox proteins during differentiation of the ES-cells reflects a different pattern for Sox7 and Sox18 on the one side and for Sox17 on the other side (4.2.2.). While the latter one is already expressed at day 4 of embryoid body (EB) differentiation, Sox7 and Sox18 appear at day 6, in parallel with VE-Cadherin, which is, like Tie-1, a marker for mature endothelial cells (Gory et al., 1999; Korhonen et al., 1995). This is not surprising, as both transcription factors are known to play a decisive role in vasculogenesis and angiogenesis during embryogenesis and in the adult organism (Pennisi et al., 2000). Sox18 is expressed in the yolk-sac blood islands, the developing endothelial cells and in the presumptive endocardial cells and has been demonstrated to be absent from endoderm (Pennisi et al., 2000), in contrast to Sox17, which is involved in endoderm development (Kanai-Azuma et al., 2002). Therefore, the co-expression of Sox18 with an endothelial marker (VE-Cadherin) is reasonable. The fact that Sox17 comes up in parallel with the early mesodermal markers, in conjunction with the observation that SOX17 is expressed in vascular smooth muscle cells of adult mice, leads to the hypothesis that this factor might be implicated in the differentiation of the VSMCs. As vascular SMCs are known to derive from different origins, like from proepicardial cells in the case of coronary artery SMCs, or from the neural crest in case of the SMCs of the aortic arch and thoracic aorta, they can also differentiate from early mesodermal cells (Landerholm et al., 1999; Mikawa et al., 1996; Ito et al., 1993; Topouzis et al., 1996; Bergwerff et al., 1998). One needs to consider that Sox17 is described as an early endodermal marker, being crucial for the differentiation of the endoderm, as mice deficient for Sox17 show as phenotype a

depletion of the definitive gut endoderm (Kanai-Azuma et al., 2002). This correlates with the finding that the Sox17 orthologues in *Xenopus* (Xsox17alpha, Xsox17beta) and the one of zebrafish (Zsox17) are specifically expressed in the endoderm during gastrulation and play a key role in endoderm formation (Hudson et al., 1997; Clements and Woodland, 2000; Alexander and Stainier, 1999). Sox17 expression is detectable in the definitive endoderm in the early headfold stage in the mouse embryo at day 8, but has moreover been shown to be expressed at day 5 to 7 (egg-cylinder stage) in the mesendoderm (Matsuura et al., 2005; Leahy et al., 1999). Thereby, one has to take into account that the examinations of Matsuura et al. were done by immunohistochemistry, detecting the protein. In our assays, we detected the mRNA, which was transcribed in cDNA for the quantitative PCR analysis. This could explain the difference of the time point of detecting Sox17 *in vivo* and *in vitro*. Also if both situations have been shown to overlap, there might also be some differences concerning the time point of first expression of different markers (Matsuura et al., 2005; Leahy et al., 1999; Choi et al., 2003).

An argument, which could support the possibility that Sox17 is involved in the differentiation of vascular SMCs, is the fact, that Laminin α1 is a target gene of Sox17 (Niimi et al., 2004). Laminin α1, like TGF-β1, is known to induce smooth muscle cell differentiation during embryonic development. A possible way to find out if Sox17 influences the differentiation of vascular SMCs at least in part, would be to knock out Sox17 transiently via RNAi technology and to look for the expression of smooth muscle marker genes, like SMactin, smoothelin, myocardin, SM22-α. On the other hand one could also do gain-of-function analyses, trying to influence the differentiation in the direction of vascular SMCs. Another possibility would be the isolation of a smooth muscle cell specific lineage, e.g. by isolating SM-actin positive cells that come up during ES-cell differentiation.

5.2.2. Klf4 is supposed to bind to Sox17 in embryoid bodies at day 4 of ES-cell differentiation

The fact that Sox17 seems to bind to Klf4 in differentiating mouse ES cells at day 4 supports the possibility of an involvement of Sox17 in the differentiation of vascular SMCs, as a possible direct interaction of both factors is also detected in vascular

smooth muscle cells. On the other hand, both proteins might be involved in endoderm differentiation processes in conjunction with each other, where they might act as activator of genes like laminin α1, and fibronectin, or as repressor of β-catenin-mediated signaling and thereby influence the wnt-signaling pathway (Niimi et al., 2004; Piccinni et al., 2004). In favor of this hypothesis, Sox17, as well as Klf4, have been detected in the developing endoderm, where they share one common target, the Laminin α1, and both of them are known to interact with β-catenin, repressing the expression of wnt-responsive genes (Zorn et al., 1999; Zhang et al., 2006).

In this light, the three factors could also act in concert for directing the differentiation of vascular smooth muscle cells. This notion is strengthened by the fact that TGF-β1, another inducer of SMC differentiation, is regulated by Egr-1 (Liu et al., 1996).

To answer the question in which cell type specification the protein complex might be involved one could select the ES-cells for different cell-specific cell surface markers. Therefore one could use antibody-coupled magnetic dynabeads directed against cell-specific surface antigens, like SM-actin, as already mentioned before. By this method, purified cell lines could be generated and afterwards precipitated for the complex.

5.2.3. Sox17 binds to β-catenin and Egr-1 at day 4 of ES-cell differentiation

The notion that Sox17 binds to β-catenin and Egr-1 at day 4 of ES cell differentiation could support the hypothesis that the resulting complex could inhibit in concert the wnt-signaling pathway and therefore direct the differentiation of a particular cell-specific lineage. The fact that Sox17 and Klf4 have been shown to bind to β-catenin could implicate the protein complex in the development of endodermal lineages, as the binding to β-catenin was not observed in human coronary artery smooth muscle cells. To answer this open question, one would have to isolate endoderm-specific cells at day 4 of embryonic development and examine these ones for the presence and activity of a transcriptional complex comprising Sox17, Egr-1, Klf4 and β-catenin. Moreover, one would have to assay if Egr-1 also binds to β-catenin and if this interaction is absent in undifferentiated ES-cells, as Egr-1, Klf4, and β-catenin are

already expressed at this time point, whereas Sox17 is expressed up from day 4 in embryonic development *in vitro*.

The fact that Sox17 binds to Klf4 and Egr-1 in CASMCs and in differentiating ES-cells leads to the conclusion that this complex might have an important regulatory role in different cell types, and even in different organisms, meaning a conserved mechanism, as the CASMCs are of human origin, wherase the ES-cells derive from mouse.

6. Summary

The development of the vascular network comprises tightly regulated processes, involving vasculogenesis and angiogenesis. The cells, which are mainly participating in these processes, are endothelial cells and vascular smooth muscle cells, the latter ones especially being important for the stability of blood vessels. Uncontrolled proliferation of VSMCs contributes crucially to the development of vascular disease, e.g. in the case of atherosclerosis. Two main initiator factors of these processes are Low Densitiy Lipoprotein (LDL) and Platelet Derived Growth Factor-BB (PDGF-BB). For this reason, the VSMCs and the transcriptional regulation of their proliferation, in response to LDL and PDGF-BB, build an important target for therapeutical interventions.

Sox17, a member of subgroup F of the Sox family proteins, was for the first time detected in vascular smooth muscle cells in different mouse tissues, like liver, brain, heart, lung, spleen and kidney *in vivo* and in human coronary artery smooth muscle cells *in vitro*. Moreover, a new possible protein complex, consisting of SOX17, KLF4 and EGR-1, was found in human coronary artery smooth muscle cells, as well as in 4 day old embryoid bodies. All members of this complex are induced by PDGF-BB, a growth factor which becomes activated in angiogenesis and pathological vascular conditions, stimulating the migration and proliferation of vascular SMCs. By this the complex might be involved in migration and proliferation of vascular SMCs, and moreover in pathological vascular conditions, like the progression of atherosclerosis. EGR-1 is known to be the key player in mediating the transcriptional responses to PDGF-BB and LDL and has already been implicated in progression of atherosclerosis. Because of the fact, that a complex, consisting of Sox17, Klf4 and Egr-1, was also observed to be formed in differentiating ES-cells (day 4), supports a broader role of this protein complex in the differentiation of cell-specific lineages during development, in particular vascular smooth muscle cells and endoderm lineages. The complex might have an inhibitory, as well as an activating role, as Sox17, Klf4, and Egr-1 are known to behave bifunctional. Besides, Sox17 seems to bind to β-catenin during EB formation. At least this could be an indication for an involvement of the complex in modulating the wnt-signaling pathway during embryonic development.

7. Literature

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8. Abbreviations

- ALP   Alkaline phosphatase
- ANG   Angiopoietin
- ApoE   Apolipoprotein A
- APS   Ammonium peroxide sulfate
- αSMA   alpha smooth muscle actin
- BMP   Bone morphogenetic protein
- Ca   Calcium
- cAMP   cyclic adenosine monophosphate
- CASMCs   Coronary artery smooth muscle cells
- ChIP   Chromatinimmunoprecipitation
- Cx43   Connexin 43
- dpc   days post coitum
- DTT   Dithiothreitol
- EB   Embryoid body
- EC   Endothelial cells
- eEPCs   embryonic endothelial progenitor cells
- EGR   Early growth response factor
- eNOS   Endothelial specific NO-synthetase
- Eph   Ephrin
- ERK   extracellular signal-regulated kinase
- ES-cells   Embryonic stem cells
- FCS   Foetal Calf Serum
- FGF   Fibroblast growth factor
- FITC   Fluoresceinisothiocyanat
- FLK-1   Fetal liver kinase – 1
- Flt-1   fms-like tyrosine kinase – 1
- Fox   Forkhead box protein
- FRP   Frizzled related protein
- FunGenES   Functional Genomics in Engineered ES-cells
- Fz   Frizzled
- GKLF   Gut-enriched KLF
- Gsc   Goosecoid
- HGF   Hepatocyte growth factor
- HMG   High mobility group
- HIF-1 α   Hypoxia-inducible factor - 1alpha
- Hox   Homeobox protein
- HUVECs   Human umbilical vein endothelial cells
- ICM   Inner cell mass
- ID   Inhibitor of differentiation
- IFN   Interferone
- IL   Interleukin
- IP   Immunoprecipitation
- KLF   Krüppel-like factor
- LDL   Low density lipoprotein
- LIF   Leukemia inhibitory factor
- LPS   Lipopolysaccharids

- LRP — Lipoprotein related protein receptor
- LSEC — Liver sinusoidal endothelial cells
- MAPK — Mitogen-activated protein kinase
- MEK — Mitogen activated kinase kinase
- MEM — Non-essential amino acids
- MMP — Matrix-metalloproteinase
- Nab — NGFI-A-binding protein
- NCDI — Nab conserved domain 1
- NF-κB — Nuclear factor-kappaB
- NLS — Nuclear localization signal
- NP-40 — Nonidet P-40
- Oct3/4 — Octamer-binding transcription factor ¾
- PAI — Plasminogen Activator Inhibitor
- PBS — phosphate buffered saline
- PCR — Polymerase chain reaction
- PDGF — Platelet derived growth factor
- Pecam-1 — Platelet endothelial cell adhesion molecule-1
- PlGF — Placental-like growth factor
- Sca-1 — Spinocerebellar ataxia-1
- SDS — Sodium dodecyl sulfate
- Shh — Sonic hedgehog
- SMCs — Smooth muscle cells
- SMMHC — Smooth muscle myosin heavy chain
- SOX — Sry-box; Capital letters = human origin
- Sox — small letters = murine origin
- SRF — Serum response factor
- SSEA — Stage specific embryonic antigen
- STAT — Signal transducer and activator of transcription
- TCE — TGF-beta control element
- TCF — T-cell factor
- TGF-β1 — Transforming growth factor - beta1
- TF — Tissue Factor
- Tie — Tyrosine kinase receptor
- TLR — Toll-like receptor
- TNF-α — Tumor necrosis factor – alpha
- TRA-1 — Tumor rejection antigen-1
- U-PA — Urokinase-type plasminogen activator
- VCAM-1 — Vascular cell adhesion molecule – 1
- VEGF — Vascular endothelial growth factor
- VSMCs — Vascular smooth muscle cells

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