# Functional characterization of the transcription factor HOXA1 and the Rho-dependent kinases ROCK I//II function during angiogenesis

**INAUGURAL-DISSERTATION**
zur
Erlangung der Doktorwürde
der Naturwissenschaftlich-Mathematischen Gesamtfakultät
der
Ruprecht-Karls-Universität
Heidelberg
**2012**

vorgelegt von
Diplom-Nanostrukturwissenschaftlerin

**Míriel Tonja Teichmann**
aus Ludwigsburg

Tag der mündlichen Prüfung:

Gutachter: Prof. Dr. Thomas Wieland
Institut für Experimentelle und Klinische Pharmakologie
und Toxikologie der Medizinischen Fakultät Mannheim,
Ruprecht-Karls-Universität Heidelberg

PD Dr. Jens Kroll
Forschungsbereich der Vaskulären Biologie der
Medizinischen Fakultät Mannheim,
Ruprecht-Karls-Universität Heidelberg
und des Deutschen Krebsforschungszentrum, Heidelberg

Die vorliegende Arbeit wurde in der Abteilung für „Vaskuläre Biologie und Tumorangiogenese“ am Zentrum für Biomedizin und Medizintechnik (CBTM) im Zeitraum vom 1. Dezember 2008 bis 30. November 2011 durchgeführt.

# Eigenständigkeitserklärung

Ich versichere, dass ich die hier vorgelegte Dissertation selbständig angefertigt habe. Die benutzten Quellen und Hilfsmittel wurden vollständig angegeben und diejenigen Stellen der Arbeit (einschließlich Abbildungen), die anderen Werken im Wortlaut oder Sinn entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht. Diese Dissertation hat noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen.

Mannheim, 28. Februar 2012

_____

Míriel Tonja Teichmann

**Für meine Eltern**

## Danksagung

An dieser Stelle möchte ich mich ganz herzlich bei allen bedanken, die zum Gelingen dieser Arbeit beigetragen haben.

Bei PD Dr. Jens Kroll bedanke ich mich für die Aufnahme in seine Gruppe und für die Überlassung meines Promotionsthemas, für die langjährige Betreuung meiner Arbeit, den regelmäßigen wissenschaftlichen Austausch und die konstruktive Kritik.

Prof. Dr. Thomas Wieland danke ich für die Möglichkeit, meine Dissertation an der Mathematisch-Naturwissenschaftlichen Gesamtfakultät der Ruprecht-Karls-Universität in Heidelberg durchzuführen und für seine wissenschaftliche sowie persönliche Unterstützung.

Prof. Dr. Hellmut Augustin danke ich dafür, dass ich in seiner Arbeitsgruppe der „Vaskulären Biologie und Tumorangiogenese“ arbeiten konnte.

Prof. Dr. Ilse Hofmann bin ich dankbar, dass sie im entscheidenden Moment für mich da war und die richtigen Worte gefunden hat.

Prof. Dr. Hans-Peter Hammes bin ich dankbar dafür, dass ich im Rahmen des GRK 880 viele wertvolle Erfahrungen machen durfte und für sein positives Feedback. Sigrid Englert danke ich für die Hilfe und Unterstützung bei allen administrativen und organisatorischen Fragen.

Der gesamten Arbeitsgruppe „Vaskuläre Biologie und Tumorangiogenese“ danke ich für die wunderbare Atmosphäre und ein freundliches Arbeitsklima, für die Unterstützung sowie das Gefühl, von Anfang an dazuzugehören. Insbesondere danke ich Susanne Bartsch für ihre experimentelle Unterstützung und die Einarbeitung in die Zellkultur. Katrin Bennewitz danke ich für die Einarbeitung in der Fischanlage und Dr. Sandra Kühl danke ich, dass sie mir beim Start im Labor so gut geholfen hat. Regina Fischer-Kešo danke ich für ihre unermüdliche Hilfe bei der Auswertung des Microarray. Martin Teichert danke ich für die Bereitstellung der Auswertungssoftware. Allen Mitdoktoranden danke ich für viele wissenschaftliche Diskussionen und experimentelle Hilfestellungen und Ideen.

Für das Korrekturlesen dieser Arbeit bedanke ich mich bei Sonia Loewen-Pandji und Kathrin Schäker.

Des Weiteren danke ich Prof. Dr. Rob Henning und seiner Arbeitsgruppe aus Groningen. Besonders Dr. Hendrik Buikema und Maaike Goris verdanke ich eine wundervolle Zeit, die ich nie vergessen werde.

Bei Haniyeh, Vanessa und Anna bedanke ich mich, dass sie mich mit Kaffee und Kopfschmerztabletten versorgt haben, mit mir die Pausen verbracht haben und mich immer wieder motiviert haben.

Ein besonderer Dank gilt Dr. Melanie Bausen für die Unterstützung in den letzten Jahren. Vielen Dank für Deine Freundschaft und Deine Hilfe! Ohne Dich wäre wahrscheinlich manches anders ausgegangen.

Bei meinem gesamten Freundeskreis möchte ich mich besonders für das Verständnis bedanken, dass ich mich in den letzten Jahren ziemlich rar gemacht habe. Ihr hättet alle mehr Zeit verdient, die ich leider nicht hatte.

Der größte Dank gilt jedoch meinen Eltern, die mir diesen Weg ermöglicht haben und mich immer bedingungslos unterstützt haben, und jede Entscheidung von mir mitgetragen haben.

"So eine Arbeit wird eigentlich nie fertig, man muss sie für fertig erklären, wenn man nach der Zeit und den Umständen das Möglichste getan hat."

Johann Wolfgang von Goethe, Italienische Reise, 1787

# Table of contents

# List of Figures and Tables

# Zusammenfassung

Der Transkriptionsfaktor HOXA1 gehört zur Familie der Homeobox-Transkriptionsfaktoren, die eine wichtige Rolle in der Regulation der Morphogenese sowie während der Embryonalentwicklung einnehmen. Über die zugrundeliegenden Signalwege und Wirkungsmechanismen ist hingegen wenig bekannt. Die Expression von HOXA1 wurde im Zebrafisch hauptsächlich in vorne liegenden Regionen wie dem Kiemenbogen und im Gehirn nachgewiesen. Darüber hinaus konnte eine starke HOXA1 Expression in Brustkrebs Läsionen nachgewiesen werden.

In dieser Arbeit wird gezeigt, dass HOXA1 in Endothelzellen exprimiert wird. In verschiedenen *in vitro* Assays konnte gezeigt werden, dass eine reduzierte HOXA1 Expression in humanen Nabelschnur-Endothelzellen (HUVEC) zu einer verminderten Sprossung, Migration und Netzwerkbildung führt. Mittels Microarray Analyse von HOXA1 defizienten HUVEC konnte E-Selektin als Zielgen identifiziert werden. E-Selektin ist ein Adhäsions-Molekül das in der Signaltransduktion involviert ist. Die Expression von E-Selektin ist stark reduziert, was durch RT-PCR validiert wurde, indem gezeigt wurde, dass die E-Selektin Expression stark reduziert war, nachdem die Expression von HOXA1 mittels siRNA-Transfektion reduziert wurde.

Um eine *in vivo* Relevanz zu zeigen wurden ebenfalls Experimente im Zebrafisch durchgeführt. Die reduzierte Expression von HOXA1 führte zu vaskulären Defekten sowohl in den Schwanzgefäßen als auch in den Kopfgefäßen. Des Weiteren zeigen die Zebrafische Kopfblutungen.

In diesem Teil der Arbeit wurde somit die Bedeutung von HOXA1 für die Blutgefäßbildung gezeigt sowie eine regulatorische Beziehung zwischen HOXA1 und E-Selektin.

Im zweiten Teil dieser Arbeit wird gezeigt, dass die Rho Kinasen ROCK I und II einen anti-angiogenetischen Einfluss auf die Neovaskularisierung der Retina haben. Eine Isolektin-färbung der Retina nach der Inhibierung von ROCK I und II mit pharmakologischen Inhibitoren zeigte ein dichteres vaskuläres Netzwerk.

Des Weiteren wurde eine Herzinfarktstudie durchgeführt, in der ebenfalls ROCK I und II pharmakologisch inhibiert wurden. Diese Studie zeigte allerdings keine Unterschiede im Hinblick auf Prozesse der Angiogenese.

# Summary

The transcription factor HOXA1 belongs to the family of homeobox transcription factors, which takes up an important role in the regulation of morphogenesis as well as during embryonic development. The underlying signaling pathways and mechanisms are relatively unknown. The expression of HOXA1 in zebrafish was reported mainly in anterior regions like in the pharyngeal arch and brain regions. Moreover a high HOXA1 expression was demonstrated in breast cancer lesions.

It is shown in this thesis that HOXA1 is expressed in endothelial cells. It was demonstrated in different *in vitro* assays that a reduced HOXA1 expression in human umbilical vein endothelial cells (HUVEC) leads to reduced sprouting, migration and network formation.

A microarray analysis with HOXA1 deficient HUVEC identified E-selectin as a target gene. E-selectin is an adhesion molecule which is involved in signal transduction processes. The expression of E-selectin was highly reduced and this was validated with RT-PCR, which showed that the expression of E-selectin was strongly reduced after the expression of HOXA1 was reduced using siRNA transfection.

To show *in vivo* relevance additional experiments in zebrafish were done. A reduced HOXA1 expression led to vascular defects in the trunk vasculature as well as in the head vasculature. Furthermore the zebrafish show head bleeding.

Thus in this part of the thesis a relevance of HOXA1 for the formation of the vasculature was shown as well as a regulatory relationship between HOXA1 and E-selectin.

In the second part of this thesis it was shown that the Rho kinases ROCK I and II have an anti-angiogenic influence on the neovascularization of the retina. Isolectin staining of the retina after ROCK I and II inhibition with pharmacological inhibitors showed a more compact vascular network.

Furthermore a myocardial infarction study was performed in which also ROCK I and II were inhibited pharmacological. This study shows no difference with regard to angiogenesis.

# 1. Introduction

## 1.1. The development of the vascular system

The first organ system that is developed during embryogenesis is the vascular system. It is important to supply the developing embryo with nutrients, fluids and signalling molecules (Adams and Alitalo, 2007). At the beginning of development the embryo obtains oxygen and nutrients through diffusion because the organism is small and oxygen is able to diffuse across short distances of about 100 – 200 μm. However if the embryo grows, it becomes harder to maintain this supply and this requires the formation of the cardiovascular system. The cardiovascular system consists of blood cells, a complex vascular network and the heart (dela Paz and D'Amore, 2009). The development of the vascular system is subdivided into two major processes, called vasculogenesis and angiogenesis. Vasculogenesis is the *de novo* synthesis of blood vessels from endothelial cells, whereas angiogenesis stands for the growth of new blood vessels from pre-existing ones (Semenza, 2007).

### 1.1.1. Vasculogenesis

Blood vessels develop from angioblasts, also known as endothelial progenitor cells (EPCs). These endothelial progenitor cells differentiate from mesoderm cells just like hematopoietic progenitors, which are a link between the blood and the vascular systems. The same origin from EPCs and hematopoietic progenitors is important for angiogenesis throughout life. The EPCs aggregate to blood islands, fusing to assemble the primary vascular network of small capillaries; a process entitled vasculogenesis (Figure 1 and Adams and Alitalo, 2007; Carmeliet, 2005; Risau and Flamme, 1995). Angioblasts emerge to be induced by fibroblast growth factor (FGF) subsequently followed by assembly to the primary vascular network, which is achieved by cell migration and cohesion. Endothelial cell migration is additionally controlled by vascular endothelial growth factor (VEGF) (Poole *et al.*, 2001). Following these formations of major vessels and the capillary plexus, development and maturation of the vascular system occur. This formation of blood vessels from pre-existing ones is called angiogenesis (1.1.2. and Figure 1).

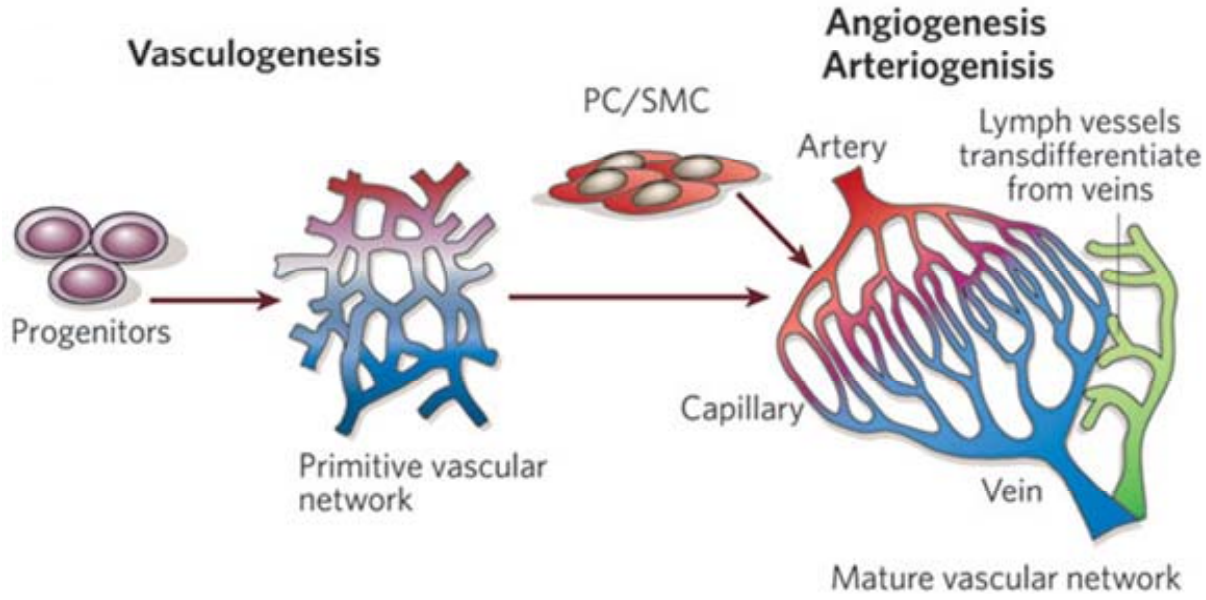



**Figure 1: Formation of the vascular network.** Endothelial progenitor cells form a primitive vascular plexus (vasculogenesis) and the network expands by blood vessel formation of pre-existing ones (angiogenesis). The mature vascular network consists of veins, arteries and capillaries, by recruiting pericytes (PC) and smooth muscle cells (SMC). Lymph vessels can be formed by transdifferentiation from veins. Reprinted with permission from Macmillan Publishers Ltd: [Nature] (Carmeliet, 2005), copyright (2005).

### 1.1.2. Angiogenesis

The vascular plexus gradually expands by means of sprouting angiogenesis and non-sprouting angiogenesis. Sprouting angiogenesis is defined as the sprouting of capillaries from pre-existing vessels, whereas non-sprouting angiogenesis, also called intussusception, is characterized by the splitting of already existing vessels along their longitudinal axis (Burri *et al.*, 2004; Risau, 1997).

Vessel sprouting is stimulated by growth factors like VEGF or FGF. These growth factors activate their receptors on the endothelial cells (ECs) of existing blood vessels. Then the ECs start with secretion of several proteases which degrade the vessel basement membrane. This results in a sprout and this sprout is guided by an EC at the tip of the sprout and therefore is called tip cell. The tip cell migrates toward the VEGF gradient (Gerhardt *et al.*, 2003). This VEGF gradient is secreted from not vascular tissue. Behind the tip cell the ECs proliferate to form the capillary lumen. The cells which follow the tip cell are called stalk cells. Finally the phalanx cells and the stalk cells form the vascular lumen and a tight barrier. The phalanx cells extend only a few filopodia and migrate weakly in response to VEGF (Mazzone *et al.*, 2009 and Figure 2).

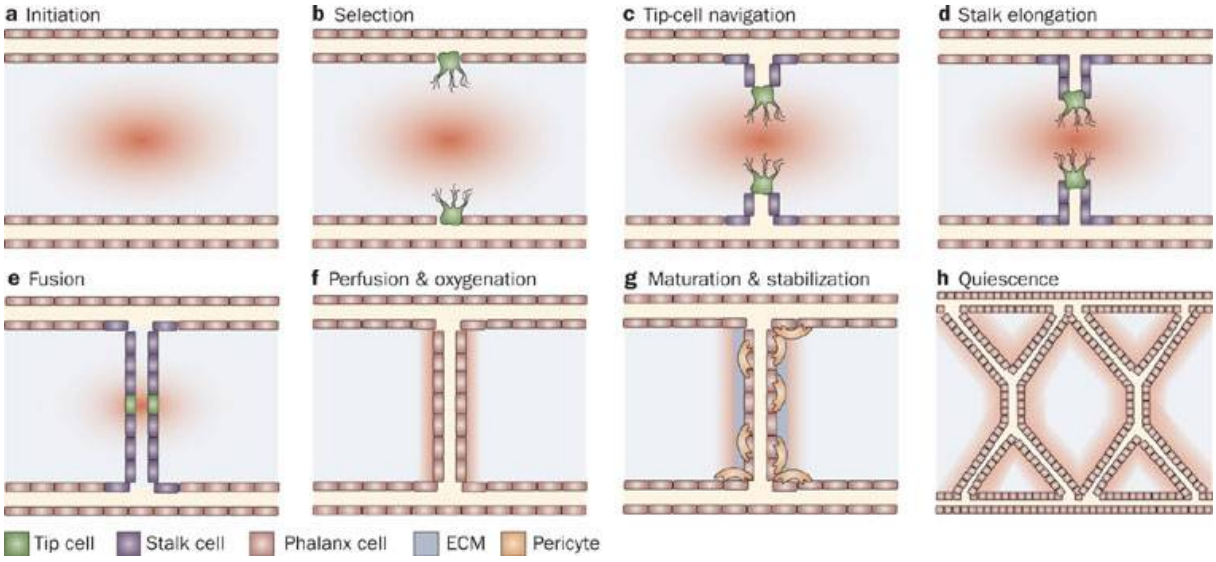



**Figure 2: Sequenced steps of sprouting angiogenesis.** (a) Sprouting starts with an initiation phase where nonvascularized cells deposit VEGF. (b) The endothelial cell near the highest concentration of VEGF becomes a tip cell. (c) The tip cell leads the sprout and the surrounding tissue invades by expanding filopodia. (d) The sprout elongates through proliferation of endothelial cells, called stalk cells. (e) Two tip cells fused to one vessel. (f) This formed vascular lumen leads to initiation of blood flow and tissue oxygenation reduces the VEGF levels. (g) To stabilize and mature the plexus recruit pericytes and form an extracellular matrix. (h) In the terminated vasculature the endothelial cells reconvert to a phalanx phenotype. Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Clinical Oncology] (Carmeliet, 2009), copyright (2009).

The other possibility in angiogenesis is splitting angiogenesis; referred to as intussusception. In this type of angiogenesis the capillary wall extends into the vascular lumen splitting a single vessel into two vessels. The intussusception process consists of four phases. First, two opposite capillary walls come into contact. Second, the EC junctions reorganize to allow growth factors and cells to invade into the vascular lumen. Third, an intrusion is formed at the area of contact and is filled with pericytes and myofibroblasts. These cells apply collagen fibres in order to make an extracellular matrix available. This extracellular matrix provides growth of the vessel lumen. The last phase is that the basal membrane and the ECs perforate in the centre and the newly formed vessels increase (Burri *et al.*, 2004).

The advantage of intussusception is that it is a reorganization of already existing cells. Therefore, intussusception makes it possible for an awesome number of new capillaries to grow without a corresponding increase of endothelial cells. This is especially important during embryonic development because there is not enough supply to form the vasculature with new cells.

### 1.1.3. Arteriogenesis

Arteriogenesis adverts to an increase in the width of pre-existing arterial vessels. For this to occur EC channels become covered with smooth muscle cells (SMCs) and pericytes. The pericytes surround the vessel wall and promote maturation and quiescence of the vessel. Arteriogenesis is correlated to increased pressure resulting in an increase of radial wall stress and higher blood flow. These two events lead to an endothelial surface stress. The blood vessels enlarge in width and length until the stress factors are normalized (Prior *et al.*, 2004). The process of arteriogenesis is associated with upregulation of cytokines and cell adhesion receptors. MCP-1 MMP, bFGF and TNF-α show increased levels caused by an intensification of shear stress. In short, MCP-1 is responsible for monocytes being able to attach to the cell wall. TNF-α offers an inflammatory milieu for the cells and bFGF induces mitosis in ECs. The last step is that MMPs alter the space around the arteries to make expansion possible (Van Royen *et al.*, 2001). It is also known that nitric oxide is a factor in increasing vessel diameter as a response to increased flow (Tronc *et al.*, 1996).

### 1.1.4. Physiological and pathological angiogenesis

Also in the adult organism angiogenesis occurs in a physiological and a pathophysiolocial way. Angiogenesis is important during the ovarian cycle and is induced in embryonic development (Augustin, 2005). Also in wound healing and tissue regeneration angiogenesis is an important factor to achieve healing (Carmeliet, 2005).

Despite this, various pathological developments are known in which angiogenic progression plays an important role. In the case of a disordered balance between stimulators and inhibitors, angiogenesis becomes excessive. Many diseases are known to be affected by excessive angiogenesis; like asthma, cirrhosis, diabetes, obesity, multiple sclerosis, bacterial infections and autoimmune diseases (Carmeliet, 2005). In multiple sclerosis patients, the blood vessel density is increased in lesions (Holley *et al.*, 2009).

Also tumours induce angiogenesis at the point when the tumour reaches a dimension that is too large for oxygen diffusion. Tumours need a high amount of oxygen and nutrition and this can only be supplied by a formed tumour blood vessel network. Besides, the vascular system is also a circuit in which metastasis can crop up (Prager *et al.*, 2011).

In other diseases the angiogenic switch is insufficient and this causes a dysfunction of endothelial cells. Diseases occurring from insufficient angiogenesis are for example hypertension, diabetes, atherosclerosis, stroke and Alzheimer's disease (Carmeliet, 2005).

This described involvement of angiogenesis in various disease patterns leads to an immense interest in the development of anti-angiogenic pharmaceuticals. The most promising drug is Avastin which is an antibody against VEGF. Angiogenesis and also vasculogenesis can be inhibited with the selective binding of Avastin to VEGF. Avastin leads to a better survival of patients with tumours (Jain *et al.*, 2006). Worldwide 30% of deaths are caused by cardiovascular diseases and 13% caused by cancer (WHO, 2011), so it is important to do research on angiogenesis and find potential therapeutic targets.

## 1.2. Angiogenesis regulation mechanisms

It is very important to analyse the mechanisms that control blood vessel formation in order to understand the process of angiogenesis. The main regulators of angiogenesis are growth factors and their corresponding receptors, which activate the angiogenic signalling cascades (Carmeliet, 2003). Aside from migration processes; protein translation and folding, RNA metabolism, chromatin remodelling and very importantly transcriptional regulations (De Val *et al.*, 2009) have been characterized as regulators for angiogenic processes.

### 1.2.1. Hypoxia

Many signals which lead to new blood vessel formation are a physiological response to hypoxia (Hadjipanayi *et al.*, 2010). To guarantee a sufficient supply of oxygen hypoxia induces vessel growth. In the case of decreased oxygen levels in the tissue the transcription factor hypoxia inducible factor 1 (HIF-1) gets activated. HIF-1 further induces the expression of other different genes like VEGF or inducible nitric oxide synthase (iNOS). Also heme oxygenase 1 (HO1) is induced by HIF-1 and heme oxygenase 1 synthesizes the vasoactive molecules NO and CO (Kikuchi *et al.*, 2005). So, one could say that HIF-1 promotes several processes in the field of angiogenesis like erythropoiesis and vasodilation (Semenza, 1998). Licht *et al.* showed in 2005 that the transgenic HIF-$1^{-/-}$ as well as the HIF-$2^{-/-}$ knock-out mice are embryonic lethal due to cardiovascular defects which indicates the important role of HIF. Taking all this into account, it would be a milestone in the therapy of cancer or ischemia if HIF activity could be manipulated either pharmalogically or with gene therapy approaches (Semenza, 2004).

## 1.2.2. Vascular guidance

Angiogenesis is mainly driven by signalling cascades, which are often caused by ligand and receptor interactions. The most investigated signalling family is the VEGF family, which includes the neuropilins. Angiopoietins (Ang) with their receptors of the Tie family are also important. A third ligand-receptor family is the Eph-family with Eph Receptors and Ephrin Ligands. The netrins and SLITs are guidance cues which also play a role during angiogenesis. And finally the Delta-Notch signalling pathway has a key function in these processes.

In general VEGF means the prototype of this gene family, VEGF-A. This family includes VEGF-A (Ferrara, 2002), VEGF-B (Olofsson *et al.*, 1996), VEGF-C (Joukov *et al.*, 1996), VEGF-D (Achen *et al.*, 1998) and placenta growth factor (PIGF) (Maglione *et al.*, 1991). The growth of vascular endothelial cells which derive from arteries and veins, is promoted by VEGF (Ferrara and Davis-Smyth, 1997). Several studies have investigated VEGF signalling *in vitro* as well as *in vivo*. VEGF is involved in all major angiogenic processes like sprouting, proliferation, migration and cell survival. VEGF binds to the two receptor tyrosine kinases VEGFR-1 (also Flt-1) and VEGFR-2 (also KDR, Flk-1) which are present at the surface of vascular endothelial cells (Vaisman *et al.*, 1990). The importance of VEGF and its receptors in regulating angiogenesis are shown in transgenic mice. Flk-$1^{-/-}$ mice are embryonic lethal because of the absence of vasculogenesis and the inability to organize blood vessels (Shalaby *et al.*, 1995). Additionally to the tyrosine kinase receptors, VEGF can also bind to neuropilin-1 (NRP-1) and neuropilin-2 (NRP-2). Especially on the cell surface of different tumour cell lines NRP-1 and 2 are expressed and bind VEGF (Klagsbrun and Eichmann, 2005). The importance of NRP-1 for the development of the vascular system was shown in NRP-1 null mice. These mice die between embryonic day 10.5 and 14.5 caused by brain and vascular defects (Kawasaki *et al.*, 1999).

Following the VEGF-system, the second important vascular-specific ligand-receptor based system is the Ang-Tie signalling system (Suri *et al.*, 1996). Tie1 and Tie2 are expressed by endothelial cells and hematopoietic stem cells (Partanen *et al.*, 1992). Ang1 is expressed in several cell types, like pericytes, smooth muscle cells, fibroblasts and in some tumour cell lines (Stratman *et al.*, 1998). It was shown that Tie1 expression is upregulated at sites of atherosclerotic plaques indicating that Tie1 plays a role in the regulation of ECs function in response to changes in flow (Porat *et al.*, 2004). Observations in Ang1 or Tie2 deficient mice showed that these mice are able to form immature vessels during embryonic development, but

they fail to develop mature vessel because they cannot recruit pericytes (Thurston, 2003 and Yancopoulos *et al.*, 2000).

The largest sub-family of the receptor tyrosine kinases are the Eph receptors with its Ephrin ligands. They were identified to work as guidance cues in several developmental processes including cell migration, axonal outgrowth and angiogenesis (O'Leary and Wilkinson 1999). Eph-ephrin complexes regulate cell adhesion, leading to increased or decreased adhesion depending on the cell type and the Eph-ephrin complex. EphrinB2 and EphB4 deficient mice die around E10.5 due to impaired vascular differentiation (Wang *et al.*, 1998 and Gerety *et al.*, 1999). This verifies that EphB4-ephrinB2 is an important regulator of vascular morphogenesis.

Further guidance cues, which were initially identified in the nervous system, are netrins and SLITs. Besides their function in the nervous system, there is evidence that they also are involved in angiogenesis. Netrins are expressed on endothelial cells and act through the receptor UNC5B. UNC5B is expressed on tip cells and UNC5B signalling leads to a repulsion of the tip cell (Lu *et al.*, 2004 and Wilson *et al.*, 2006). Additionally, there is data existing that it also can promote angiogenesis (Epting *et al.*, 2010).

SLIT ligands and their corresponding ROBO receptors are known to play a role during neuronal migration. In particular ROBO4 is a vascular specific receptor and is expressed on endothelial cells indicating an involvement during angiogenesis (Huminiecki *et al.*, 2002). In 2003 Wang *et al.* could show that recombinant SLIT2 induce migration and also tube formation *in vitro*. Additionally they found SLIT2 expression in many types of solid tumours. Bedell *et al.* (2005) investigate on ROBO4 expression in zebrafish and they discovered lace-like intersomitic vessels (ISV) after ROBO4 knockdown and misdirecting ISV after ROBO4 overexpression. ROBO4 is abundantly expressed in endothelial cells of tumours and SLIT2 inhibition blocks migration of endothelial cells by ERK/FAK (Seth *et al.*, 2005).

The last example that should be mentioned here is the angiogenic ligand-receptor system Dll4-Notch. Inhibition or blocking of the Notch pathway results in an uncontrolled hypersprouting (Siekmann and Lawson, 2007) indicating its involvement in angiogenic processes. Arterial-venous differentiation is also Notch signalling dependent (Lawson *et al.*, 2001). The Delta/Notch system regulates the communication between the tip cells and the stalk cells and thereby this system promotes the sprouting activity (Hofmann and Iruela-Arispe, 2007). The regulation of the Notch pathway is partially coupled to the VEGF pathyway (Lobov *et al.*, 2007).

## 1.2.3. Transcriptional regulation in angiogenic processes

In 1.2.2. it is shown how angiogenesis is regulated on the level of ligand-receptor signalling. But what about the remaining questions; like the gene expression downstream of those signalling pathways and also the regulation of receptor and ligand expression, about which still so little is known? The approach of investigating transcriptional regulation could provide some answers.

A few transcription factors regulating endothelial cell development have been identified, for example Ets, Forkhead (FOX), GATA, Krüppel-like and Sox. All these transcription factor families indicate an involvement in vasculogenesis and angiogenesis (Figure 3).

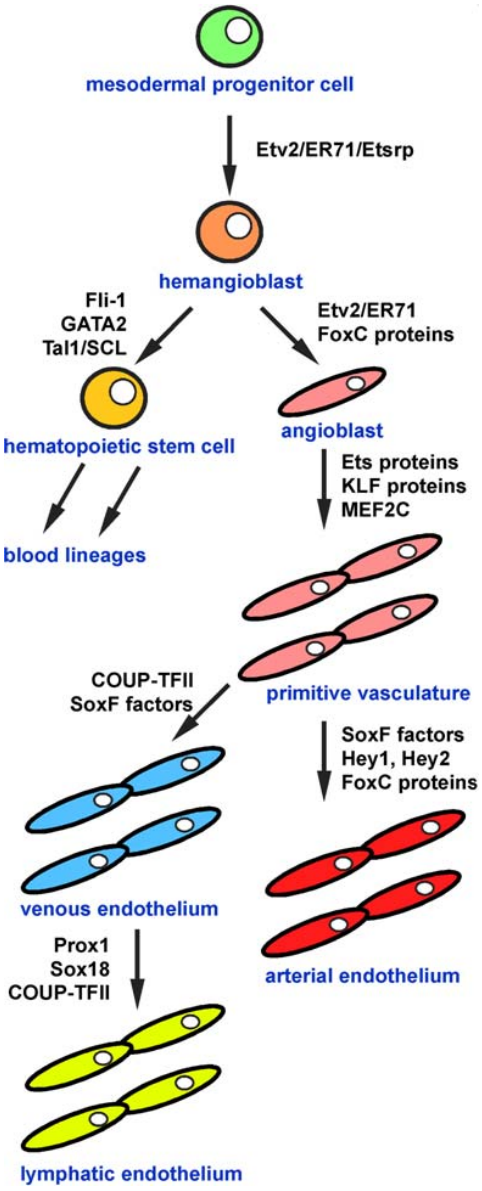



**Figure 3: Transcription factors involved in endothelial development.** This scheme shows the development from mesodermal progenitors to a primitive vasculature which develops into the arterial, venous and lymphatic endothelium. The transcription factors that are known to be involved in these processes are registered. Reprinted by permission from Elsevier [Developmental Cell] (De Val *et al.*, 2009), copyright (2009).

The transcription factors of the Ets family play a role in endothelial gene expression. Some Ets family members are expressed very early in embryogenesis and in haemangioblasts (Patterson and Patient, 2006). Pham *et al.* (2007) silenced 4 Ets using a morpholino injection on zebrafish and found out that a combinatorial network of Ets factors are important to form a physiological vascular network. Inhibition of all 4 Ets genes resulted in a total loss of endothelial differentiation (Pham *et al.*, 2007).

The most investigated regulators of endothelial transcription are the members of the Forkhead (Fox) family. They are expressed in endothelial cells as well as in their precursors, so-called endothelial progenitor cells. Indeed the expression is not specific to ECs and their precursors although they play an important role in endothelial transcription and vascular development. Key players are the members of the FoxC, FoxF, FoxH and FoxO subfamilies (Papanicolaou *et al.*, 2008).

FoxC1 and FoxC2 are essential for vascular development indicated by data obtained from $FoxC1^{-/-}$ and $FocC2^{-/-}$ mice. These FoxC deficient mice showed many vascular defects (Kume *et al.*, 2001). Additionally, these two FoxC proteins are involved in arterial and lymphatic EC differentiation. And there are some indicators that FoxC1 and FoxC2 are downstream effectors of the Notch signalling pathway (Hayashi and Kume, 2008 and Seo *et al.*, 2006).

Inactivation of FoxF1 also leads to a severe vascular phenotype resulting in embryonic lethality. This phenotype includes for example the absence of vasculatization in the allantois as well as in the yolk sac (Mahlapuu *et al.*, 2001). On the other hand, FoxH1 is known to play an inhibitory role in vascular development because FoxH1 overexpression in zebrafish leads to an impaired vascular system (Choi *et al.*, 2007).

It is clear that FoxO1 is important during vascular development, but up until now the mechanism behind is unclear. Interestingly, it is know that FoxO1 can act as a positive regulator of transcription as well as a negative regulator, indicating that FoxO1 may function as a transcriptional switch in the endothelium (Daly *et al.*, 2004 and Paik *et al.*, 2007).

GATA2 is a zinc finger transcription factor which is an important regulator of endothelial genes (Lee *et al.*, 1991 and de Val *et al.*, 2009) since many endothelial enhancers include GATA binding sites.

Krüppel-like factor (KLF) transcription factors function in EC after differentiation and specification. This was shown amongst others by Kuo *et al.* (1997) where they observed death of $KLF2^{-/-}$ mice on E14.5 due to incorrect vessel stabilization.

Sox transcription factors like Sox7, Sox17 and Sox18 are known to be needed for arteriovenous specification. Knockdown of Sox7 and Sox18 in zebrafish leads to vascular

defects (Cermenati *et al.*, 2008). This vascular phenotype consists of fusions between the major axial vessels, errors in arteriovenous specification and the lack of trunk and tail circulation.

Additionally, the transcription factors of the HOX family are important during angiogenesis and vasculogenesis as described in the next section.

## 1.3. The family of Homeobox transcription factors

Homeobox (HOX) genes encode transcription factors. They are known to regulate cell proliferation and migration. HOX genes play an important role in the development of the cardiovascular system during embryogenesis. During embryogenesis the HOX genes are important regulators to develop and form the anterior-posterior axis. Beside this function HOX genes are needed during the development of body patterns and organogenesis (Gorski and Walsh, 2000). Some HOX genes are oncogenes and thus they play a role in tumour development (Sha und Sukumar, 2010). Many HOX genes play also a role in angiogenesis which will be discussed in 1.3.1. The regulatory mechanisms of HOX gene expression are only partly understood. It is known that HOX expression can be induced by retinoic acid, FGF and steroid hormones (Svingen and Tonissen, 2006).

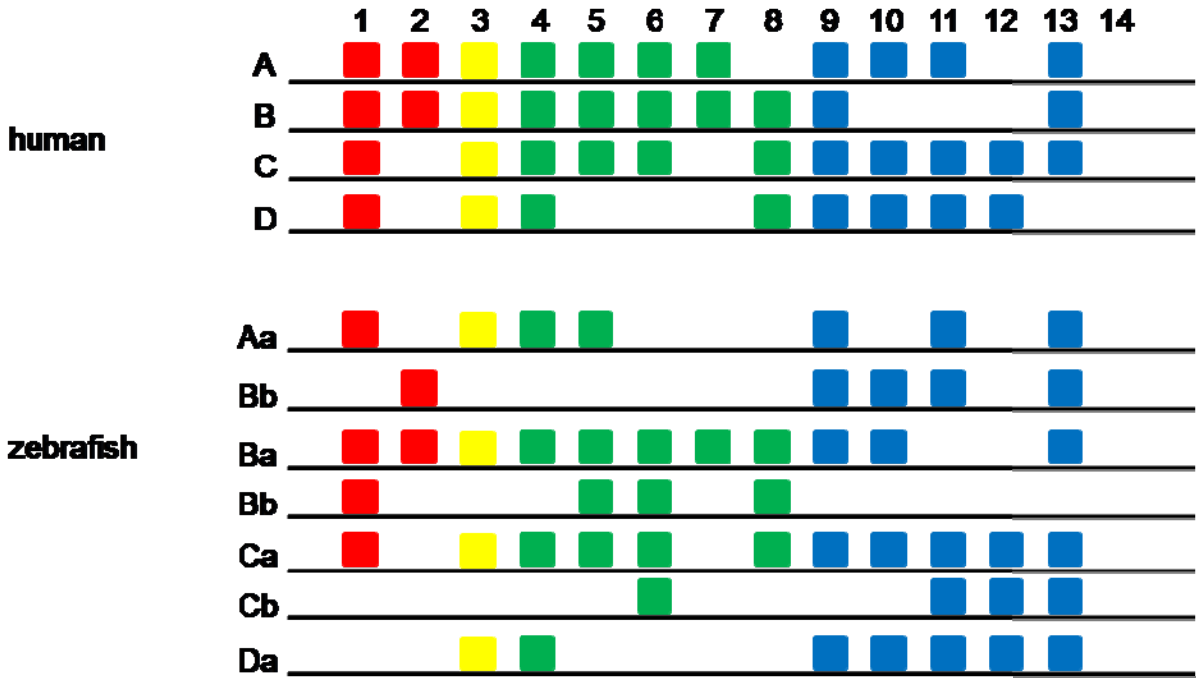



**Figure 4: HOX genes in the human and zebrafish species.** The characters indicate the cluster and the number the position in the anterior-posterior axis. Gene duplication in the zebrafish genome leads to a higher number of HOX genes in the zebrafish. Adapted and modified by permission from Ivyspring International Publisher [International journal of biological science] (Monteiro *et al.*, 2006), copyright (2006).

All HOX genes share a 183 base-pair respectively 61 amino-acid DNA-binding domain called a homeobox. This homeobox contains of a helix-turn-helix motif which can interact with DNA (Cantile *et al.*, 2008) The HOX-genes of mammals are organised in four clusters named A through D. Each cluster contains a maximum of 13 HOX genes named HOX1 through HOX13 and in combination with its cluster they are named HOXA1 through HOXD13. HOX genes with the same number share a high homology and not every number is present in every cluster (Figure 4; Monteiro *et al.*, 2006). Because of gene duplications in the zebrafish more HOX genes are found in the zebrafish genome.

### 1.3.1. The role of HOX-genes in angiogenesis

In 1996 Miano *et al.* published for the first time that HOX-genes play a role in angiogenesis. They reported that HOXA5, HOXA11, HOXB1, HOXB7 and HOXC9 are expressed in human smooth muscle cells (SMC). These HOX-genes are able to distinguish between foetal and adult smooth muscle cells thereby modulating vascular functions (Figure 5).

The paralogous HOX-genes HOXA3, HOXB3 and HOXD3 have overlapping as well as complementary roles during angiogenic processes (Cantile *et al.*, 2008). In $HOXA3^{-/-}$ knock-out mice cardiogenesis is impaired due to short or absent arteries (Chisaka *et al.*, 2005). HOXB3 expression leads to an increased capillary morphogenesis whereas HOXD3 overexpression generates haemangiomas (Boudreau *et al.*, 1997 and Myers *et al.*, 2000).

Another important HOX-gene during angiogenesis is HOXA5. It had been shown that an "increased HOXA5 expression blocks angiogenesis *in vivo* and cell migration *in vitro*" (Rhoads *et al.*, 2005). The anti-angiogenic effect resulted eventually from an interaction between HOXA5 and the vascular endothelial growth factor receptor-2 (VEGFR-2) as Coultas *et al.* suggested in 2005.

HOXA9 is a regulator of angiogenesis by acting as a proinflammatory factor. HOXA9 mediates cytokine induction of E-selectin (Bandyopadhyay et al., 2007) and is able to bind to the VEGFR-2 promoter and to the promoter of the endothelial nitric oxide synthase (eNOS) (Rössig, Urbich *et al.*, 2005). Studies by the group of S. Dimmeler showed that overexpression of HOXA9 leads to a higher EphB4 gene expression (Bruhl *et al.*, 2004).

The last gene of the HOXA locus, namely HOXA13, interacts with EphA7 and EphA4 (Stadler *et al.*, 2001).

The most investigated HOX locus is the HOXB locus, because 8 out of 10 HOXB genes are known to be expressed in human umbilical vein endothelial cells (HUVEC) indicating an important role during new blood vessel formation (Belotti *et al.*, 1998).

HOXB2 induces inhibition of proliferation shown in *in vitro* studies in HUVEC by Liu *et al.* (2004). HOXB5 transactivates the VEGFR promoter (Wu *et al.*, 2003) and HOXB7 can transactivate several pro-angiogenic factors for example FGF, CXCL1, Ang2 and MMP (Caré *et al.*, 1996).

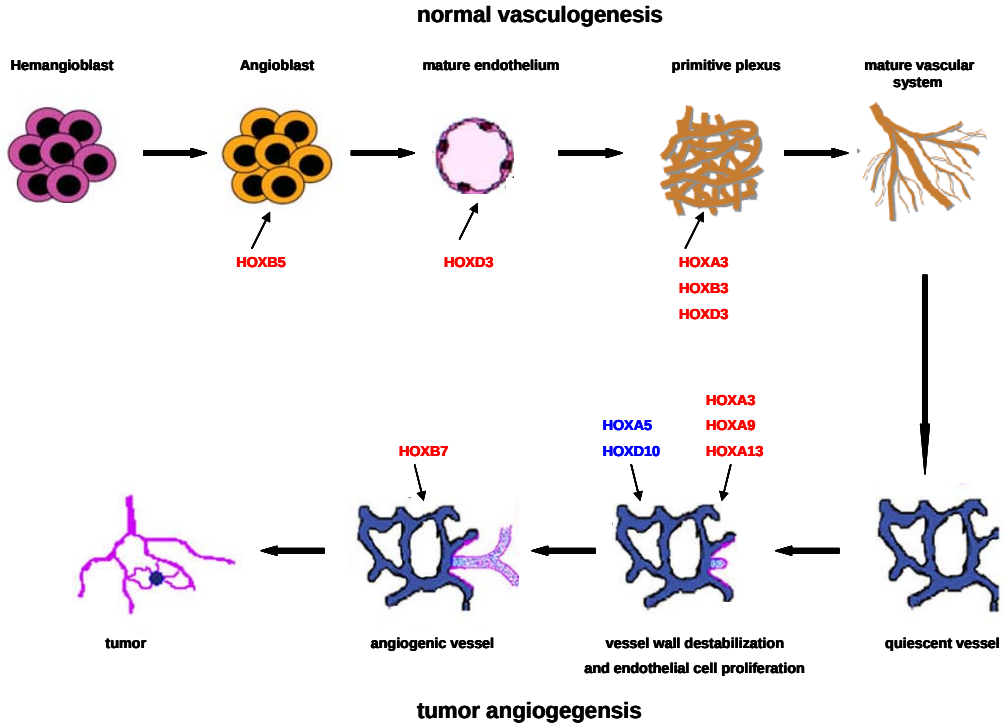



**Figure 5: The role of HOX genes during normal and pathological vessel formation.** At the top physiological vessel formation is shown. At the bottom pathological angiogenesis is illustrated. The HOX genes involved in those processes are shown in red (proangiogenic) and blue (antiangiogenic). Adapted and modified by permission from Elsevier: [Nutrition, Metabolism and Cardiovascular Diseases] (Cantile *et al.*, 2008), copyright (2008).

Loss of function of HOXC5 and the paralogous genes HOXA5 and HOXB5 leads to the formation of an additional pharyngeal arch containing an aortic arch artery. This novel aortic arch artery is completely independent and has a normal cardiac outflow (Kirby *et al.*, 1997).
HOXC9 is strongly expressed in quiescent endothelial cells and regulate vascular function via inhibition of Il-8. Otherwise HOXC9 is downregulated in active endothelial cells. And it was shown that HOXC9 is restrained under hypoxic conditions (Stoll *et al.*, 2011).
HOXD3 seems to be the most involved HOX-gene in angiogenesis. In addition to the function described above HOXD3, expression induces the production of collagen A1 and $\beta_3$ integrin to enhance wound healing (Uyeno *et al.*, 2001). Increased levels of angiogenic processes and cerebral blood flow are the result of ectopic HOXD3 expression in the mouse brain (Chen *et al.*, 2004). HOXD3 is able to enhance the transcription of the integrin $\alpha v\beta_3$ in endothelial cells (Boudreau *et al.*, 2004).

Finally HOXD10 indicates an anti-angiogenic effect, resulting in blocked angiogenesis and migration in the endothelium and also in human breast cancer cells (Myers *et al.*, 2002).

### 1.3.2. The role of HOXA1

In recent years many aspects of the role of HOXA1 were discussed. In 1991 Lufkin *et al.* showed for the first time that HOXA1$^{-/-}$ knock-out mice died shortly after birth due to anoxia and several other defects concerning delayed hindbrain neural tube closure, malformed inner ears and the absence of appointed cranial nerves and ganglia, whereas heterozygous mice developed normally and stayed alive. These findings were approved in 1992 by Chisaka *et al.*, where they specified this phenotype. First they described an ear phenotype where they could show that both inner ear compartments were poorly developed in HOXA1$^{-/-}$ mice. Also the middle ear and the external ear were gravely affected in HOXA1$^{-/-}$ mice. This implies in detail that the formation of the cochlea and the vestibule were malformed, the cochlear and the vestibular nerves were totally absent (Figure 6A). Focusing on the external ear it was showed that the auricle as well as the external acoustic meatus is deformed. Additionally the homozygous HOXA1$^{-/-}$ mice showed defects concerning the cranial nerves. Moreover the glossopharyngeal ganglion (GIX) and the vagus ganglion (GX) are poorly developed which results in an absence of preganglionic connections with the brainstem. The ganglions VII/VIII (facial ganglion/vestibulocochlear ganglion) and the trigeminal ganglion (GV) are particularly displaced resulting in the fusion of these ganglions (Figure 6B).

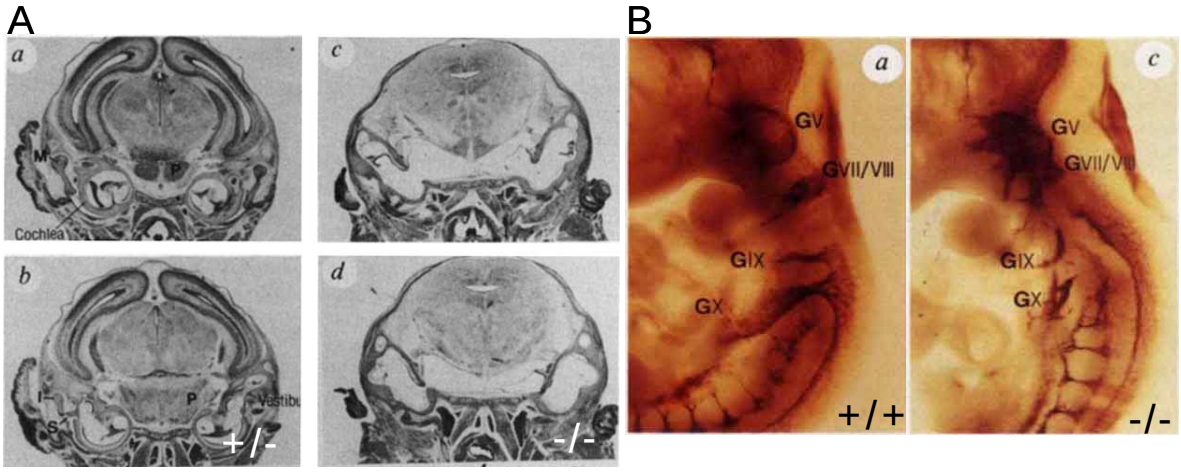



**Figure 6: Ear defects and malformed cranial nerves in HOXA1$^{-/-}$ mice.** (A) Frontal sections of inner ear compartments of newborn mice show severe defects in the HOXA1 deficient mice (c,d). The inner ear of homozygous mice looks normal (a,b) (B) The ganglion IX and X are poorly developed in the HOXA1 deficient mice (c) in comparison to wildtype mice (a). GV and GVII/GVIII are displaced in the HOXA1 deficient mice which results in a fusion of these ganglions. Reprinted by permission from Macmillan Publishers Ltd: [Nature] (Chisaka *et al.*, 1992), copyright (1992).

HOXA1 mutations in human individuals cause a broad spectrum of disease patterns. In 2005 Tischfield *et al.* described homozygous truncating mutations in HOXA1 in different, genetically isolated, populations. In this study the authors observed five consanguineous families, four families from Saudi Arabia and one family from turkey. The defects in HOXA1 are the reason for the Bosley-Salih-Alorainy syndrome (BSAS). This disease is characterized by horizontal gaze abnormalities (Figure 7A), facial weakness, deafness, mental retardation, autism spectrum disorder and hypoventilation. And this syndrome includes vascular malformations of the internal carotid arteries and cardiac outflow tract (Figure 7B).

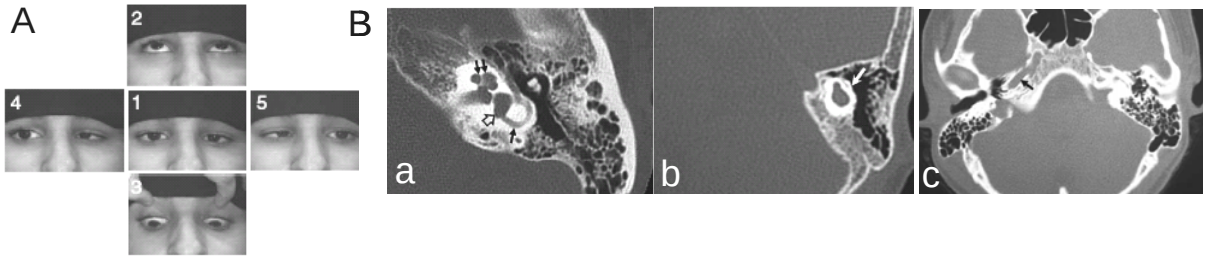



**Figure 7: Disease pattern of human individuals with HOXA1 mutations.** (A) Horizontal gaze eye movement in a person affected with BSAS. (B) (a) Computed tomography of the temporal bone and the left inner ear with a normal developed cochlea, vestibule and lateral semicircular canal. (b) Computed tomography of the same area as in (a) of an individual with BSAS showing poorly developed inner ear and a cavity. (c) Computed tomography of the skull base with an absent left carotid canal. Reprinted by permission from Macmillan Publishers Ltd: [nature] (Tischfield et al., 2005), copyright (2005).

In 1996 another syndrome which is called Athabaskan brainstem dysgenesis syndrome or Navajo brainstem syndrome was already described (Friedman *et al.*). This syndrome is also a result from mutations in HOXA1 and the disease pattern is similar to the BSAS.

## 1.4. Selectins and their role in angiogenesis

Three different selectins are known to be expressed in human individuals: E-selectin, P-selectin and L-selectin. These selectins are adhesion molecules and are type-I transmembrane glycoproteins which are expressed on leukocytes and cancer cells. Selectins bind to carbohydrate ligands in a calcium-dependent manner. Selectins and their ligands are involved in signal transduction to regulate several cell functions.

E-selectin is present in the plasma membrane of endothelial cells, P-selectin is expressed by endothelial cells and also by platelets and L-selectin is produced by leukocytes.

E-selectin, also known as CD62-antigen-like family member E (CD62E), ELAM-1 or LECAM2, is a cell adhesion molecule, which is only expressed on endothelial cells, and is

activated by cytokines. E-selectin is encoded by the *SELE* gene. E-selectin has a C-type lectin domain on the amino-terminus, an EGF-like domain, six short consensus repeat domains, a transmembrane domain and a short cytoplasmic tail at the carboxy-terminus (Springer, 1994 and Figure 8). Soluble E-selectin can be found in serum and plasma and is released by activated or damaged human endothelial cells (Leeuwenberg *et al.*, 1992 and Pigott *et al.*, 1992).

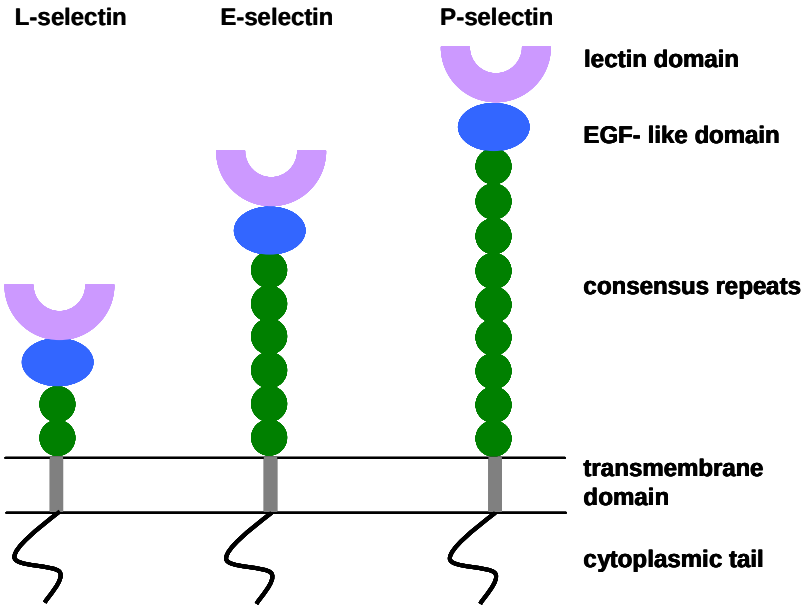



**Figure 8: Structure of the three different selectins: L-selectin, E-selectin and P-selectin.** All three selectins have a lectin domain (lavender) and an EGF-like domain (blue). L-selectin consists of two consensus repeat domains (green), E-selectin of six and P-selectin of nine. The transmembrane domain and the cytoplasmic tail at the carboxy terminus are identical in all three selectins. Adapted and modified from Hill, 2011.

### 1.4.1. E-selectin and its regulatory factors

Several transcription factors such as TNF-α, IL-1, NF-κB and activator protein 1 are known to regulate the transcription of E-selectin (Collins *et al.*, 1995 and Higai *et al.*, 2006). There exist four regulatory elements in the human E-selectin promoter, three NF-κB binding sites and one activation transcription factor (ATF) binding site (Kaszubska *et al.*, 1993). The expression of E-selectin can be induced by shear stress, VEGF, high mobility group 1 B-box (HMGB1) and monocytes (Gaucher *et al.*, 2007; Stannard *et al.*, 2007; Treutiger *et al.*, 2003; Rainger *et al.*, 1996). The activation of Rho family GTPases by different stimuli leads to an upregulation of E-selectin (Cernuda-Morollón *et al.*, 2006). E-selectin expression through TNF- α stimulation is inhibited by IL-4 and STAT6, which antagonizes the binding of NF-κB (Bennett *et al.*, 1997). The expression of E-selectin can be blocked by inhibitors of transcription and

translation such as actinomycin D and chyclohexamide, cytokines, and glucocorticoids (Cronstein *et al.*, 1992).

### 1.4.2. Role of E-selectin in angiogenesis and tumour differentiation

E-selectin plays an important role in several angiogenic processes and it is also involved in cancer metastasis. Possible functions of E-selectin in cardiovascular diseases, like diabetes and hypertension, were reported (Roldán *et al.*, 2003). In patients with these disease patterns the expression level of E-selectin is higher than in healthy persons. Increased levels of E-selectin were also found in patients with acute ischemic stroke (Fassbender *et al.*, 1995).

During inflammation E-selectin is responsible for the recruitment of leukocytes to the place of injury. The cytokines IL-1 and TNF in the area of wounded cells induces the overexpression of E-selectin on endothelial cells of blood vessels near the site of injury (Jubeli *et al.*, 2011).

In the case of an enhanced blood glucose level, for example during sepsis, the expression of E-selectin is higher which results in a higher microvascular permeability. This higher permeability causes edema resulting in skeletal muscle ischemia and finally necrosis (Visser *et al.*, 2006).

Selectins regulate the extravasation of tumour cells and the selectin ligands are involved in cancer metastasis. In patients of colorectal cancer E-selectin was detected in endothelial cells nearby cancer nodules (Kiriyama *et al.*, 1995). In gastric cancer, especially in highly vascularised tumour areas, *de novo* expression of E-selectin was found (Mayer *et al.*, 1998). Moreover, E-selectin expression is significantly higher in malignancies tumours than in benign tumours of head, neck and breast cancer (Renkonen *et al.*, 1999 and Nguyen *et al.*, 1997).

The adhesion of cultured human epithelial cancer cell lines of the pancreas, lung, liver and colon origin on the endothelium, occurs in an E-selectin-dependent pathway (Takada *et al.*, 1993).

E-selectin-deficient mice are viable and showed no obvious developmental defects. These knock-out mice show no changes “in the trafficking of neutrophils in models of inflammation” (Labow *et al.*, 1994). Furthermore the study of Labow *et al.* showed that E-selectin and P-selectin are functionally redundant and it seems that a functional neutrophil migration needs an endothelial selectin either P-selectin or E-selectin.

The development of pharmaceuticals that can inhibit the binding of E-selectin could disrupt the metastasis progression. The limitation of this strategy therein is that the selectins play an important role in several physiological processes. Furthermore the selectins interact with

many ligands as well as other adhesion molecules and all these interactions have to be considered when developing pharmaceuticals. In the past many clinical trials for treatment of diseases, such as arthritis, asthma and atherosclerosis were performed, but all these trials did not achieve successful results (Hill, 2011).
Traditional Chinese herbal medicines such as berberin are known to downregulate E-selectin (Hu *et al.*, 2009).

## 1.5. Role of the RhoGTPases in angiogenesis

Important regulators of many diverse developmental and cellular processes are the small guanosine triphosphatases (GTPases). The Rho/Rac family proteins are involved in cell division, cell proliferation, gene expression, apoptosis and transcription factor activity in normal conditions as well as during pathological ones (Bryan and D'Amore, 2007). At present 23 members of the RhoGTPases have been identified and some proteins like RhoA, Rac1 and Cdc42 have been extensively characterized (Bustelo *et al.*, 2007). Several studies have implicated in the last years that RhoA-GTPases are involved in the regulation of several angiogenic processes for example proliferation, migration, morphogenesis and vascular permeability. And they were identified as downstream effectors of VEGF signalling. The majority of these proteins behave as "molecular switchers" which means that they fluctuate between an inactive and an active state. The conformations depend on the binding of either GDP or GTP to the protein. This cycle is controlled by guanosine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) (Beckers *et al.*, 2010; Rossman *et al.*, 2005 and Tcherkezian *et al.*, 2007).

### 1.5.1. Cellular functions of ROCK I/II

The Rho kinases I and II referred to as ROCK I and II, are protein serine/threonine kinases of around 160 kDa. Two isoforms exists: ROCK I and ROCK II. Both ROCK I and ROCK II are ubiquitously expressed across human, rat and mouse tissues. ROCK I is more prominent in the liver, testis and kidney, whereas ROCK II is more pronounced in the brain and in skeletal muscle. Both ROCK I and ROCK II are expressed in vascular smooth muscle and in the heart. These kinases consist of an amino-terminal kinase domain, a potential coiled-coil-forming region including the Rho-binding domain (RBD), a pleckstrin homology (PH) domain, which has an internal cysteine-rich domain (Figure 9A). The amino-acid sequences of the two ROCK isoforms have 65% overall identity, and they share 92% identity in their kinase domains (Riento and Ridley, 2003). In the inactive form, the PH domain and the RBD of

ROCK bind to the amino-terminal region of the protein, which forms an autoinhibitory loop. Activated, GTP-bound Rho binds to RBD of ROCK, which results in an open conformation of the kinase and sets the catalytic activity free. Additionally, ROCK can be set in motion by arachidonic acid, which binds to the PH domain of ROCK, which also results in an open conformation of the kinase. The isoform ROCK I can also be activated by caspase-3-mediated cleavage next to the carboxyl terminus (Figure 9B).

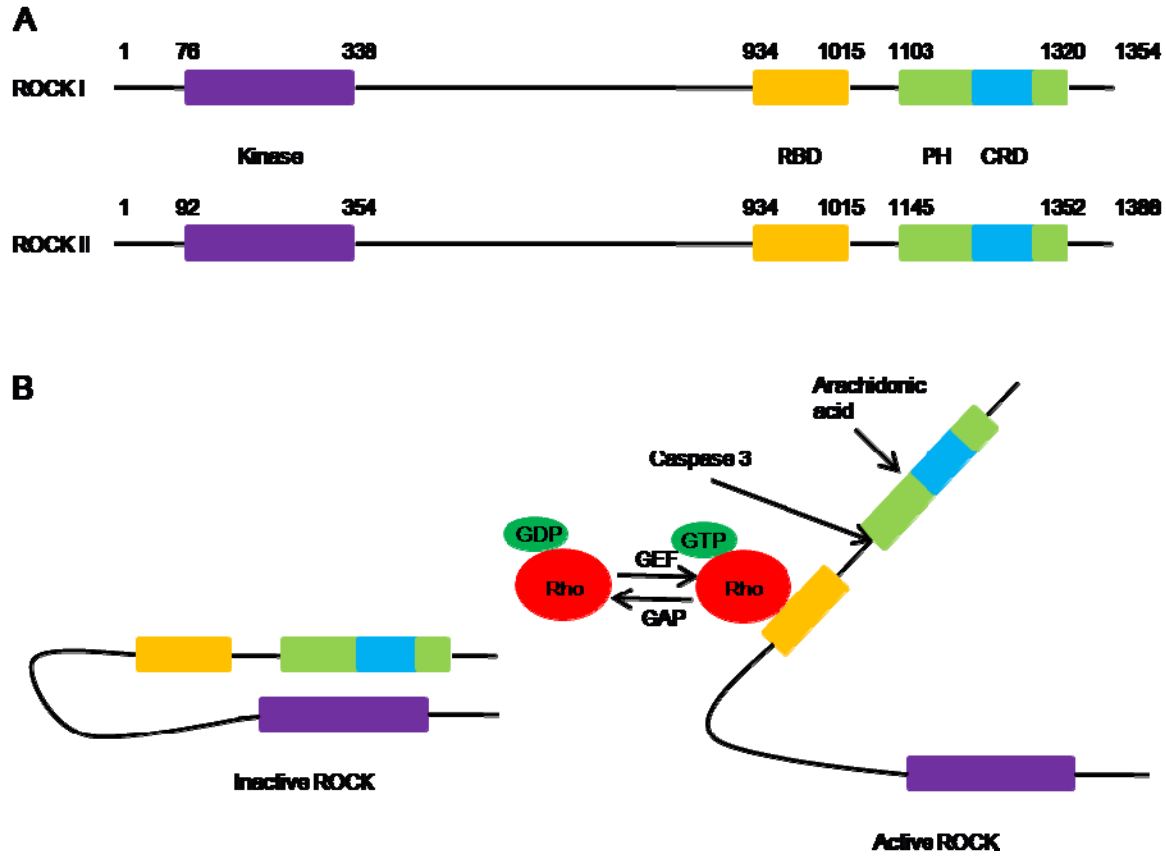



**Figure 9: Structure and regulation of ROCK I and II.** (A) ROCK I/II consist of a kinase domain at the amino terminus, a Rho binding domain (RBD) and a pleckstrin homology (PH) domain including a cysteine rich domain (CRD). (B) Inactive ROCK forms an autoinhibitory loop by binding of RBD and PH to the kinase domain (left). ROCK can be activated via three possible scenarios: GTB-bound Rho binds to RBD, arachidonic acid bind to PH or caspase-3 slice near the carboxyl terminus. Adapted and modified by permission from Macmillan Publishers Ltd: [Nature Reviews Molecular Cell Biology] (Riento and Ridley, 2003), copyright (2003).

ROCKs phosphorylate several substrates leading to various cellular responses (Figure 10). ROCK phosphorylates ERM (ezrin-radixin-moesin) and these proteins are responsible for actin-membrane linkage. Phosphorylated LIM (Lin11, Isl1 and Mec3) kinase is responsible for actin-filament stabilization through cofilin inhibition. MLCP (myosin light chain phosphatase) is responsible for the dephosphorylation of MLC (myosin light chain), and so negatively controls actomyosin-based contractility (Riento and Ridley, 2003).

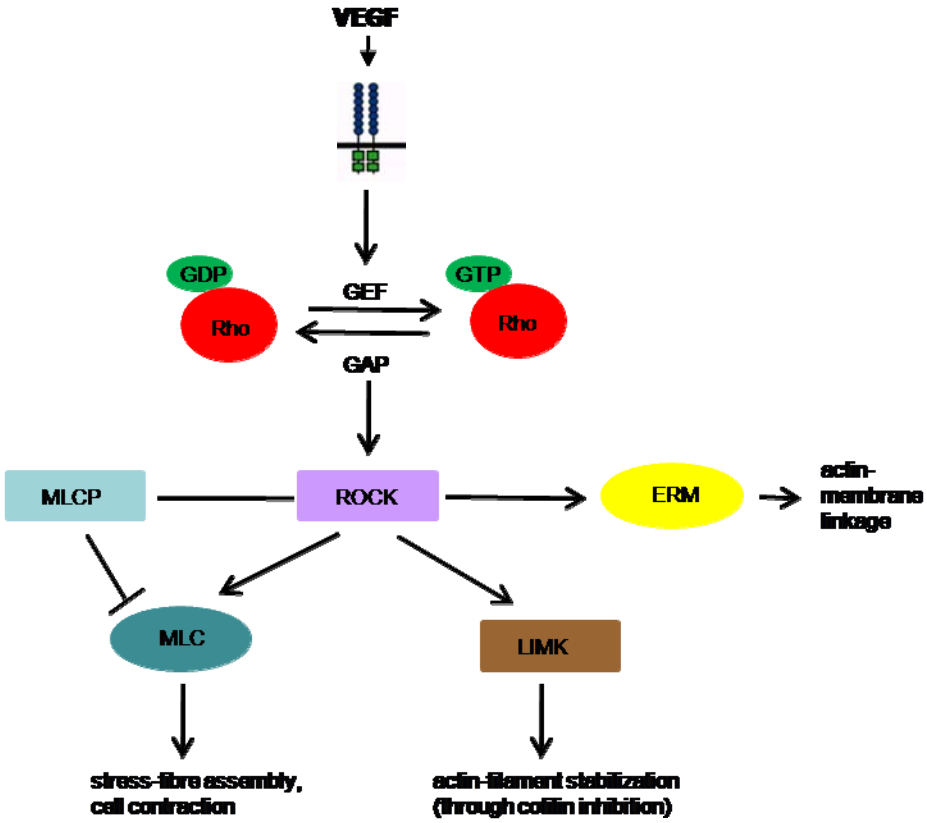



**Figure 10: ROCK targets with their contribution in cellular functions.** GTP-bound Rho subsequently activates ROCK and activated ROCK leads to phosphorylation of several target genes. Adapted and modified by permission from Macmillan Publishers Ltd: [Nature Reviews Molecular Cell Biology] (Riento and Ridley, 2003), copyright (2003).

The inhibitors used to investigate ROCK function are not specific for the isoforms ROCK I and II. But *in vivo* studies with ROCK I or ROCK II deficient mice showed that the isoforms have different functions because their phenotypes are altered. ROCK $I^{-/-}$ mice showed an omphalocele phenotype caused by a disorganization of actin filament in the epithelial cells of the umbilical ring. Also due to disorganized actin filament in the eyelid the mice display eyelid open at birth (Shimizu *et al.*, 2005). ROCK $II^{-/-}$ mice are embryonic lethal and this is caused by a dysfunction of the placenta. Additionally the intrauterine are strongly growth retarded caused by thrombus formation in the placenta (Thumkeo *et al.*, 2003). This ROCK $II^{-/-}$ phenotype indicates that the loss of ROCK II cannot be compensated by ROCK I.

## 1.5.2. ROCKs and their vascular and cardiovascular functions

A lot of investigation was done to develop inhibitors for Rho kinases ROCK I and II, gaining new treatment options for cardiovascular disorders such as hypertension and ischemic stroke

(Shimokawa *et al.*, 2007). Several *in vitro* and *in vivo* studies showed that inhibition of ROCK I and II provide a strategy to inhibit angiogenesis (Hata *et al.*, 2008; Pang *et al.*, 2009; Uchida *et al.*, 2000). On the other hand, our group was able to show that inhibition of Rho kinases with H1152 promotes angiogenesis *in vitro*. ROCK inhibition activates VEGF-induced sprouting of endothelial cells and increases VEGF-induced angiogenic signalling (Kroll *et al.*, 2009). It was also shown that inhibition of Rho kinases improves the function of the endothelium in patients with coronary artery disease (Nohria *et al.*, 2006). These two different findings suggest a dual role of Rho kinases during angiogenesis. Van Hinsbergh and van Nieuw Amerongen tried to bring these opposite effects together. They proposed a model that ROCK I and II can promote cortical tension and tail retraction. Cortical tension leads to less branching and finally inhibits or blocks angiogenesis. Tail retraction on the other hand leads to more sprouting and thereby more angiogenesis (van Nieuw Amerongen and van Hinsbergh, 2009).

## 1.6. Aim of the thesis

To understand the complex mechanisms behind blood vessel formation and function it is very important to comprehend the transcriptional regulation of angiogenesis (see also 1.2.3.). As stated earlier several transcription factors have been described as regulators of angiogenic processes. Certainly, the knowledge regarding their precise mechanisms is not fully understood. The transcription factor HOXA1 is highly conserved between the human, mouse and zebrafish genomes. Studies about HOXA1 mutations in human individuals indicate a possible function of HOXA1 during angiogenesis (1.3.2.). Sprouting and migration are influenced by HOXA1.
This work was aimed to characterize the function of HOXA1 in angiogenesis and the involvement of HOXA1 in the regulatory mechanisms of angiogenesis. To answer these questions *in vivo* studies in zebrafish were performed and several *in vitro* angiogenesis assays were performed to characterize the function of HOXA1 in cultured human umbilical vein endothelial cells (HUVECs). Finally a microarray analysis was arranged to find potential target genes of HOXA1.

A second part of my PhD thesis was to contribute to the very controversial issue of whether the Rho kinases I and II (ROCK I/II) are pro- or anti-angiogenic. Previous studies from our group showed that the inhibition of ROCK I and II leads to higher neovascularisation in

mouse retina and to more sprouting in HUVECs (Kroll *et al.*, 2009). For the investigation of its endothelial function, both kinases were pharmacologically inhibited in this thesis with the specific inhibitor H-1152 and the unspecific inhibitors Fasudil and Y-27632. An OIR-study was performed to investigate the retinal neovascularisation and a myocardial infarction study focused on the involvement of ROCK I and II in heart regeneration.

# 2. Material and Methods

## 2.1. Material

### 2.1.1. Equipment

| | |
|---|---|
| A1R confocal laser scanning system | Nikon |
| Agarose gel documentation system | Intas |
| Analytical balance (ABS) | Kern |
| Blotting chamber | Peqlab |
| Boyden chamber | Neuro Probe |
| C1-CLEM confocal laser scanning system | Nikon |
| Cell counting chamber | Neolab |
| Centrifuge (Rotina™ 420R) | Hettich |
| Chemiluminescence-imaging system | Peqlab |
| $CO_2$ cell culture incubator (HERA cell 150®) | Thermo Scientific |
| Confocal microscope DMIRE2 (TCS SP2) | Leica |
| PCR Cycler | Bio-Rad |
| Electrophoresis chamber | Peqlab |
| Electrophoresis power supply | Consort |
| Fluorescence microscope Axio Imager 2 | Zeiss |
| Fluorescence microscope DMI 6000 B | Leica |
| Incubation shaker (CERTOMAT®IS) | Sartorius |
| Incubator | Memmert |
| Inverted light microscope Type 090-135.001 | Leica |
| Microbiological Safety Cabinets (HERAsafe®) | Thermo Scientific |
| Microcentrifuge (Mikro™ 200R) | Hettich |
| Minicentrifuge (Rotilabo® Uni-fuge) | Carl Roth |
| Olympus IX50 microscope | Olympus |
| Pipets | Gilson |
| Photometer | Eppendorf |
| Precision balance (PC2200) | Mettler |
| Rocking shaker | Neolab |
| Thermo Mixer (MHR11) | HLC BioTech |
| Water bath | Julabo |

## 2.1.2. Chemicals

All chemicals that are used in the described experiments – as not indicated otherwise – were purchased from the following companies:

AppliChem GmbH
Carl Roth GmbH
Fermentas GmbH
Merck AG
Roche Diagnostics GmbH
Sigma Aldrich Chemie GmbH

## 2.1.3. Animals

### 2.1.3.1. Zebrafish lines

For all experiments the AB wildtype line, the *tg(fli1:EGFP)* line (Lawson and Weinstein, 2002) and the *tg(fli1:EGFP/tg(gata1:dsRed)* line were used.

### 2.1.3.2. Mouse

| | |
|---|---|
| C57BL/6 wildtype line | Harlan Winkelmann GmbH |

### 2.1.3.3. Rat

| | |
|---|---|
| Male wistar rats | Harlan Laboratories, Netherlands |

## 2.1.4. Morpholinos

The Morpholinos were obtained from GeneTools, LLC and the following morpholinos were used:

HOXa1a-TB-Mo: 5'- CTAAGAATGTGCTCATTGTGTCATC -3'
Standard Control-Mo (Co-Mo): 5'- CCTCTTACCTCAGTTACAATTTATA -3'

## 2.1.5. Inhibitors and growth factors

| | |
|---|---|
| Desferrioxamine mesylate (Dfx) | Sigma-Aldrich |
| Fasudil (HA-1077) | LC Laboratories |
| Fibroblast growth factor (FGF) | Relia Tech Cat |
| H1152 dihydrochloride (ROCK I/II inhibitor) | Tocris Bioscience |

| | |
|---|---|
| Vascular endothelial growth factor (VEGF) | R&D Systems |
| Y-27632 dihydrochloride | Tocris Bioscience |

## 2.1.6. Plasmids

| | |
|---|---|
| HOXA1 in pDONR221 | DKFZ core facility |
| pAd/CMV/V5-DEST | Invitrogen |

## 2.1.7. Bacteria

| | |
|---|---|
| MAX Efficiency® DH5α™ Competent Cells | Invitrogen |
| MAX Efficiency® Stbl2™ Competent Cells | Invitrogen |
| One Shot® Stbl3™ Chemically Competent *E. coli* | Invitrogen |
| One Shot® TOP10 Chemically Competent *E. coli* | Invitrogen |

## 2.1.8. RT-PCR and PCR

### 2.1.8.1. Reagents, buffers, nucleotides

| | |
|---|---|
| BufferS | Peqlab |
| dNTPs (100 pmol) | Peqlab |
| Taq-Polymerase | Peqlab |

### 2.1.8.2. Primer

All primers were purchased from MWG (www.mwg-biotech.com)

| target gene | species | sequence |
|---|---|---|
| HOXA1 | human | 5’ CCC ATG GAG GAA GTG AGA AA 3’<br>5’ CGC GTC AGG TAC TTG TTG AA 3’ |
| HOXa1a | zebrafish | 5’ ATG AGC ACA TTC TTA GAT TTT TCG 3’<br>5’ TTA ATT AGA GGA GTA TGC CTC AAC A 3’ |
| Actin | human | 5’ GGC ATC CTC ACC CTG AAG TA 3’<br>5’ GGG GTG TTG AAG GTC TCA AA 3’ |
| Actin | zebrafish | 5’ CTT GCG GTA TCC ACG AGA 3’<br>5’ GCG CCA TAC AGA GCA GAA 3’ |
| E-selectin | human | 5’ AGC CCA GAG CCT TCA GTG TA 3’<br>5’ CCT TTT GGA TCC CTT CAA CA 3’ |

| | | |
|---|---|---|
| T7 long | human | 5’ CGTAATACGACTCACTATAGGG 3’ |
| pENTR forward | human | 5’ TGGCCTTTTTGCGTTTCTAC 3’ |

## 2.1.9. Kits

| | |
|---|---|
| Caspase-Glo® 3/7 Assay System | Promega |
| Illustra plasmidPrep Mini Spin Kit | GE Healthcare |
| RNeasy | Qiagen |
| SuperScript®First-Strand Synthesis System for RT-PCR | Invitrogen |
| Wizard® SV Gel and PCR Clean-Up System | Promega |
| ABC Vectastain Kit | Vector Laboratories |

## 2.1.10. Cell culture

### 2.1.10.1. Cells

| | |
|---|---|
| 293A cells | Invitrogen |
| HUVEC (human umbilical vein endothelial cells) | Umbilical cords were obtained from the Universitätsklinikum Mannheim and freshly isolated |
| BAEC (bovine aortic endothelial cells) | Invitrogen |

### 2.1.10.2. Media and reagents

| | |
|---|---|
| Collagen type I | isolated from rat tails |
| Chemotaxis collagen | Sigma |
| Dulbecco’s Modified Eagel’s Medium (DMEM) | Gibco |
| Endothelial Cell Growth medium (ECGM) | Promocell |
| Endothelial Cell Basal Medium | Promocell |
| Fibronectin | Sigma-Aldrich |
| Foetal Calf Serum (FCS) | Biochrom |
| Matrigel | BD Bioscience |
| Medium 199 | Sigma-Aldrich |
| Methylcellulose (Methocel) | Sigma-Aldrich |
| OPTI-MEM | Gibco |
| Penicillin-Streptomycin (P/S) | Invitrogen |

| | |
|---|---|
| Phosphate buffered saline (PBS) | Gibco |
| Trypsin-EDTA | Gibco |

#### 2.1.10.3. Transfection reagents

| | |
|---|---|
| Oligofectamin | Invitrogen |

#### 2.1.10.4. Small interfering RNA (siRNA)

| | |
|---|---|
| HOXA1 siRNA (ID: 114539)<br>(sense: 5' CCAUAGGAUUACAACUUUCtt 3' ;<br>antisense: 5' GAAAGUUGUAAUCCUAUGGtc 3') | Ambion |
| HOXA1 siRNA (ID: 3467)<br>(sense: 5' GGAAUCCUCAGAGAAGUCCtt 3' ;<br>antisense: 5'GGACUUCUCUGAGGAUUCCtc 3') | Ambion |
| non-targeting negative control 1 siRNA (AM4635) | Ambion |

In this thesis the siRNA with ID 114539 is called siHOXA1_A and the siRNA with ID 3467 siHOXA1_B.

#### 2.1.10.5. Consumables

| | |
|---|---|
| Cell scraper (28 cm) | Greiner |
| Conical tubes (15 and 50 ml) | Falcon |
| Petri dishes (10cm, round) | Greiner |
| Petri dishes (12 cm, quadratic) | Greiner |
| Reaction tubes (0.5, 1.5 and 2 ml) | Eppendorf |
| Serological pipettes (50, 25, 10 and 5 ml) | Greiner |
| T75 flask | Greiner |
| Tissue culture plates (96-, 48-, 24-, 6-well) | Greiner |

### 2.1.11. Antibodies

#### 2.1.11.1. Primary antibodies

| | |
|---|---|
| Goat-anti-human Actin (I-19) | Santa Cruz Biotechnology |
| Goat-anti-human HoxA1 (AF5014) | R & D Systems |
| Rabbit-anti-GFP (A-11122) | Invitrogen |
| Isolectin B4 (L2895) | Sigma-Aldrich |

#### 2.1.11.2. Secondary antibodies

| | |
|---|---|
| Donkey-anti-goat Alexa 488 (A-11055) | Molecular Probes |
| Rabbit-anti-goat Alexa 546 (A-21085) | Molecular Probes |
| Rabbit-anti-goat HRP (P0160) | DAKO |

#### 2.1.11.3. Nuclei staining

| | |
|---|---|
| DAPI B2883-25mg (stock: 10mg/ml) | Sigma-Aldrich |

#### 2.1.11.4. Additional staining reagents

| | |
|---|---|
| DAB-tablet, 10mg | Sigma-Aldrich |
| Fluorescent Mounting Medium | DAKO |
| Hydrogen peroxide, 30% | Roth |

### 2.1.12. DNA and protein markers

| | |
|---|---|
| GeneRuler™ DNA Ladder Mix | Fermentas (SM0331) |
| PagerRuler™ Protein Ladder | Fermentas (SM1811) |

### 2.1.13. Western Blot reagents

| | |
|---|---|
| Nitrocellulose membrane | Whatman |
| ECL Western blot detection reagent | Thermo scientific |

### 2.1.14. Commercial enzymes and buffers

| | |
|---|---|
| Alkaline phosphatase CIP | New England Biolabs |
| 10x BSA | New England Biolabs |
| Restriction buffers | New England Biolabs |
| Restriction enzymes | New England Biolabs |
| Proteinase K | Marcherey-Nagel |
| Quick ligase | Takara |
| Quick ligase buffer | Takara |

## 2.1.15. Solutions and buffers

| | |
|---|---|
| 10 x blotting buffer | 30.28 g Tris<br>106.6 g Glycine<br>ad 1 l MilliQ water |
| 1 x blotting buffer | 1 l 10x blotting buffer<br>ad 10 l MilliQ water |
| DAB/TrisHCl staining solution | 1 ml 1 M Tris/HCl pH 7.4<br>0.1% Tween20<br>500 µl 10 mg/ml DAB solution<br>ad 10 ml MilliQ water |
| 5 x DNA sample buffer | 0.2% Bromphenol Blue solution (v/v)<br>50% Glycerol (v/v)<br>10 mM EDTA |
| E3 (“eggwater”) | 3 g Red Sea Salt<br>ad 10 l MilliQ water |
| 10 x electrophoresis buffer | 144 g Glycine<br>30 g Tris<br>10 g SDS<br>ad 1 l MilliQ water |
| 1 x electrophoresis buffer | 1 l 10 x electrophoresis buffer<br>ad 10 l MilliQ water |
| FACS buffer | 0.5% FCS<br>1 mM EDTA in PBS |
| FACS washing buffer | 2.5% FCS in PBS |
| Giemsa | obtained from Sigma |

| | |
|---|---|
| LB agar | 35 g LB-agar (Sigma)<br>ad 1 l MilliQ water |
| LB-Medium | 20 g LB-Broth (Sigma)<br>ad 1 l MilliQ water |
| modified Ringer solution | 116 mM NaCl<br>2.9 mM KCl<br>5 mM HEPES, pH 7.2 |
| NP-40 lysis buffer | 150 mM NaCl<br>50 mM Tris / HCl pH 7.4<br>10 mM EDTA<br>1% NP-40 (v/v)<br>10% Glycerol (v/v)<br>1% Protease Inhibitor Cocktail (Sigma-Aldrich) (v/v) |
| 10 x PBS | 400 g NaCl<br>10 g KCl<br>57.5 g $Na_2HPO_4$<br>10 g $KH_2PO_4$<br>pH 6.8<br>ad 5 l MilliQ water |
| 1 x PBS | 1 l 10 x PBS + 9 l MilliQ water |
| PBST | 500 µl Tween<br>ad 1 l 1x PBS |
| PBSTT | 500 µl Tween<br>500 µl Triton-X<br>ad 1 l 1x PBS |

| | |
|---|---|
| 4% PFA | 16 g PFA dissolved in 400ml PBS while boiling and stirring, when dissolved cooled on ice |
| 10% PFA | 40 g PFA dissolved in 400ml PBS while boiling and stirring, when dissolved cooled on ice |
| 5 x protein sample buffer | 250 mM Tris / HCl, pH 6.8<br>0.5 M DTT (1.4-Dithiothreitol)<br>10% SDS (v/v)<br>0.5% Bromphenol Blue solution (v/v)<br>50% Glycerol (v/v) |
| 1-phenyl-2-thiourea (PTU, 5 x stock) | 304 mg PTU<br>ad 1 l MilliQ water |
| Resolving gel solution (12% acrylamide) | 6.4 mL Rotiphorese Gel 30<br>4 mL 1.5 M Tris, pH 8.8<br>5.32 mL MilliQ water<br>160 µL 10 % SDS<br>160 µL 10 % APS<br>40 µL TEMED |
| SOC medium | obtained from Invitrogen |
| Soerensen A | 9.08 g $KH_2PO_4$<br>ad 1 l MilliQ water |
| Soerensen B | 11.8 g $Na_2HPO_4$<br>ad 1 l MilliQ water |

| | |
|---|---|
| Stacking gel solution | 1.36 ml Rotiphorese Gel 30<br>700 µl 1.5 M Tris, pH 6.8<br>5.72 ml MilliQ water<br>80 µl 10 % SDS<br>80 µl 10 % APS<br>20 µl TEMED |
| Stripping solution | 0.375 g Glycine<br>500 µl fuming HCl<br>ad 50 ml MilliQ water |
| 50 x TAE-buffer | 400 mM Tris<br>20 mM $NaCH_3COO$<br>10 mM $Na_2EDTA$<br>pH 7.8<br>ad 1 l MilliQ water |
| 1 x TAE buffer | 300 ml 50 x TAE buffer<br>ad 5 l MilliQ water |
| 100mM TrisHCl buffer/Tween20 | 10 ml 1 M Tris/HCl pH 7.4<br>10 µl Tween20<br>ad 100 ml MilliQ water |

### 2.1.16. Miscellaneous

| | |
|---|---|
| Microscope glass slide | Thermo Scientific |
| Microscope coverslip | Thermo Scientific |
| Nucleopore Track-Etch Membrane | Whatman |
| Nuclease free water | Ambion |
| Microlance syringe | BD Bioscience |

# 2.2. Methods

## 2.2.1. Molecular biology

### 2.2.1.1. RNA isolation

The RNeasy kit from Qiagen was used for the RNA isolation. First the cells were washed with PBS and RLT buffer from the kit containing 10 µl/ml β-mercaptoethanol was used to lysate the cells. The cells were cultivated in 6-well plates and therefore 250 µl RLT buffer was used per well. For optimal decomposition the plates were frozen at -20°C or -80°C for minimal 30 min up to three days. The plates were then thawed, scraped and transferred into reaction tubes. Then the lysates were treated according to the manufacturer´s instructions. Finally the RNA was eluted in 30 µl nuclease free water from the kit.

The zebrafish embryos have to be dechorinated and deyolked by pipeting with a 200 µl pipet up and down. Then the embryos were centrifuged (5 min, 14000 rpm, 4°C) and resuspended in 600 µl (maximum of 60 embryos) RLT buffer from the kit containing 10 µl/ml β-mercaptoethanol. For optimal decomposition the embryos were incubated on ice for 30 min on a rotation platform. To homogenize the embryos a BD microlance syringe (0.9 x 40 mm) were used. Then the lysates were treated according to the manufacturer´s instructions. Finally the RNA was eluted in 30 µl nuclease free water from the kit.

To evaluate the quality and the concentration of the RNA, the concentration was measured with a photometer and 1% agarose gels (Sambrook and Russell, 2001) were performed. The RNA was stored at -80°C.

### 2.2.1.2. Reverse transcription

To produce first-strand cDNA 1 to 5 µg total cellular RNA was used. The transcription was performed according to the SuperScript®First-Strand Synthesis System instruction manual.

The RNA was mixed with 1 µl Oligo$(dT)_{12-18}$ Primer (0.5 µg/µl) and 1 µl dNTPmix (10 mM) and was adjusted to a total volume of 10 µl by adding DEPC-treated water. After incubation at 65°C for 5 min the probes were refrigerated on ice for one minute. Then the RT-reaction buffer was added: 2 µl 10x RT buffer, 4µl $MgCl_2$ (25 mM), 2µl DTT (0.1 M) and 1 µl RNaseOUT recombinant ribonuclease inhibitor. After preincubation (42°C, 2 min) 1 µl SuperScript® II RT (50 U/µl) was added and incubated at 42°C for additional 50 min. The reverse transcription was stopped by heating to 70°C for 15min, followed by a 1 min on ice.

In a final step the RNA was digested by added 1 µl RNaseH (2 U/µl) and incubated for 20 min at 37°C. The cDNA was stored at -20°C.

#### 2.2.1.3. Polymerase chain reaction (PCR)

Polymerase chain reaction was accomplished using 2 µl cDNA, 2 µl Buffer S, 0.4 µl dNTPs, 1 µl of sense and antisense Primer and 0.3 µl Taq-Polymerase. The total volume was 20 µl and was adjusted with deionised water.

The amplification reaction was performed as follows:

| | |
|---|---|
| Initialization step | 95°C, 2 min |
| Denaturation | 95°C, 30 min |
| Annealing | primer dependent temperature, 30 sec |
| Elongation | 72°C, product size dependent time |
| Final extension | 72°C, 5 min |

For zebrafish Hoxa1a and zebrafish actin 33 cycles from denaturation to elongation were performed, for human HoxA1 35 cycles and for human actin 26 cycles. The annealing temperature for human HoxA1 and actin was 56°C and for zebrafish Hoxa1a and actin 54°C. The E-selectin PCR was done with 35 cycles and an annealing temperature of 57°C. The specific elongation time was 30 sec -1 min for all approaches.
The PCR-product was stored at 8°C and a 1% agarose gel was performed to evaluate the result.

#### 2.2.1.4. Restriction of DNA and dephosphorylation of DNA-ends

To clone DNA fragments in plasmids, restriction enzymes were used. In general up to 200 ng DNA were used for restriction in a total volume of 20 µl. The approach was: DNA, 2 µl buffer (type dependent on enzyme), 2 µl 1x BSA, 1 µl enzyme, ad 20 µl sterile water. For a higher amount of DNA, for a preparative restriction, the approach was: 3 µg DNA, 5 µl buffer (type dependent on enzyme), 5 µl 1x BSA, 1 µl enzyme, ad 50 µl sterile water. The restrictions were performed for 3 h in a water bath at 37°C. A 1% agarose gel was performed to evaluate the result.

To prevent the religation of the plasmids the DNA-ends were dephosphorylated. Therefore 1 µl alkaline phosphatase CIP was added to the approach following by an additional incubation time for 30 min at 37°C.

### 2.2.1.5. Gel electrophoresis

The agarose gels were performed in a concentration of 1% agarose in TAE-buffer which were run at 100 V in TAE-buffer in an electrophoresis chamber.

### 2.2.1.6. Extraction of DNA from agarose gels

The Wizard® SV Gel and PCR Clean-Up System kit was used to extract DNA from the agarose gel. The desired fragments were cut out of the agarose gel with a scalpel and subsequently transferred in Membrane Binding Solution from the kit to dissolve the gel. According to the manufacturer´s instructions the extraction was performed and finally the respective DNA was eluted in 30 µl nuclease free water from the kit.

### 2.2.1.7. Ligation and Transformation in *E.coli*

To perform a ligation of DNA fragments into linearized plasmid the Quick ligase was used. To gain an optimal ligation result three times more insert-DNA fragments than linearized plasmids were used. To calculate the amount of DNA the following formula was applied:

$$\frac{\text{(x) amount of plasmid [ng] x length of insert [kb]}}{\text{length of plasmid [kb]}} \times 3 = \text{(y) amount of insert [ng]}$$

The amount of plasmid and insert (x and y) were adjusted with sterile water to a volume of 10 µl. Then 10 µl of Quick ligase buffer and 1 µl Quick ligase was added. The complete ligation mix was incubated 30 min up to 5 h at 25°C or RT.

The plasmids were transformed into *E. coli* to amplify the ligated plasmid. Different *E. coli* strains such as DH5α, stbl2, stbl3 and TOP10 were used. 50 µl of the respective *E. coli* stock were thawed on ice. 3 – 20 µl of the ligation approach were given to the *E. coli*, mixed with caution and left for 20 min on ice. Then the bacteria were treated with a heat-shock for 90 sec in a 42°C water bath and then the approach were immediately kept on ice for 2 min. In the next step 1 ml of SOC medium was added and the approach was incubated for 45 min in case of ampicillin resistance or 1 h in case of kanamycin resistance at 37°C and 200 rpm. After this incubation the bacteria were centrifuged 5 min and 6000 rpm and then 900 µl of the supernatant was removed. The bacteria pellet were resuspended in the remaining supernatant and

plated on a LB-agar plate containing ampicillin (100 µg/ml) or kanamycin (25 µg/ml). The plates were incubated overnight at 37°C in an incubator.

#### 2.2.1.8. Isolation of plasmids from *E.coli*

Single colonies from LB plates (see 2.2.1.7) were picked and transferred to a 3 ml culture composed of LB medium with ampicillin (100 µg/ml) or kanamycin (25 µg/ml).The cultures were incubated at 37°C and 200 rpm over night. The plasmids were isolated from this culture using the Illustra plasmidPrep Mini Spin Kit according to the manufacturer´s instructions. Finally the plasmid was eluted in 30 µl nuclease free water.

#### 2.2.1.9. Cloning procedures and adenovirus production

The human HoxA1 full length clone in pDONR221 was cloned via gateway cloning into the pAd/CMV/V5-DEST vector using the gateway clonase from Invitrogen. The cloning step was verified via test restriction analysis and sequencing (performed by the DKFZ core facility). The adenovirus pAd-HoxA1 was produced using the ViraPower™ Adenoviral Expression System according to the manufacturer´s protocol from invitrogen.

### 2.2.2. Biochemical analysis

#### 2.2.2.1. Extraction of protein lysates from HUVE cells and zebrafish

To analyze the protein levels *in vitro* cells were washed with cooled PBS and the lysate was produced with NP-40 lysis buffer, 250 µl per 6-well were used. For optimal decomposition the plates were frozen at -20°C for minimal 30 min up to three days. The plates were then thawed, scraped and transferred into reaction tubes. Finally the lysates were centrifuged 5 min at full speed (14.000 rpm) at 4°C and the supernatant was transferred to a new reaction tube. To perform a SDS-PAGE 50 µl from this supernatant were mixed with 5 x Laemmli buffer and boiled 5 min at 95°C.

To analyze the protein levels in zebrafish embryos first the embryos were dechorinated and deyolked by pipeting up and down with a 200 µl pipet. Normally 30 – 50 embryos per condition were used. After a centrifugation step for 5 min at full speed (14.000 rpm) at RT the embryos were resuspended in 100 µl NP-40 lysis buffer and then the embryos were incubated on ice on a rotating platform for 30 min. Then the embryos were homogenized with a BD microlance syringe (0.9 x 40 mm) and finally the lysates were centrifuged 5 min at full speed at 4°C. The supernatant was transferred into a new reaction tube and 50 µl from this

supernatant were mixed with 5 x Laemmli buffer to perform a SDS-PAGE. Finally the approach was boiled 5 min at 95°C.

#### 2.2.2.2. Western blot analysis

Firstly the proteins were separated using a 12% SDS-PAGE, therefore an aliquot of the lysate was boiled with 5x Laemmli sample buffer for 10 min at 95°C. The proteins were transferred to a nitrocellulose membrane (2 h, 100 V) and blocked in 3% BSA for 1 h at RT on a rotating platform. The membrane was incubated with the primary antibody in a final concentration of 1 µg/ml (solved in blocking solution) at 4°C overnight. After three times washing with PBST, the membrane was incubated with an HRP conjugated secondary antibody for 30 min and finally washed three times with PBST. The protein of interest was visualized by enhanced chemoluminescence (ECL reagent) and images were made with the Chemi-Capt 5000 software. For a loading control actin was used and on that account the membrane has to be stripped. The membrane was incubated with the stripping solution for 30 min at RT on a rotating platform and subsequently washed three times with PBST. The actin antibody was used in a final concentration of 0,2 µg/ml (solved in PBST) and the membrane was incubated with this antibody for 2 h at RT on a rotating platform. After three times washing with PBST, the membrane was incubated with an HRP conjugated secondary antibody for 30 min and finally washed three times with PBST. Finally the protein was visualized by ECL reagent and Chemie-Capt 5000 software. The predicted product size for HOXA1 is 35 kDa and for actin 42 kDa. The signals were quantified using the Gel-Pro Analyzer 6.0 and the signals were normalized to the actin loading control.

### 2.2.3. Cell culture methods

#### 2.2.3.1. Cell culture conditions

For thawing the cells a vial of frozen cells was transferred in a 37°C pre-warmed water bath and immediately mixed with ECGM. To remove DMSO from the cells a centrifugation step was performed and the supernatant was discarded and replaced against fresh ECGM. The resuspended cells were then plated in a T75 flask or 10 cm petri dish.

To culture human umbilical vein endothelial cells (HUVEC) endothelial cell growth medium (ECGM) supplemented with 10% heat-inactivated foetal calf serum (FCS), 1% penicillin/streptomycin (P/S) and the corresponding supplements was used. For the assays HUVEC between passage 2 and 7 were used.

293A cells for the production of the adenovirus and the following experiments with adenovirus were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% FCS and 1% P/S.

To gain good stimulations in some angiogenic assay the HUVECs were starved over night before the assay was set up. Therefore the cells were cultured in basal medium without supplements, but with 2.5% FCS and 1%P/S.

All cells were cultured in a humidified incubator at 37°C and 5% $CO_2$. The cells were split 1:3 using trypsin to passage the cells. For the HUVE cells T75 flask and for the 293A cells petri dishes (10cm, round) were used.

#### 2.2.3.2. Transfection of endothelial cells with siRNA

In each case 120.000 HUVECs were seeded on a 6-well and incubated 24 h. On the second day they cells were transfected using oligofectamin. 10 µl (correspond 200 pmol) siRNA and 90 µl OPTI-MEM were mixed and incubated for 10 min at RT. Additionally 6 µl oligofectamin mixed with 94 µl OPTI-MEM were incubated for 10 min at RT. After that time the siRNA and the oligofectamin mix were mixed and incubated for another 30 min at RT. Then the probes were adjusted to 1 ml OPTI-MEM. The HUVECs were washed twice with OPTI-MEM. Then the cells were transfected with the siRNA/oligofectamin-mix and incubated for 4 h in the humidified incubator. After this incubation the medium has to be changed to ECGM. After 24 h or 48 h the transfected cells were used for cellular or angiogenesis assays.

#### 2.2.3.3. Transfection of endothelial cells with adenovirus

In each case 300.000 HUVECs were seeded on a 6-well and incubated 24 h. On the subsequent day cells were transfected with the HoxA1-adenovirus using a multiplicity of infection (MOI). For this transfection medium was extracted from the cells leaving 1 ml per well. 30 µl to 500 µl of virus lysate was added to the cells for a MOI of 35. After incubation time of 24 h in the humidified incubator the cells were washed twice with PBS and 2 ml ECGM was applied. Cellular and angiogenesis assay were performed either immediately or after additionally 24 h.

## 2.2.4. Cellular and angiogenesis assays

### 2.2.4.1. Apoptosis assay

This assay was performed with the Promega Caspase-Glo® 3/7 Assay System to investigate cell apoptosis via measurement of the Caspase 3 and 7 activities. For this HUVECs were transfected with HOXA1_A and HOXA1_B siRNA according to 2.2.3.2. or transduced with HOXA1-adenovirus according to 2.2.3.3. and 24 h after this transfection the cells were seeded in 96-wells with 10.000 cells per well in 100 µl ECGM. After additional 24 h the positive control cells were stimulated with 250 nM staurosporin for 2 h at 37°C in the humidified incubator. Then the medium was removed and the cells were lysed with 100 µl per well Glo-Substrate solution for 1 h at RT. Finally the luminescence was measured with an ELISA reader. The assay was performed with triplicates for each condition and was performed minimum three times. The values were normalized to the control (con-siRNA or cherry-adenovirus) transfected/transduced cells and not stimulated with staurosporin.

### 2.2.4.2. Chemical hypoxia treatment

150.000 HUVECs were seeded in a 6-well and treated with Dfx in a final concentration of 75 µM or $CoCl_2$ in a final concentration of 125 µM for 8 or 24 h. After 24 h hypoxia the cells were washed with PBS and new ECGM was added without Dfx or $CoCl_2$ for additional 8, 24 and 48 h to recover. To analyse the experiment, western blot lysates were made at the different time points focusing on HOXA1 expression levels. Untreated cells function as controls and were lysed at the beginning, at the middle and at the end of the experiment.

### 2.2.4.3. Migration assay

The migration assay was performed as a modified Boyden chamber assay. At the beginning the cells were transfected with the HOXA1_A or HOXA1_B siRNA and were 48 h after transfection starved overnight in starving medium. Also the nucleopore Track-Etch membrane was coated in 20 ml PBS + 100 µl 1 M NaOH + 200 µl chemotaxis collagen and was tried overnight. On the next day, the starving medium was incubated 30 min at 37°C in the humidifier incubator to avoid gas cavities. In the wells of the lower part of the Boyden chamber the medium was filled in, basal without and for stimulation with 25 ng/ml VEGF. The wells have to be filled until a meniscus is formed above the well. The membrane was placed with the brilliant side down onto the lower part of the chamber. Then the silicon layer

and the upper part of the chamber were placed above. HUVECs were adjusted to 450.000 cells per ml and 52 µl of this cell suspension was given in triplicates to the wells on the upper part of the Boyden chamber. That the cells could migrate through the membrane the chamber was incubated for 3 h at 37°C in the humidifier incubator. To stain the migrated cells the membrane was first carefully removed from the chamber and fixed with the brilliant side up in 100 % ethanol for 10 min at RT. Afterwards the membrane was washed twice in MilliQ-water and stained for 1 h at RT in staining solution. The staining solution exists of 9.9 ml Soerensen A + 15.1 ml Soerensen B + 1.25 ml Giemsa. Finally the membrane was washed twice and then placed with the brilliant side down on an object slide. To remove the non-migrated cells the matt side of the membrane was cleaned with a cotton bud. The migrated cells were counted under the microscope, therefore 5 power fields per well were counted and the average was calculated. Average and standard deviation from the triplicates were calculated. The assay was performed four times and mean-values were calculated.

#### 2.2.4.4. Stimulation with growth factors

This experiment was performed to investigate the effect of growth factors like VEGF or FGF on the HoxA1 expression level. 200.000 cells were seeded in a 6-well and incubated for 24 h by 37°C in the humidifier incubator. Then the cells were starved in starving medium overnight. At the next day the cells were stimulated with 25 ng/ml VEGF or FGF. After a stimulation time of 12 h respectively 24 h the cells were lysated and a Western Blot was performed.

#### 2.2.4.5. Sprouting assay

For this assay HUVECs were transfected with HOXA1_A and HOXA1_B siRNA according to 2.2.3.2. or transduced with HOXA1-adenovirus according to 2.2.3.3. The sprouting assay was started 48 h after the transfection/transduction and was performed as described by Korff and Augustin (1998). Firstly the HUVECs were trypsinized and counted. 160.000 cells were resuspended in 10 ml ECGM containing 20% methocel. The cells were seeded in non-adherent plastic plates with approximately 400 cells per 25 µl drop. The plates were turned to allow the cells to form spheroids in hanging drops. The hanging drops were incubated in a humidifier incubator for 24 h at 37°C.

On the next day the spheroids were harvested by washing with PBS and centrifugation at 1000 rpm for 5 min and then the supernatant was aspirated. The spheroid-pellet was resuspended with 3 ml methocel containing 20% FCS. This solution was mixed with a collagen solution (3 ml Collagen + 375 µl Medium 199, pH 7.4 adjusted with 0.2% NaOH) and rapidly transferred into a 24-well suspension plate (1 ml per well). The gels have to polymerize for 30 min at 37°C in the humidifier incubator and then two wells per condition were stimulated with 25 ng/mL VEGF on top of the gel. Finally the assay was incubated for 24 h in the humidifier incubator. The spheroids were fixed with 1 ml 10% PFA per well.
*In vitro* angiogenesis was digitally quantified by measuring the length of the sprouts that had grown out of each spheroid (cumulative sprouting length). The sprouting length was measured using the Olympus IX50 microscope and the cell-P software analyzing at least 10 spheroids per experimental group and experiment. The experiment was performed in duplicates at least three times and mean-values were calculated.

#### 2.2.4.6. Tube formation assay

At the beginning the cells were transfected with the HOXA1_A or HOXA1_B siRNA or transduced with HOXA1-adenovirus as described above. The assay was performed 48 h after the transfection or transduction of the cells.
For this tube formation assay Matrigel has to thawed on ice and 150 µl chilled Matrigel was filled per 48-well. To get a solid matrigel the plate was incubated in a humidifier incubator for 30 min at 37°C. Then 25.000 HUVECs in 250 µl ECGM were seeded on the solid matrigel per well. The medium contained 100 ng/ml VEGF which results in a final concentration of 25 ng/ml. To form a network the assay was incubated for 24 h at 37°C in the humidifier incubator. After this time the formation of the network was stopped fixing with 240 µl 4% PFA. The cumulative sprouting length and the branching points were evaluated by using the Olympus IX50 microscope and the cell-P software. The assay was performed twice and mean-values were calculated.

#### 2.2.4.7. Viability assay

To investigate if the transfection with HOXA1 siRNAs or the transduction with HOXA1-adenovirus has an effect on the viability of the cells this assay was used for.
For the investigation of the effects of the siRNA, HUVECs were transfected with HOXA1_A and HOXA1_B siRNA according to 2.2.3.2. To have enough cells two wells per condition

were transfected. 48 h after transfection the cells were seeded in 24-wells with 30.000 cells per well. 24, 48 and 72 h after seeding the cells were trypsinized and counted. The assay was performed in triplicates, the mean-values were calculated and a growth curve was designed. For the investigation of the effects of the adenovirus, HUVECs were transduced with HOXA1-adenovirus according to 2.2.3.3. but only 120.000 cells were seeded per 6-well. One day later, 24 h after the transduction, the cells were trypsinized and counted. The other cells were washed with PBS and new ECGM was added and were incubated for additional 24 h or 48 h. So 48 h and 72 h after transduction the cells were trypsinized and counted. The assay was also performed in triplicates, the mean-values were calculated and a growth curve was designed.

## 2.2.5. Animal experiments

### 2.2.5.1. Zebrafish

#### 2.2.5.1.1. Zebrafish maintenance

The Zebrafish lines were raised and staged as described (Kimmel *et al.* 1995) under standard husbandry conditions (Lawson and Weinstein, 2002) in the Center for Biomedicine and Medical Technology, Mannheim, Division for Vascular Biology and Tumorangiogenesis. Embryos were kept in E3 solution at 28.5°C with or without 0.003% 1-phenyl-2-thiourea (PTU) to suppress pigmentation and staged according to somite number or hours post-fertilization (hpf).

#### 2.2.5.1.2. Morpholino injection

The morpholino was solved in MilliQ water in a stock solution of 16 µg/µl. The morpholino was diluted in 0.1 M KCl to concentrations of 2 to 8 µg/µl. The dilution was incubated for 5 min at 65°C. 1 nl of the respective dilution was injected through the chorion into the yolk sac of 1-cell stage embryos.

### 2.2.5.2. Mouse

#### 2.2.5.2.1. Mouse maintenance

The care and experimental use of the mice were in accordance with institutional guidelines and in compliance with the Association for Research in Vision and Ophthalmology (ARVO)

statement. Female C57BL/6 mice were purchased and housed in a 12 h light/dark cycle with free access to food and drinking water.

2.2.5.2.2. Oxygen-induced retinopathy (OIR) mouse model

The oxygen-induced retinopathy (OIR) mouse model was performed as described in Kroll *et al.* (2009). At postnatal day 7, newborn mice were exposed to 75% oxygen for 5 days with their nursing mothers, and subsequently returned to room air. All animals were weighted and randomly divided into groups (DMSO, H-1152, Fasudil and Y27632) and once per day injected intraperitoneally for five consecutive days from postnatal day 12 to 16. At postnatal day 17, the animals were sacrificed, and one eye per mouse was fixed in 4% formalin and the second eye was frozen in $N_2$ at -80°C. From the eye fixed in formalin serial paraffin sections (6 µm) were stained with periodic acid-Schiff and hematoxylin. The numbers of nuclei in preretinal neovascularizations were counted.

**2.2.5.3. Rat**

2.2.5.3.1. Rat maintenance

Ten week old male Wistar rats (n = 40) were staged under standard conditions in groups, 4 per cage, at the animal facility of the University of Groningen and had free access to food and drinking water throughout the study. All animal experiments were accomplished in approval with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Committee for Animal Experiments of the University of Groningen, Netherlands.

2.2.5.3.2. Myocardial infarction rat model

The myocardial infarction study was performed as described in Lutgens et al. (1999), by permanent ligation of the left coronary artery. Rats were anesthetized and the thorax was opened in the fourth intercostals space and the muscles overlying this region were dissected. The main left anterior coronary artery was ligated. Directly after the induced myocardial infarction the rats get an osmotic minipump in the neck. The rats were divided into three groups: (i) sham group (control I; n = 8), (ii) control group II (water; n = 16) and (iii) treatment group (H1152 (3 mg/kg/day); n = 16).

## 2.2.6. Microarray analysis

The microarray analysis was performed using HUVECs transfected with control- or HOXA1_A or HOXA1_B siRNAs. RNA was isolated from the cells 48 hours after the transfection. The RNA samples were sent to the Core Facility "Genom and Proteom" of the DKFZ, which performed the microarray analysis and sent afterwards the results. This analysis was carried out in biological quadruples.

## 2.2.7. Flow cytometry

This technique was used to analyze the expression of HOXa1a in GFP positive cells. Therefore *tg(fli1:EGFP)* embryos were injected as described in 2.2.5.1.2. At 48 hpf the embryos were anesthetized and dechorinated. Then the embryos were incubated in modified Ringer solution and deyolked by pipeting up and down with a 200 µl pipet. Before the lysis the embryos were washed in PBS and then lysed in 0.25% trypsin for 20 min. The reaction was stopped with PBS containing 10% FCS and 2 mM $CaCl_2$. Finally the cells were centrifuged and resuspended in FACS buffer. Using a FACScan flow cytometer with CellQuest software (Becton Dickinson) GFP positive cells were sorted. These positive cells were then used for PCR analysis.

## 2.2.8. Imaging

### 2.2.8.1. Immunocytochemical staining

Before the cell staining a transfection with siRNAs as described in 2.2.3.2 was performed. 48 h after the transfection the cells were seeded in a 24-well adhesion plate. Cover slips were coated with fibronectin in PBS (1:200) for 30 min at 37°C in the humidifier incubator. Then the fibronectin was removed from the slips and 50.000 cells were seeded per well onto the cover slips and incubated over night in the humidifier incubator. On the next day the cells were washed twice with PBS and then fixed with 4% PFA for 30 min at RT. The cells were subsequently permeabilized in 0.3% TritonX-100 in PBS for 10 min followed by a washing step with PBS. Then the blocking solution, 3% BSA in PBS, was applied for 30 min. The primary antibody was diluted in blocking solution and was used in a final concentration of 5 µg/ml. The cells were incubated with the primary antibody for 1 h at RT. Then the cells were washed three times in PBS and then the secondary antibody was applied for 30 min. The secondary antibody was diluted in PBS and used in a final concentration of 8 µg/ml. To visualize the nuclei the cells were counterstained with DAPI (final concentration: 2 µg/ml) for

10 min and then the cover slips were removed from the 24-well plate and embedded with Fluoromount Medium on an object slide.

#### 2.2.8.2. Immunohistochemical staining

To stain retinas with Isolectin B4, the eyes were incubated with 4% PFA over night at 4°C and were then washed three times for 10 min with PBS. Then the retinas were dissected from the eyes and were then blocked in steril filtered 1% BSA in PBS with 0.5% Triton-X-100. The retinas were blocked over night at 4°C. On the third day the retinas were washed five times for 10 min with PBS. Then the retinas were incubated with pBLEC (0.1 mmol $CaCl_2$, 0.1 mmol $MgCl_2$, 0.1 mmol $MnCl_2$, 1% Triton-X-100 in PBS; pH = 6.8) two times for 1 hour. The retinas were stained with Isolectin B4 at 4°C over night in a concentration of 1:100. Finally on the fourth day, the retinas were washed 5 times for 1 h with PBS followed by the embedding with Fluoromount Medium on an object slide. The branching points were counted physically without a software program.

#### 2.2.8.3. Whole mount staining of zebrafish

To enhance the GFP-signal a whole mount antibody staining against GFP was performed. All steps were performed on a rotating platform. *Tg(fli1:EGFP)* embryos were fixed in 4 % PFA for 2 h at RT or overnight at 4°C. Then the embryos were washed 3 times 5 min in PBST, were dehydrated in methanol (25%, 50% and 75% methanol, 5 min each) and stored at -20°C (minimum 2 h). To stain the embryos were first rehydrated (75%, 50% and 25% methanol, 10 min each) and washed 4 times 5 min in PBST. Then the embryos were permeabilize using 10 µg/ml Proteinase K in PBST. 48 hpf embryos were treated 20 min whereas 72 hpf embryos were treated 1 h. After washing 2 times 5 min in PBST they were fixed again with 4% PFA for 15 min at RT. Then the embryos were washed again 4 x 5 min in PBST and equilibrated in PBSTT for 5 min. The embryos were then blocked in 1% BSA in PBSTT containing 2% sheep serum. The anti-GFP antibody was diluted in blocking solution in a final concentration of 10 µg/ml and was applied at 4°C over night. On the following day the embryos were washed 2 times 5 min in PBSTT and then minimum 6 times for 1 h. The secondary antibody was taken from the ABC Vectastain kit and added to the following solution: 2 ml PBST + 30 µl blocking serum from rabbit + 10 µl secondary antibody. The embryos were also incubated with the secondary antibody at 4°C over night. On the next day the embryos were washed again 2 x 1min and 1 x 30 min with PBSTT. The ABComplex from the Vectastain kit was

incubated 30 min during this time and then applied to the embryos for an incubation time of 1 h. Before quenching the embryos were washed 2 x 1 min and 3 x 20 min with PBSTT, then the endogenous peroxidise was quenched using 1% $H_2O_2$ in PBSTT for 15 min and subsequently washed 3 x 5min in PBSTT. Then an equilibration step followed for 15 min in 100 mM TrisHCl buffer/Tween20 and afterwards the embryos were preincubated with DAB/TrisHCl staining solution for 10 min. Then 0.012% $H_2O_2$ was added and the embryos were incubated in the dark for approximately 10 min until the desired staining intensity was reached. The reaction was stopped by washing 4 x 10 min with PBST and then the embryos were post-fixed in 4% PFA over night at 4°C. Finally the stained embryos were washed with PBST 3 times for 5 min and analyzed under the microscope. The embryos were stored in 100% glycerol at 4°C.

#### 2.2.8.4. Microscopy

Angiogenesis *in vitro* assays (described in 2.2.4.5. and 2.2.4.6.) were analyzed using the Olympus IX50 microscope and the cell-P software.

The *in vivo* images from the trunk with the *tg(fli1:EGFP)* embryos were made using the confocal microscope DMIRE2 with Leica TCS SP2 True Confocal Scanner (Leica Microsystems). Confocal images of the zebrafish head vasculature were made with the Nikon C1-CLEM confocal laser scanning system and the Nikon A1R confocal laser scanning microscope. Therefore the embryos were dechorinated, anesthetized with 0.003% tricaine and embedded in 1% low-temperature melting agarose.

Overview pictures of embryos, and mouse tissues as well as HUVEC were analyzed with the fluorescence microscope DMI 6000 B (Leica), the fluorescence microscope Axio Imager 2 (Zeiss) and the inverted light microscope Type 090-135.001 (Leica).

### 2.2.9. Statistical and phylogenetic analysis

All results are expressed as mean ± standard error of the mean (SEM). To define significant differences of experimental groups, the two-tailed student t-test was used. $p<0.05$ was considered as statistically significant. For protein alignment analysis and phylogenetic relationship the sequences of HoxA1 proteins from invertebrates and vertebrates the bioinformatic program ClustalW was used (Thompson et al., 2002). To analyse the results of the microarray the CSC Chipster software package and the Ingenuity Pathway Analysis software were used.

# 3. Results – HOXA1

## 3.1. Expression of HOXA1

During a number of angiogenic processes, for example sprouting and migration, normal angiogenesis as well as pathological angiogenesis, several members of the HOX transcription factor family play a crucial role (Cantile *et al.* 2008). HOXA1 was reported to be expressed in anterior regions mainly in the pharyngeal arch and brain regions, predominantly hindbrain and tegmentum, during zebrafish development (Thisse *et al*., 2005). From mouse embryonic day 7.75 to 8.5, HOXA1 is highly expressed in the neuroectoderm and mesoderm during hindbrain development (Murphy and Hill, 1991). Moreover HOXA1 expression was also detected in a diversity of human breast cancer lesions, suggesting that HOXA1 may be required for the establishment of breast cancer cells (Chariot and Castronovo, 1996). If HOXA1 is expressed in endothelial cells and the role of HOXA1 during angiogenesis was unknown until now.

### 3.1.1. HOXA1 is an evolutionary conserved protein

HOXA1 is ubiquitously expressed among species and is highly conserved. Figure 11A shows the evolutionary relationship between the HOXA1 proteins found in vertebrates. HOXA1 consists of a homeobox domain. This homeobox domain is 183 bp long, 61 amino acids respectively and encodes a protein domain, which can bind DNA. The identity between the amino acids sequences of human and mouse HOXA1 is 93%. Between human and zebrafish the identity of the amino acids sequence is 56% and also 56% between mouse and zebrafish (Figure 11B). The homeodomain of all three species exhibits 100% identity in the amino acids sequence. The human HOXA1 linear DNA have 10014 bp, the linear mRNA consists of 2517 bp, the complete coding region (cds) consists of 1008 bp and the protein consists of 335 amino acids, which results in 36 kDa. The zebrafish HOXa1a gene consists of 990 bp and the protein of 36 kDa (329 amino acids).

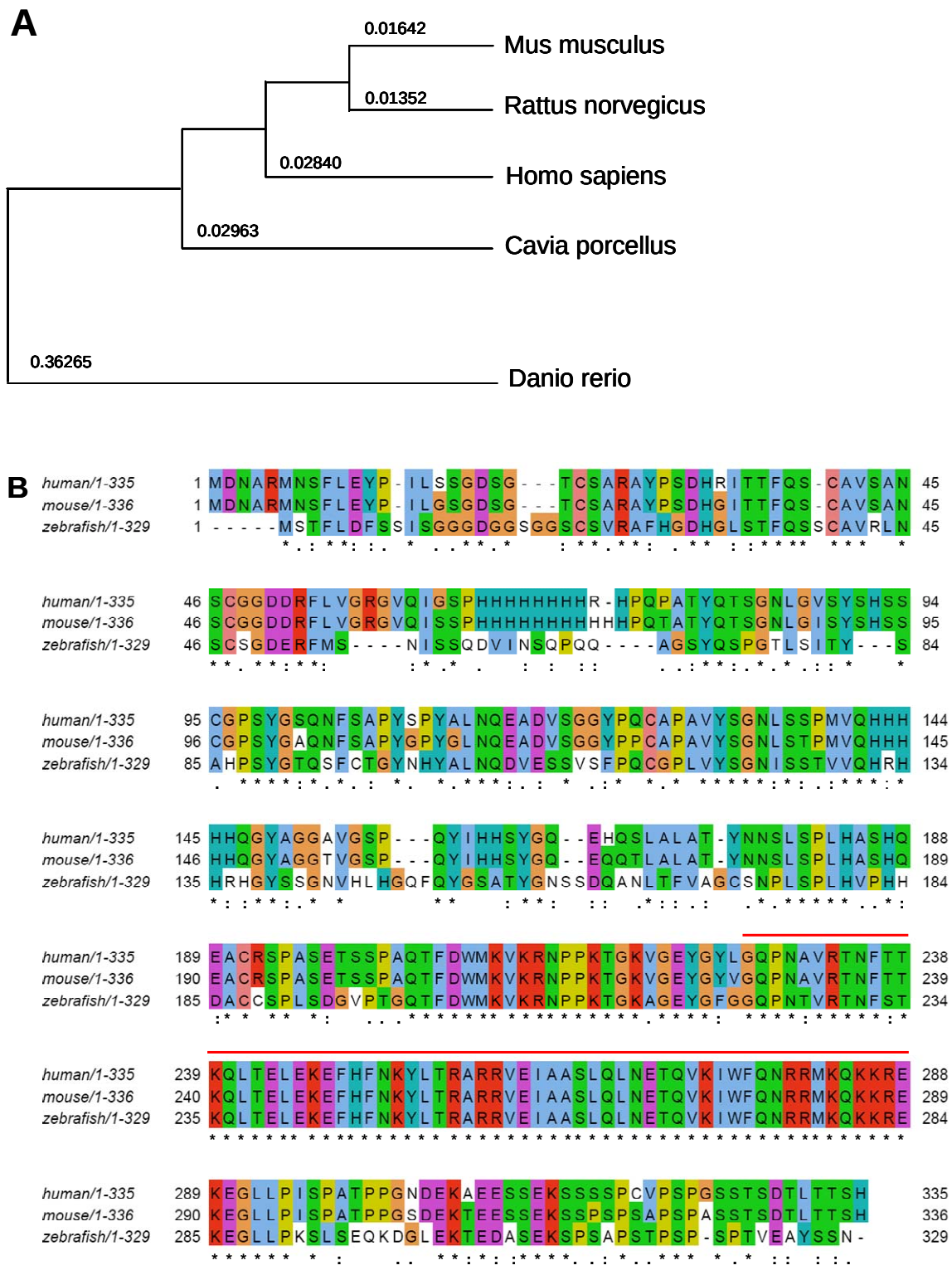



**Figure 11: HOXA1 is highly conserved during vertebrate development.** (A) Phylogenetic analysis of HOXA1 proteins from different vertebrates. This analysis shows that HOXA1 is highly conserved throughout evolution. The numbers indicates the relative evolutionary distance. (B) Multiple amino acid comparison of human (Homo sapiens), mouse (Mus musculus) and zebrafish (Danio rerio) sequences of HOXA1. The numbers indicates the amino acid positions. Conserved amino acids are labelled with an asterisk. The red line represents the homeobox domain. Accession codes: Homo sapiens (NP_005513.1), Mus musculus (NP_034579.3), Danio rerio (Q98SI1.1), Rattus norvegicus (NP_037207.1), Cavia procellus (XM_003467943.1).

### 3.1.2. HOXa1a is expressed in zebrafish

To analyse HOXa1a expression in zebrafish, mRNA was isolated or lysates for Western Blot analysis were extracted. RNA was isolated 24, 48, 72 and 120 hours post fertilization and RT-PCR was performed. figure 12a shows that HOXa1a is highly expressed during zebrafish development. Also on protein level (Figure 12b) HOXa1a is expressed from 24 hpf until 96 hpf.

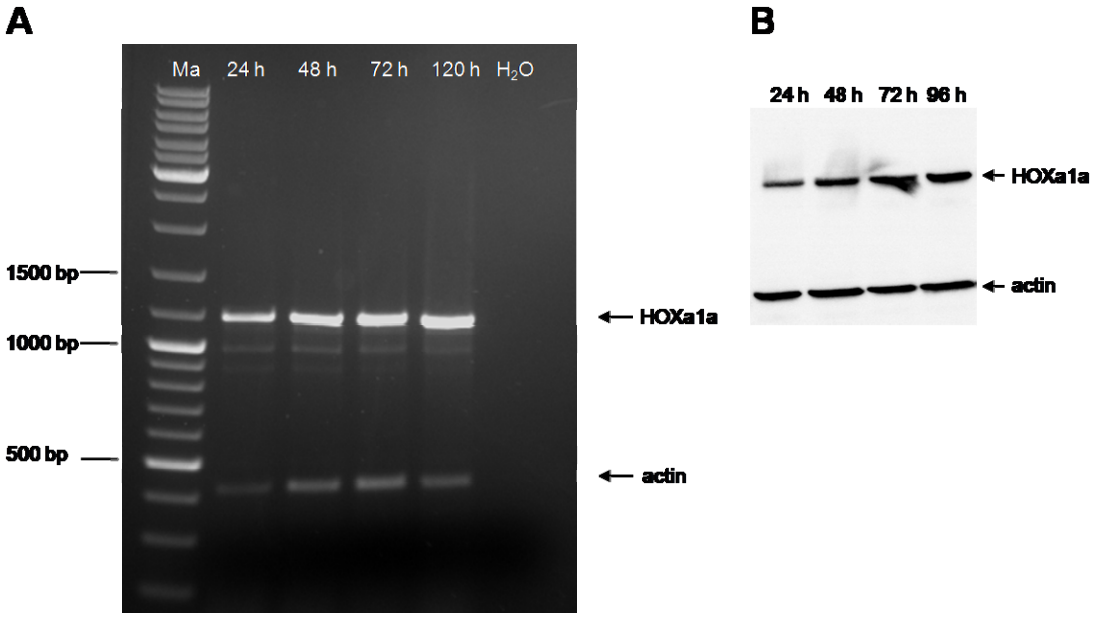



**Figure 12: HOXa1a is expressed in zebrafish.** (A) PCR shows HOXa1a expression 24 hpf, 48 hpf, 72 hpf and 120 hpf. (B) Western Blot analysis shows HOXa1a expression 24 hpf, 48 hpf, 72 hpf and 96 hpf as well.

Endothelium specific HOXa1a expression was analysed with FACS and RT-PCR. Wildtype and *tg(fli1:EGFP)* zebrafish embryos were trypsinized 48 hours post fertilization and the cells were sorted for GFP-positive cells explicitly endothelial cells. This shows that HOXa1a is expressed in zebrafish endothelial cells (Figure 13).

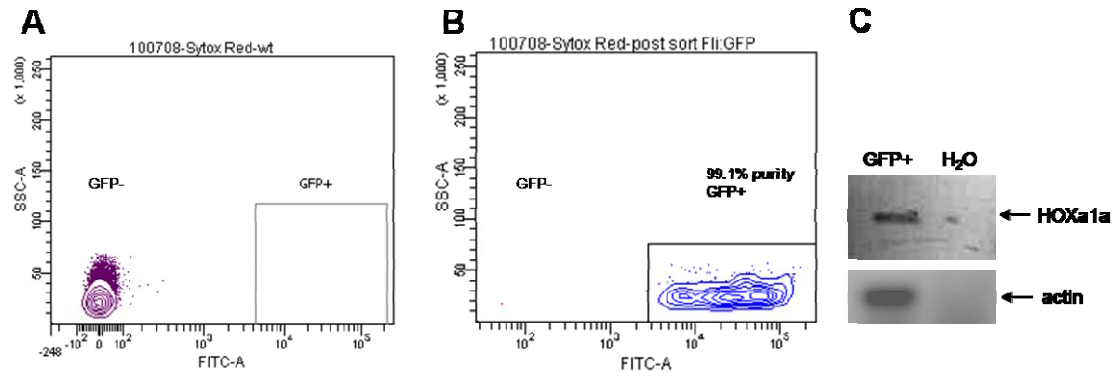



**Figure 13: HOXa1a is expressed in endothelial cells in *tg(fli1:EGFP)* zebrafish.** (A) Control from wildtype zebrafish embryos. All cells are GFP negative. (B) *tg(fli1:EGFP)* zebrafish embryos show GFP positive cells with 99.1% purity during FACS. (C) PCR with GFP positive cells showing HOXa1a expression.

### 3.1.3. HOXA1 is expressed in human umbilical vein endothelial cells and bovine aortic endothelial cells

Human umbilical vein endothelial cells (HUVEC) and bovine aortic endothelial cells (BAEC) were stained for HOXA1 to study its expression in the cells. The cells were additionally stained with DAPI to investigate the localization of HOXA1 in the nucleus. In agreement to HOXA1 function as a transcription factor the protein is localised in the nucleus (Figure 14).

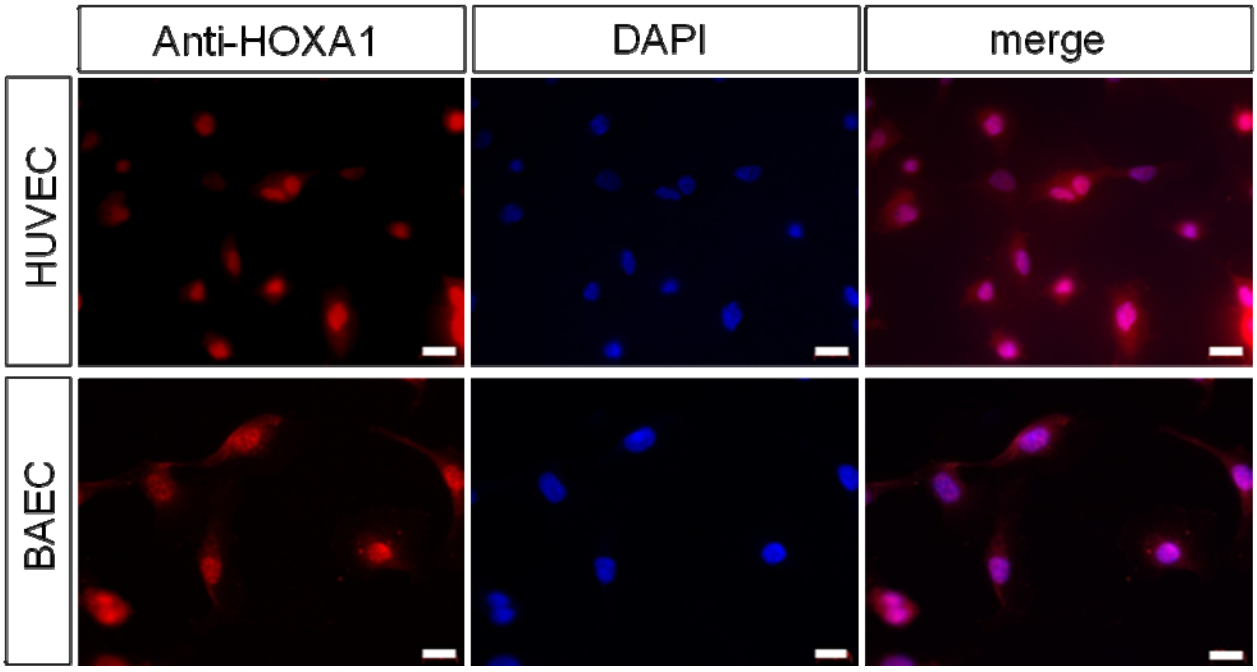



**Figure 14: HOXA1 is expressed in HUVECs and BAECs.** HOXA1 is localized in the nucleus. Cells were stained with an Anti-HOXA1 antibody and DAPI. Scale bar: 20 µm.

## 3.2. Zebrafish show vascular defects after HOXa1a silencing

Experiments were performed to focus on the *in vivo* relevance of HOXa1a using zebrafish studies. In order to silence HOXa1a in zebrafish a translational blocking morpholino (TB-Mo) was used. These translational blocking morpholinos block the "translation initiation in the cytosol by targeting the 5' UTR through the first 25 bases of coding sequence" (www.gene-tools.com). First a Western Blot analysis was performed to prove the functionality of the HOXa1a morpholino. Therefore zebrafish embryos were injected in the 1-cell stage with 2 ng and 4 ng of the HOXa1a-TB-Mo and after 24 h, 48 h, 72 h and 96 h the embryos were lysed. The Western Blot shows that the morpholino works until 96 hpf (Figure 15) because the intensity of HOXa1a expression is lower in the lysates from HOXa1a-TB-Mo injected zebrafish.

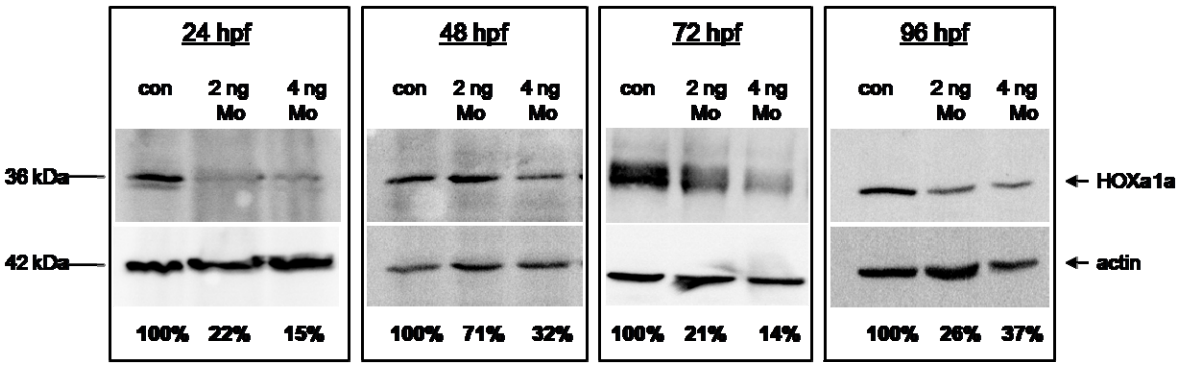



**Figure 15: Western Blot analysis shows that the HOXa1a-TB-Mo downregulates HOXa1a level after 24 hpf until 96 hpf.** The figure shows one Western Blot per time point.

## 3.2.1. Zebrafish show vascular defects in the trunk vasculature after HOXa1a silencing

HOXa1a silencing via morpholino injection led to an impaired blood vessel formation. Some intersomitic vessels (ISV) are not completely developed and some ISVs do not even begin to sprout from the dorsal aorta. The formation of the dorsal longitudinal anastomotic vessel (DLAV) is also affected. Mostly the impaired DLAV formation is a subsequent result of the impaired ISV formation. The morphology of the zebrafish looks normal so side effects of the injection can be excluded (Figure 16).

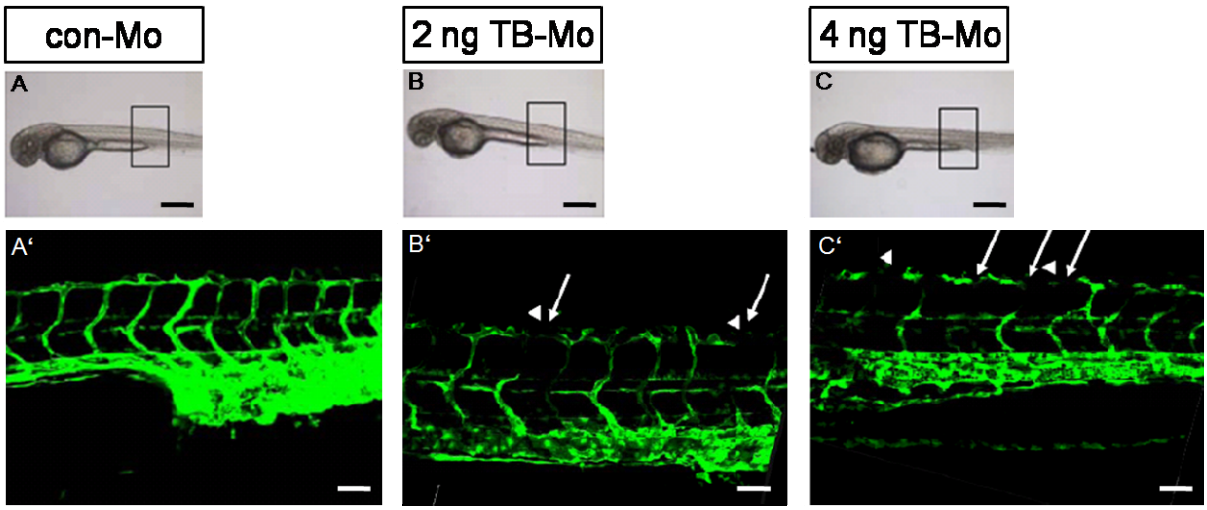



**Figure 16: Specific phenotype in the zebrafish trunk vasculature after HOXa1a silencing with morpholino injection in a concentration of 2 ng respectively 4 ng. A, A':** The con-Mo injected embryo showed a normal morphology (A) and a normal vascular system (A'). The black box marks the area which is shown in A'. **B, B':** The HOXa1a-Mo injected embryos with an injection volume of 2 ng showed a normal morphology (B) but defects in the vasculature (B'). Impaired ISV formations are indicated by arrows and breaks in the DLAV are marked with arrowheads. The black box marks the area which is shown in B'. **C, C':** The HOXa1a-Mo injected embryos with an injection volume of 4 ng showed a normal morphology (C) but defects in the vasculature (C'). Impaired ISV formations are indicated by arrows and breaks in the DLAV are marked with arrowheads. The black box marks the area which is shown in C'. Black scale bar: 500 µm. White scale bar: 50 µm.

To quantify this phenotype a DAB staining, to enhance the GFP signal, was performed, in which 40 zebrafish in each group were analysed. After 48 h 62% of the embryos injected with 2 ng of HOXa1a morpholino showed defects in the ISVs and 67.5% of the embryos with 4 ng injection volumes. After 72 h the results were nearly the same, 70% (2 ng) and respectively 67.5% (4 ng) of fish were affected (Figure 17).

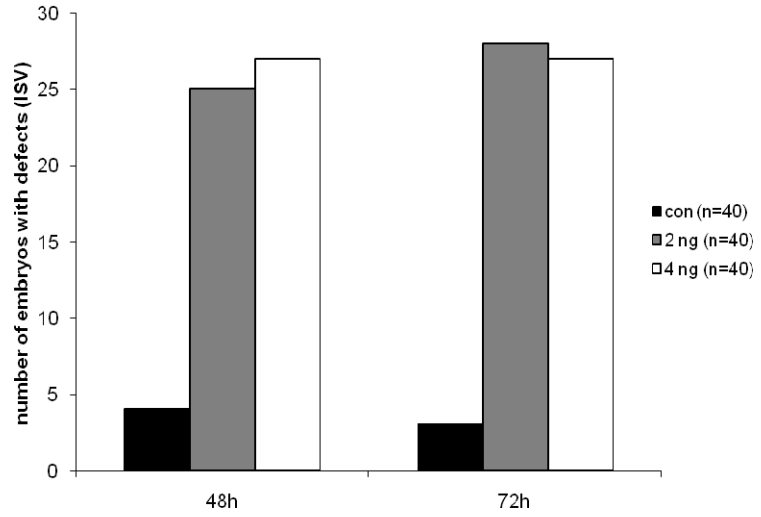



**Figure 17: Quantification of the phenotype of impaired ISV formation.** A number of 40 embryos were analysed and more than a half of the morpholino injected fish showed impaired vessel formation.

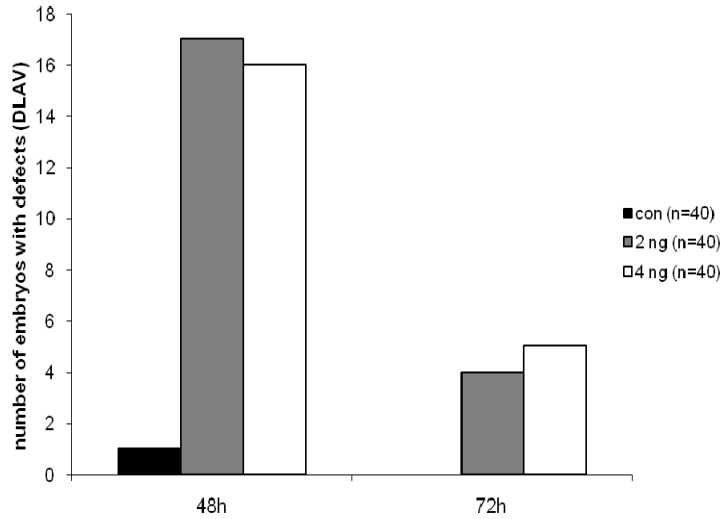



**Figure 18: Quantification of the phenotype of impaired DLAV formation.** A number of 40 embryos were analysed and after 48 hpf the half of the morpholino injected fish showed impaired vessel formation. After 72 hpf only a few injected fish showed defects in the DLAV.

## 3.2.2. Zebrafish show vascular defects and cranial haemorrhages in the head vasculature after HOXa1a silencing

The expression level of HOXa1a in anterior regions is higher than in the rest of the organism (see 3.1. and introduction), suggesting a phenotype in the head of the zebrafish. After

HOXa1a silencing cranial haemorrhages arise in zebrafish (Figure 19). Nevertheless the embryos survive until the observation time of 120 hpf and their morphology looks normal (see also 3.2.1. and Figure 16). The head bleeding starts at approximately 24 hpf and this phenotype is not reversible. The fish with bleedings after 24 hpf were separated and at 48 hpf all bleedings were still there. The fish were observed until 120 hpf and until this point in time, the head bleeding does not regress. Table 1 shows the quantification of the cranial haemorrhages. After 48 hpf around 20% of the fish observed were affected by head bleeding. After 72 hpf around 30% of the fish suffer from head bleeding. As described before, all fish displaying head bleeding after 48 hpf also had head bleeding after 72 hpf.

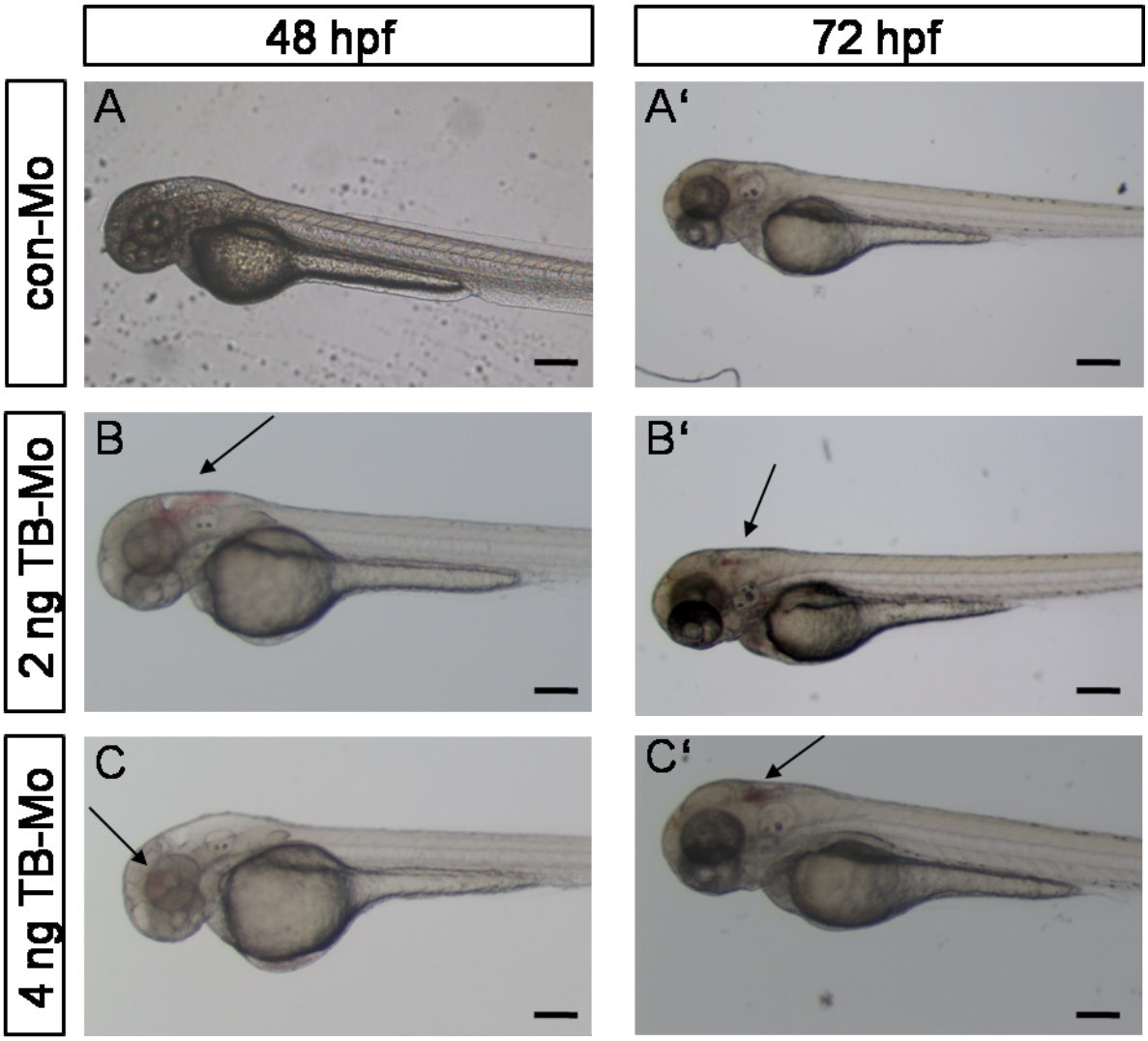



**Figure 19: Cranial haemorrhages in zebrafish after HOXa1a silencing. (A)** Zebrafish at 48 hpf injected with con-Mo. **(A')** Zebrafish at 72 hpf injected with con-Mo. (**B**) Zebrafish at 48 hpf with an injected volume of 2 ng HOXa1a-Mo. (**B'**) Zebrafish at 72 hpf with an injected volume of 2 ng HOXa1a-Mo. (**C**) Zebrafish at 48 hpf with an injected volume of 4 ng HOXa1a-Mo. (**C'**) Zebrafish at 72 hpf with an injected volume of 4 ng HOXa1a-Mo. Arrows mark the positions of cranial haemorrhages. Black scale bar: 500 µm.

**Table 1: Quantification of zebrafish after HOXa1a silencing shows that a loss of function of HOXa1a leads to cranial haemorrhages.** Zebrafish were observed after 48 hpf and 72 hpf. Zebrafish injected with control morpholino show a lower number of zebrafish with bleedings than zebrafish injected with HOXa1a-Mo. At 72 hpf more zebrafish are affected from cranial haemorrhages. At 48 hpf the zebrafish with bleeding were separated and all these fish show cranial haemorrhages at 72 hpf.

**48 hpf**

| | con-Mo 2 ng | con-Mo 4 ng | HOXa1a-Mo 2 ng | HOXa1a-Mo 4 ng |
|---|---|---|---|---|
| total | 131 | 138 | 112 | 165 |
| - bleeding | 129 | 129 | 88 | 148 |
| + bleeding | 2 | 9 | 24 | 17 |
| % bleeding | 1,53 | 6,52 | 21,43 | 10,30 |

**72 hpf**

| | con-Mo 2 ng | con-Mo 4 ng | HOXa1a-Mo 2 ng | HOXa1a-Mo 4 ng |
|---|---|---|---|---|
| total | 130 | 138 | 104 | 159 |
| - bleeding | 115 | 115 | 72 | 113 |
| + bleeding | 15 | 23 | 32 | 46 |
| % bleeding | 11,54 | 16,67 | 30,77 | 28,93 |

To further investigate the influence of HOXa1a silencing in the zebrafish, confocal images of the head were made to investigate vessel formation. The vessels in the head vasculature are also severely affected after HOXa1a silencing. Especially in the hindbrain vasculature, the central arteries are absent or malformed (Figure 20). The central arteries are sub-divided into three groups: Cerebellar central artery (CCtA), middle mesencephalic central artery (MMCtA) and posterior (caudal) mesencephalic central artery (PMCtA) (Isogai *et al.*, 2001). All three types of central arteries are affected after HOXa1a silencing. Besides absent or malformed central arteries, small fine sprouts can also be observed. These sprouts grow unregulated in every direction, but this phenomenon is only seen at 48 hpf.

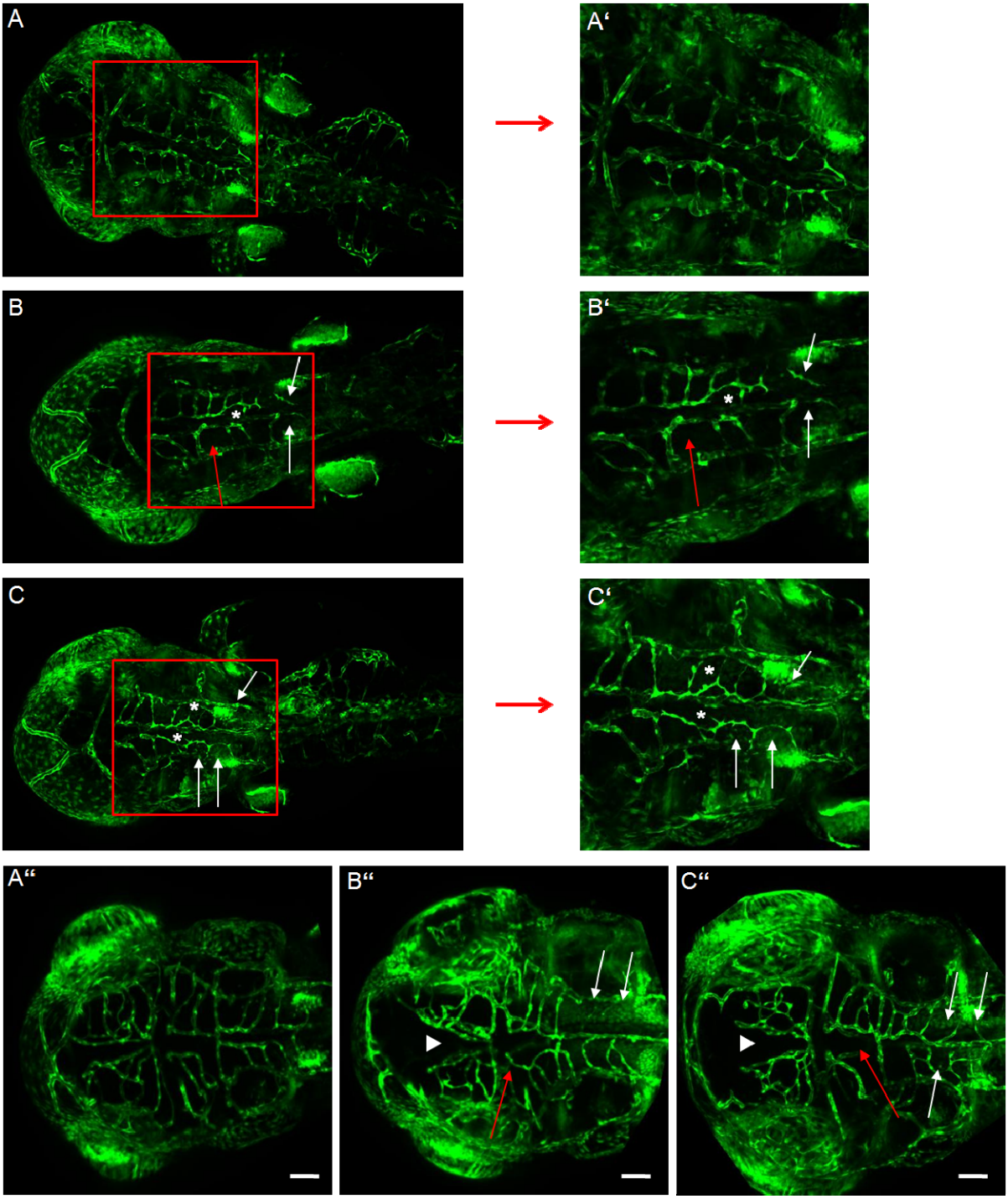



**Figure 20: Confocal images of the head vasculature of zebrafish at 48 hpf (A, B, C) and at 72 hpf (A'', B'', C'') show defects in the central arteries. The red boxes mark the areas shown in A', B' and C'.** White arrows indicate the absent or malformed CCtA, red arrows indicate the absent or malformed PMCtA and the arrowheads the malformed MMCtA. The asterisks show the regions of abnormal sprouting.

## 3.3. HOXA1 is a positive regulator of angiogenic processes in vitro

To confirm the phenotype from the zebrafish experiments *in vitro* experiments were performed which is described in the following sections. For the following *in vitro* experiments the siRNAs HOXA1_A and HOXA1_B were used and a control-siRNA (consi) was used.

Therefore HUVEC-staining and Western Blot analysis after siRNA transfection were performed to investigate the quantity of HOXA1 downregulation. The qualitative analysis with HUVEC-staining show a downregulation of HOXA1 after siRNA transfection (Figure 21A) as does the quantitative Western Blot analysis (Figure 21B). The siHOXA1_A leads to a downregulation of 62% and siHOXA1_B of 68%.

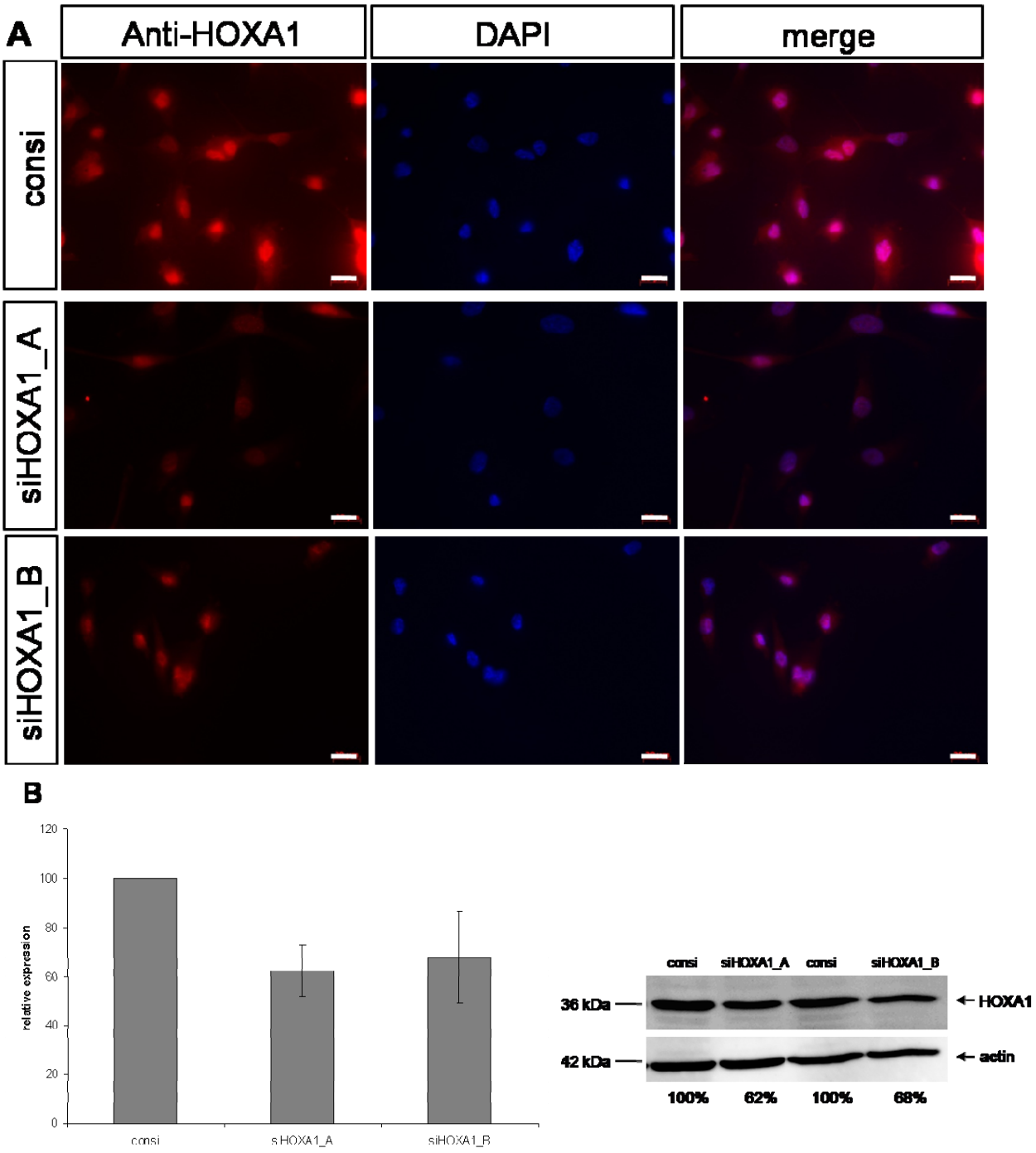



**Figure 21: HOXA1 is downregulated after siHOXA1 transfection.** (A) HUVECs were stained with Anti-HOXA1 antibody and DAPI after transfection with consiRNA, siHOXA1_A and siHOXA1_B. Merge pictures show the localization of HOXA1 in the nucleus. Scale bar: 20 µm (B) Western Blots show the downregulation of HOXA1 after siRNA transfection. The bar graph shows the quantification as an average of three independent experiments.

## 3.3.1. Downregulation of HOXA1 inhibits migration of HUVEC in a VEGF independent manner

One of the major processes in angiogenesis is cell migration. Migration of HUVEC was analysed by the modified Boyden chamber assay. Quantification was calculated by migrated cells per power field to test the effect of HOXA1 on this angiogenic process. Therefore, siHOXA1_A and siHOXA1_B transfected cells were examined and compared to consiRNA control cells (Figure 22). Downregulation of HOXA1 in HUVEC showed a significantly reduced migration. This result suggests that HOXA1 is a proangiogenic regulator. But HOXA1 does not regulate VEGF dependent angiogenesis because this reduced migration was seen in VEGF stimulated cells as well as in unstimulated cells.

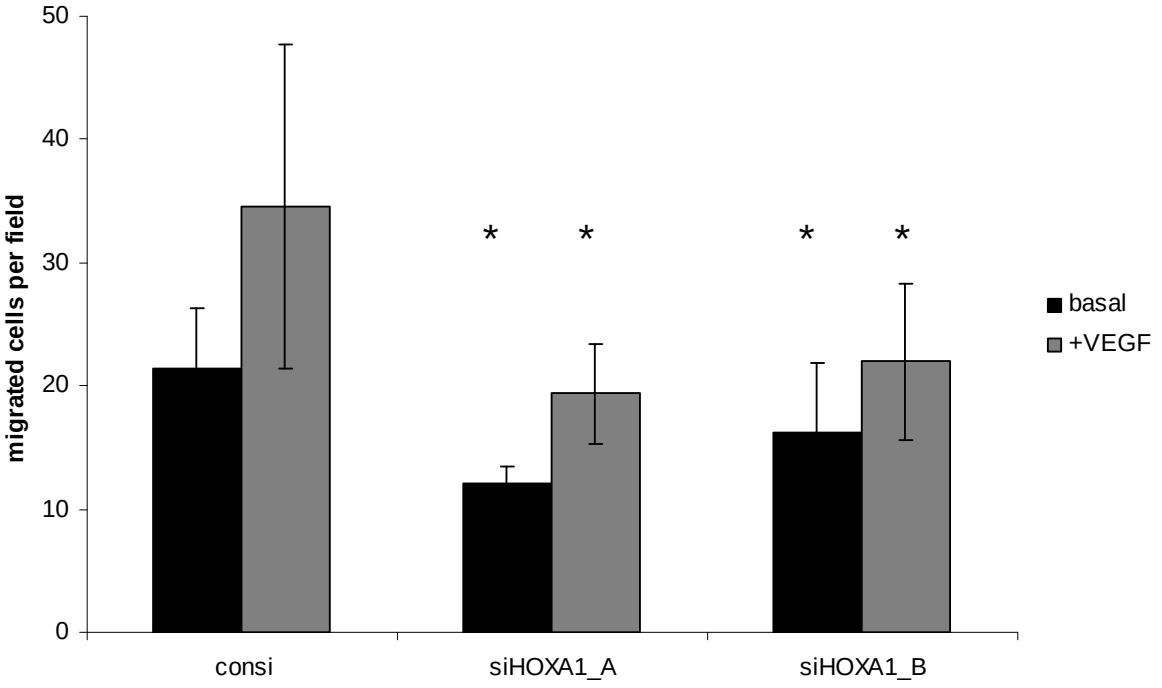



**Figure 22: HOXA1 promotes migration in HUVECs.** siRNA mediated Hoxa1 downregulation significantly inhibited basal and VEGF-driven endothelial cell migration in the modified Boyden chamber assay. Mean values of four independent experiments. * $P < 0.05$ compared with control basal.

## 3.3.2. Downregulation of HOXA1 inhibits sprouting of HUVEC

Another important process during angiogenesis is sprouting. To study the impact of HOXA1 on sprouting, a sprouting assay was performed. The siHOXA1_A and siHOXA1_B transfected cells were examined and compared to consiRNA control cells (Figure 23). Downregulation of HOXA1 in HUVEC showed a significantly reduced sprouting. This enforces the conclusion in 3.3.1 that HOXA1 is a proangiogenic regulator. In the sprouting

assay, it was also shown that HOXA1 does not regulate VEGF dependent angiogenesis because this reduced sprouting was seen in VEGF stimulated cells as well as in unstimulated cells.

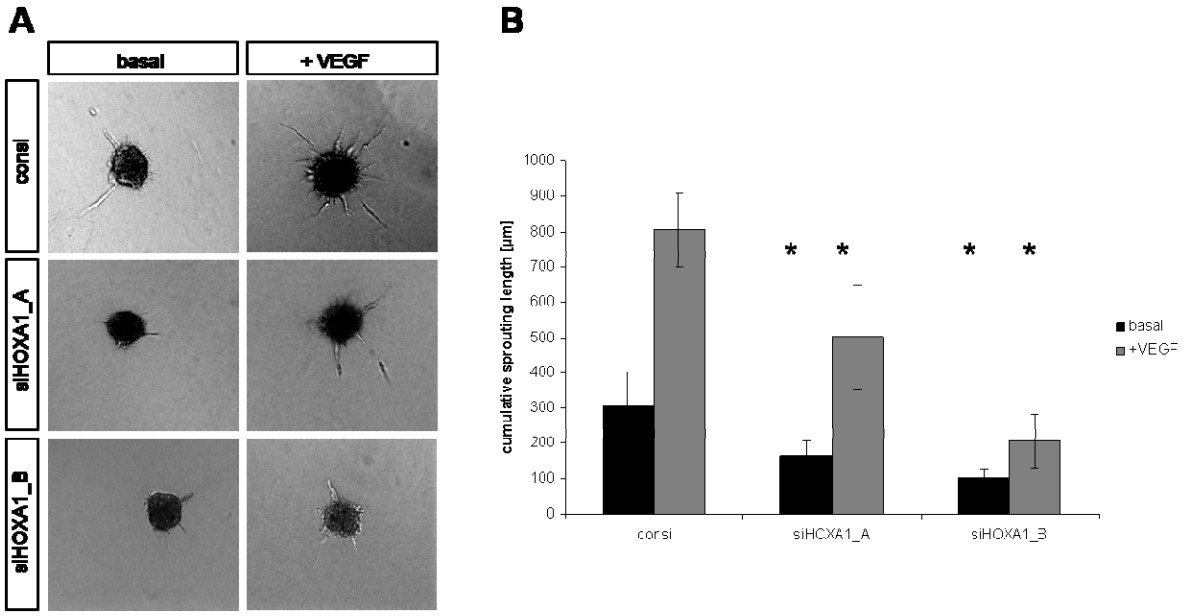



**Figure 23**: **HOXA1 is an important factor during sprouting processes.** (A) siRNA mediated HOXA1 down-regulation strongly decreases endothelial sprouting. (B) Quantification of the experiment shown in (A). Mean values of six independent experiments. * $P < 0.05$ compared with control basal.

## 3.3.3. Downregulation of HOXA1 does not lead to apoptosis and does not inhibit viability of HUVEC

The viability of HUVEC was analysed to exclude unspecific effects of the siRNA transfection, which can influence the results of the migration and sprouting assays. The assay was performed as described in 2.2.4.7 and a growth curve was developed (Figure 24). HUVEC were counted at the beginning of the experiment and after 24 h, 48 h and 72 h. The growth curves show that all samples, the control siRNA and both HOXA1 siRNAs revealed continuous growth and no symptom of cell death during the experiment. The treatment of cells with siRNA does not influence the growth curves of the cells, excluding cytotoxic side effects of the siRNA.

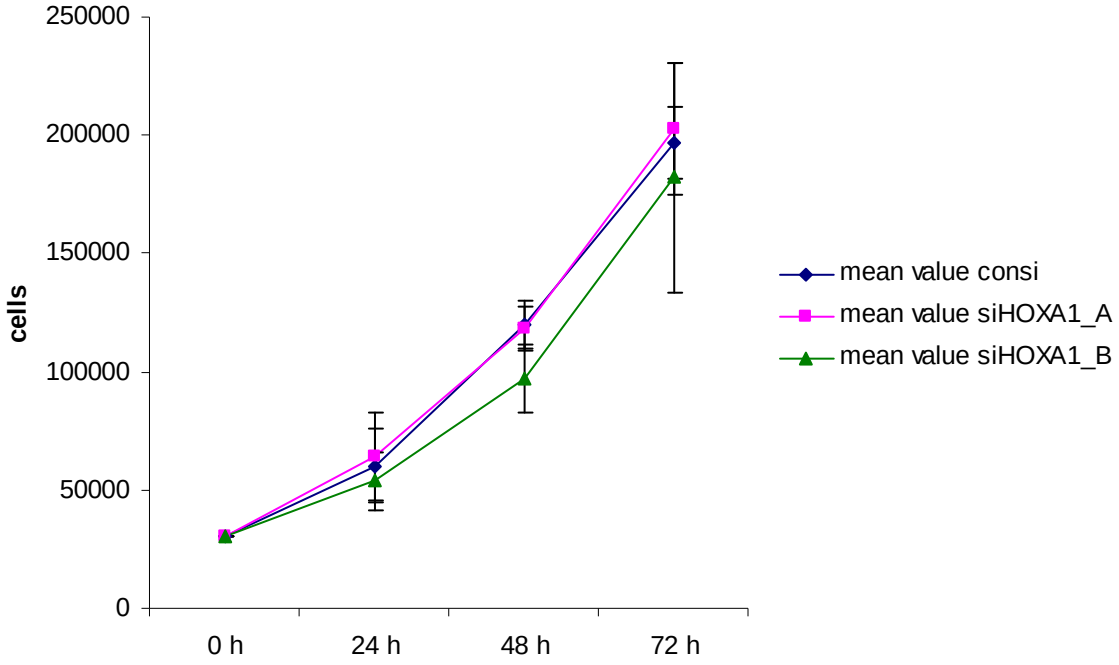



**Figure 24: siRNA mediated HOXA1 down-regulation does not influence viability of HUVEC.** The cell number was counted at the indicated time points after transfection with consi, siHOXA1_A and siHOXA1_B. Mean values of three independent experiments are shown.

Secondly the effect of siRNA transfection on cell apoptosis was analysed. HUVECs were transfected with consiRNA, siHOXA1_A and siHOXA1_B and the caspase activity was measured as described in 2.2.4.1. As a control for apoptosis, HUVECs were treated with 250 nM staurosporin to induce cell apoptosis. As figure 25 shows, transfection of siHOXA1_A or siHOXA1_B did not provoke cell apoptosis.

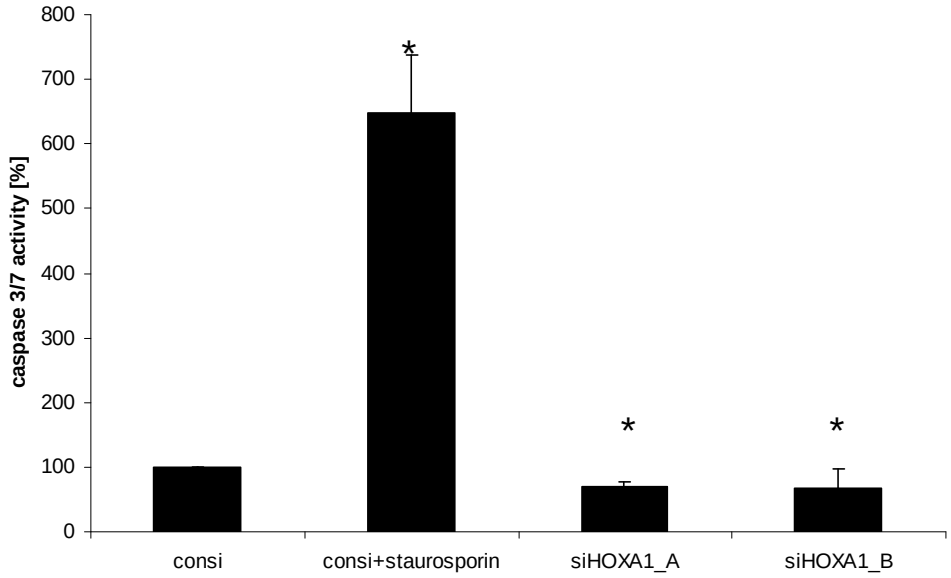



**Figure 25: siRNA mediated HOXA1 downregulation does not induce cell death in HUVEC.** Mean values of three independent experiments are shown. * $P < 0.05$ compared with consi.

### 3.3.4. Downregulation of HOXA1 shows less tube formation in HUVECs

Aside from the basic cellular processes of sprouting and migration, it is also possible to study the mechanism of angiogenesis with the tube formation assay. HUVECs were transfected with HOXA1-siRNAs and seeded on a matrigel. The cells were stimulated with VEGF to reflect the *in vivo* situation. The cumulative sprouting length and the number of branching points were measured and are shown in figure 26. This figure also illustrates representative pictures of the network in each condition.

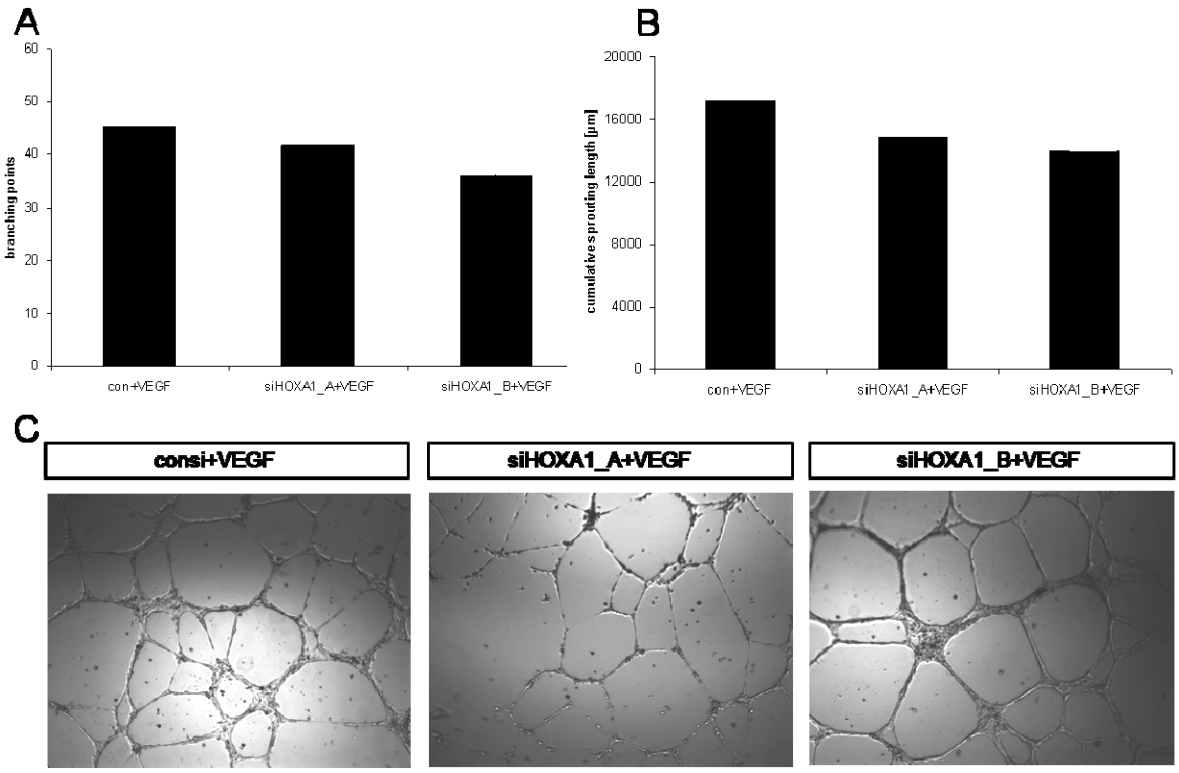



**Figure 26: HOXA1 knock-down with siRNA transfection leads to an incomplete network formation in HUVECs.** (A) Number of branching points in the control and after HOXA1_A and HOXA1_B transfection. (B) Cumulative sprouting length was measured in the control and after HOXA1_A and HOXA1_B transfection. (C) Representative pictures show the network formation in the afore mentioned conditions. Mean values of two independent experiments are shown.

The branching points, as well as the cumulative sprouting length, are influenced by HOXA1-siRNA transfection. Although the number of branching points and the cumulative sprouting length is lower, this effect is not significant. Furthermore, a statistic analysis is impossible as only two experiments were performed. Only two experiments were performed because no significant effect was observed. Nevertheless the tube formation assay gives an indication that HOXA1 is a pro-angiogenic factor.

### 3.3.5. HOXA1 is a regulator of angiogenic processes independently of the VEGF-pathway and is not influenced under hypoxic conditions

To confirm the data resulting from the sprouting assay and the migration assay, a stimulation experiment with growth factors was performed. HUVECs were stimulated with either VEGF (25 ng/ml) or FGF (25 ng/ml) for 12 h (data not shown) respectively 24 h (Figure 27) followed by a Western Blot analysis with an HOXA1 antibody. The levels of HOXA1 did not change in comparison to the control, which was not stimulated (Figure 27). So it seems that HOXA1 is not influenced by VEGF signalling and concordantly to the angiogenesis assays, where it was shown that migration and sprouting are reduced in a VEGF-independent manner (see 3.3.1. and 3.3.2.).

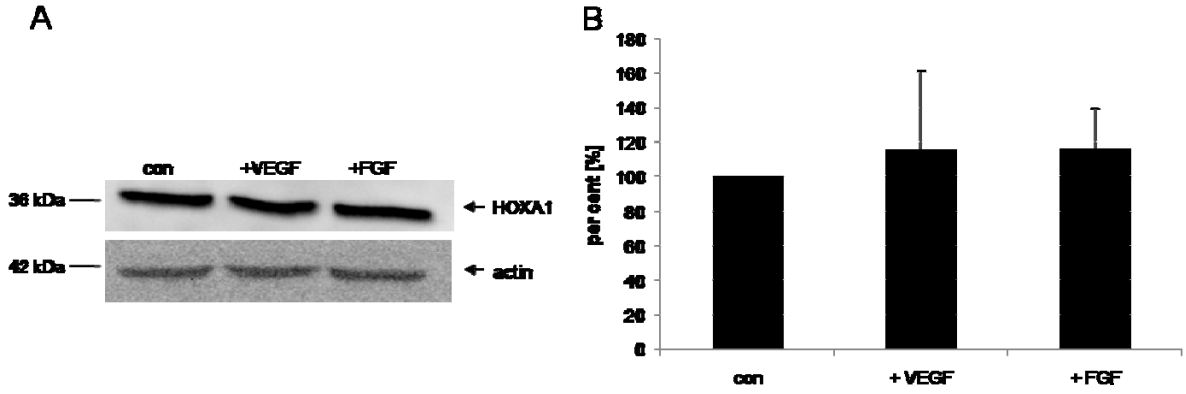



**Figure 27: Western Blot after HUVEC stimulation with 25 ng/ml VEGF or 25 ng/ml FGF. (A)** Actin functions as a loading control. There are no appreciable differences between the bands of the different conditions. One representative Western Blot is shown. **(B)** Statistical analysis show mean values of three independent experiments.

To investigate hypoxic influence on HOXA1 expression HUVECs were chemically treated (Wang and Semenza, 1993). HOXA1 is not influenced under hypoxic conditions with Dfx or $CoCl_2$. Western Blot analysis shows no alterations between the probes, neither during hypoxia nor at different time points after recovery (Dfx treatment: Figure 28; $CoCl_2$ treatment: data not shown).

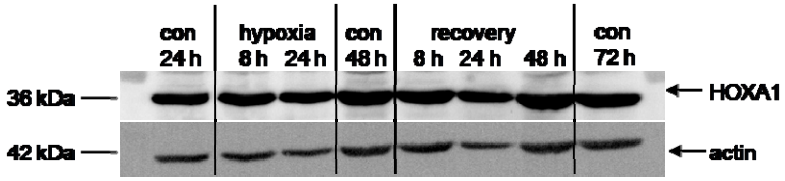
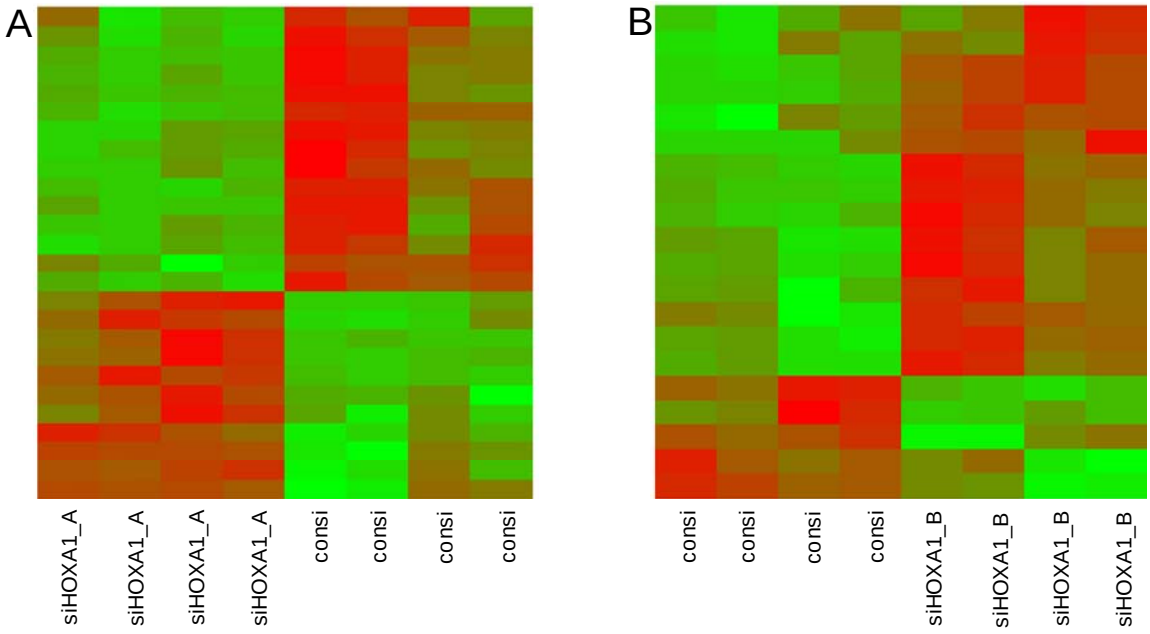



**Figure 28: HOXA1 is not influenced with Dfx treatment.** HUVECs were treated for 8 or 24 h with Dfx and recovered for 8 or 24 h in normoxic conditions. Untreated cells served as controls (0 h, 48 h and 72 h). The Western blot shows no differences of the expression levels of HOXA1.

## 3.4. Identification of HOXA1 target genes

Microarray analysis was performed to identify potential target genes of HOXA1. The microarray was executed 48 hours after siRNA transfection, thus at that point in time the *in vitro* angiogenesis assays were performed. The microarray was carried out using triplicates to assure validated results. Few genes were significantly up- or downregulated. The microarray was analysed using the CSC Chipster software package. A significant regulation was defined as 0.005 and with this threshold 26 genes were significantly regulated after HOXA1 knockdown with siHOXA1_A (Figure 29A). In the case of knockdown with siHOXA1_B, 20 genes were significantly regulated (Figure 29B). Among these genes the SELE gene, which encodes for E-selectin, was highly downregulated after HOXA1 silencing (Figure 29C).




C

| | | |
|---|---|---|
| SELE | E-selectin | 0.39 |
| HOXB8 | homeobox B8 | 0.54 |
| CXCL2 | chemokine (C-X-C motif) ligand 2 | 0.57 |
| SLC2A3 | solute carrier family 2 (facilitated glucose transporter), member 3 | 0.58 |
| TNFSF18 | tumor necrosis factor (ligand) superfamily, member 18 | 0.61 |
| HOXA1 | homeobox A1 | 1.01 |
| ALCAM | activated leukocyte cell adhesion molecule | 1.76 |
| IL6ST | interleukin 6 signal transducer | 1.80 |
| PGF | placental growth factor | 1.84 |
| CD24 | CD24 molecule | 1.85 |
| CXCR4 | chemokine (C-X-C motif) receptor 4 | 1.85 |

**Figure 29: Global gene expression analysis after downregulated HOXA1 expression.** (A+B) Data representation of the microarray results by a heat map using the CSC Chipster software package. Upregulated genes are labelled in different shades of red and downregulated genes are labelled in shades of green. n = 3 experiments were analysed. (C) Interesting genes of endothelial cell biology which are regulated by HOXA1.

Interestingly, the majority of the regulated genes are involved in cell-to-cell signalling and interaction (Figure 30). Also in an inflammatory response and in cancer, the regulated genes involved indicate the potential role of HOXA1 in these processes.

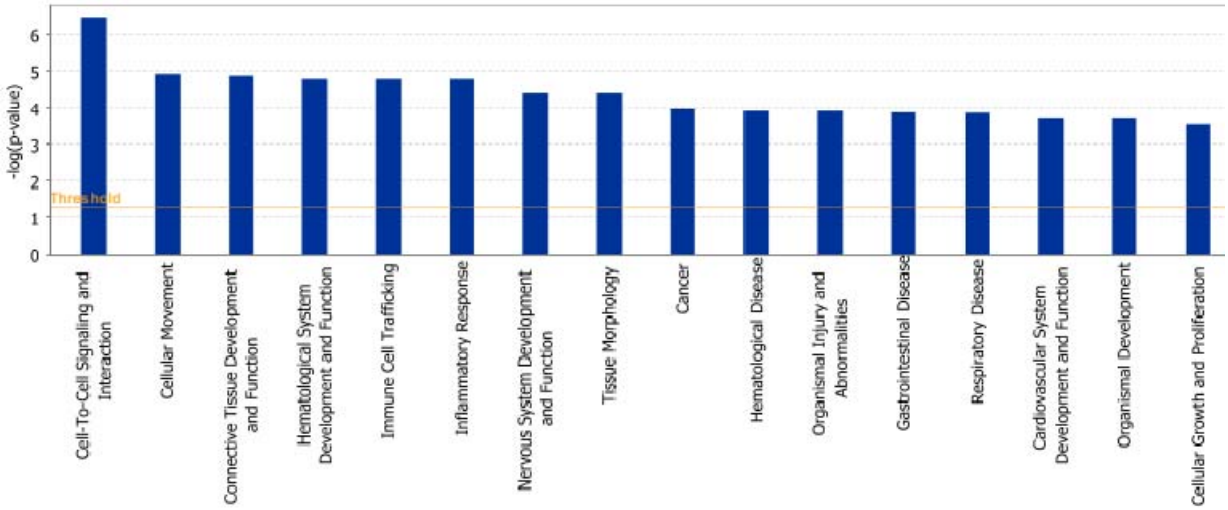



**Figure 30: The majority of regulated genes in the microarray are involved in cell-to-cell signalling.** The regulated genes were attributed to their functions using the Ingenuity Pathway Analysis software. HOXA1 regulated genes are mainly involve in cell-to-cell signalling and interaction.

### 3.4.1. E-selectin as a potential target gene of HOXA1

E-selectin is downregulated after HOXA1 downregulation in HUVECs (Figure 29C). The expression of E-selectin is more than 50% lower in comparison to the control. To confirm the data from the microarray analysis, a RT-PCR was performed. First HOXA1 was downregulated with siRNA transfection and after 48 h (according to the procedure used in the microarray) the cells were lysed. After RNA and cDNA production a PCR for E-selectin was done (Figure 31A). The quantitative analysis showed that E-selectin is expressed only 33% and 37% respectively of the expression level in the control (Figure 31B). HOXA1 siRNAs lead to lower cell densities and this could lead to a lower expression of the adhesion molecule E-selectin, an additional control was used. As an additional control a siRNA which downregulates the BTB-kelch-protein KLEIP was used because this siRNA is known to lead to a lower cell density. To exclude that E-selectin, which is an adhesion molecule, is downregulated due to missing cell junctions, the KLEIP siRNA was used. The KLEIP siRNA probe showed no difference in the expression level of E-selectin.

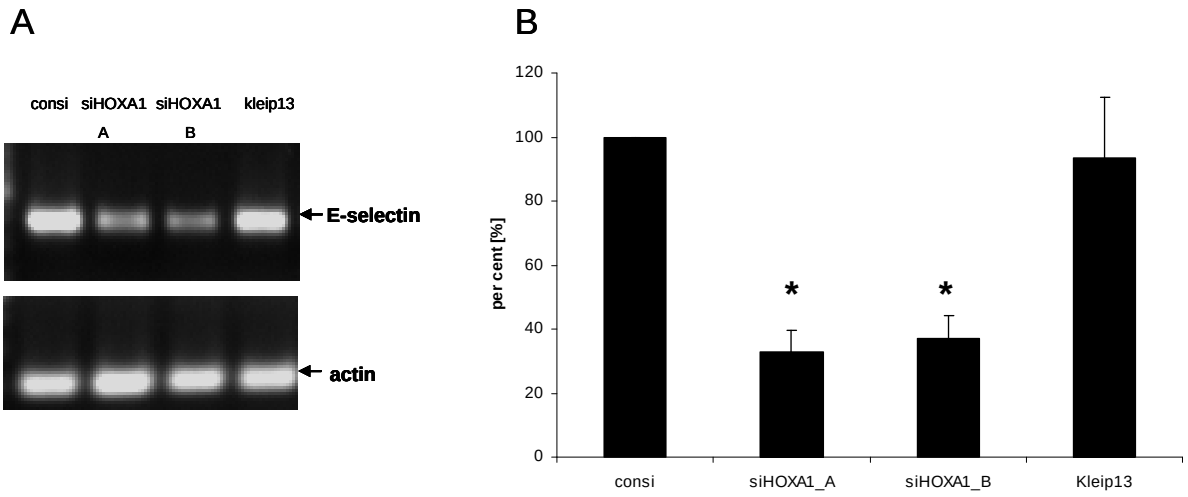



**Figure 31: E-selectin is downregulated after HOXA1 downregulation. (A)** Agarose-Gel after RT-PCR showed clearly that in the siHOXA1 transfected probes E-selectin is highly downregulated compared to the control. **(B)** Quantitative analysis of the experiment shown in A. E-selectin expression was only 33% respectively 37% compared to the E-selectin expression in the control. Mean values of three independent experiments are shown. * $P < 0.05$ compared to consi.

# 4. Results – Rho-dependent kinases ROCK I/II

The small GTPase RhoA activates the serine/threonine kinases ROCK I and ROCK II (see 1.5.). Inhibition of the Rho kinases with different inhibitors, leads to contrary findings *in vivo* and *in vitro* which are controversially discussed. In this study the Rho kinases where inhibited with H1152, Fasudil and Y27632 in the oxygen-induced retinopathy (OIR) mouse model (4.1.) and with H1152 in the myocardial infarction study (4.2.).

## 4.1. Inhibition of Rho-dependent kinases with different inhibitors in the oxygen-induced retinopathy (OIR) mouse model

The OIR model was used to investigate angiogenic processes in the retina. In the OIR mouse model the mice were exposed to 75% oxygen at postnatal day 7 till postnatal day 12. At postnatal day 12 the mice were returned to room air and treated with the inhibitors from the 12 till day 16 (see also Material and Methods). In this study, all three Rho kinases inhibitors were used: the very specific inhibitor H1152, the inhibitor Fasudil and the inhibitor Y27632. The experiment was performed according to the procedures described in Kroll *et al.*, 2009 (2.2.5.3.2.) but more Rho kinase inhibitors were tested.

### 4.1.1. Inhibition of ROCK I/II with H1152 and Fasudil shows higher neo-vascularization

The inhibition of Rho kinases I and II was shown to advance endothelial function in patients with coronary artery diseases (Nohria *et al.*, 2006). The OIR model is appropriate to investigate the mechanisms of endothelial cell activation and angiogenesis (Feng *et al.*, 2007). Mice injected with H1152 and Fasudil showed an increase of pre-retinal new vessels in comparison with the control mice injected with DMSO (Figure 32A). Injection with Y27632 shows no variance in the amount of pre-retinal new vessels (Figure 32B).

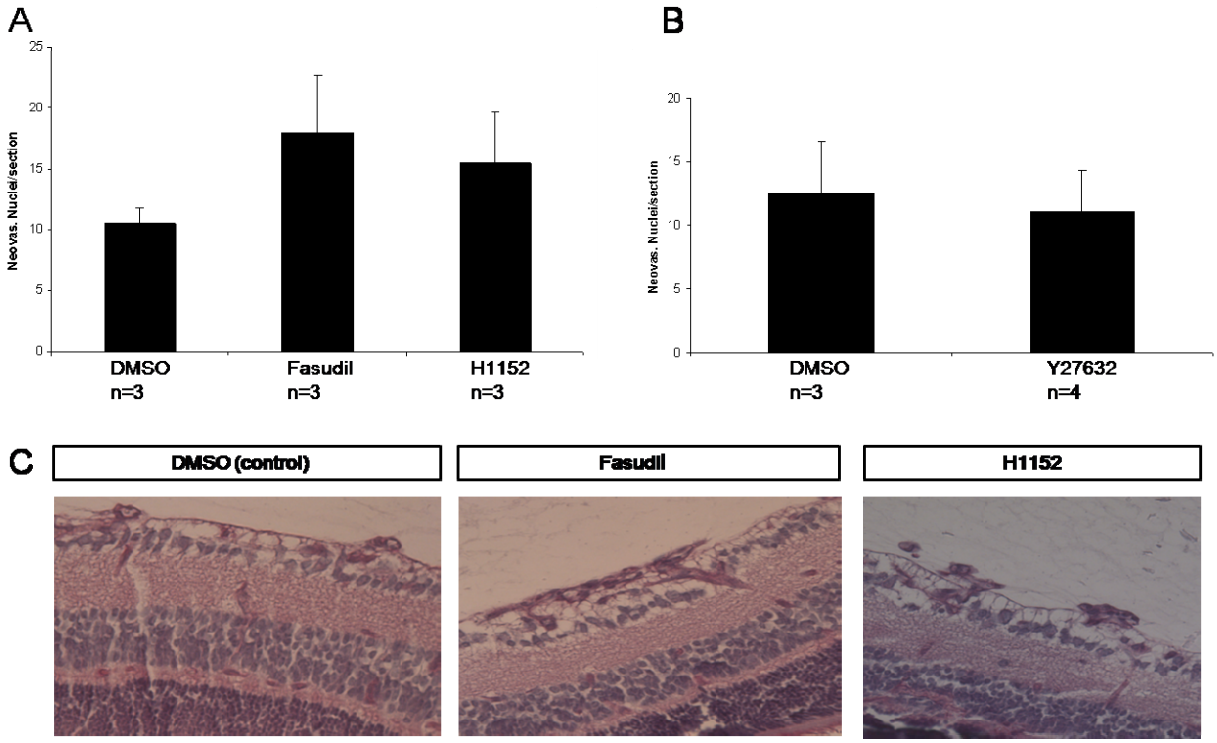



**Figure 32: Pharmacological inhibition of Rho kinases increase angiogenesis in mouse retinas.** (A) Injection with Fasudil showed an increase of 50% and injection with H1152 an increase of 25%. Mean values of three independent experiments are shown. From each retina 10 sections were counted. (B) Injection with Y27632 showed no effect. Mean values of three respectively four independent experiments are shown. From each retina 10 sections were counted. (C) Representative pictures of retinas injected with DMSO (control), H1152 and Fasudil.

### 4.1.2. Isolectin staining shows higher retinal vascularization after ROCK I//II inhibition

An isolectin staining was performed to investigate at the growth and formation of endothelial cells in the retina after the OIR experiment (Figure 33). The retinas of mice injected with H1152 showed more vascularization. The network formed consists of more branching points in comparison to the control retinas of mice injected with DMSO (Figure 34). So more vessels developed, which indicates an anti-angiogenic effect of the Rho kinases. Fasudil showed a higher density of the blood vessels in comparison to the control, but the effect is not as strong as in the retinas of the H1152 injected mice. The retinas after being injected with Y27632, showed no differences in the density of the vascular network. However the network did show disruptions, which resulted in an incomplete and potentially non-functional network.

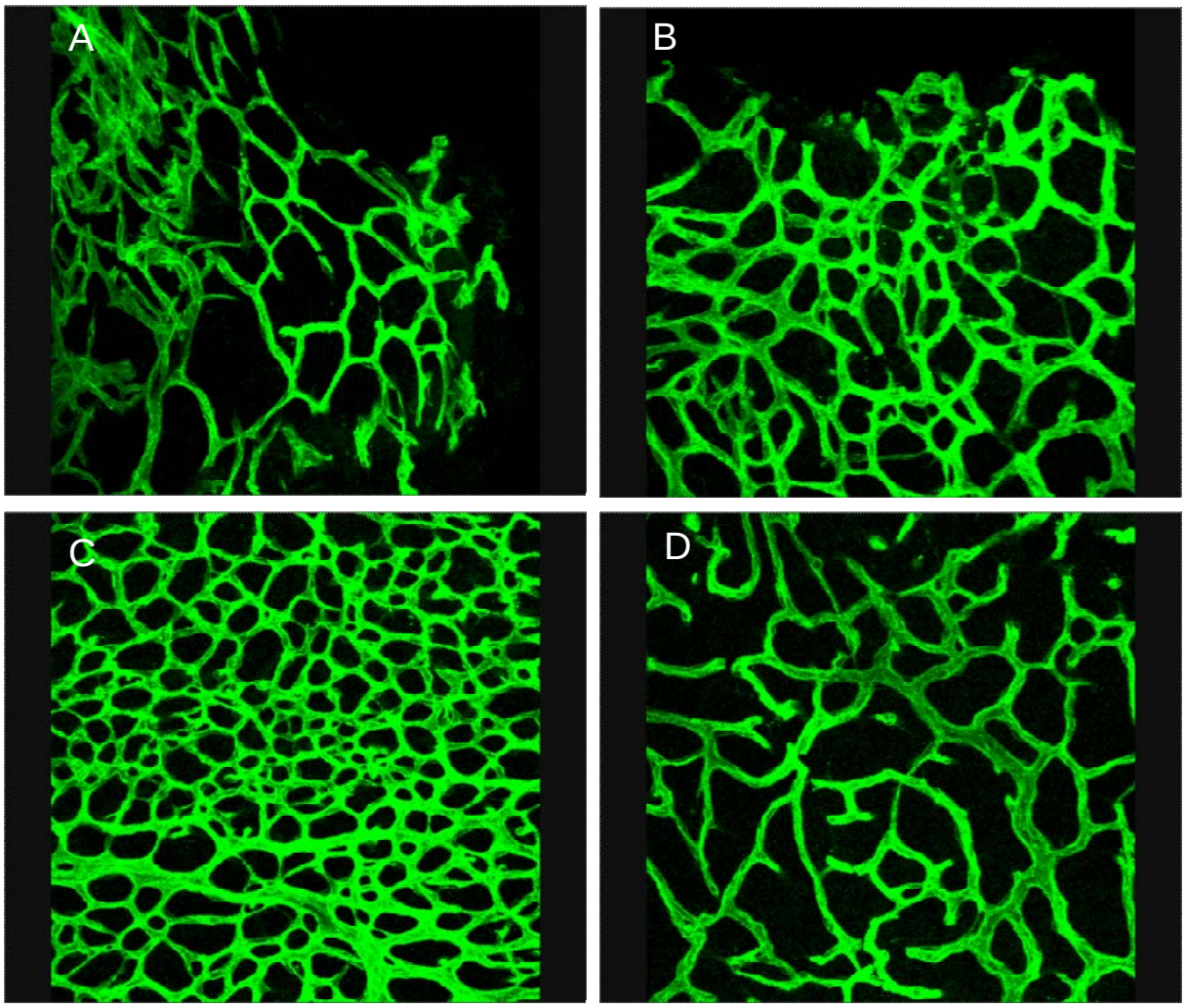



**Figure 33: Retinas of H1152 and Fasudil injected mice showed a higher neovascularization in the isolectin staining whereas after an Y27632 injection the vascular network showed disruptions.** (A) Control retina injected with DMSO. (B) Retina of a mouse injected with Fasudil. (C) Retina of a mouse injected with H1152. (D) Retina of a mouse injected with Y27632.

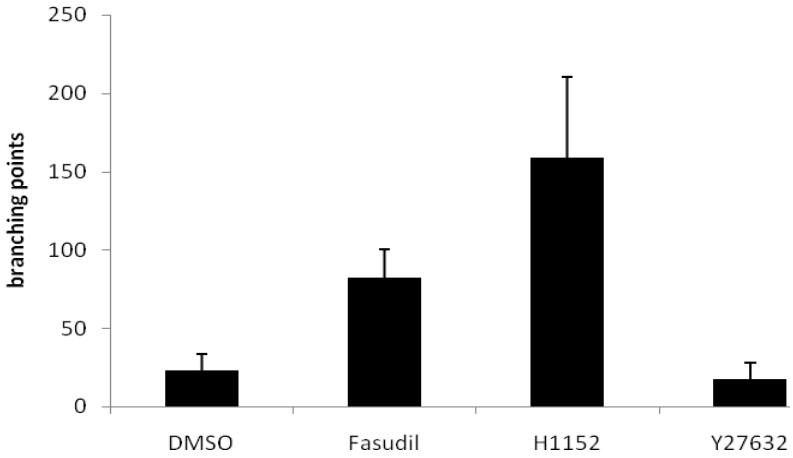



**Figure 34: Quantification of three pictures per group show more branching points in retinas from H1152 and Fasudil injected mice whereas after Y27632 injection less branching points can be observed.** The inhibition with Fasudil and H1152 increased the branching points (factor 3.61 respectively 6.99) whereas the inhibition with Y27632 led to a decreased number of branching points (factor 0.78). Mean values of three independent experiments are shown.

## 4.2. Inhibiton of Rho-dependent kinases in the myocardial infarction (MI) rat model

Constitutive of the data in the OIR model, the hypothesis was developed that inhibition of ROCK I and II can activate angiogenesis in relation with cardiovascular diseases. A myocardial infarction study in rats was performed in cooperation with the working group of Prof. Henning (clinical pharmacology) at the Rijksuniversiteit Groningen, Netherlands. For this study the specific inhibitor of Rho kinases H1152 was used and the operations were done according to Zhao *et al*., 2009 (see also 2.2.5.3.2.). The myocardial infarction was performed by permanent ligation of the left coronary artery. Rats were anesthetized and the thorax was opened in the fourth intercostals space and the muscles overlying this region were dissected. Then the main left anterior coronary artery was ligated. With this ligation the myocardial infarction was induced and directly after the infarction the rats get an osmotic minipump in the neck. The rats were divided into three groups: (i) sham group (control I; n = 8), (ii) control group II (water; n = 16) and (iii) treatment group (H1152 (3 mg/kg/day); n = 16).

### 4.2.1. Cardiac function measurements show no different levels between the different study-groups

The rats were sacrificed seven days after the induced myocardial infarction. At this day the weight of the rats was higher than at the operation day (Figure 35A). Between the three study groups no differences in weight was observed. This indicates that all animals overcome the operation in the same positive way. In figure 35B two exemplarily hearts of rats are shown. It is clear to see, that below the ligation the tissue in the infarcted heart is much brighter than that in the sham heart. This means that a myocardial infarction was induced and the tissue is partly destroyed.

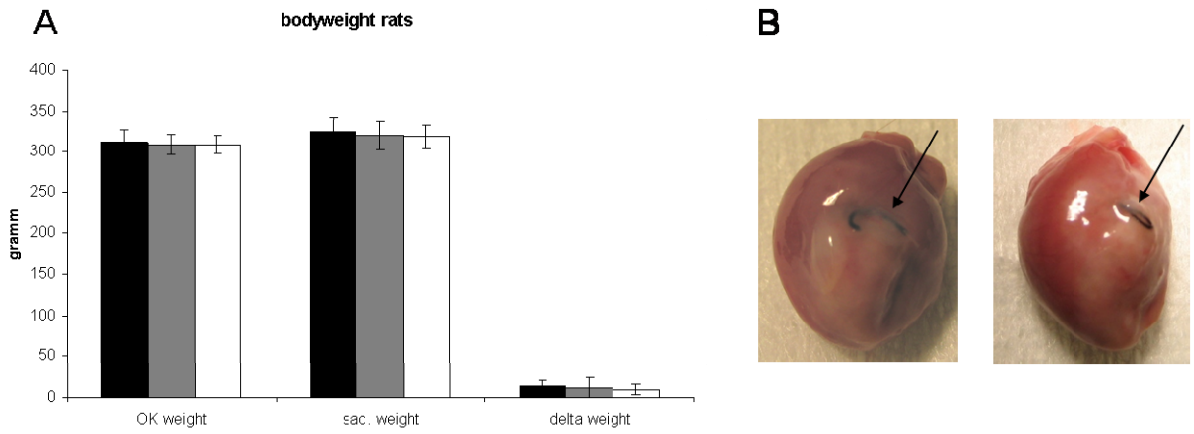



**Figure 35: Rats weights and ligated hearts** (A) Weight of the rats on the sacrificing day, seven days after the operation (sac. weight), is higher than on the day of the operation (OK weight). There is no difference between the study groups (delta weight); black: sham group (n = 8), grey: control group MI water (n = 16) and white: treatment group MI H1152 (n = 16). (B) Below the ligation (arrow) the destroyed tissue is brighter in the infarcted heart (right) than in comparison to the sham heart (left).

Figure 36 shows that the measurements of the weights of heart, left kidney and right kidney differ insignificantly. The organ weights were normalized as the respective organ was divided by the body weight (Figure 36B).

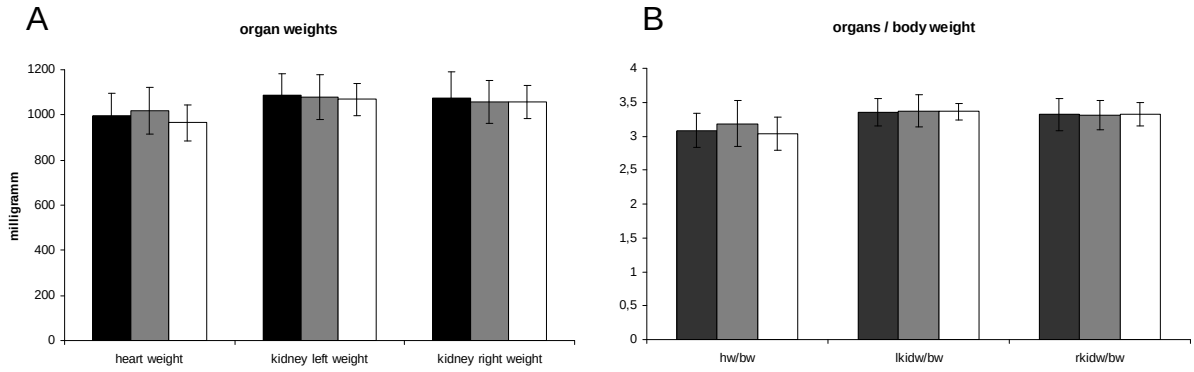



**Figure 36: The organ weights show no significant differences.** (A) The weight of heart, left kidney and right kidney differ rarely between the study groups. (B) Organ weights were normalized to the body weight. The weight between the groups also differed rarely. hw/bw: heart weight divided by body weight. lkidw/bw: left kidney weight divided by body weight. rkidw/bw: right kidney weight divided by body weight. black: sham group (n = 8), grey: control group MI water (n = 16) and white: treatment group MI H1152 (n = 16).

To investigate if the myocardial infarction has any influence on hemodynamic functions like blood flow or pressure multiple hemodynamic measurements were performed. These hemodynamic measurements showed only minimal differences between the three study groups as figure 37 shows. Additionally the aorta heart rate (Figure 37A) and the left ventricle heart rate (Figure 37C) were measured, as were the blood pressure (Figure 37B) and the systolic pressure of the left ventricle (Figure 37D).

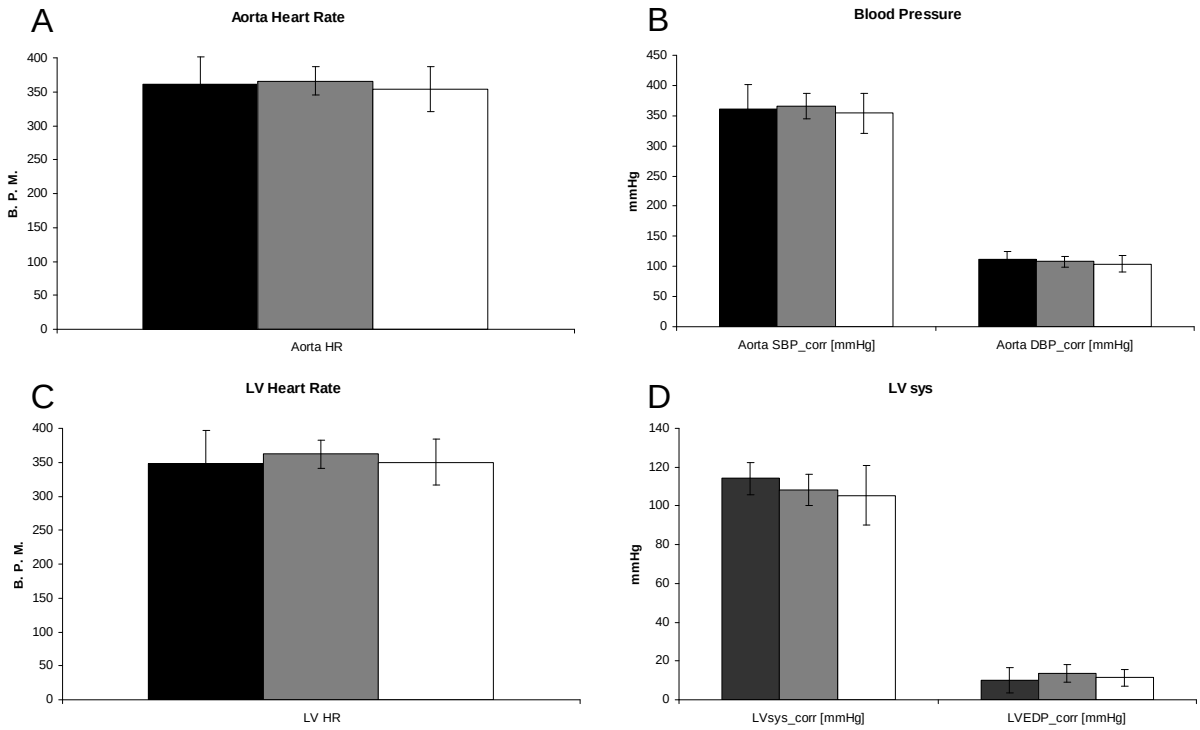



**Figure 37: Hemodynamic measurements seven days post operation show no significant differences between the study groups. (A)** Heart rate (B.P.M.= beats per minute) **(B)** Aortic blood pressure (SBP = systolic blood pressure and DBP = diastolic blood pressure; mmHg = millimetre of mercury) **(C)** left ventricle heart rate (B.P.M.= beats per minute) **(D)** systolic pressure of the left ventricle (LVsys) and enddiastolic pressure of the left ventricle (LVEDP) (mmHg = millimetre of mercury. black: sham group (n = 8), grey: control group MI water (n = 16) and white: treatment group MI H1152 (n = 16).

These experiments showed that the inhibition of Rho-dependent kinases with the inhibitor H1152 has no influence on the recovery of rats after an induced myocardial infarction. All measured parameters showed no significantly differences between the study groups.

The literature dissociate between rats with a MI-size of more than 20% or less than 20%. The rats with a MI-size over 20% have been shown to develop heart failure. Table 2 showed the number of rats with a MI-size of more than 20% and the mean value. The body weight of the rats with the MI-size > 20% is lower in comparison to the sham heart. So one can clearly see that the rats with a bigger infarcted area recovery not in the same way than the sham animals. Also significant is the higher enddiastolic pressure of the left ventricle in the MI water group in comparison to the sham group. The enddiastolic pressure of the left ventricle is also higher in the MI H1152 group but this is not significant.

**Table 2: Rats with a MI-size > 20% show differences in body weight and enddiastolic pressure of the left ventricle (LVEDP).** Only animals with an MI-size > 20% were analysed. Increase in body weight was 14.5 g in the sham group and only 3.6 g in the MI water group and 7.0 g in the MI H1152 group. The LVEDP is higher in the MI water group (13.8 mmHg) and the MI H1152 group (11.5 mmHg) compared with the control (sham, 8.4 mmHg) * $P < 0.05$ compared to sham.

| | sham | MI water | MI H1152 |
|---|---|---|---|
| Number | 7 | 8 | 11 |
| MI-size [%] | - | 39.9 ± 4.9 | 34.93 ± 2.5 |
| Range MI-size [%] | | 23 - 61 | 23 - 46 |
| | | | |
| BW [g] | 325.9 ± 7.2 | 313.3 ± 6.1 | 314.5 ± 3.2 |
| Increase in BW [g] | 14.5 ± 2.7 | 3.6 ± 7* | 7.0 ± 1.0 |
| | | | |
| Heart rate [mmHg] | 362 ± 16 | 361 ± 7 | 348 ± 10 |
| SBP [mmHg] | 111.7 ± 5.4 | 106.5 ± 3.5 | 102.6 ± 4.7 |
| LVEDP [mmHg] | 8.4 ± 1.7 | 13.8 ± 1.8* | 11.5 ± 1.4 |

The results shown in table 2 indicate impaired cardiac functions like contractility and relaxation in rats with MI-size > 20%. Also the lower body weight of rats with myocardial infarction is a sign of impaired cardiac functions.

# 5. Discussion

## 5.1. HOXA1 is a proangiogenic factor

The expression of the transcription factor HOXA1 can be seen in the pharyngeal arch and brain regions in zebrafish (Thisse *et al.*, 2005) and also in the neuroectoderm and mesoderm during hindbrain development in mice (Murphy and Hill, 1991), but not directly in endothelial cells. Chung *et al.* (2009) screened 37 HOX genes for changes in their expression patterns during differentiation of bone-marrow derived mesenchymal stem cells into endothelial cells. This screening identified angiogenesis-relevant HOX genes; e.g. the expression of HOXA7 and HOXB3 was highly increased and the expression of HOXA3 and HOXB13 was strongly decreased during the process of differentiation (Chung *et al.*, 2009). HOXB1, HOXB4, HOXB8 and HOXB9 were identified as being expressed in human umbilical vein endothelial cells (Belotti *et al.*, 1997 and Belotti *et al.*, 1998). The expression of HOXA1 in endothelial cells was clearly shown in this work. Immunocytochemical staining, Western Blot analyses and FACsorting showed that HOXA1 is expressed in human umbilical vein endothelial cells, bovine aortic endothelial cells and zebrafish endothelial cells, indicating a potential vascular function.

Loss-of-function experiments were performed *in vivo* as well as *in vitro* as support for the role of HOXA1 in angiogenic processes. Loss-of-function experiments *in vitro* showed an inhibition of sprouting and migration. Possibly, the downregulation of HOXA1 leads to a decreased tube formation. HOXA1 silencing in zebrafish further supported these findings. The vascular network in the trunk was impaired and also the head vasculature was malformed resulting in cranial hemorrhages. Together all these results suggest a pro-angiogenic role of HOXA1.

Interestingly, the basal and the stimulated migration as well as sprouting were reduced after HOXA1 siRNA-transfection to the same extent (see 3.3.1. and 3.3.2.) indicating that HOXA1 signalling is not affected by the VEGF-pathway. This is also supported by the stimulation experiment. HOXA1 expression is not influenced by stimulation with growth factors like VEGF or FGF (see 3.3.5.). In several anti-tumour therapy strategies the VEGF-pathway is targeted and a variety of drugs base their concept on targeting this pathway (Palazzo *et al.*, 2010). The problem with this therapy concept is that there exists a VEGF resistance and so a number of studies have come out dealing with the development of VEGF independent targets

(Ferrara, 2010). The fact that HOXA1 is a positive regulator of angiogenesis independent of VEGF could be an advantage in developing anti-tumour therapies.

The results showing that HOXA1 regulates angiogenesis fit together with other research findings in the field of HOX gene biology. Initially HOX genes were described as regulating morphogenesis, but many HOX genes are also able to regulate angiogenic processes (see also 1.3.1.). HOXA9 promotes migration and also tube formation in endothelial cells (Bruhl *et al.*, 2004). HOXB2 was shown to be necessary for endothelial cell proliferation (Liu *et al.*, 2004) and HOXD3 is active during tube formation of endothelial cells (Boudreau *et al.*, 1997). These findings led to the consolidation of our hypothesis that HOXA1 can act as a pro-angiogenic factor.

## 5.2. E-selectin is regulated by HOXA1 in endothelial cells

So far only some of the HOX transcription factors direct target genes are known. EPHB4 was identified as a direct target gene of HOXA9 (Bruhl *et al.*, 2004), ß3-integrin is a target gene of HOXA10 (Daftary *et al.*, 2002) and Flk1 is a target gene of HOXB5 (Wu *et al.*, 2003). In this thesis a microarray was performed to identify possible HOXA1 target genes (see 3.4.). This microarray revealed E-selectin as a very promising HOXA1 target. With RT-PCR this result was proved and the experiment clearly showed that E-selectin is highly downregulated after HOXA1 downregulation. The fact that the cranial haemorrhages in zebrafish did not regress, implicates that wound healing would not be possible under HOXA1 silencing conditions. Furthermore E-selectin is known to play a crucial role during wound healing (Jubeli *et al.*, 2011), so it makes sense that E-selectin is not fully functional after HOXA1 silencing and thus head bleeding cannot regress.

Another interesting point is that HOXA1 expression has been detected in human breast cancer lesions (Chariot and Castronovo, 1996). E-selectin is highly expressed in breast cancer tissue (Nguyen *et al.*, 1997) which gives an indication of possible interactions between HOXA1 and E-selectin during breast cancer development. This leads to the perspective that the inhibition of either HOXA1 or E-selectin or both could stop breast cancer development. And it would be interesting to look on the other regulated genes from the microarray analysis. If there is another gene or genes which are also involved in breast cancer development, it would be interesting to look if there are interactions either with HOXA1 or E-selectin.

## 5.3. Further perspectives – HOXA1

In this work, an influence on angiogenesis by HOXA1 was shown. Furthermore E-selectin was identified as a target gene of HOXA1. The interaction between HOXA1 and E-selectin needs further investigation. It will be important to investigate the expression of HOXA1 and E-selectin in different tumour phases, as the expression of both genes were shown in breast cancer tissue. Another interesting point to investigate would be HOXA1 and its target gene in the zebrafish model, as there is no homolog of E-selectin in the zebrafish.

It would also be interesting to investigate other genes, which are regulated in the microarray (see 3.4.) e.g. CXCL2, PGF or CXCR4. The chemokine ligands and receptors could be especially interesting, because it's known that HOXC9 regulates vascular development in an IL8 dependent way (Stoll *et al.*, 2011).

Taken together, this work offers new possibilities to understand angiogenic processes and suggests a new interaction pair: HOXA1 and E-selectin. This work also opens new perspectives in therapy of various diseases in which E-selectin is involved.

## 5.4. ROCK I/II functions as positive and negative regulators of angiogenic processes

The analysis after the OIR model and H1152/Fasudil treatment showed that ROCK I/II inhibition leads to an increased neovascularisation in mouse retinas. Previous studies revealed that the inhibition of ROCK I/II, with pharmacological inhibitors, led to contradictory results. Different studies were performed with the inhibitor Y27632, where it was shown that even with the usage of the same concentration the results showed opposite effects. The inhibition of ROCK I/II with Y27632 enhanced migration and sprouting in the study of Mavria *et al.* (2006). Van Nieuw Amerongen *et al.* (2003) showed that the inhibition of ROCK I/II with Y27632 stunted migration and sprouting. In both studies 10 μM Y27632 was used for the experiments. The same circumstances can be seen in case of fasudil usage. One report by Yin *et al.* (2007) showed that usage of fasudil led to inhibition of angiogenesis, whereas the report by Nohria *et al.* (2006) described a positive impact on endothelial function after fasudil treatment. In our case, Y27632 showed no effect on retinal neovascularisation.

A limiting factor of the inhibitors, are that these inhibitors do not distinguish between the two isoforms ROCK I and ROCK II. To generate conditional knock-out, it would be of interest to study the different functions of the isoforms on mice.

In summary, this part of the project indicates that inhibition of ROCK I/II can promote and enhance respectively angiogenesis. In turn this implies that inhibition of the Rho kinases is a potential therapeutic target.

## 5.5. ROCK I/II cannot act as therapeutic targets in cardiovascular diseases

The myocardial infarction study implicates that the inhibition of ROCK I/II with the inhibitor H1152 has no positive impact on the recovery process after the infarct. The survival rate of the rats doesn't differ between the study groups. Therefore, the inhibition of ROCK I/II with the inhibitor H1152 has neither a positive nor a negative effect on the survival rate. The measurement of the MI-size showed that the induced myocardial infarction was successful. This myocardial infarction induces angiogenesis at the border zone of the affected tissue. CD31 stainings showed this effect (data not shown), however it is an effect independent of H1152. Nevertheless, H1152 has no negative effect on angiogenesis. This study attested that

the inhibition of ROCK I/II with H1152 is not sufficient as a therapy after a myocardial infarction.

## 5.7. Future perspectives – ROCK I/II

In this part of the thesis, the impact of ROCK I and ROCK II in angiogenesis were shown. The results of the Isolectin staining in the OIR treated eyes showed that ROCK I and II are negative regulators of angiogenesis. This phenotype is similar to the observed phenotype from the Notch-signaling-pathway. If the Notch pathway is inhibited, an increased blood vessel formation can be observed (Siekmann and Lawson, 2007). For this reason, it would be interesting to concentrate further investigations on tip cell formation after ROCK I/II inhibition, as well as conducting NG2 staining to investigate pericyte coverage.

In addition to the stainings mentioned in 5.6., an investigation on macrophages and eNOS could be of importance. Macrophages are involved in the development of VEGF, and eNOS plays an important role in the regulation of blood pressure and the functionality of blood vessels. Less activity or a dysfunction of eNOS promotes atherosclerosis. Another interesting point would be to analyse the level of ROCK I and ROCK II in different organs after the myocardial infarction, especially in the heart, and to analyse if H1152 regulates the level of the Rho kinases.

Taken together, this part of the thesis shows some evidence that ROCK I/II are negative regulators of angiogenesis.

# List of abbreviations

**A**

| | |
|---|---|
| Ang | angiopoietin |
| APS | ammonium persulfate |

**B**

| | |
|---|---|
| BAEC | bovine aortic endothelial cells |
| bp | basepairs |
| BSA | bovine serum albumin |
| BTB | broad-complex, tramtrack and bric-a-brac |
| BW | body weight |

**C**

| | |
|---|---|
| $CaCl_2$ | Calcium chloride |
| CD31 | cluster of differentiation 31 |
| cDNA | complementary DNA |
| CIP | calf intestinal alkaline phosphatase |
| cm | centimetre |
| CO | carbon monoxide |
| $CO_2$ | carbon dioxide |
| $CoCl_2$ | Cobalt(II)chloride |
| con | control |
| CXCL | CXC ligand |
| CXCR | CXC receptor |

**D**

| | |
|---|---|
| DAB | 3,3'-Diaminobenzidine |
| DAPI | 4',6-diamidino-2-phenylindole |
| Dfx | desferrioxamine mesylate |
| DKFZ | Deutsches Krebsforschungszentrum |
| DLAV | dorsal longitudinal anastomotic vessel |
| Dll4 | delta-like 4 |

| | |
|---|---|
| DMEM | Dulbecco's modified eagle medium |
| DMSO | dimethylsulfoxid |
| DNA | deoxyribonucleic acid |
| dNTP | equal molar mix of the deoxy-nucleotides dATP, dCTP, dGTP and dTTP |
| DTT | dithiothreitol |

**E**

| | |
|---|---|
| EC | endothelial cell(s) |
| ECGM | endothelial cell growth medium |
| ECL | enhanced chemoluminescence |
| EDTA | ethylenediaminetetraacetic acid |
| *E. coli* | Escherichia coli |
| EGFP | enhanced green fluorescent protein |
| eNOS | endothelium nitric oxide synthase |
| EPC | endothelial progenitor cells |
| Eph | ephrin receptor tyrosine kinase |
| ERK | extracellular signal-regulated kinase |
| et al. | and others |
| Ets | E-twenty-six |

**F**

| | |
|---|---|
| FACS | fluorescence-activated cell sorting |
| FAK | focal adhesion kinase |
| FCS | foetal calf serum |
| FGF (bFGF) | fibroblast growth factor (basic fibroblast growth factor) |
| Fli1 | friend leukemia virus integration |
| Flk1 | foetal liver kinase-1 |
| Flt | fms-like tyrosine kinase |
| FOX | forkhead box transcription factor |

**G**

| | |
|---|---|
| g | gram |
| GATA | G-A-T-A binding protein |

| | |
|---|---|
| GDP | guanosine diphosphate |
| GEF | guanine exchange factor |
| GFP | green fluorescent protein |
| GTP | guanosine triphosphate |
| GTPase | guanosine triphosphatase |

**H**

| | |
|---|---|
| h | hour(s) |
| HCl | hydrochloric acid |
| HIF | hypoxia-inducible factor |
| hpf | hours post fertilization |
| $H_2O_2$ | hydrogen peroxide |
| HRP | horse radish peroxidise |
| HUVEC(s) | human umbilical vein endothelial cell(s) |
| HOX | homeobox gene |

**I**

| | |
|---|---|
| Il1 | interleukin 1 |
| iNOS | cytokine-inducible nitric oxide synthase |
| ISV | intersomitic vessel |

**K**

| | |
|---|---|
| kb | kilobase |
| KCl | kaliumchloride |
| kDa | kilo Dalton |
| KDR | kinase insert domain receptor |
| kg | kilogram |
| KLEIP | Kelch-like Ect-2 interacting protein |

**L**

| | |
|---|---|
| LB | lysogeny broth |

**M**

| | |
|---|---|
| MCP-1 | Monocyte chemotactic protein-1 |
| µg | microgram |
| mg | milligram |
| MI | myocardial infarction |
| min | minutes |
| ml | millilitre |
| µl | micorlitre |
| mm | millimetre |
| µm | micrometre |
| mM | millimolar |
| µM | micromolar |
| mmHg | millimetre of mercury |
| MMP | matrix metalloproteinase |
| Mo | morpholino |
| MOI | multiplicity of infection |
| mRNA | messenger ribonucleic acid |

**N**

| | |
|---|---|
| n | number of samples |
| $N_2$ | nitrogen |
| NaOH | sodium hydroxide |
| NF-κB | nuclear factor 'kappa-light-chain-enhancer' of activated B-cells |
| ng | nanogram |
| nl | nanoliter |
| nM | nanomolar |
| NO | nitric oxide |
| NP-40 | Nonidet P-40 |
| Nrp | neuropilin |

**O**

| | |
|---|---|
| OIR | Oxygen-induced retinopathy |

**P**

| | |
|---|---|
| P | p(probability)-value |
| PCR | polymerase chain reaction |
| PBS | phosphate buffered saline |
| PBST | phosphate buffered saline with 0.1 % Tween 20 |
| PBSTT | phosphate buffered saline with 0.1 % Tween 20 and 0.1% Triton X |
| PCR | polymerase chain reaction |
| PFA | paraformaldehyde |
| PGF | placental growth factor |
| pH | pH value |
| PH | pleckstrin homology |
| pmol | pico-mol |
| P/S | penicillin/streptomycin |
| PTU | 1-phenyl-2-thiourea |

**R**

| | |
|---|---|
| RAC | ras-related C3 botulinum toxin substrate |
| RBD | Rho-binding domain |
| Rho | ras homologous |
| RNA | ribonucleic acid |
| ROBO | roundabout |
| ROCK | Rho-associated coiled-coil forming protein kinases |
| rpm | rotations per minute |
| RT | room temperature |
| RT-PCR | reverse transcription PCR |

**S**

| | |
|---|---|
| SDS | sodium dodecyl sulphate |
| SDS-PAGE | sodium dodecyl sulphate polyacrylamid gel electrophoresis |
| sec | seconds |
| siRNA | small interfering RNA |
| SMC | smooth muscle cell(s) |
| SOC | super optimal broth with glucose |

**T**

| | |
|---|---|
| TAE | Tris base, acetic acid and EDTA |
| TB | translational blocking |
| TEMED | N, N, N', N'-tetramethylethylenediamine |
| tg | transgenic |
| Tie | tyrosine kinase with immunoglobulin and epidermal growth factor (EGF) homology domains |
| TNF-α | tumour necrosis factor alpha |
| Tris | Tris(hydroxymethyl)-aminomethan |

**U**

| | |
|---|---|
| U | Unit |
| UTR | untranslated region |

**V**

| | |
|---|---|
| V | Volt |
| VEGF | vascular endothelial growth factor |

| | |
|---|---|
| °C | degree Celsius |
| % | per cent |

# References

Achen, M. G., Jeltsch, M., Kukk, E., Makinen, T., Vitali, A., Wilks, A. F., Alitalo, K., Stacker, S. A. 1998. Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). *Proc Natl Acad Sci USA* 95:548–553.

Adams, R. H., Alitalo, K. 2007. Molecular regulation of angiogenesis and lymph-angiogenesis. *Nat Rev Mol Cell Biol* 8(6): 464-478.

Augustin, H. G. 2005. Angiogenesis in the female reproductive system. In: Clauss, M., Breier, G. (eds) Mechanisms of angiogenesis. Birkhäuser, Basel, pp 35-52.

Augustin-Voss, H. G., Pauli, B. U. 1992. Quantitative analysis of autocrineregulated, matrix-induced, and tumor cell-stimulated endothelial cell migration using a silicon template compartmentalization technique. *Exp Cell Res.* 198: 221–227.

Bandyopadhyay, S., Ashraf, M.Z., Daher, P., Howe, P. H., DiCorleto, P.E. 2007. HOXA9 participates in the transcriptional activation of E-selectin in endothelial cells. *Mol Cell Biol* 27: 4207-4216.

Bedell, V. M., Yeo, S. Y., Park, K. W., Chung, J., Seth, P., Shivalingappa, V., Zho, J., Obara, T., Sukhatme, V. P., Drummond, I. A., Li, D. Y., Ramhandran, R. 2005. Roundabout4 is essential for angiogenesis in vivo. *Proc Natl Acad Sci USA* 2005; 102: 6373–6378.

Beckers, C. M. L., van Hinsbergh, V. W. M., Amerongen, G. P. V. 2010. Driving Rho GTPase activity in endothelial cells regulates barrier integrity. *Thromb Haemostasis* 103, 40-55.

Belotti, D., Clausse, N., Alami, Y., Daukandt, M., Deroanne, C., Faiella, A., Flagiello, D., Poupon, M. F., Castronovo, V. 1997. Homeobox genes expression in endothelial cells and their potential role In angiogenesis. *European Journal of Cancer* 33, Supplement 8: S44.

Belotti, D., Clausse, N., Flagiello, D., Alami, Y., Daukandt, M., Deroanne, C. 1998. Expression and modulation of homeobox genes from cluster B in endothelial cells. *Lab Invest* 78: 1291-1299.

Bennett, B. L., Cruz, R., Lacson, R. G., Manning, A. M. 1997. Interleukin-4 suppression of tumor necrosis factor alphastimulated E-selectin gene transcription is mediated by STAT6 antagonism of NF-kappaB. *J Biol Chem* 272: 10212-10229.

Boudreau, N., Andrews, C., Srebrow, A., Ravanpay, A., Cheresh, D. A. 1997. Induction of the angiogenic phenotype by Hox D3. *J Cell Biol* 139: 257-264.

Boudreau, N. J., Varner, J. A. 2004. The homeobox transcription factor Hox D3 promotes integrin alpha5beta1 expression and function during angiogenesis. *J Biol Chem* 279: 4862-4868.

Brühl, T., Urbich, C., Aicher, D., Acker-Palmer, A., Zeiher, A. M., Dimmeler, S. 2004. Homeobox A9 transcriptionally regulates the EphB4 receptor to modulate endothelial cell migration and tube formation. *Circ Res* 94:743-751.

Bryan, B.A., D'Amore, P.A. 2007. What tangled webs they weave: Rho-GTPase control of angiogenesis. *Cell Mol Life Sci* 64, 2053-2065.

Burri, P. H., Hlushchuk, R., Djonov, V. 2004. Intussusceptive angiogenesis: its emergence, its characteristics, and its significance. *Dev Dyn* 231: 474-488.

Bustelo, X. R., Sauzeau, V., and Berenjeno, I. M. 2007. GTP-binding proteins of the Rho/Rac family: regulation, effectors and functions in vivo. *Bioessays* 29: 356-370.

Cantile, M., Schiavo, G., Terracciano, L, Cillo, C. 2008. Homeobox genes in normal and abnormal vasculogenesis. *Nutrition, Metabolism & Cardiovascular Diseases* 18: 651-658.

Care´, A., Silvani, A., Meccia, E., Mattia, G., Stoppacciaro, A., Parmiani, G. 1996. HOXB7 constitutively activates basic fibroblast growth factor in melanomas. *Mol Cell Biol* 16: 4842-4851.

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

Carmeliet, P. 2005. Angiogenesis in life, disease and medicine. *Nature* 438(7070): 932-936.

Carmeliet, P., De Smet, F., Loges, S., Mazzone, M. 2009. Branching morphogenesis and antiangiogenesis candidates: tip cells lead the way. *Nat Rev Clin Oncol* 6(6): 315-326.

Cermenati, S. Moleri, S. Cimbro, S., Corti, P., Del Giacco, L., Amodeo, R., Dejana, E., Koopman, P., Cotelli, F., Beltrame, M. 2008. Sox18 and Sox7 play redundant roles in vascular development. *Blood* 111: 2657–2666.

Cernuda-Morollón, E., Ridley, A. J. 2006. Rho GTPases and leukocyte adhesion receptor expression and function in endothelial cells. *Circ Res* 98: 757-767.

Chen, Y., Xu, B., Arderiu, G., Hashimoto, T., Young, W. L., Boudreau, N. 2004. Retroviral delivery of homeobox D3 gene induces cerebral angiogenesis inmice. *J CerebBlood FlowMetab* 24: 1280-1287.

Chisaka, O., Kameda, Y. 2005. Hoxa3 regulates the proliferation and differentiation of the third pharyngeal arch mesenchyme in mice. *Cell Tissue Res* 320: 77-89.

Chisaka, O., Musci, T. S., Capecchi, M. R. 1992. Developmental defects of the ear, cranial nerves and hindbrain resulting from targeted disruption of the mouse homeobox gene Hox-1.6. *Nature* 355: 516-520.

Choi, J., Dong, L., Ahn, J., Dao, D., Hammerschmidt, M., Chen, J. N. 2007. FoxH1 negatively modulates flk1 gene expression and vascular formation in zebrafish. *Dev. Biol.* 304: 735–744.

Chung, N., Jee, B. K., Chae, S. W., Jeon, Y. W., Lee, K. H., Rha, H. K. 2009. HOX gene analysis of endothelial cell differentiation in human bone marrow-derived mesenchymal stem cells. *Mol Biol Rep* 36(2): 227-235.

Collins, T., Read, M. A., Neish, A. S., Whitley, M. Z., Thanos, D., Maniatis, T.: Transcriptional regulation of endothelial cell adhesion molecules: NF-kappa B and cytokine-inducible enhancers. *FASEB J* 9: 899-909.

Coultas, L., Chawengsaksophak, K., Rossant, J. 2005. Endothelial cells and VEGF in vascular development. *Nature* 438: 937-945.

Cronstein, B. N., Kimmel, S. C., Levin, R. I., Martiniuk, F., Weissmann, G. 1992. A mechanism for the antiinflammatory effects of corticosteroids: the glucocorticoid receptor regulates leukocyte adhesion to endothelial cells and expression of endothelial-leukocyte adhesion molecule 1 and intercellular adhesion molecule 1. *Proc Natl Acad Sci USA* 89: 9991-9995.

Daftary, G. S., Troy, P. J., Bagot, C. N., Young, S. L., Taylo,r H. S. 2002. Direct regulation of beta-3-integrin subunit gene expression by HOXA10 in endometrial cells. *Mol Endocrinol* 16: 571–579.

Daly, C., Wong, V., Burova, E., Wei, Y., Zabski, S., Griffiths, J., Lai, K. M., Lin, H. C., Ioffe, E., Yancopoulos, G. D. 2004. Angiopoietin-1 modulates endothelial cell function and gene expression via the transcription factor FKHR (FOXO1). *Genes Dev.* 18: 1060–1071.

Epting, D., Wendik, B., Bennewitz, K., Dietz, C. T., Driever, W., Kroll, J. 2010.The Rac1 regulator ELMO1 controls vascular morphogenesis in zebrafish. *Circ Res* 107(1): 45-55.

Fassbender, K., Mössner, R., Motsch, L., Kischka, U., Grau, A., Hennerici, M. 1995. Circulating selectin- and immunoglobulin-type adhesion molecules in acute ischemic stroke. Stroke 26: 1361-1364.

Feng, Y., vom Hagen, F., Pfister, F., Djokic, S., Hoffmann, S., Back, W., Wagner, P., Lin, J., Deutsch, U., Hammes, H. P. 2007. Impaired pericyte recruitment and abnormal retinal angiogenesis as a result of angiopoietin-2 overexpression. *Thrombosis and haemostasis* 97: 99-108.

Ferrara, N., Davis-Smyth, T. 1997. The biology of vascular endothelial growth factor. *Endocr Rev* 18: 4–25.

Ferrara, N. 2002. VEGF and the quest for tumour angiogenesis factors. *Nat Rev Cancer* 2: 795–803.

Ferrara, N. 2010. Vascular endothelial growth factor and age-related macular degeneration: from basic science to therapy. *N. Nat Med.* 16(10): 1107-1111.

Friedman, B. D., Tarby, T. J., Holve, S. 1996. Congenital horizontal gaze palsy, deafness, central hypoventilation, and developmental impairment: a brain stem syndrome prevalent in the Navajo population. *Am J Hum Genet* 59: 37

Gaucher, C., Devaux, C., Boura, C., Lacolley, P., Stoltz, J. F., Menu P. 2007. In vitro impact of physiological shear stress on endothelial cells gene expression profile. *Clin Hemorheol Microcirc* 37, 99-107.

Gerety, S. S., Wang, H. U., Chen, Z. F., Anderson, D. J. 1999. Symmetrical mutant phenotypes of the receptor EphB4 and its specific transmembrane ligand ephrin-B2 in cardiovascular development. *Mol Cell* 4: 403–414.

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

Gorski, D. H., Walsh, K. 2000. The Role of Homeobox Genes in Vascular Remodeling and Angiogenesis. *Circ Res* 87: 865-872.

Hata, Y., Miura, M., Nakao, S., Kawahara, S., Kita, T., Ishibashi, T. 2008. Antiangiogenic properties of fasudil, a potent Rho-kinase inhibitor. *Jpn J Ophthalmol* 52: 16–23.

Hayashi, H., Kume, T. 2008. Foxc transcription factors directly regulate Dll4 and Hey2 expression by interacting with the VEGF-Notch signaling pathways in endothelial cells. *PLoS ONE* 3: 2401.

Higai, K., Shimamura, A., Matsumoto, K. 2006. Amadori-modified glycated albumin predominantly induces E-selectin expression on human umbilical vein endothelial cells through NADPH oxidase activation. *Clin Chim Acta*, 367, 137-143.

Hill St., C.A. 2001. Interaction between endothelial selectins and cancer cells regulate metastasis. *Frontiers in Biosciences* 16, 3233-3251.

Hofmann, J. J., Iruela-Arispe, M.L. 2007. Notch signaling in blood vessels: who is talking to whom about what? *Circ Res* 100(11): 1556-1568.

Holley, J. E., Newcombe, J., Whatmore, J. L., Gutowski, N. J. 2009. Increased blood vessel density and endothelial cell proliferation in multiple sclerosis cerebral white matter. *Neuroscienc Letters* 470(1): 65-70.

Hu, Y., Chen, X., Duan, H., Hu, Y., Mu, X. 2009. Chinese herbal medicinal ingredients inhibit secretion of IL-6, IL-8, E-selectin and TXB2 in LPS-induced rat intestinal microvascular endothelial cells. *Immunopharmacol Immunotoxicol.* 31(4): 550-555.

Huminiecki, L., Gorn, M., Suchting, S., Poulsom, R., Bicknell, R. 2002. Magic roundabout is a new member of the roundabout receptor family that is endothelial specific and expressed at sites of active angiogenesis. *Genomics* 79: 547–552.

Isogai, S., Horiguchi, M., Weinstein, B. M. 2001. The Vascular Anatomy of the Developing Zebrafish: An Atlas of Embryonic and Early Larval Development. *Developmental Biology* 230: 278–301.

Jain, R. K., Duda, D. G., Clark, J. W., Loeffler, J. S. 2006. Lessons from phase III clinical trials on anti-VEGF therapy for cancer. *Nature Clinical Practice Oncology* 3: 24-40.

Joukov, V., Pajusola, K., Kaipainen, A., Chilov, D., Lahtinen, I., Kukk, E., Saksela, O., Kalkkinen, N., Alitalo, K. 1996. A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. *EMBO J* 15: 1751.

Jubeli, E., Moine, L., Vergnaud-Gauduchon, J., Barratt, G. 2011. E-selectin as a target for drug delivery and molecular imaging. *J Control Release*

Kaszubska, W., Hooft van Huijsduijnen, R., Ghersa, P., DeRaemy-Schenk, A. M., Chen, B. P., Hai, T., DeLamarter, J. F., Whelan, J. 1993. Cyclic AMP-independent ATF family members interact with NF-kappa B and function in the activation of the E-selectin promoter in response to cytokines. *Mol Cell Biol* 13, 7180-7190.

Kawasaki, T., Kitsukawa, T., Bekku, Y., Matsuda, Y., Sanbo, M., Yagi, T., Fujisawa, H. 1999. A requirement for neuropilin-1 in embryonic vessel formation. *Development* 126:4895–4902.

Kikuchi, G., Yoshida, T., Noguchi, M. 2005. Heme oxygenase and heme degradation. *Biochem. Biophys. Res. Commun.* 338 (1): 558–567.

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B., Schilling, T. F. 1995. Stages of embryonic development of the zebrafish. *Dev Dyn.* 203(3): 253-310.

Kirby, M. L., Hunt, P., Wallis, K., Thorogood, P. 1997. Abnormal patterning of the aortic arch arteries does not evoke cardiac malformations. *Dev Dyn* 208(1): 34-47.

Kiriyama, K., Ye, C. 1995. E-selectin expression in serum and tissue correlates with distant metastasis of colorectal cancer. *Nippon Rinsho* 53(7): 1760–1764.

Klagsbrun, M., Eichmann, A. 2005. A role for axon guidance receptors and ligands in blood vessel development and tumor angiogenesis. *Cytokine Growth Factor Rev* 16: 535–548.

Kroll, J., Epting, D., Kern, K., Dietz, C. T., Feng, Y., Hammes, H. P., Wieland, T., Augustin, H. G. 2009. Inhibition of Rho-dependent kinases ROCK I/II activates VEGF-driven retinal neovascularization and sprouting angiogenesis. *Am J Physiol Heart Circ Physiol* 296: 893-899.

Kume, T., Jiang, H., Topczewska, J. M., Hogan, B. L. 2001. The murine winged helix transcription factors, Foxc1 and Foxc2, are both required for cardiovascular development and somitogenesis. *Genes Dev* 15: 2470–2482.

Kuo, C.T., Veselits, M.L., Barton, K.P., Lu, M.M., Clendenin, C., Leiden, J. M. 1997. The LKLF transcription factor is required for normal tunica media formation and blood vessel stabilization during murine embryogenesis. *Genes Dev.* 11: 2996–3006.

Lawson, N. D., Scheer, N., Pham, V. N., Kim, C.-H., Chitnis, A. B., Campos-Ortega, J. A., Weinstein, B. 2001. Notch signaling is required for arterial-venous differentiation during embryonic vascular development. *Development* 128: 3675-3683.

Lawson, N. D., Weinstein, B. M. 2002. In vivo imaging of embryonic vascular development using transgenic zebrafish. *Dev Biol.* 248(2): 307-318.

Lbow, M. A., Norton, C. R., Rumberger, J. M., Lombard-Gillooly, K. M., Shuster, D. J., Hubbard, J., Bertko, R., Knaak, P. A., Terry, R. W., Harbison, M. L. 1994. Characterization of E-selectin-deficient mice: demonstration of overlapping function of the endothelial selectins. *Immunity* 1(8): 709-720.

Lee, M. E., Temizer, D. H., Clifford, J. A., Quertermous, T. 1991. Cloning of the GATA-binding protein that regulates endothelin-1 gene expression in endothelial cells. *J. Biol. Chem.* 266**:**16188–16192.

Leeuwenberg, J. F., Smeets, E. F., Neefjes, J. J., Shaffer, M. A., Cinek, T., Jeunhomme, T. M. Ahern T. J., Buurman, W.A. 1992. E-selectin and intercellular adhesion molecule-1 are released by activated human endothelial cells in vitro. *Immunology*, 77, 543-549.

Licht, A. H., Müller-Holtkamp, F., Flamme, I., Breier, G. 2005. Inhibition of hypoxia-inducible factor activity in endothelial cells disrupts embryonic cardiovascular development. *Blood* 107: 584-590.

Liu, X. S., Zhang, X. Q., Liu, L., Ming, J., Xu, H., Ran, X. Z., Cheng, T. M. 2004. The role of homeobox B2 gene in vascular endothelial proliferation and the protective effects of VEGF on the endothelia against radiation injury. *Zhonghua Shao Shang Za Zhi* 20(5): 287-291.

Lobov, I. B., Renard, R. A., Papadopoulos, N., Gale, N. W., Thurston, G., Yancopoulos, G. D., Wiegand, S. J. 2007. Delta-like ligand 4 (Dll4) is induced by VEGF as a negative regulator of angiogenic sprouting. *Proc Natl Acad Sci USA* 104(9): 3219-3224.

Lu, X., Le Noble, F., Yuan, L., Jiang, Q., De Lafarge, B., Sugiyama, D., Bréant, C., Claes, F., De Smet, F., Thomas, J. L., Autiero, M., Carmeliet, P., Tessier-Lavigne, M., Eichmann, A. 2004. The netrin receptor UNC5B mediates guidance events controlling morphogenesis of the vascular system. *Nature* 432(7014): 179-186.

Lufkin, T., Dierich, A., LeMeur, M., Mark, M., Chambon, P. 1991. Disruption of the Hox-1.6 Homeobox gene results in defects in a region corresponding to its rostral domain of Expression. *Cell* 66: 1105-1119.

Lutgens, E., Daemen, M. J., de Muinck, E. D., Debets, J., Leenders, P., Smits, J. F. 1999. Chronic myocardial infarction in the mouse: cardiac structural and functional changes. *Cardiovasc Res.* 41(3): 586-593.

Maglione, D., Guerriero, V., Viglietto, G., Delli-Bovi, P., Persico, M.G. 1991. Isolation of a human placenta cDNA coding for a protein related to the vascular permeability factor. *Proc Natl Acad Sci USA* 88: 9267–9271.

Mahlapuu, M., Ormestad, M., Enerback, S., Carlsson, P. 2001. The forkhead transcription factor Foxf1 is required for differentiation of extra-embryonic and lateral plate mesoderm. *Development* 128: 155–166.

Mavria, G., Vercoulen, Y., Yeo, M., Paterson, H., Karasarides, M., Marais, R., Bird, D., Marshall, C. J. 2009. ERK-MAPK signaling opposes Rho-kinase to promote endothelial cell survival and sprouting during angiogenesis. *Cancer Cel.* 9(1): 33-44.

Mayer, B., Spatz, H., Funke, I., Johnson, J. P., Schildberg, F. W. 1998. De novo expression of the cell adhesion molecule E-selectin on gastric cancer endothelium. *Langenbecks Arch. Surg.* 383(1): 81–86.

Mazzone, M., Dettori, D., Leite de Olivera, R., Loges, S., Schmidt, T., Jonckx, B., Tian, Y. M., Lanahan, A. A., Pollard, P., Ruiz de Almodovar, C., De Smet, F., Vinckier, S., Aragones, J., Debackere, K., Luttun, A., Wyns, S., Jordan, B., Pisacane, A., Gallez, B., Lampugnani, M. G., Dejana, E., Simons, M., Ratcliffe, P., Maxwell, P., Carmeliet, P. 2009. Heterozygous deficiency of PHD2 restores tumor oxygenation and inhibits metastasis via endothelial normalization. *Cell* 136, 839–851.

Miano, J. M., Firulli, A. B., Olson, E. N., Hara, P., Giachelli, C. M., Schwartz, S. M. 1996. Restricted expression of homeobox genes distinguishes fetal from adult human smooth muscle cells. *Proc Natl Acad Sci USA* 93: 900-905.

Murphy, P., Hill, R. E. 1991. Expression of the mouse labial-like homeobox-containing genes, Hox 2.9 and Hox 1.6, during segmentation of the hindbrain. *Development* 111(1): 61-74.

Myers, C., Charboneau, A., Boudreau, N. 2000. Homeobox B3 promotes capillary morphogenesis and angiogenesis. *J Cell Biol* 148: 343-351.

Myers, C., Charboneau, A., Cheung, I., Hanks, D., Boudreau, N. 2002. Sustained expression of homeobox D10 inhibits angiogenesis. *Am J Pathol* 161: 2099-2109.

Nacak, T.G., Alajati, A., Leptien, K., Fulda, C., Weber, H., Miki, T., Czepluch, F. S., Waltenberger, J., Wieland, T., Augustin, H. G., Kroll, J. 2007. The btb-kelch protein kleip controls endothelial migration and sprouting angiogenesis. *Circ Res*. 100: 1155-1163.

Nguyen, M., Corless, C. L., Kraling, C. L., Tran, C., Atha, T., Bischoff, J., Barsky, S. H. 1997. Vascular expression of E-selectin is increased in estrogen-receptor-negative breast cancer: a role for tumor-cell-secreted interleukin-1 alpha. *Am. J. Pathol.* 150(4): 1307–1314.

Nieuw Amerongen van, G. P., Koolwijk, P., Versteilen, A., van Hinsbergh, V. W. 2003. Involvement of RhoA/Rho kinase signaling in VEGF-induced endothelial cell migration and angiogenesis in vitro. *Arterioscler Thromb Vasc Biol.* 23(2): 211-217.

Nieuw Amerongen van, G. P., van Hinsbergh, V. W. 2009. Role of ROCK I/II in vascular branching. *Am J Physiol Heart Circ Physiol* 296: 903-905.

Nohria, A., Grunert, M. E., Rikitake, Y., Noma, K., Prsic, A., Ganz, P., Liao, J. K., Creager, M. A. 2006. Rho kinase inhibition improves endothelial function in human subjects with coronary artery disease. *Circ Res.* 99(12): 1426-1432.

O'Leary, D. D., Wilkinson, D. G. 1999. Eph receptors and ephrins in neural development. *Curr Opin Neurobiol* 9: 65–73.

Olofsson, B., Pajusola, K., Kaipainen, A., von Euler, G., Joukov, V., Saksela, O., Orpana, A., Pettersson, R. F., Alitalo, K., Eriksson, U. 1996. Vascular endothelial growth factor B, a novel growth factor for endothelial cells. *Proc Natl Acad Sci USA* 93: 2576–2581.

Paik, J. H., Kollipara, R., Chu, G., Ji, H., Xiao, Y., Ding, Z., Miao, L., Tothova, Z., Horner, J. W., Carrasco, D. R. 2007. FoxOs are lineage-restricted redundant tumor suppressors and regulate endothelial cell homeostasis. *Cell* 128: 309–323.

Palazzo, A., Iacovelli, R., Cortesi, E. 2010. Past, present and future of targeted therapy in solid tumors. *Curr Cancer Drug Targets* 10(5): 433-461.

Pang, X., Yi, T., Yi, Z., Cho, S. G., Qu, W., Pinkaew, D., Fujise, K., Liu, M. 2009. Morelloflavone, a biflavonoid, inhibits tumor angiogenesis by targeting rho GTPases and extracellular signal-regulated kinase signaling pathways. *Cancer Res.* 69: 518-525.

Papanicolaou, K. N., Izumiya, Y., Walsh, K. 2008. Forkhead transcription factors and cardiovascular biology. *Circ. Res.* 102: 16–31.

Partanen, J., Armstrong, E., Makela, T. P., Korhonen, J., Sandberg, M., Renkonen, R., Knuutila, S., Huebner, K., Alitalo, K. 1992. A novel endothelial cell surface receptor tyrosine kinase with extracellular epidermal growth factor homology domains. *Mol Cell Biol* 12:1698–1707.

Patterson, L.J., Patient, R. 2006. The "Ets" factor: vessel formation in zebrafish — the missing link? *PLoS Biol.* 4(1): 24.

Paz dela, N. G., D'Amore P. A. 2009. Arterial versus venous endothelial cells. *Cell Tissue Res.* 335(1): 5-16.

Pham, V. N., Lawson, N. D., Mugford, J. W., Dye, L., Castranova, D., Lo, B., Weinstein, B. M. 2007. Combinatorial function of ETS transcription factors in the developing vasculature. *Dev. Biol.* 303(2): 772–783.

Pigott, R., Dillon, L. P., Hemingway, I. H., Gearing, A. J.: Soluble forms of E-selectin, ICAM-1 and VCAM-1 are present in the supernatants of cytokine activated cultured endothelial cells. *Biochem Biophys Res Commun* 187, 584-589.

Poole, T. J., Finkelstein, E. B., Cox C. M. 2001. The role of FGF and VEGF in angioblast induction and migration during vascular development. *Dev Dyn.* 220(1): 1-17.

Porat, R. M., Grunewald, M., Globerman, A., Itin, A., Barshtein, G., Alhonen, L., Alitalo, K., Keshet, E. 2004. Specific induction of tie1 promoter by disturbed flow in atherosclerosisprone vascular niches and fl ow-obstructing pathologies. *Circ Res* 94: 394–401.

Prager, G. W., Poettler, M. 2011. Angiogenesis in cancer. Basic mechanisms and therapeutic advances. *Hamostaseologie* 32(2).

Prior, B. M., Yang, H. T., Terjung, R. L. 2004. What makes vessels grow with exercise training? *J App Physiol* 97: 1119-1128.

Rainger, G. E., Wautier, M. P., Nash G. B., Wautier, J. L. 1996. Prolonged E-selectin induction by monocytes potentiates the adhesion of flowing neutrophils to cultured endothelial cells. *Br J Haematol* 92: 192-199.

Renkonen, J., Makitie, A., Paavonen, T., Renkonen, R. 1999. Sialyl-Lewis(x/a)-decorated selectin ligands in head and neck tumours. *J. Cancer Res. Clin. Oncol.* 125(10): 569–576.

Rhoads, K., Arderiu, G., Charboneau, A., Hansen, S.L., Hoffman, W., Boudreau, N. 2005. A role for Hox A5 in regulating angiogenesis and vascular patterning. *Lymphat Res Biol* 3: 240-252.

Riento, K., Ridley, A. J. 2003. Rocks: multifunctional kinases in cell behaviour. *Nat Rev Mol Cell Biol.* 4(6): 446-456.

Risau, W. 1997. Mechanisms of angiogenesis. *Nature* 386: 671-674.

Risau, W., Flamme, I. 1995. Vasculogenesis. *Annu Rev Cell Dev Biol*. 11: 73-91.

Rössig, l., Urbich, C., Brühl, T., Dernbach, E., Heeschen, C., Chavakis, E. 2005 Histone deacetylase activity is essential for the expression of HoxA9 and for endothelial commitment of progenitor cells. *J Exp Med* 20: 1825-1835.

Roldán, V., Martin, F., Lip, G. Y., Blann, A. D. 2003. Soluble E-selectin in Cardiovascular disease and its risk factors. A review of the literature. *Thromb Haemost* 90: 1007-1020.

Rossman, K. L., Der, C. J., Sondek, J. 2005. GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors. *Nat Rev Mol Cell Biol* 6, 167-180.

Royen van, N., Piek, J. J., Buschmann, I., Hoefer, I., Voskuil, M., Schaper, W. 2001. Stimulation of arteriogenesis: a new concept for the treatment of arterial occlusive disease. *Cardiovasc Res* 49: 543–553.

Sambrook, J., Russell, D. W. 2001. Molecular Cloning, 3rd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

Semenza, G. L. 1998. Hypoxia-inducible factor 1: master regulator of O-2 homeostasis. *Curr Opin Genet Dev* 8: 588-594.

Semenza G. L. 2004. Hydroxylation of HIF-1: oxygen sensing at the molecular level. *Physiology* 19: 176-182.

Semenza, G. L. 2007. Vasculogenesis, angiogenesis, and arteriogenesis: mechanisms of blood vessel formation and remodeling. *J Cell Biochem* 102(4): 840-847.

Seo, S., Fujita, H., Nakano, A., Kang, M., Duarte, A., Kume, T. 2006. The forkhead transcription factors, Foxc1 and Foxc2, are required for arterial specification and lymphatic sprouting during vascular development. *Dev. Biol.* 294: 458–470.

Shah, N., Sukumar, S. 2010. The Hox genes and their roles in oncogenesis. *Nature reviews* 10: 361-371.

Shalaby, F., Rossant, J., Yamaguchi, T. P., Gertsenstein, M., Wu, X. F., Breitman, M. L., Schuh, A. C. 1995. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* 376: 62–66.

Shimizu, Y., Thumekeo, D., Keel, J., Ishizaki, T., Oshima, H., Oshima, M. Noda, Y., Matsumura, F., Taketo, M. M., Narumiya, S. 2005. ROCK-I regulates closure of the eyelids and ventral body wall by inducing assembly of actomyosin bundles. *J Cell Biol* 168:941-953.

Shimokawa, H., Rashid, M. 2007. Development of Rho-kinase inhibitors for cardiovascular medicine. *Trends Pharmacol Sci.* 28: 296-302.

Siekmann, A. F., Lawson, N. D. 2007. Notch signalling limits angiogenic cell behaviour in developing zebrafish arteries. *Nature* 445: 781-784.

Springer, T. A. 1994. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* 76: 301-314.

Stadler, H. S., Higgins, K. M., Capecchi, M. R. 2001. Loss of Eph-receptor expression correlates with loss of cell adhesion and chondrogenic capacity in Hoxa13 mutant limbs. *Development* 128 :4177-4188.

Stannard, A. K., Khurana, R., Evans, I. M., Sofra, V., Holmes, D. I., Zachary, I. 2007: Vascular endothelial growth factor synergistically enhances induction of E-selectin by tumor necrosis factor-alpha. *Arterioscler Thromb Vasc Biol* 27, 494-502.

Stoll, S. J., Bartsch, S., Augustin, H. G., Kroll, J. 2011. The Transcription Factor HOXC9 Regulates Endothelial Cell Quiescence and Vascular Morphogenesis via Inhibition of Interleukin 8. *Circ Res* 108 (11): 1367-1377.

Stratmann, A., Risau, W., Plate, K. H. 1998. Cell type-specific expression of angiopoietin-1 and angiopoietin-2 suggests a role in glioblastoma angiogenesis. *Am J Pathol* 153: 1459-1466.

Suchting, S., Heal, P., Tahtis, K., Stewart, L. M., Bicknell, R. 2005. Soluble Robo4 receptor inhibits in vivo angiogenesis and endothelial cell migration. *Faseb J* 19: 121–123.

Suri, C., Jones, P. F., Patan, S., Bartunkova, S., Maisonpierre, P. C., Davis, S., Sato, T. N., Yancopoulos, G. D. 1996. Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell* 87: 1171–1180.

Svingen, T., Tonissen, K. F. 2006. Hox transcription factors and their elusive mammalian gene targets. *Heredity* 97: 88-96.

Takada, A., Ohmori, K., Yoneda, T., Tsuyuoka, K., Hasegawa, A., Kiso, M., Kannagi, R. 1993. Contribution of carbohydrate antigens sialyl Lewis A and sialyl Lewis X to adhesion of human cancer cells to vascular endothelium. *Cancer Res.* 53(2): 354–361.

Tcherkezian, J., Lamarche-Vane, N. 2007. Current knowledge of the large RhoGAP family of proteins. *Biol Cell* 99, 67-86.

Thisse, B., Thisse, C. 2004. Fast Release Clones: A High Throughput Expression Analysis. ZFIN Direct Data Submission (http://zfin.org).

Thompson, J. D., Gibson, T. J., Higgins, D. G. 2002. Multiple sequence alignment using ClustalW and ClustalX. *Curr Protoc Bioinformatics* Chapter 2: Unit 2.3.

Thumkeo, D., Keel, J., Ishizaki, T., Hirose, M., Nonomra, K., Oshima, H., Oshima, M., Taketo, M. M., Narumiya, S. 2003. Targeted disruption of the mouse rho-associated kinase 2 gene results in intrauterine growth retardation and fetal death. *Mol Cell Biol* 23:5043-5055.

Thurston, G. 2003. Role of angiopoietins and Tie receptor tyrosine kinases in angiogenesis and lymphangiogenesis. *Cell Tissue Res* 314: 61-68.

Tischfield, M. A., Bosley, T. M., Salih, M. A., Alorainy, I. A., Sener, E. C., Nester, M. J., Oystreck, D. T., Chan, W. M., Andrews, C., Erickson, R. P., Engle, E. C. 2005. Homozygous HOXA1 mutations disrupt human brainstem, inner ear, cardiovascular and cognitive development. *Nat Genet* 37:1035-1037.

Treutiger, C. J., Mullins, G. E., Johansson, A. S., Rouhiainen, A., Rauvala, H. M., Erlandsson-Harris, H., Andersson, U., Yang, H., Tracey, K. J., Andersson J., Palmblad, J. E. 2003. High mobility group 1 B-box mediates activation of human endothelium. *J Intern Med* 254: 375-385.

Tronc, F., Wassef, M., Exposito, B., Henrion, D., Glagov, S., Tedgui, A. 1996. Role of NO in flow-induced remodeling of the rabbit common carotid artery. *Arterioscler Thromb Vasc Biol* 16: 1256–1262.

Uchida, S., Watanabe, G., Shimada, Y., Maeda, M., Kawabe, A., Mori, A., Arii, S., Uehata, M., Kishimoto, T., Oikawa, T., Imamura, M. 2000. The suppression of small GTPase rho signal transduction pathway inhibits angiogenesis in vitro and in vivo. *Biochem. Biophys. Res. Commun.* 269: 633–640.

Uyeno, L. A., Newman-Keagle, J.A., Cheung, I., Hunt, T. K., Young, D.M., Boudreau, N. 2001. Hox D3 expression in normal and impaired wound healing. *J Surg Res* 100: 46-56.

Vaisman, N., Gospodarowicz, D., Neufeld, G. 1990. Characterization of the receptors for vascular endothelial growth factor. *J Biol Chem* 265:19461–19466.

Val de, S., Black, B. L. 2009. Transcriptional Control of Endothelial Cell Development. *Developmental Cell* 16: 180-195.

Visser, L. H. 2006. Critical illness polyneuropathy and myopathy: clinical features, risk factors and prognosis. *Eur J Neurol.* 13(11): 1203-1212.

Wang, B., Xiao, Y., Ding, B. B., Zhang, N., Yuan, X., Gui, L., Qian, K. X., Duan, S., Chen, X., Rao, Y., Geng, J. G. 2003. Induction of tumor angiogenesis by Slit-Robo signaling and inhibition of cancer growth by blocking Robo activity. *Cancer Cell* 4: 19–29.

Wang, G. L., Semenza, G. L. 1993. Desferrioxamine induces erythropoietin gene expression and hypoxia-inducible factor 1 DNA-binding activity: implications for models of hypoxia signal transduction. *Blood* 82(12): 3610-3615.

Wang, H. U., Chen, Z. F., Anderson, D. J. 1998. Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. *Cell* 93:741–753.

WHO 2011. Global Burden of Diseases.

Wilson, B. D., Ii, M., Park, K. W., Suli, A., Sorensen, L. K., Larrieu-Lahargue, F., Urness, L. D., Suh, W., Asai, J., Kock, G. A., Thorne, T., Silver, M., Thomas, K. R., Chien, C. B., Losordo, D. W., Li, D. Y. 2006.Netrins promote developmental and therapeutic angiogenesis. *Science* 313(5787): 640-644.

Wu, Y., Moser, M., Bautch, V. L., Patterson, C. 2003. Hox B5 is an upstream transcriptional switch for differentiation of vascular endothelium from precursor cells. *Mol Cell Biol* 23: 5680-5691.

Yancopoulos, G. D., Davis, S., Gale, N. W., Rudge, J. S., Wiegand, S. J., Holash, J. 2000. Vascular-specific growth factors and blood vessel formation. *Nature* 7: 242-248.

Yin, L., Morishige, K., Takahashi, T., Hashimoto, K., Ogata, S., Tsutsumi, S., Takata, K., Ohta, T., Kawagoe, J., Takahashi, K., Kurachi, H. 2007. Fasudil inhibits vascular endothelial growth factor-induced angiogenesis in vitro and in vivo. *Mol Cancer Ther* 6(5): 1517-1525.

# Appendix

## Overexpression of HOXA1 with adenovirus showed different results

A HOXA1-adenovirus was prepared to analyse angiogenic processes like sprouting or migration in a HOXA1 overexpressed manner. The function of the adenovirus-construct was analysed in RT-PCR and Western Blot analyses. HUVEC was transduced with the adenovirus and lysed after an incubation period (see Material and Methods). After the RT-PCR, an upregulation of HOXA1 can be seen in the HOXA1 overexpressing samples. However, this upregulation is not very strong (Figure 38A). In the Western Blot analysis no regulation of HOXA1 was seen (Figure 38B). These results provoked the statement that the adenovirus is not functional. Mutations in the adenovirus-construct can be excluded because the sequence was checked several times. It could be possible that the protein structure of the adenovirus is folded in a different manner than the native protein. This could be the reason why, the HOXA1 antibody cannot bind to the HOXA1 adenovirus. Structural analysis, like crystallography, can help to clarify if the proteins are folded in a different way.

Functional assays which were done with the HOXA1 adenovirus delivered positive results. In the sprouting assay, it was shown that after HOXA1 overexpression sprouting was highly increased (Figure 38C). On the other hand, the rescue experiment, where the cells were firstly treated with HOXA1 siRNA and then treated with the HOXA1 adenovirus, showed similar results to the experiments displaying overexpression (Figure 38C).

To confirm the data from the microarray analysis, a RT-PCR with HOXA1 overexpressing cells was performed. In this experiment it can be seen that E-selectin is highly upregulated after HOXA1 overexpression. This fits together with the data of E-selectin regulation after HOXA1 downregulation (Figure 38D and also 3.4.1.).

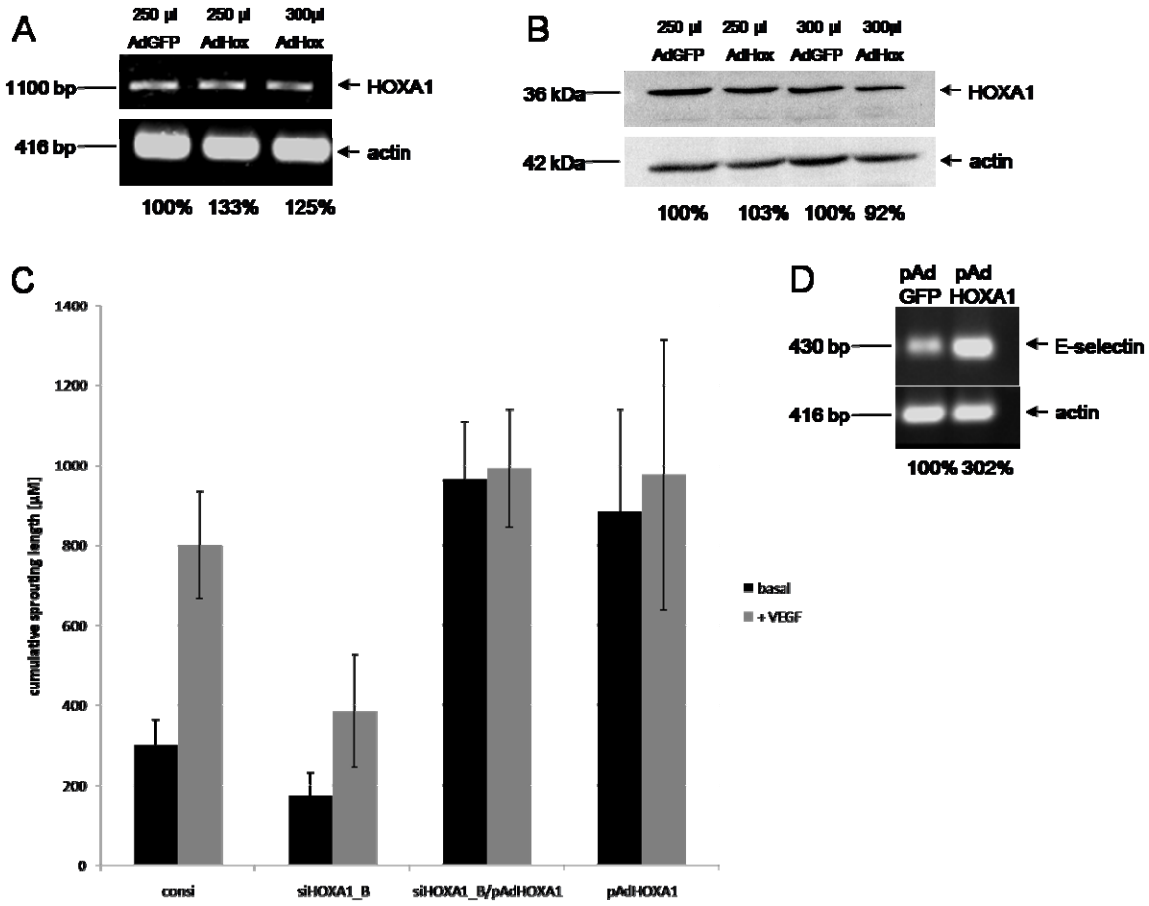



**Figure 38: Overexpression with adenovirus showed positve and negative results.** (**A**) Agarose-Gel after RT-PCR showed a higher HOXA1 expression after pAdHOXA1 transduction. (**B**) Western Blot analysis showed no difference in HOXA1 expression levels between pAdGFP and pAdHOXA1 transduced HUVEC. (**C**) pAdHOXA1 mediated upregulation increases in endothelial sprouting. The rescue-experiment looks similar to the experiment displaying overexpression. Mean values of three independent experiments are shown. (**D**) Agarose-Gel after RR-PCR showed that E-selectin is upregulated after HOXA1 upregulation.