Functional analysis of angiogenic factors in tumor cells and endothelia

Funktionelle Analyse angiogener Faktoren in Tumorzellen und Endothelien

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1 Summary

Tumor angiogenesis is essential for the growth of solid tumors as their proliferation and survival is dependent on consistent oxygen and nutrient supply. Anti-angiogenic treatments represent a therapeutic strategy to inhibit tumor growth by preventing the formation of new blood vessels leading to starvation of the tumor. One of the best characterized anti-angiogenic therapeutics is the monoclonal antibody bevacizumab (Avastin), which targets and neutralizes VEGF leading to disruption of the VEGF signaling pathway. Until today, bevacizumab has found its way into clinical practice and has gained approval for treatment of different types of cancer including colorectal cancer, non-small cell lung cancer, breast cancer and renal cell carcinoma. Signaling of VEGF is mediated through VEGF receptors, mainly VEGFR2, which are primarily located on the cell surface of endothelial cells. However, there has been evidence that expression of VEGF receptors can also be found on tumor cells themselves raising the possibility of autocrine and/or paracrine signaling loops. Thus, tumor cells could also benefit from VEGF signaling, which would promote tumor growth. The aim of this study was to investigate if bevacizumab has a direct effect on tumor cells *in vitro*. To this end, tumor cell lines from the NCI-60 panel derived from four different tumor types were treated with bevacizumab and angiogenic gene and protein expression as well as biological outputs including proliferation, migration and apoptosis were investigated. Most of the experiments were performed under hypoxia to mimic the *in vivo* state of tumors. Overall, there was a limited measurable effect of bevacizumab on treated tumor cell lines according to gene and protein expression changes as well as biological functions when compared to endothelial controls. Minor changes in terms of proliferation or gene regulation were evident in a single tumor cell line after VEGF-A blockade by bevacizumab, which partially demonstrated a direct effect on tumor cells. However, the overall analysis revealed that tumor cell lines are not intrinsically affected in an adverse manner by bevacizumab treatment.

Besides the functional analysis of tumor cells, embryonic stem cell derived endothelial cells were characterized to delineate vascular Hey gene functions. Hey and Hes proteins are the best characterized downstream effectors of the evolutionary conserved Notch signaling pathway, which mainly act as transcriptional repressors regulating downstream target genes. Hey proteins play a crucial role in embryonic development as loss of Hey1 and Hey2 in mice *in vivo* leads to a severe vascular phenotype resulting in early embryonic lethality. The major aim of this part of the thesis was to identify vascular Hey target genes using embryonic stem cell derived endothelial cells utilizing a directed endothelial differentiation approach, as ES cells and their differentiation ability provide a powerful *in vitro* system to study developmental processes. To this end, Hey deficient and Hey wildtype embryonic stem cells were stably transfected with an antibiotic selection marker driven by an endothelial specific promoter,

which allows selection for endothelial cells. ESC-derived endothelial cells exhibited typical endothelial characteristics as shown by marker gene expression, immunofluorescent staining and tube formation ability. In a second step, Hey deficient ES cells were stably transfected with doxycycline inducible Flag-tagged Hey1 and Hey2 transgenes to re-express Hey proteins in the respective cell line. RNA-Sequencing of Hey deficient and Hey overexpressing ES cells as well as ESC-derived endothelial cells revealed many Hey downstream target genes in ES cells and fewer target genes in endothelial cells. Hey1 and Hey2 more or less redundantly regulate target genes in ES cells, but some genes were regulated by Hey2 alone. According to Gene Ontology term analysis, Hey target genes are mainly involved in embryonic development and transcriptional regulation. However, the response of ESC-derived endothelial cells in regulating Hey downstream target genes was rather limited when compared to ES cells, which could be due to lower transgene expression in endothelial cells. The limited response also raises the possibility that target gene regulation in endothelial cells is not only dependent on Hey gene functions alone and thus loss or overexpression of Hey genes in this *in vitro* setting does not influence target gene regulation.

2 Zusammenfassung

Tumorangiogenese ist essential für das Wachstum von Tumoren, da ihr Überleben und ihre Proliferation von einer dauerhaften Versorgung mit Sauerstoff und Nährstoffen abhängig sind. Anti-angiogene Therapeutika inhibieren das Wachstum von Tumoren, indem sie die Bildung von neuen Blutgefäßen unterbinden, was zu einem „Verhungern“ des Tumors führt. Zu den am besten untersuchten anti-angiogenen Therapeutika gehört der monoklonale Antikörper Bevacizumab (Avastin), welcher den Wachstumsfaktor VEGF bindet und neutralisiert, was schließlich zu einer Unterbrechung des VEGF-Signalweges führt. Bevacizumab wird aktuell zur Behandlung verschiedener Tumortypen, unter anderem Colonkarzinom, nicht kleinzelliges Lungenkarzinom, Brustkrebs und Nierenzellkarzinom in der Praxis angewandt. VEGF bindet an VEGFR2 und andere VEGF-Rezeptoren, welche primär an der Oberfläche von Endothelzellen lokalisiert sind. Dennoch gibt es Hinweise, dass die Expression von VEGF-Rezeptoren nicht ausschließlich auf Endothelzellen begrenzt ist, sondern auch auf Tumorzellen nachgewiesen werden konnte. Die tumorale Expression von VEGF-Rezeptoren ermöglicht eine direkte autokrine und/oder parakrine Stimulation von Tumorzellen. Tumorzellen könnten dadurch selbst vom VEGF-Signalweg profitieren, was das Tumorwachstum fördern würde. Das Ziel dieser Arbeit war es, zu untersuchen, ob Bevacizumab *in vitro* einen direkten Einfluss auf Tumorzellen ausübt. Dazu wurden verschiedene Tumorzelllinien von vier unterschiedlichen Tumortypen aus der NCI-60 Sammlung mit Bevacizumab behandelt und anschließend die Gen- und Proteinexpression von angiogenen Markern sowie biologische Funktionen wie Proliferation, Migration und Apoptose untersucht. Die Experimente wurden hauptsächlich unter Hypoxie durchgeführt, um den *in vivo* Status von Tumoren nachzuahmen. Insgesamt war ein direkter Effekt von Bevacizumab auf Tumorzellen im Vergleich zu Endothelzellen nicht nachweisbar. Gen- und Proteinexpression sowie biologische Funktionen waren in Tumorzellen nach Bevacizumab-Behandlung bis auf wenige Ausnahmen unverändert. Geringe Veränderungen in der Proliferationsrate sowie in der Genregulation nach Inhibition von VEGF durch Bevacizumab konnten jeweils in einer einzelnen Zelllinie nachgewiesen werden, wodurch zumindest teilweise eine direkte Wirkung von Bevacizumab auf Tumorzellen gezeigt werden konnte. Dennoch zeigt die umfassende Analyse der verschiedenen Tumorzellen, dass die Bevacizumab-Behandlung sich insgesamt nicht negativ auf Tumorzellen auswirkt und diese nicht intrinsisch beeinflusst werden.

Neben der funktionellen Analyse von Tumorzellen wurden Endothelzellen, die aus embryonalen Stammzellen gewonnen wurden, charakterisiert, um anhand derer die Rolle der Hey Gene in der vaskulären Entwicklung näher zu bestimmen. Hey und Hes Proteine sind die am besten charakterisierten nachgeschalteten Effektoren des evolutionär konservierten Notch-Signalweges. Als Transkriptionsfaktoren sind Hey Proteine an der Regulation von

Zielgenen beteiligt, wobei sie meist als transkriptionelle Repressoren fungieren. Hey Proteine spielen eine sehr wichtige Rolle während der Embryonalentwicklung, da der gemeinsame Verlust von Hey1 und Hey2 in Mäusen *in vivo* einen vaskulären Phenotyp hervorruft, der in sehr früher embryonaler Letalität endet. Das Hauptziel dieser Arbeit war es, vaskuläre Hey Zielgene mit Hilfe von Endothelzellen, die aus embryonalen Stammzellen differenziert wurden, zu identifizieren. Die Fähigkeit embryonaler Stammzellen verschiedene Zelltypen zu bilden, liefert dabei ein wertvolles *in vitro* System, um entwicklungsbiologische Prozesse zu analysieren. Dazu wurden Hey-defiziente und Hey-Wildtyp embryonale Stammzellen mit einem antibiotischen Selektionsmarker versehen, dessen Expression von einem Endothel-spezifischen Promoter kontrolliert wird und daher die Selektion von Endothelzellen erlaubt. Die differenzierten Endothelzellen wiesen typische endotheliale Charakteristika auf, was durch Markergen-Expression, Immunfluoreszenzfärbungen und der Bildung netzwerkähnlicher Strukturen gezeigt werden konnte. In einem zweiten Schritt wurden Doxyzyklin-induzierbare Flag-markierte Hey1 bzw. Hey2 Konstrukte stabil in Hey-defiziente Zellen integriert, um Hey1 bzw. Hey2 kontrollierbar zu re-exprimieren. Die Zielgensuche mittels RNA-Seq Analyse lieferte viele Hey Zielgene in embryonalen Stammzellen, jedoch weitaus weniger Zielgene in ausdifferenzierten Endothelzellen. Zusammengefasst zeigten Hey1 und Hey2 eine redundante Regulation von Zielgenen in embryonalen Stammzellen, wobei einige wenige Gene alleine durch Hey2 reguliert wurden. Die Gen-Ontologie Analyse zeigte, dass diese Zielgene hauptsächlich an der embryonalen Entwicklung und der transkriptionellen Regulation von anderen Genen beteiligt sind. Dennoch war die Anzahl der Hey regulierten Gene in Endothelzellen weitaus geringer als in embryonalen Stammzellen. Das könnte auf eine geringere Transgen-Expression in Endothelzellen im Vergleich zu embryonalen Stammzellen zurückzuführen sein. Die weitaus geringere Anzahl an Hey Zielgenen in Endothelzellen lässt außerdem vermuten, dass die Regulation von Zielgenen in Endothelzellen nicht ausschließlich von der Funktion der Hey Gene abhängig ist. Die Regulation von Hey Zielgenen in Endothelien wurde durch den Verlust bzw. die Überexpression von Hey1 und Hey2 in dem angewandten *in vitro* System nur geringfügig beeinflusst.

3 Introduction

3.1 Anti-angiogenic therapy of human tumor cells

3.1.1 Tumor angiogenesis

The growth of tumor cells is dependent on consistent oxygen and nutrient supply, which are provided by blood vessels. The formation of blood vessels is a two-stage process, involving vasculogenesis and angiogenesis. During vasculogenesis the primary vascular plexus is formed from the mesoderm by differentiation of angioblasts, which subsequently build primitive blood vessels. Vasculogenesis mainly occurs during early development. After vasculogenesis the primary plexus is remodeled by a process named angiogenesis. During angiogenesis more endothelial cells are generated and new capillaries are formed e.g. by sprouting from pre-existing vessels (Risau, 1997). The formation of blood vessels form pre-existing ones is not only restricted to early development, but also occurs in adult organisms regulating physiological processes, e.g. wound-healing. Angiogenesis also occurs in pathological processes leading to a severe formation of vessels, e.g. in tumor growth. Tumor growth is dependent on persistent blood vessel growth, which allows tumors also to metastasize and to spread. Tumor fragments or tumor cells, which are placed into avascular sites, are able to attract new capillaries from the existing host vascular network, which demonstrates the dependency of tumor cell growth from blood vessels (Hanahan and Folkman, 1996, Gimbrone et al., 1972). Inadequate blood and thus oxygen supply can lead to tissue hypoxia, a massive reduction of the normal tissue oxygen tension. Tissue hypoxia often emerges as a consequence of acute and chronic vascular diseases as well as cancer. Most solid tumors develop low oxygen regions and are able to adapt to the hypoxic environment in contrast to healthy tissue. Therefore, tumor cells undergo genetic and adaptive changes, which allow them to survive and to proliferate even under hypoxic conditions. Low intratumoral oxygen levels can affect several biological parameters and often lead to a more aggressive and malignant phenotype of tumors. Because of its strong association with tumor progression and malignancy, tumor hypoxia has evolved to an important therapeutic target in cancer treatment (Harris, 2002, Hockel and Vaupel, 2001). In response to the hypoxic environment a variety of biological processes including cell proliferation, cell migration, apoptosis, switch from an aerobic to an anaerobic metabolism as well as angiogenesis are affected. In tumor cells, one of the main responses to hypoxia is the production of growth factors, which induce angiogenesis (Harris, 2002). Hypoxia-induced biological processes are controlled by HIF-1 (Hypoxia-inducible factor-1), which is the major transcription factor regulating gene expression in mammalian cells in response to hypoxia. This basic-helix-loop-helix transcription factor consists of HIF-1α and HIF-1β subunits, which activate transcription of hypoxic sensitive genes upon heterodimerization under hypoxia

(Wang et al., 1995). One of the major hypoxic sensitive genes is vascular endothelial growth factor (VEGF), which is strongly induced under hypoxa (Harris, 2002). Besides VEGF other angiogenic genes like VEGF receptors, VEGFR1 (FLT-1) and VEGFR2 (FLK-1), as well as the co-receptor Neuropilin1 (NRP1) are involved in the process of tumor angiogenesis and thus of particular interest in this study.

3.1.2 Angiogenic pathway molecules

Vascular endothelial growth factor A (VEGF-A) is the major inducer of angiogenesis and also plays an important and essential role in tumor angiogenesis. Signaling through its receptors presents the best characterized pathway in angiogenesis (Ferrara and Kerbel, 2005). VEGF was identified in 1989 by Ferrara and collaborators, which described VEGF as a secreted molecule mediating endothelial cell growth and angiogenesis (Leung et al., 1989, Ferrara and Henzel, 1989, Keck et al., 1989). The VEGF family consists of seven subtypes including VEGF-A, VEGF-B, placental growth factor (PLGF), VEGF-C and VEGF-D from mammals as well as parapoxvirus genome encoded VEGF (viral VEGF, also denoted as VEGF-E) and snake venom-derived VEGF, also referred as VEGF-F. All VEGF family members are secreted glycoproteins, which contain a cystein knot motif with three intertwined disulfide bridges. VEGF members function as ligands and are able to bind to corresponding tyrosine kinase receptors like VEGFR1 (FLT-1), VEGFR2 (FLK-1) and VEGFR3 (FLT-4) and non-tyrosine kinase receptors, Neuropilin1 (NRP1) and Neuropilin2 (NRP2). NRP1 and NRP2 serve as co-receptors for VEGF binding. Binding of VEGF subtypes is highly selective for specific receptors and occurs with different binding affinities, which reflects the diversity of the VEGF family in biological functions (Yamazaki and Morita, 2006).

The best characterized and most important member of the VEGF family is VEGF-A. Human VEGF-A is located on chromosome six and contains eight exons. Due to alternative splicing different isoforms of VEGF-A with varying exon content are generated, which can differ in their functional properties as well as in their expression pattern (VEGF-A_{121}, VEGF-A_{145}, VEGF-A_{148}, VEGF-A_{165}, VEGF-A_{183}, VEGF-A_{189} and VEGF-A_{206}). Amongst all isoforms of VEGF-A, VEGF-A_{165} is known as the predominant and best characterized isoform. In general, expression of VEGF-A is often increased in several pathologies including cancer and thus is investigated in detail for its function in cancer progression (Harper and Bates, 2008). VEGF-A has been described as the major ligand for VEGFR2 (FLK-1, KDR) and signaling through VEGFR2 plays an essential role in the development of the vascular system and the process of angiogenesis (Millauer et al., 1993). VEGFR2 is a 200-230 kDa tyrosin kinase receptor, which is primarily located on the cell surface of endothelial cells. According to its high binding affinity for VEGF-A it is the major mediator of the mitogenic, angiogenic and permeability-enhancing effects of VEGF-A (Ferrara and Kerbel, 2005). VEGFR2 contains a series of immunoglobulin-like domains in the extracellular region and a conserved

intracellular tyrosine kinase domain (Lohela et al., 2009). Phosphorylation of the two main autophosphorylation sites of VEGFR2, Tyr1175 and Tyr1214, leads to activation of downstream pathways of the VEGF-A-VEGFR2 signaling axis. Activation of downstream intracellular signaling pathways are involved in the regulation of biological processes like migration, vascular permeability, survival and proliferation as illustrated in Fig. 1 (Takahashi and Shibuya, 2005).

Another tyrosine kinase receptor involved in angiogenesis is VEGFR1 (FLT-1). VEGFR1 is a 180 kDa protein, which is also activated through binding of VEGF-A, but in addition can also be stimulated by PLGF. VEGFR1 is expressed on vascular endothelial cells, but also on non-endothelial cells like macrophages and monocytes as well as haematopoietic stem cells (Takahashi and Shibuya, 2005). Although expression of VEGF receptors in general is mainly found on endothelial cells, there has also been evidence for expression on tumor cells for non-small cell lung cancer (NSCLC), colorectal cancer (CRC) and breast cancer (BC) (Duff et al., 2006, Seto et al., 2006, Ghosh et al., 2008). Tumor cells expressing VEGF receptors on their cell surface could benefit from autocrine or paracrine signaling loops of VEGF-A signaling to enhance tumor growth and survival. A co-receptor of the VEGF pathway, Neuropilin1 (NRP1), is a 130 – 135 kDa cell surface glycoprotein, which is activated by binding of specific isoforms of VEGF-A (Soker et al., 1996). Binding of VEGF-A_{165}, but not VEGF-A_{121}, is able to activate NRP1 signaling, which indicates that signaling through NRP1 is isoform-dependent on VEGF-A. Besides VEGF-A other members of the VEGF family like PLGF can function as ligands for NRP1. In addition, as a co-receptor for VEGF signaling, NRP1 is able to enhance VEGF-A binding to VEGFR2. Expression of NRP1 is not primarily present on endothelial cells, but also can be found to a great extent on the cell surface of tumor cells of breast and prostate carcinoma as well as melanoma (Soker et al., 1996, Soker et al., 1998).

A scheme of VEGF-A signaling through its receptors VEGFR1 and VEGFR2 as well as its co-receptor NRP1 is presented in Fig. 1.

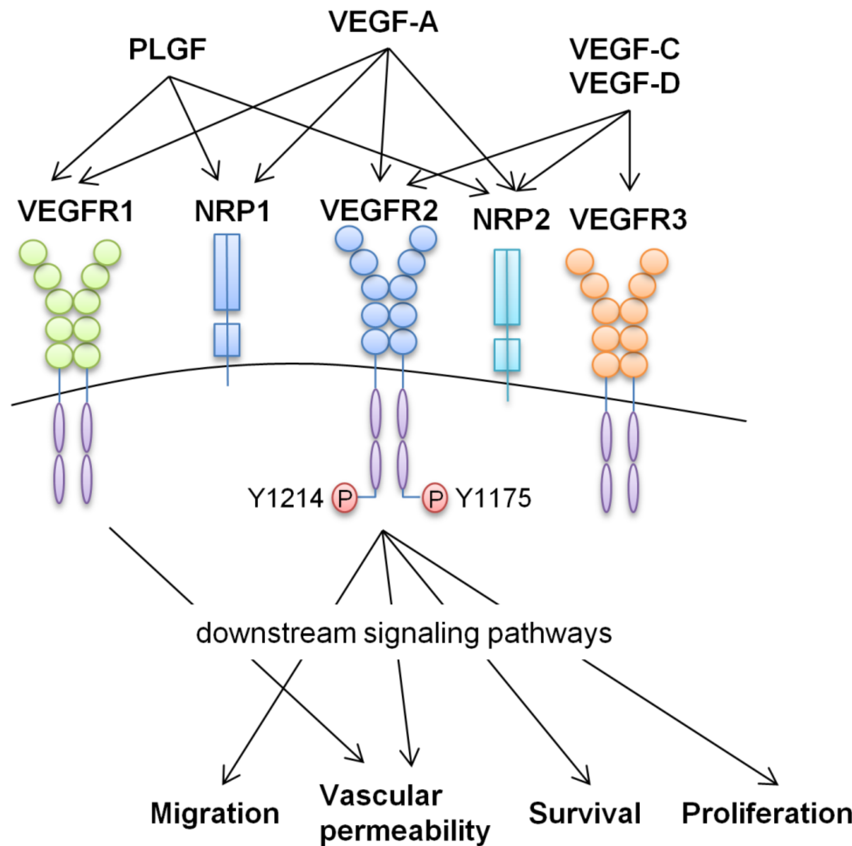


Fig. 1 Interactions of VEGF family members with VEGF receptors and NRP co-receptors (modified from Takahashi and Shibuya, 2005). VEGF family members bind to their receptors with different binding specificity (indicated by the arrows). Binding of VEGF to VEGFR2 leads to dimerization of the receptor and to autophosphorylation of the main phosphorylation sites (Y1214, Y1175) inducing downstream signaling pathways, which are involved in regulation of cell migration, vascular permeability, cell survival and cell proliferation (detailed downstream signaling pathways are presented in a review from Takahashi and Shibuya, 2005).

3.1.3 Biological function of VEGF-A

VEGF plays a very crucial role during angiogenesis and in normal blood vessel development as shown by VEGF deletion studies. (Carmeliet et al., 1996, Ferrara et al., 1996). VEGF deletion in mice revealed that already the loss of a single allele of VEGF resulted in early lethality between embryonic day 11 and 12 due to impaired angiogenesis and reduced blood-island formation. Furthermore, endothelial cell differentiation is delayed in VEGF knockout mice and most steps of early vascular development are impaired (Ferrara et al., 1996). Besides vascular development and blood vessel formation, VEGF is also involved in many pathological conditions like the growth of solid tumors, hematological malignancies, inflammatory disorders and pathologies of the female reproductive tract (Ferrara, 2004). A functional involvement of VEGF in angiogenesis and tumorigenesis was shown by VEGF depletion in embryonic stem cells and subsequent injection into nude mice. VEGF deficient cells were impaired in their growth *in vivo* and exhibited a decreased ability to form tumors in nude mice, which indicated that even in a pluripotent system VEGF is essential for tumorigenesis (Ferrara et al., 1996). Involvement of VEGF in tumorigenesis is also apparent

from in *vitro studies*, as several tumor cell lines secrete VEGF-A in cell culture (Ferrara et al., 1992). Secretion of VEGF from tumor or endothelial cells supports the theory of an autocrine or paracrine stimulation of neighboring cells. Paracrine stimulation enhances angiogenesis by secretion of angiogenic growth factors or cytokines like PDGF, EGF, TNF-α and IL-1β from endothelial cells, which leads to induced VEGF mRNA transcription in tumor cells (Ferrara and Davis-Smyth, 1997). Subsequently, the release of growth factors from tumor cells like VEGF can activate VEGF signaling on host cells, e.g. endothelial cells. Spread and proliferation of endothelial cells lead to vessel extension and recruitment of supporting cells, like pericytes, to form a new vascular network (McMahon, 2000). In contrast, autocrine stimulation can exert a direct effect on tumor cells. If tumor cells express VEGF receptors on their cell surface, autocrine stimulation can lead to enhanced VEGF signaling, which in turn promotes tumor growth (McMahon, 2000). Besides secretion of VEGF from tumor and endothelial cells, VEGF expression is also demonstrated in tumor tissue of human tumors including lung and breast cancer cells by immunohistochemistry. Intensity of VEGF staining is variable between different tumor samples from very high to very low expression, but there is no significant correlation between the amount of VEGF expression and the degree of tumor progression (Volm et al., 1997, Yoshiji et al., 1996). Furthermore, the possibility of an autocrine VEGF signaling loop is supported by VEGF receptor expression on the cell surface of tumor cells and thus not only restricted to the surrounding vasculature.

3.1.4 The complexity of cancer

Besides cardiovascular diseases, cancer is the second most common death in Germany. According to data from the Federal Statistical Office more than 26 % of all deaths in Germany in 2011 were related to cancer. As the number of new cases is still increasing, the search for therapeutic agents targeting cancer is an essential component of today's research to promote prevention and treatment of cancer. The major problem of treating cancer is its complexity when compared to other diseases, as it does not underlie a universal defined cause and as it can originate in almost every organ of the human body. Different organs of the human body are affected to varying degree and also differentially affected in males and females. The most common cancer disease worldwide in males is lung cancer and in females breast cancer. Colorectal cancer is the third most commonly diagnosed cancer in males and the second one in females, while renal cell carcinoma occur less frequently in both, males and females (Jemal et al., 2011). Because of differences in origin and progression of cancer it is impossible to treat it with a universal therapy. In addition, cancer cells are often able to metastasize and to spread all over the body, which makes therapy even more difficult. Initial assumptions about the cause of cancer were described by Theodor Boveri in 1914, who linked chromosomal changes, as numerical and structural aberrations of chromosomes, to the origin of cancer (Boveri, 2008). Besides chromosomal aberrations also

genomic changes like mutations in oncogenes and tumor suppressor genes have been described as a major cause of cancer. Oncogenes and tumor suppressor genes mainly function as important regulators of cell division, cell proliferation, apoptosis and cell cycle control and thus, mutations can result in deregulation and uncontrolled proliferation of cancer cells (Croce, 2008, Levine and Oren, 2009). Alterations in cell physiological processes like self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, tissue invasion and metastasis as well as sustained angiogenesis have also been linked to the complexity of cancer (Hanahan and Weinberg, 2000). Tumor cells have developed resistance against tightly controlled physiological processes, which facilitates their proliferation and tumor growth.

Although no unique therapy to cure cancer is available, a common useful therapeutic approach is chemotherapy. Chemotherapy aims at either killing cancer cells or preventing cancer cells from proliferation. The large disadvantage of chemotherapeutic agents is their impact on malignant as well as healthy tissue, which has led to the discovery and the development of novel anticancer drugs. Anticancer drugs specifically inhibit molecules of pathways, which are essential for tumor progression. These new approaches of targeting cancer, e.g. by preventing the building of new vessels has extended the traditional view of targeting cancer only by chemotherapy (Ramaswami et al., 2013). Targeting angiogenesis is one of the novel tumor therapy approaches, which has already been investigated over the past decade (Ferrara et al., 2004).

3.1.5 Bevacizumab – a monoclonal antibody for anti-angiogenic treatment and cancer therapy

Anti-angiogenic therapy was first described as a novel cancer treatment approach targeting angiogenesis by Judah Folkman about 40 years ago (Folkman, 1971). Even earlier it was evident that the growth of solid tumors is closely connected to new vessel growth and vascular supply (Algire et al., 1950). The association between growing malignant tumors and new vessel growth has led to intensive research on anti-angiogenic targets as well as on the development of anti-angiogenic drugs. The first anti-angiogenic agent targeting blood vessel supply of tumors was bevacizumab, a humanized monoclonal antibody against VEGF. Bevacizumab was developed by Ferrara et al. in 1997 by humanization of the mouse anti-VEGF monoclonal antibody A.4.6.1. It binds and neutralizes all VEGF-A isoforms and bioactive proteolytic fragments, thereby limiting the interaction between VEGF and VEGF receptors. The impaired interaction of VEGF and VEGF receptors results in disruption of the VEGF pathway and thus tumor angiogenesis. Since 1997 bevacizumab has been passed through several clinical trials from phase I to phase III studies and was finally approved by the Food and Drug Administration as a first-line treatment for metastatic colorectal cancer in February 2004. In several phase II studies bevacizumab was combined with

chemotherapeutic agents, e.g. 5-fluoruracil/leucovorin and irinotecan, which resulted in prolonged survival of patients with colorectal and non-small cell lung cancer (Ellis and Hicklin, 2008). Until today, bevacizumab has proven efficacy in clinical applications in combination with chemotherapy for colorectal cancer (Hurwitz et al., 2004), non-small cell lung cancer (Sandler et al., 2006), breast cancer (Miller et al., 2007) and in combination with interferon treatment for renal cancer (Escudier et al., 2007). Clinical trials and case studies for treatment of patients with bevacizumab and chemotherapeutic agents lead to prolonged survival and progression free survival but do not increase overall survival of patients. Several open-label phase I and phase II studies identified a number of adverse side effects including thrombosis, bleeding, proteinuria and hypertension, which were caused by bevacizumab treatment, but overall it is well tolerated by the patients (Ferrara et al., 2005). Although application of bevacizumab could efficiently improve response rates and prolong survival of patients, further research is needed in the field of anti-angiogenic treatments to enhance overall survival. Tyrosine kinase inhibitors of VEGFR1 and VEGFR2 (sorafenib, sunitinib) are already under investigation and part of clinical trials and applications (Escudier et al., 2009, Motzer et al., 2009). Targeting other members of the VEGF pathway, e.g. PLGF and NRP1, could also improve efficacy in case of resistance that develop from existing treatments (Ellis and Hicklin, 2008). Combination therapies simultaneously targeting ligands and receptors of the VEGF signaling pathway would be beneficial for cancer therapy. In addition, the identification of new biomarkers and the development of new drugs targeting angiogenic pathway molecules are required to improve anti-angiogenic treatment.

3.1.6 Tumor cell lines for the experimental research of cancer

A useful tool to study the impact of anticancer drugs *in vitro* is the availability of a wide variety of human tumor cell lines. A group of specific immortal human tumor cell lines derived from nine different tissues, known as the NCI-60 panel from the National Cancer Institute, build the basis of tumor related cell culture research since decades. Cell lines from the NCI-60 panel were systemically characterized according to their genetics and genomics by the Cancer Genome Project to improve their utility and interpretation of data in terms of drug discovery. Furthermore, the experimental use of tumor cell lines from the NCI-60 panel provides the possibility to relate recently achieved data with new data. To this end, experiments in this study were performed with tumor cell lines derived from the NCI-60 panel. More detailed information is provided at http://dtp.nci.nih.gov/branches/btb/characterizationNCI60.html. An overview of all 60 tumor cell lines of the panel is given at: http://cancer.sanger.ac.uk/cell_lines/cbrowse/nci.

3.1.7 Aim of the thesis – part I

The major goal of this part of my thesis was to investigate potential direct effects of bevacizumab on human tumor cells *in vitro*. As VEGF receptors can be present on the cell surface of human tumor cells, secretion of VEGF-A could lead to an autocrine or paracrine stimulation of tumor cells. Anti-angiogenic agents like bevacizumab could therefore have additional antitumoral effects besides their impact on endothelial cells.

Tumor cell lines from the NCI-60 panel derived from four different tumor types (NSCLC, CRC, RCC and BC) were used to study changes in angiogenic gene and protein expression after bevacizumab exposure. In addition, alterations of cellular responses like proliferation, migration and apoptosis, due to bevacizumab treatment, were measured in a larger number of tumor cell lines.

3.2 The role of Hey genes during vascular development

3.2.1 Blood vessel development and function of the endothelium

The first organ to develop during embryogenesis is the blood vessel system. The formation of blood vessels is a two-stage process, including vasculogenesis and angiogenesis. During vasculogenesis the primary vascular plexus is formed from the mesoderm by differentiation of angioblasts into blood islands. Central cells of the blood islands give rise to embryonic blood cells, while peripheral cells of the blood islands differentiate into endothelial cells. Subsequently, endothelial cells build the primary vascular plexus, which is remodeled to form the extraembryonic yolk sac vasculature. Intraembryonic vascular development is initiated by differentiation of hemangioblasts into the vascular primary plexus, which builds the aorta and the cardinal vein. After vasculogenesis, a more complex vascular network is formed by interaction of endothelial cells with mural cells and by generation of new capillaries, e.g. by sprouting of vessels from pre-existing ones, a process called angiogenesis. The complete vasculature is formed by intensive remodeling after specification of arterial and venous blood vessels (Risau, 1997, Flamme et al., 1997, Choi, 2002, Park et al., 2013).

The inner surface of blood vessels is lined with vascular endothelial cells. Endothelial cells mainly function as a physical barrier between vessel walls and the intravascular space controlling the exchange of oxygen and nutrients between blood and tissues. A precise organ-specific oxygen supply results from the selective permeability of endothelial cells, which is due to their heterogeneity. Endothelial cells can mainly be sub-divided into three different phenotypes according to their morphology and their vascular permeability: continuous, fenestrated and discontinuous endothelium. Continuous endothelial cells are connected to each other with tight junctions and exchange of substances between blood and tissue mainly occurs through specific transport mechanisms. Fenestrated and discontinuous capillaries exhibit a higher permeability. The different phenotypes of endothelial cells exhibit various functions and show a different organ distribution. Continuous endothelium for example can be found in the central nervous system, where it functions as the so-called blood-brain barrier controlling diffusion of molecules into the brain. Discontinuous endothelial cells are primarily found in tissues with high filtration, secretion and absorption, e.g. in the kidney, and are more permeable for low molecular hydrophilic molecules. Thus, the differentiation into a specific endothelial phenotype is adapted to the specific needs of the organ and occurs through interaction of endothelial cells and the surrounding tissue (Risau, 1995).

3.2.2 The canonical Notch signaling pathway

The Notch signaling pathway is a central regulator of gene expression, which controls cell fate determination and cell differentiation processes. It plays a very crucial role in embryonic

development as demonstrated by several knockout studies. Loss of ligands and receptors of the Notch signaling pathway often result in early lethality of the mouse embryo. In humans, mutations of Notch ligands and Notch receptors are associated with several diseases like Alagille syndrome, CADASIL, T-cell leukemia, aortic valve calcification and other cardiovascular diseases (Fischer and Gessler, 2007). Notch signaling is highly conserved throughout different species from *Caenorhabditis elegans*, *Drosophila melanogaster* to vertebrates and mammals. In mammals, there are four Notch receptors (Notch1-4), while *Caenorhabditis elegans* exhibits only two (LIN-12, GLP-1) and *Drosophila melanogaster* only one receptor (Notch). Notch receptors are transmembrane proteins, which are activated by binding of a ligand, another transmembrane protein, of a neighboring cell (Delta, Serrate, Jagged). Thus, activation of Notch signaling requires direct interaction between two cells. The interaction with Notch ligands on the neighboring cell is mediated by epidermal growth factor (EGF)-like repeats. The binding of a ligand to the Notch receptor on the neighboring cell results in two proteolytic cleavages. The first cleavage is catalyzed by an ADAM metalloprotease, while the second cleavage is mediated by γ-secretase, which leads to the release of the Notch intracellular domain (NICD). γ-secretase is an enzyme complex consisting of presenilin, the catalytic subunit, and three transmembrane proteins, nicastrin, APH1 and PEN-2. The Notch intracellular domain is translocated to the nucleus, where it interacts with a CSL DNA-binding protein complex named CBF1/RBPkJ in mammals (LAG-1 in *Caenorhabditis elegans*, Su(H) in *Drosophila melanogaster*). In addition, co-activators like Mastermind in *Drosophila melanogaster* and mammals (LAG-3 in *Caenorhabditis elegans)* as well as the histone acetylase p300 and the Ski-interacting protein (SKIP) are recruited by Notch to promote transcription of target genes (Bray, 2006). Transcription of target genes is suppressed by association of the RBPkJ complex with co-repressors. To initiate transcription of target genes co-repressors are released upon binding of the Notch intracellular domain. Amongst others, the most and best studied downstream target genes of Notch signaling are Hairy and Enhancer-of-split genes in *Drosophila melanogaster* and Hes and Hey genes in mammals (Fischer and Gessler, 2007).

A scheme of the canonical Notch signaling pathway is presented in Fig. 2.

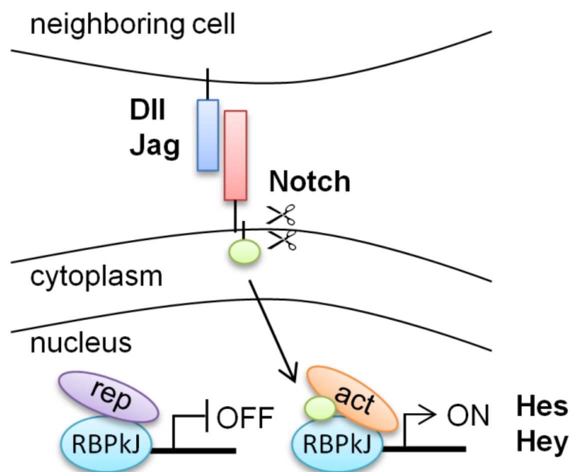


Fig. 2 Scheme of Notch signaling (modified from Fischer and Gessler, 2007). Ligands of the Delta (Dll) orJagged (Jag) family induce intramembrane cleavage of the Notch receptor (Notch). The intracellular domain (green colored circle) replaces transcriptional co-repressors (rep) with activators (act) enabling transcription of Hes and Hey genes by RBPkJ.

3.2.3 Hey basic helix-loop-helix transcription factors

Two of the best characterized downstream target genes of the canonical Notch signaling pathway are the mammalian Hes and Hey genes. Hes and Hey genes are closely related to the Hairy and Enhancer-of-split genes in *Drosophila melanogaster* according to their domain organization (Fischer and Gessler, 2007). All members contain a basic helix-loop-helix domain and an Orange domain. Within the basic domain, Hey proteins differ from Hes proteins, as they contain a glycine residue instead of a proline residue. Because of this amino acid change Hey proteins cannot bind to N-box sequences (CACNAG) in promoters and preferentially bind to E-box sequences (CACGTG) instead (Nakagawa et al., 2000). The C-terminal part of Hey proteins contains a YRPW (Y) motif, followed by a TEIGAF (T) motif and thus differs from Hes proteins, which contain a WRPW (W) motif. The structure of Hes and Hey proteins and their domain organization is shown in Fig. 3.

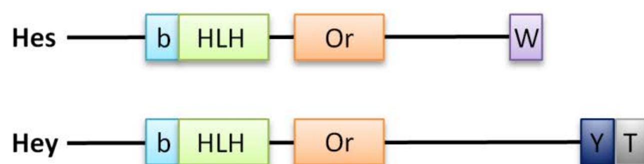


Fig. 3 Structure of Hes and Hey proteins and their domain organization (modified from Fischer and Gessler, 2007). Hes and Hey proteins contain a basic (b) helix-loop-helix (HLH) domain and an Orange (Or) domain. They differ in their C-terminal part as Hes proteins contain a WRPW (W) motif and Hey proteins contain a YRPW (Y) motif followed by a TEIGAF (T) motif.

In mammals, a new subfamily of the hairy-related basic helix-loop-helix (bHLH) proteins with three members, Hey1, Hey2 and HeyL, was identified in 1999 by Leimeister et al. Mammalian Hey proteins are also known as Hrt, Hesr, Herp and Chf (Leimeister et al., 1999, Nakagawa et al., 1999, Kokubo et al., 1999). Hey genes encode transcriptional regulators,

which mainly function as repressors. Regulation of transcription occurs by interaction of Hey proteins with other basic helix-loop-helix proteins or by recruitment of additional co-repressors. For transcriptional regulation Hey proteins can bind to the DNA, which is mediated by their basic domain, whereas their helix-loop-helix domain is essential for homo- and heterodimerization (Massari and Murre, 2000). Both, Hey and Hes proteins, can form homo- as well as heterodimers and bind to similar DNA-sequences to repress target gene transcription, whereby heterodimers show higher and stronger DNA binding affinity and repression activity than homodimers (Iso et al., 2001). The stability of heterodimers can be increased by the Orange domain, which is also necessary for additional protein interactions (Leimeister et al., 2000). In Hes proteins, transcriptional repression is mainly mediated by the WRPW (W) motif in the C-terminal part, as it can recruit the co-repressor TLE (Grbavec and Stifani, 1996). In contrast, the C-terminal YRPY (Y) motif of Hey proteins is not able to mediate transcriptional repression. Repressive functions of Hey proteins are mainly caused through direct interaction between the helix-loop-helix domain and other repressive proteins like the mSin3 complex, which includes HDAC1 and HDAC2. This complex is associated with additional co-repressors like N-CoR and SMRT (Iso et al., 2001). Furthermore, transcriptional repression can also be mediated by interaction with SIRT1, which has been shown at least for Hey2 and Hes1 (Takata and Ishikawa, 2003). Any function of the C-terminal TEIGAF (T) motif has not been elucidated so far.

3.2.4 Expression and function of Hey genes during cardiovascular development

As downstream effectors of the Notch signaling pathway, Hey genes play an important role during embryonic development as shown by several knockout studies in mice. The single loss of Hey2 in mice reveals a strong cardiac phenotype, which leads to embryonic lethality during the first ten days of life. Hey2 knockout mice show ventricular and atrioventricular septum defects with massive hypertrophy and cardiomyopathy (Donovan et al., 2002, Gessler et al., 2002, Sakata et al., 2002). In contrast, deletion of either Hey1 or HeyL does not result in any cardiac phenotype, but the combined loss of Hey1 and HeyL in turn shows a similar phenotype as the single loss of Hey2 (Fischer et al., 2007). Besides cardiac phenotypes, Hey deletion also causes vascular phenotypes in mice. The combined loss of Hey1 and Hey2 leads to early lethality at embryonic day 9.5 due to impaired angiogenic remodeling and impaired arterial cell fate determination. Hey1 and Hey2 deficient embryos suffer from massive bleeding in the trunk and in the head region and show truncated and less well organized blood vessels. The size of large blood vessels in knockout embryos is often strongly reduced or the dorsal artoae and the cardinal veins are even absent (Fischer et al., 2004). The lethal vascular phenotype as a result of the combined loss of Hey1 and Hey2 emphasizes their important role in embryonic vascular development. At later developmental stages Hey1 and Hey2 expression in blood vessels is restricted to endothelial

cells of the arteries and to smooth muscle precursor cells, but no expression can be found in endothelial cells of veins. As downstream effectors of active Notch signaling Hey proteins are involved in arterial cell fate determination and the complex process of the development of the vascular system (Fischer et al., 2002).

3.2.5 The role of Notch signaling and Hey genes in endothelial cell specification

Loss of Hey1 and Hey2 in mice leads to a lack of arterial cell fate determination, which emphasizes the importance of Hey proteins in determining endothelial cell specification (Fischer et al., 2004). Arterial cell determination is mediated by Hey proteins as shown by overexpression of Hey2 in zebrafish (Zhong et al., 2001) as well as in human endothelial cells (Chi et al., 2003). Overexpression of Hey2 diminishes vein formation and induces expression of artery-specific genes. The arterialization process initiated by Notch signals is counteracted by CoupTFII, also known as Nr2f2. CoupTFII is a major regulator of venous endothelial cells, which inhibits Notch signaling and the expression of arterial specific genes. In contrast, loss of CoupTFII leads to the induction of Notch signaling and the expression of arterial marker genes in veins (You et al., 2005). However, little is known from the literature about the antagonism and a possible direct interaction between CoupTFII and Hey proteins.
Differentiation of arterial endothelial cells is stimulated by VEGF, which may act upstream of Notch signaling. VEGF binds to the VEGF receptor 2 and leads to expression of Dll4, which activates Notch signaling (Lawson et al., 2002). As a result of activated Notch signaling expression of its downstream target genes Hey1 and Hey2 is induced. Expression of Hey1 and Hey2 in arterial cells has been linked to the hypoxic response, as VEGF was strongly increased in Hey1/2 double knockout embryos compared to controls indicating that these embryos suffer from hypoxia (Fischer et al., 2004). In endothelial cells and endothelial progenitor cells hypoxia leads to induction of Hey1 and Hey2 as well as to the induction of the Notch ligand Dll4 (Diez et al., 2007). Increasing amounts of Dll4 enhance Notch signaling and Hey gene expression, which determines the arterial phenotype of endothelial cells. Furthermore, Hey expression is also able to suppress CoupTFII and thus venous cell fate determination (Diez et al., 2007). The converse negative regulation of Hey and CoupTFII emphasizes an important role of both pathways in regulating arterial versus venous endothelial cell fate determinations (Wiese et al., 2010). A schematic model of arterial versus venous endothelial cell specification is shown in Fig. 4.

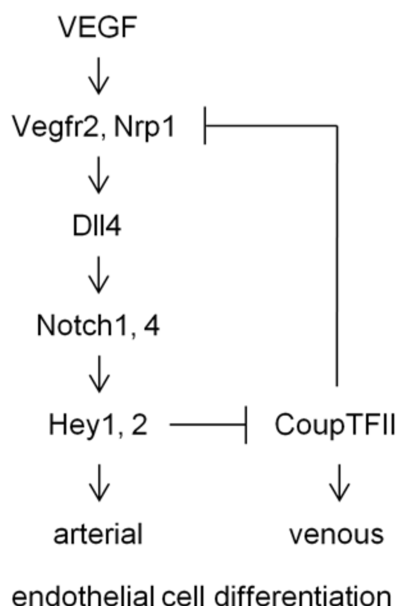


Fig. 4 Schematic model of arterial versus venous endothelial cell specification (modified from Wiese et al., 2010). VEGF binds to the Vegfr2/Nrp1 heterodimer that leads to expression of Dll4 and activation of Notch receptors. The Notch target genes Hey1 and Hey2 are induced upon active Notch signaling leading to arterial endothelial cell specification by additionally suppressing venous endothelial cell development. In contrast, expression of CoupTFII is able to suppress Nrp1 leading to reduced Notch signaling.

3.2.6 Notch signaling and Hey gene functions in embryonic stem cells and endothelial cells

The combined loss of Hey1 and Hey2 leads to a severe vascular phenotype during embryonic development and results in early lethality *in vivo.* The lethal phenotype *in vivo* requires a system to study the role of Hey genes during development *in vitro.* A commonly used system to study developmental processes dependent on specific gene functions is based on the use of embryonic stem cells and their differentiation ability. Embryonic stem cell cultures were first established in the 1980s. Stem cells were isolated from the inner cell mass of a developing blastocyst from mice and could be further cultured *in vitro* (Evans and Kaufman, 1981). Embryonic stem cells exhibit two major characteristics, namely their capacity of self-renewal and their pluripotent ability to differentiate into cells from all three germ layers including ectoderm, mesoderm and endoderm, which reflects *in vivo* embryonic development (Doetschman et al., 1985). To study developmental processes *in vitro*, embryonic stem cells can be used for the differentiation into specific cell types. Thereby, the use of genetically modified or knockout cells allows the investigation of the differentiation of ES cells in dependence of a specific gene. For this purpose, Hey deficient and Hey re-expressing embryonic stem cells were generated to study specific Hey gene functions during differentiation. Results obtained from earlier *in vitro* studies using Hey deficient ES cells further supported knockout studies of Hey deficient mice according to deregulation of cardiac related genes (Fischer et al., 2005). Furthermore, it has been shown that inhibition of

Notch signaling influences VEGF-induced arterialization and endothelial differentiation, which results in a phenotypic switch from arterial to venous endothelial cells (Lanner et al., 2007).

3.2.7 Aim of the thesis – part II

The major goal of the second part of my thesis was to identify vascular Hey target genes using an *in vitro* differentiation system with embryonic stem cells. The combined loss of Hey1 and Hey2 leads to a severe vascular phenotype and early embryonic lethality *in vivo*. Thus, an *in vitro* system is needed to study Hey gene functions during vascular development. Hey1/2 deficient embryonic stem cells were stably transfected with an antibiotic resistance gene under the control of endothelial specific promoters (VE-Cadherin, Tie1) to establish a selection-based differentiation method for endothelial cells in a chemically defined medium. In a second step, Hey1 and Hey2 inducible plasmids were used to re-express either Hey1 or Hey2 in embryonic stem cells to study endothelial cell differentiation in the absence or presence of Hey genes. Finally, whole transcriptome analysis was performed in embryonic stem cells as well as in embryonic stem cell (ESC) derived endothelial cells to identify possible Hey downstream target genes.

4 Material

4.1 Chemicals

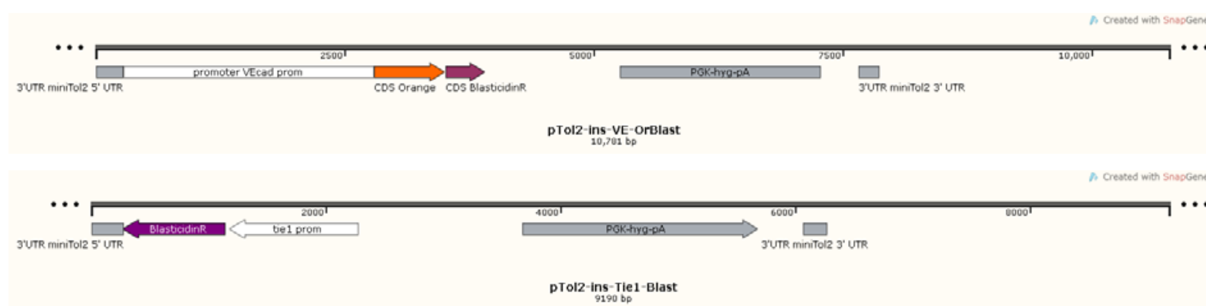
Chemicals were purchased from Roth GmbH, Sigma-Aldrich GmbH or AppliChem GmbH unless otherwise indicated.

peqGOLD TriFast™	PeqLab
SYBR Green	Ambrex Bio Science

4.2 Plasmids

pTol2-ins-VE-Or-Blast and pTol2-ins-Tie1-blast

Expression construct with miniTol2 flanking sites for target gene integration of VE-Cad-bsd or Tie1-bsd.

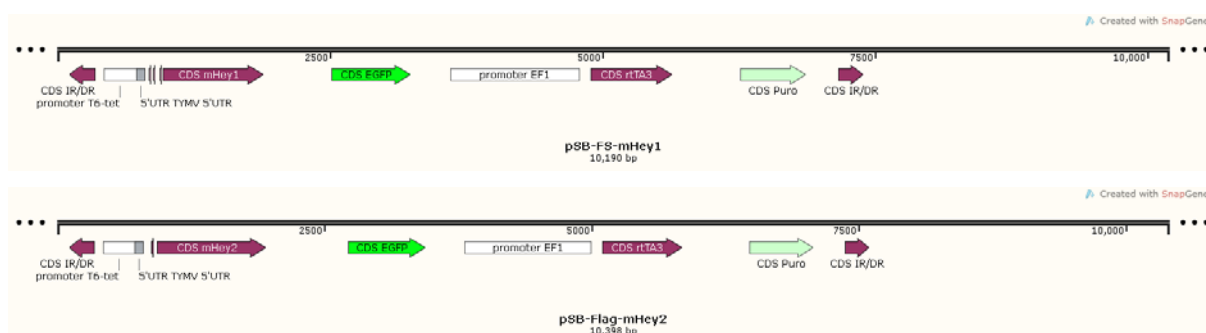


pKate-NI-Tol2

Expression construct for Tol2 transposase.

pSB-FS-mHey1 and pSB-Flag-mHey2

Expression construct with a doxycycline dependent transactivator (TA) to induce target gene expression upon addition of doxycycline. Inverted terminal repeats (IR/DR) are the recognition sites for the SB100X transposase for integration to a genomic target site.



pCMV-(CAT)-T7-SB100X

Expression construct for SB100X transposase.

4.3 Cell culture material

4.3.1 Cell culture media and supplements

0.25 % Trypsin-EDTA solution	Sigma-Aldrich GmbH
AccutaseTM	Sigma-Aldrich GmbH
Alpha-MEM	Sigma-Aldrich GmbH
bFGF	Miltenyi Biotec GmbH
BMP4	Miltenyi Biotec GmbH
DMEM	Sigma-Aldrich GmbH
ECGS	Sigma-Aldrich GmbH
FBS	PAN Biotech
Glutamax (100x)	Life Technologies GmbH
Heparin	Sigma-Aldrich GmbH
Knockout-DMEM	Life Technologies GmbH
KnockOut™ Serum Replacement	Life Technologies GmbH
Leukemia inhibitory factor (LIF)	Developmental Biochemistry, University of Wuerzburg
M199	Sigma-Aldrich GmbH
Matrigel	BD Biosciences
$mVEGF_{164}$	Developmental Biochemistry, University of Wuerzburg
Non-essential amino acids (100x)	Sigma-Aldrich GmbH
Nutridoma-CS	Roche Diagnostics GmbH
Penicillin/streptomycin	PAA Laboratories GmbH
RPMI-1640	Sigma-Aldrich GmbH
ß-Mercaptoethanol	PAN-Biotech GmbH

4.3.2 Eukaryotic cell lines

4.3.2.1 Human tumor cell lines

Human tumor cell lines were obtained from American Type Culture Collection (ATCC).

Non-small cell lung cancer	Colorectal cancer	Renal cell carcinoma	Breast cancer
A549	COLO 205	786-0	MCF7
EKVX	HCC-2998	A498	MDA-MB-231
HOP-62	HCT-116	ACHN	HS-578T
HOP-92	HCT-15	CAKI-1	BT-549
NCI-H226	HT29	RXF-393	T-47D
NCI-H23	KM12	SN12C	MDA-MB-468
NCI-H460	SW-620	TK-10	
NCI-H522	KM20L2	UO-31	
LXFL529		RXF-631	
		SN12K1	

4.3.2.2 Human endothelial cell lines

HUVEC (human umbilical vein endothelial cells)	PromoCell

4.3.2.3 Mouse embryonic stem cell lines

ΔHey1/2 (original name: A3d)	Developmental Biochemistry, University of Wuerzburg
ΔHey1/2-(VE-Cad-bsd)	
ΔHey1/2-(VE-Cad-bsd)-pSB-FS-mHey1#1	
ΔHey1/2-(VE-Cad-bsd)-pSB-Flag-mHey2#3	
E14	Prof. Dr. Albrecht Müller, MSZ, University of Wuerzburg
E14-(Tie1-bsd)	

4.3.2.4 Mouse endothelial cell lines

bEnd5	Prof. Dr. Georg Breier

4.4 Buffers and solutions

10 x PBS	1.4 M NaCl, 27 mM KCl, 80 mM Na_2HPO_4, 18 mM KH_2PO_4
10 x Repro Fast buffer	100 mM $(NH4)_2SO_4$, 200 mM Tris-HCl (pH 8.8), 100 mM KCl, 20 mM $MgSO_4$, 1 % TritonX100, 1 % BSA
10 x SDS running buffer	250 mM Tris, 192 mM Glycin, 1 % SDS, pH 8.3
2 x SDS sample buffer	0,1 M Tris-HCl (pH 6.8), 4 % SDS, 0.25 % Bromphenolblue, 25 % Glycerin, 200mM DTT
20 x SB buffer	200 mM NaOH, pH 8.0 (adjust with boric acid)
50 x Base solution	1.25 M NaOH, 10 mM EDTA, pH 12.0
50 x Neutralization solution	2M Tris-HCl, pH 5.0
50 x TAE buffer	2 M Tris-AcOH (pH 7.5 – 8.0), 50 mM EDTA
Cell lysis buffer	20mM HEPES pH 7.8, 500mM NaCl, 5mM $MgCl_2$, 5mM KCl, 0.1% Sodium deoxycholate, 0.5% Nonidet-P40, 10ug/ml Leupeptin, 10ug/ml Aprotinin, 1mM PMSF, 200uM Na_3VO_4, 0.1M NaF
DNA loading buffer	0.25 % Bromphenol blue, 10 mMEDTA, 15 % Ficoll
ECL solution	100 mM Tris-HCl pH 8,0, 250 mM Luminol, 90 mM Coumaric acid, 3 µl H_2O_2 (35 %) / 10 ml ECL
RIPA buffer	50 mM Tris (pH 8.0), 1 % Nonidet P40, 0.5 % Sodium deoxycholate, 0.1 % SDS, 150 mM NaCl, 1x Protease inhibitor mix, 50 µg/ml PMSF
TM staining solution	25 ml TM-buffer, 200 µl 10 % $MgCl_2$, 10 mg Naphtol AS-MX Phosphat, 25 mg Fast Red TR
TM buffer	30 mM Tris, pH 9.0 (adjust with 1 M maleic acid)
Transfer blotting buffer	25 mM Tris, 150 mM Glycin, 10 % Methanol (pH 8,3)

4.5 Oligonucleotides

Oligonucleotides were obtained from Sigma-Aldrich GmbH. Sequences of oligonucleotides are presented in 5‘-3‘ direction.

4.5.1 Human oligonucleotides for quantitative real-time PCR

Gene	Primer name	Primer sequence
GLUT1	huGLUT1-5'real	GCTTTGTGGCCTTCTTTGAA
	huGLUT1-3'real	CAGAACCAGGAGCACAGTGA
HPRT	hHPRT_5'real_neu2	AAGATGGTCAAGGTCGCAAG
	hHPRT_3'real_neu2	GTCAAGGGCATATCCTACAACAA
NRP1	h-NP1-real5'	CAAAACCAGCAGACCTGGAT
	h-NP1-real3'	CATTATGCCAACAGGCACAG
VEGF total	hVEGF-real1	TACCTCCACCATGCCAAGTG
	hVEGF-real2	GCTGCGCTGATAGACATCCA
$VEGFA_{121}$	hVEGFA-3-f	GTGTGTGCCCACTGAGGAG
	hVEGFA-5,8a-r	GCCTCGGCTTGTCACATTT
$VEGFA_{165}$	hVEGFA-5,7a-f	AGATAGAGCAAGACAAGAAAATCCC
	hVEGFA-7b8a-r	CTCGGCTTGTCACATCTGC
$VEGFA_{189}$	hVEGFA-6a7a-f	TATAAGTCCTGGAGCGTTCCC
	hVEGFA-7b8a-r	CTCGGCTTGTCACATCTGC
VEGFR1	hVEGFR1-real5'	CTTCACCTGGACTGACAGCA
	hVEGFR1-real3'	ACAGCTGGAATGGCAGAAAC
VEGFR2	hVEGFR2-real-f	ACAACCAGACGGACAGTGGT
	hVEGFR2-real-r	AGTCAGGCTGGAGAATCTGG

4.5.2 Murine oligonucleotides for quantitative real-time PCR

Gene	Primer name	Primer sequence
Actc1	mActc1-real-for	AGCTGTCTTCCCGTCCATC
	mActc1-real-rev	GCTCTGGGCTTCATCACCTA
C1d	mC1d-real-for	GCATCCAGTGAAGCAGGAACT
	mC1d-real-rev	TTGGCAGCCTTCTTCTTGTCT
Calcoco2	mCalcoco2-real-for	GGAACAGCTCAGTGAGGAGC
	mCalcoco2-real-rev	CCCTCTGTGTTGCTTCCAGT
CoupTFII	mCoup-TF2-real5'	AGTACTGCCGCCTCAAAAAG
	mCoup-TF2-real3'	CAGGTACGAGTGGCAGTTGA
CoupTFIIalt	mCoup-TF2-alt5'	TTTCACCCGCCAAACTAAAG
	mCoup-TF2-real3'	CAGGTACGAGTGGCAGTTGA
Cxxc1	mCxx1c-real-for	CAGGGGCTACAAGAGGCTAC
	mCxx1c-real-rev	AGCTTGCAGACTGTCCACTG
Ddit3	mDdit3-real-for	GAGCCAGAATAACAGCCGGAA
	mDdit3-real-rev	TGGACCAGGTTCTGCTTTCA

Dll4	mDll4-real3‘	AGCTGGGTGTCTGAGTAGGC
	mDll4-real5‘	AGAAGGTGCCACTTCGGTTA
Dnajc4	mDnajc4-real-for	GGCCTGCACTATGTTGCCTT
	mDnajc4-real-rev	CTGGCCCGAGTGTCATTGT
EG434280	mEG434280-real-for	TCCAGGCTAACCGGGATACA
	mEG434280-real-rev	CAGCCGCAGGTTTCCAGATA
EphB4	mEphb4-real-for	TACTGGGACATGAGCAACCA
	mEphb4-real-rev	ATTTTGAGGCTAGCGGGATT
EphrinB2	m-ephrinB2-5’real	CTCAACTGTGCCAGACCAGA
	m-ephrinB2-3’real2	TATCCAGGCCCTCCAAAGA
Esm1	mEsm1-real-for	ATGGACGGGGTCAAGTGTG
	mEsm1real-rev	CATTCCATCCCGAAGGTGCC
Fabp3	mFabp3-real-for	AGGGAGCTAGTTGACGGGA
	mFabp3-real-rev	ACGCCTCCTTCTCATAAGTCC
Fibronectin1	mFibronectin1-real5‘	AAGTGTGatccccATGAAGC
	mFibronectin1-real3‘	CAGGTCTACGGCAGTTGTCA
Hdac8	mHdac8-real-for	CCACCGAATCCAGCAAATCC
	mHdac8-real-rev	TTCCACAAACCGCTTGCATC
Hey1 (endogenous)	mHey1-5’UTR	CTGCAGTTAACTCCTCCTTGC
	clik-race	ATTCTCGTCCGCGCTCTCCTTTTCC
Hey1 (transgene)	pSB-FS-mHey1-for	GCTCAAGCCACCCAGACTAC
	clk2	CTGGCCAAAACCTGGGAC
Hey2 (endogenous)	mHey2-real-e1for	AGTAGCTGCTCCTCCTTCGTC
	mHey2-real-e2rev	GTCGGTGAATTGGACCTCAT
Hey2 (transgene)	Flag-Tag-for	GGATTACAAGGATGACGACGAT
	mHey2-real-e2rev	GTCGGTGAATTGGACCTCAT
Hprt	mHPRT-real-ex8	TGTTGTTGGATATGCCCTTG
	mHPRT-real-ex9	ACTGGCAACATCAACAGGACT
Hspg2	mHspg2-real-for	TGTGAACATTCGCAAGCCCT
	mHspg2-real-rev	GGTGGTCACCGTTACACCTT
Icam1	mIcam1-real for	TTTGAGCTGAGCGAGATCGG
	mIcam1-real rev	AGAGGTCTCAGCTCCACACT
Krt19	mKrt19-real-for	AAGCAAGACCGAAGTCACGG
	mKrt19-real-rev	CAGCTGGACTCCATAACGGG
Lefty1	mLefty1-real5’	GGCTCTGCTGGGCACTCTGGGCACT
	mLefty1-real3’	GACACCAGGAACCTGCCTGCCACCTCTC
Lefty2	mLefty2-real5‘	ACGCCGGACGGCAAGGGGCAG

	mLefty2-real3‘	CGAGGCCCCAGAAATGGCCACCCGA
Mier3	mMier3-real-for	GGAATGACAGCATGGACGGA
	mMier3-real-rev	GGCAACCGTTCTAGACCTCA
Myl9	mMyl9-real-for	GCAATGCCTTTGCCTGCTTT
	mMyl9-real-rev	TCCTCATCCGTGAATCGGTC
Nanog	mNanog-real5‘	TTGCCTAGTTCTGAGGAAGCA
	mNanog-real3‘	GAGGAAGGGCGAGGAGAG
Nptx2	mNptx2-real-for	CTTAGCCGCTCCTTGCAAAC
	mNptx2-real-rev	AGCCCAGCGTTAGACACATT
Oct4	mOct4-real5‘	CCGTGAAGTTGGAGAAGGTG
	mOct4-real3‘	GAAGCGACAGATGGTGGTCT
Perp	mPerp-real-for	CATTCTCTCGTTCTTCGCCCT
	mPerp-real-rev	TGAAGCCTGAAGGTCTGTGTG
Psmb10	mPsmb10-real-for	CTTTACTGCCCTTGGCTCTG
	mPsmb10-real-rev	AGCCCAGGTCACTCAGGAT
Ptges	mPtges-real-for	CCAGTATTACAGGAGTGACCCAG
	mPtges-real-rev	ATGAGTACACGAAGCCGAGG
Rarres1	mRarres1-real-for	GAGCAATACAACCCCGAGCA
	mRarres1-real-rev	GCCGGTCTGGGTTTTTCATTC
Serpine2	mSerpine2-real-for	TGGAACCAAAGCTTCGGCAG
	mSerpine2-real-rev	GATGGCACCTGTGGGATTGT
Skil	mSkil-real_F	TGAGGAGCAGGAGAAAATGG
	mSkil-real_R	AAGCTGCACACAGCAGACTC
Smarca2	mSmarca2-real-for	CAAACCGAGGCAAAGCCAAA
	mSmarca2-real-rev	CACGTCCAGGATGGTCACTC
Sox1	mSox1-real-for	AGTGGAAGGTCATGTCCGAG
	mSox1-real-rev	TGTAATCCGGGTGTTCCTTC
Sox2	Sox2_fwd_mus	AAGGGTTCTTGCTGGGTTTT
	Sox2_rev_mus	AGACCACGAAAACGGTCTTG
Spred3	mSpred3-real-for	CCATTGCCACAGTGGAGTCA
	mSpred3-real-rev	CAGTGAACGGTAGAAGCGGA
Tie1	mTie1-real5‘	TGCAGACTTTGGCCTTTCTC
	mTie1-real3‘	TGCCTCCAAGGCTCACTATC
Tie2	tie2-for-real	CTCGGCCAGGTACATAGGAG
	tie2-rev-real	ATAAACCCAGGAGGGCAAAT
VE-Cadherin	mVE-Cadherin_5’real	GGATGTGGTGCCAGTAAACC
	mVE-Cadherin_3’real	ACCCCGTTGTCTGAGATGAG

Vegfr2	flk1-for-real	GGCGGTGGTGACAGTATCTT
	flk1-rev-real	GTCACTGACAGAGGCGATGA

4.5.3 Additional oligonucleotides

The following oligonucleotides were used to confirm transgene integration into embryonic stem cells.

Transgene	Primer name	Primer sequence
Tie1-bsd	mTie1-prom-3‘	TCTGGTTCATTCCAGATCATTGT
	bGlob-3’UTR-rev	GAGACTCCATTCGGGTGTTC
VE-Cad-bsd	Cherry-3for	CACCATCGTGGAACAGTACG
	blasticidin_R	CAAGATGCCCCTGTTCTCAT

The following oligonucleotides were used for genotyping of embryonic stem cells (in one reaction).

Genotype	Primer name	Primer sequence
flox Hey1	clikATG	GCGGGATCCACATGAAGAGAGCTCACCCAG
	Clikseq2	TGAGATCTTGCAGATGACTGTG
	Hey1flox-wt	CACGCCGAGCACGCAAAG
	Eagrev	ACAAAGCAAAGCAGGCAGTC

4.6 Antibodies

4.6.1 Antibodies for western blot analysis

Anti-cleaved PARP (Asp214) (552596)	BD Pharmingen™
Anti-Flag M2 (F3165)	Sigma-Aldrich GmbH
Anti-Neuropilin (H-286) (sc-5541)	Santa Cruz Biotechnology
Anti-ß-Aktin (C4) (sc-47778)	Santa Cruz Biotechnology
Anti-VEGFR1 (Flt1) (C17) (sc-316)	Santa Cruz Biotechnology
Anti-VEGFR2 (55B11) (2479)	Cell Signaling
Anti-Vinculin (V9131)	Sigma-Aldrich GmbH
Anti-α-Tubulin (T6074)	Sigma-Aldrich GmbH
Goat anti-Mouse IgG, Peroxidase conjugated, H+L (AP124P)	Chemicon
Goat anti-Rabbit IgG, HRP conjugated, H+L	Chemicon

4.6.2 Antibodies for immunofluorescence

AlexaFluor488 donkey α-goat IgG (H+L) (A11055)	Life Technologies GmbH
AlexaFluor568 donkey α-goat IgG (H+L) (A11057)	Life Technologies GmbH
AlexaFluor568 goat α-rat IgG (H+L) (A11077)	Life Technologies GmbH
PECAM-1 (CD31) (sc-18916)	Santa Cruz Biotechnology

VE-Cadherin (sc-6458)	Santa Cruz Biotechnology

4.6.3 Antibodies for flow cytometry

CD304 (BDCA-4/Neuropilin-1)-APC, human	Miltenyi Biotec
CD309 (VEGFR-2/KDR)-APC, human	Miltenyi Biotec

4.6.4 Humanized monoclonal antibodies

Avastin® (bevacizumab), 25.3 mg/ml	F. Hoffmann-La Roche AG

4.7 Enzymes and Kits

Experion™ DNA 1K Reagents and Supplies	Bio-Rad Laboratories
Experion™ RNA HighSense Reagents and Supplies	Bio-Rad Laboratories
His-Taq-Polymerase, lot 10, 12	Developmental Biochemistry, University of Wuerzburg
mRNA Library Reagents Set for Illumina®	New England Biolabs
Multiplex Oligos for Illumina® Index Primer Set 1	New England Biolabs
Plasmid Midi Kit	Omega bio-tek
Plasmid Mini Kit I	Omega bio-tek
RevertAid™ First Strand cDNA Synthesis Kit	Thermo Scientific
Rneasy™ Purification Kit	Qiagen
Sera-Mag Oligo(dT)-Coated Magnetic Particles	Thermo Scientific
QIAquick Gel Extraction Kit	Qiagen
QIAquick PCR Purification Kit	Qiagen

4.8 Technical devices

Hypoxia chamber (C-Chamber und Adapter Plate)	BioSpherix
Leica DMI6000 B	Leica Microsystems
Mastercycler® ep *realplex*	Eppendorf GmbH
NanoDrop ND 1000 Spectrophotometer	Fisher Scientific
Nikon Eclipse TS100	Nikon GmbH
Proox Model 110	BioSpherix
Sunrise Absorbance Reader	Tecan Group Ltd.
Tristar LB 941	Berthold Technologies

5 Methods

5.1 Cell culture

5.1.1 Cell culture of human tumor cell lines and human endothelial cells

Human tumor cell lines selected from the NCI-60 panel (4.3.2.1) were obtained from American Type Culture Collection (ATCC) and cultured in RPMI-1640 medium supplemented with 10 % FBS, 1 % Glutamax, 1 % penicillin and 1 % streptomycin as recommended by the NCI-Frederick Cancer DCTD Tumor Cell Line Repository. Cells were passaged using trypsin at about 80 % confluency. HUVECs (human umbilical vein endothelial cells) were cultured in M199 medium with 10 % FBS, 25 µg/ml heparin, 50 µg/ml ECGS and 1 % Glutamax on plates pre-coated with 0.2 % gelatin.

5.1.2 Cell culture under hypoxic conditions and bevacizumab treatment

Most of the experiments with human tumor and human endothelial cells were performed under hypoxia at 1 % O_2. Therefore, cells were cultured in a special chamber inside the incubator (C-Chamber), which was flushed with a gas mixture of 95 % N_2 und 5 % CO_2 to reduce the oxygen level in the interior. The oxygen level was controlled by a device outside the chamber (Proox Model 110). Cells were cultured under serum-reduced conditions with 1 % FBS for up to 96 hours and treated with 250 µg/ml bevacizumab (Avastin) and/or 50 ng/ml recombinant human VEGF-A.

5.1.3 Cell culture of mouse embryonic stem cells

Mouse embryonic stem cells [E14-(Tie1-bsd), ΔHey1/2-(VE-Cad-bsd), ΔHey1/2-(VE-Cad-bsd)-Hey1, ΔHey1/2-(VE-Cad-bsd)-Hey2] were cultured in KO-DMEM medium supplemented with 15 % KnockOut™ Serum Replacement (KOSR), 1 % Glutamax, 1 % penicillin, 1% streptomycin, 1 % non-essential amino acids, 0.1 mM 2-mercaptoethanol and 2000 units/ml Leukemia Inhibiting Factor (LIF) on plates pre-coated with 0.2 % gelatin. Medium was changed daily and the cells were passaged every second day by trypsinization.

5.1.4 Establishment of stable mouse embryonic stem cell lines

For the generation of stable mouse embryonic stem cell lines different plasmids were incorporated into the cells by either electroporation (pTol2-ins-VE-Or-Blast and pTol2-ins-Tie1-blast) or by transfection (pSB-FS-mHey1 and pSB-Flag-mHey2).

5.1.4.1 Electroporation

5 x 10^6 cells were electroporated with either 2 µg pTol2-ins-VeOr-Blast or 2 µg pTol2-ins-Tie1-blast and 12 µg pKate-NI-Tol2 (transposase) using program A024 (E14) or program A023 (ΔHey1/2) of the electroporation device Amaxa Nucleofector II according to

the manufacturer's instructions. The cells were plated on two 10 cm dishes after electroporation.

5.1.4.2 Transfection

1 x 10^5 cells per well were seeded on a 12 well plate and transfected with the transfection reagent Metafectene Pro overnight. 0.6 µg of plasmid DNA (pSB-FS-mHey1 or pSB-Flag-mHey2) were co-transfected with 0.6 µg of pCMV-(CAT)-T7-SB100X (transposase) using 2.4 µl Metafectene Pro according to the manufacturer's instructions. Before the mixture of the DNA and the transfection reagent was added to the cells, the medium in the cell culture well was reduced from 1.0 ml to 0.5 ml to facilitate integration of the transgene. On the next day cells were detached from the 12 well plate using Accutase solution and re-plated on a 6 well plate. Before starting the selection for stable clones cells were again detached and transferred to a 10 cm dish to limit the cell density on the plate and to prevent overgrowth as well as uncontrolled differentiation.

5.1.4.3 Selection of stable clones

Selection of stable clones, which have integrated the transgene, was started 48 hours after electroporation or transfection using 0.5 µg/ml puromycin or 150 µg/ml hygromycin depending on the respective antibiotic resistance gene. After seven to ten days of selection, stable clones arose on the culture plate and were picked in a well of a 48 well plate. Individual clones were grown up to a 10 cm dish and tested via PCR or western blot for stable integration of the transgene.

5.1.5 Differentiation of mouse embryonic stem cells into endothelial cells

For the differentiation of mouse embryonic stem cells into endothelial cells, cell culture plates were pre-coated with 0.5 % gelatin overnight. On day 0 of the differentiation between 2 x 10^4 and 3 x 10^4 cells per well were seeded on a 6 well plate in serum-free ESC differentiation medium (induction medium) consisting of α-MEM supplemented with 20 % KnockOut™ Serum Replacement (KOSR), 1 % Glutamax, 1 % penicillin, 1 % streptomycin, 1 % non-essential amino acids, 0.05 mM 2-mercaptoethanol, 5 ng/ml BMP4 and 120 ng/ml $mVEGF_{164.}$ Cells were incubated for four days (induction phase) without changing the medium. On day four of the differentiation, medium was changed using another chemically defined medium named Ldsk as published previously (Blancas et al., 2011). Ldsk medium consists of 70 % α-MEM and 30 % DMEM supplemented with 1 % Glutamax, 1 % penicillin, 1 % streptomycin, 1 % non-essential amino acids, 0.1 mM 2-mercaptoethanol, 2 x Nutridoma-CS, 50 ng/ml bFGF and 400 ng/ml $mVEGF_{164.}$ Again, the medium was not changed for another four days until day eight of differentiation. From day eight on, Ldsk medium was supplemented with 5 µg/ml blasticidin and changed every second day. Selection with blasticidin allows the enrichment of endothelial cells, while eliminating

embryonic stem cells from the culture dish. At day 14 of the differentiation nearly all embryonic stem cells were eliminated and the resulting ESC-derived endothelial cells could be used for further experiments. For experiments with doxycycline inducible cell lines [ΔHey1/2-(VE-Cad-bsd)-Hey1, ΔHey1/2-(VE-Cad-bsd)-Hey2], cells were re-plated on a 6 well plate and cultured in Ldsk medium for 48 or 72 hours in the presence of 1 µg/ml doxycycline to induce Hey transgene expression.

5.2 Cell biological methods

5.2.1 Cell proliferation assay

Cell proliferation was assessed for up to 96 hours using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] staining as described previously (Mosmann, 1983). Briefly, between 2 x 10^3 and 5 x 10^3 cells/well (cell line/doubling time dependent) were seeded into 96 well plates and incubated overnight to adhere. Medium was then replaced by fresh medium supplemented with bevacizumab or VEGF-A at the concentrations indicated depending on the application. After 24, 48, 72 or 96 hours of treatment 20 µl of MTT (5 mg/ml in PBS) was added and incubated for two hours at 37°C. The supernatant was removed and reaction products were solubilized for 1 hour in 10 % HCl, 0.1 % NP-40 in isopropanol. Absorbance was measured at 570 nm with a reference wavelength of 650 nm.

5.2.2 Cell migration assay

Cell migration was measured using the *in vitro* scratch assay as described previously (Liang et al., 2007). Briefly, cells were grown in 6 well plates to a confluent monolayer, before scraping in a straight line using a sterile P200 pipet tip. To remove debris, cells were washed once with PBS. Medium was changed to serum reduced +/- treatment and cells were incubated for up to 24 hours. Images of the scratch width were measured using ImageJ software at the same location after 6 and 24 hours of incubation.

5.2.3 Tube formation assay

To test the functionality of endothelial cells *in vitro,* the tube formation assay was performed as described previously (Arnaoutova and Kleinman, 2010). 50 µl of Matrigel per well were loaded on a 96 well plate. The plate was incubated for 30 min at 37°C to allow the Matrigel to polymerize. 15 x 10^3 cells in a total volume of 100 µl were plated into each well. Culture medium was supplemented with 50 ng/ml bFGF and 400 ng/ml $mVEGF_{164}$. The formation of tubes was observed after four to 16 hours under an inverted microscope.

5.2.4 Immunofluorescence

For immunofluorescent staining, cells were cultured on coverslips pre-coated with 0.2 % gelatin in a 24 well plate. Fixation was done using ice-cold methanol (100 %) for ten

minutes at room temperature. Depending on the antibody staining, cells were blocked with appropriate blocking reagents for 1 hour at room temperature (CD31: 10 % goat serum in PBS, VE-Cadherin: 5 % BSA in PBS). Primary antibodies were diluted 1:100 in PBS + 1.5 % of the blocking reagent and incubated at 4 °C overnight. Secondary antibodies were diluted 1:1000 and incubated at room temperature for 1 hour in PBS + 1.5 % of the blocking reagent. Cells were washed three times with PBS after incubation of the primary and the secondary antibody for five minutes at room temperature.

5.2.5 Alkaline phosphatase staining

For alkaline phosphatase staining, embryonic stem cells were fixed with 4 % paraformaldehyd for 20 minutes at room temperature. Cells were washed three times with TM buffer and subsequently stained with TM staining solution for 20 minutes. Afterwards cells were washed with PBS and then alkaline phosphatase positive cells were investigated under an inverted cell culture microscope.

5.3 Molecular biological methods

5.3.1 Molecular cloning

Cloning of plasmids was done using standard molecular cloning methods.

5.3.2 Reverse transcription and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from subconfluent monolayers using peqGOLD TriFast according to the manufacturer's instructions. cDNA was transcribed using 1 or 2 µg total RNA with the RevertAid First Strand cDNA Synthesis Kit and diluted in 500 µl DEPC-treated water. cDNA was amplified by qRT-PCR using a two-step PCR program of 40 cycles with denaturation at 95°C for 15 s, annealing and extension at 60°C for 30 s, followed by a melting curve from 50 to 95°C using a Mastercycler ep *realplex*. The following standard PCR master mix was used.

13.30 µl	ddH_2O
1.50 µl	Ethylenglycol
2.50 µl	10 x Repro Fast buffer
0.25 µl	dNTPs (25 mM)
0.75 µl	SYBR Green (1:2000)
0.2 µl	Taq-Polymerase (15 U/µl)
0.75 µl	Forward Primer (10 pmol/µl)
0.75 µl	Reverse Primer (10 pmol/µl)
5.0 µl	cDNA

Relative quantification was done using ΔΔCt (cycle threshold) measurements on SYBR Green based fluorescence readings with HPRT as a housekeeping gene.

5.3.3 Isolation of genomic DNA and PCR analysis

For genotyping and/or verification of stable transgene integration, cells were pelletized and incubated in 1 x base solution at 96°C for 20 minutes. Between 30 and 100 µl of base solution were added according to the size of the cell pellet. After cooling down of the sample, the same amount of 1 x neutralization solution was added and 1 µl of the sample was directly used as a template in the PCR reaction. PCR reaction was performed with the a three-step PCR program of 35 cycles with denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 45 s, following a single incubation step at 72°C for 7 min.

5.3.4 Agarose gel electrophoresis

DNA fragments with a size of up to 600 bp were separated in 1 % agarose gels with SB-buffer for 15 minutes at 300 volt. For larger fragments with up to 20 kb, fractionation was done using 1 % agarose gels in TAE-buffer. Agarose gels were supplemented with 1 µg/ml ethidiumbromid and DNA was visualized under UV light.

5.3.5 RNA-Sequencing

For whole transcriptome analysis, total RNA was extracted using Rneasy™ Purification Kit according to the manufacturer's instructions. Starting material for the isolation of mRNA did contain between 1.5 and 5 µg of total RNA. Quality control of RNA was analyzed with Experion™ RNA HighSens Chips. Isolation of mRNA was done with Sera-Mag Oligo(dT)-Coated Magnetic Particles according to the Illumina transcriptome sequencing library preparation protocol. The following preparation of the cDNA library was performed with the mRNA Library Reagents Set according to the sample preparation guideline from Illumina. mRNA was fragmented into 200 bp fragments at 94°C and afterwards transcribed into cDNA using random primers. cDNA was end-repaired, dA-tailed and Illumina sequencing adapters were attached. After each reaction cDNA samples were purified using QIAquick PCR Column Purification Kit. Adaptor-ligated DNA ranging from 200 - 300 bp was excised from an agarose gel and purified using QIAquick Gel Extraction Purification Kit. The adaptor-ligated cDNA library was enriched by PCR amplification with 12 cycles. Quality and quantification of the resulting cDNA library was measured using an Experion™ DNA Chip.

For RNA-Sequencing, various samples were multiplexed and analyzed on a single flow cell v4. After de-multiplexing of the samples, data were analyzed using bowtie 1.0.0 with standard parameters and aligned to the Mus Musculus genome (NCBI37/mm9) (Langmead et al., 2009). Genes with less than ten reads per million were excluded from further analysis. Noise expression was calculated from reads mapping to non-exonic regions of the genome and a gene was considered as expressed with a value of ≥ 2.26 reads per kilo base per million (RPKM), which was ten-fold higher than RPKM values for non-exonic regions of the genome. Regulated genes were identified using the R-package Deseq (Anders and Huber,

2010) and a gene was considered to be regulated if the fold change was ≥ 1.8 between the control and the induced sample, while the RPKM value had to be ≥ 2.26 in at least one sample. Heatmaps were generated using hierarchical clustering in Cluster 3.0 (De Hoon et al., 2002, Eisen et al., 1998) and were visualized using TreeView 1.1.6r2 (Saldanha, 2004). Gene Ontology (GO) term analysis was performed with DAVID 6.7 (Huang da et al., 2009) using the functional annotation clustering method allowing only to enrich for biological processes. Clusters were named based on interpretation of enriched GO annotations. A Cluster Enrichment Score of 1.3 was considered as significant (p-value ≤ 0.05).

5.4 Protein biochemical methods

5.4.1 Western blot analysis

Cell pellets were either lysed in RIPA buffer or cell lysis buffer for up to four hours on ice or directly re-suspended in 2 x SB buffer and denaturated at 95°C for 5 minutes. Protein quantification was done using Bradford reagent, as described previously (Bradford, 1976). Proteins were separated by SDS-polyacrylamide gel electrophoresis. Usually, 12 % SDS-polyacrylamide gels were used. For separation of larger proteins (> 90 kDa), polyacrylamide gels with lower percentages were used according to the size of the appropriate protein. After separation on a SDS-PAGE, proteins were transferred to a nitrocellulose membrane using a semi-dry blot chamber for 35 min at 360 mA and 25 V per gel. Transfer of larger proteins (> 90 kDa) was done using a wet-blot chamber at 100 mA and 15 V overnight. Protein transfer was performed at 4°C.

5.4.2 Flow cytometry

Protein expression on the cell surface was determined by flow cytometry. Cells were harvested using Accutase solution and stained for Neuropilin1 or VEGFR2 with CD304- or CD309-APC conjugated antibodies according to the manufacturer's instructions and measured by a BD FACS Canto II flow cytometer. Analysis was done using FlowJo software (version 8.8.6) to determine the percentages of positive cells.

Propidium iodide stained cells were prepared by fixing the cells in 80 % ice-cold ethanol for up to 48 hours. Afterwards, cells were washed with PBS and re-suspended in 38 mM sodium citrate, 24 µg/ml Rnase A and 54 µM propidium iodide. After 30 minutes of incubation samples were measured with a flow cytometer.

6 Results

6.1 Impact of bevacizumab treatment on human tumor cell lines *in vitro*

Bevacizumab targets the vascular network of tumor cells and thus primarily acts on endothelial cells and not on tumor cells themselves. To investigate if bevacizumab also directly influences tumor cells, which express VEGF receptors, cells from different tumor entities were treated either with or without bevacizumab. In general, experiments were performed under hypoxic conditions to mimic the *in vivo* state of tumors. After bevacizumab treatment cells were analyzed for angiogenic gene and protein expression as well as for biological processes like proliferation, migration and apoptosis. According to publicly available microarray data and to preliminary gene expression data different cell lines from the NCI-60 panel, derived from four tumor types (NSCLC, CRC, RCC and BC), were selected according to high expression of angiogenic pathway genes (NSCLC: H522, HOP62; CRC: HCT-116, HT-29, KM12; RCC: A498; BC: HS-578 T, MDA-MB-231).

Below, results of NSCLC and CRC cell lines as well as the endothelial control (HUVECs) were obtained by myself. Results of RCC and BC cell lines were kindly provided by Shannon Graver and for the sake of completeness additionally presented in this thesis. All results were published together as a single publication: **Tumor cell response to bevacizumab single agent therapy *in vitro***. *Cancer Cell International* 2013 13:94 (Hein and Graver, 2013).

6.1.1 Tumor cell and cell surface expression of VEGF receptors

VEGF-A signaling is activated through different angiogenic receptors like VEGFR1, VEGFR2 and Neuropilin1. Expression of receptors by tumor cells was determined by western blot analysis with HUVECs as a control. Cells were incubated under normoxia and hypoxia (1 % O_2) and either treated with or without 250 µg/ml bevacizumab for 24 hours. Vinculin was used as a loading control and served as an internal standard between treated and untreated cells (Fig. 5).

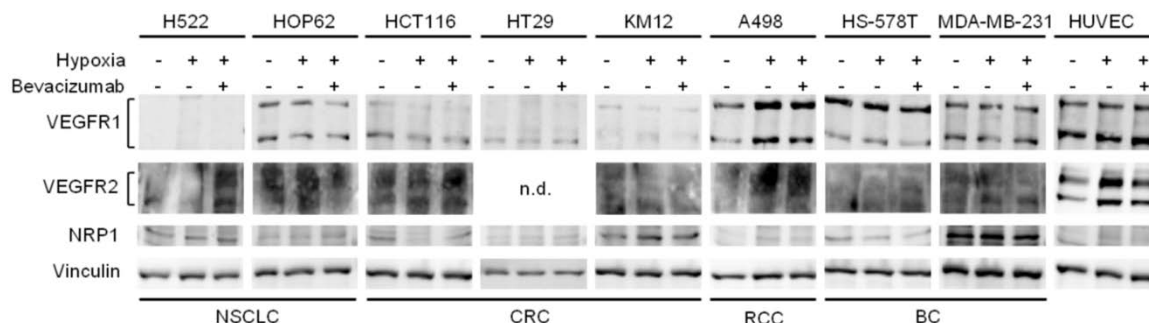


Fig. 5 Protein expression of VEGFR1, VEGFR2 and NRP1.[1] Protein expression of VEGFR1, VEGFR2 and NRP1 was determined in tumor cells and HUVECs under normoxia and after 24 hours of hypoxia with or without bevacizumab treatment. Vinculin was used as a loading control. n.d. = not determined

[1]This figure was published similarly in Hein and Graver (2013), Tumor cell response to bevacizumab single agent therapy *in vitro*. Results of RCC and BC cell lines were kindly provided by Shannon Graver and for the sake of completeness additionally presented in this thesis.

VEGFR1 was expressed by all cell lines with the exception of H522 as shown by two specific bands (Fig. 5). Incubation under hypoxia led to an increase of protein expression in the renal cell line A498, but treatment with bevacizumab did not further influence expression. In contrast, expression of VEGFR2, the main angiogenic receptor, was limited with only four tumor cell lines showing expression (H522, HOP62, HCT-116 and MDA-MB-231), while HUVECs, which served as a control, exhibited clear and strong expression of VEGFR2 (Fig. 5). However, hypoxic conditions as well as bevacizumab treatment did not alter expression of VEGFR2. Neuropilin1, which serves as a co-receptor for VEGFR1 and VEGFR2, was expressed by all cell lines investigated to varying intensities, but again bevacizumab treatment and hypoxic incubation did not change expression of Neuropilin1 (Fig. 5).

In addition, protein expression of VEGFR2 and Neuropilin1 was further evaluated on the cell surface by flow cytometry under normoxia and hypoxia with bevacizumab treatment (Fig. 6). Respective examples of unlabeled and labeled cells are shown for endothelial controls (HUVECs) and for two tumor cell lines, MDA-MB-231 (BC) and HCT-116 (CRC), in the FACS profile in Fig. 6A. Quantification of VEGFR2^{+} and NRP1^{+} cells for all tumor cell lines investigated, is presented in Fig. 6B and Fig. 6C.

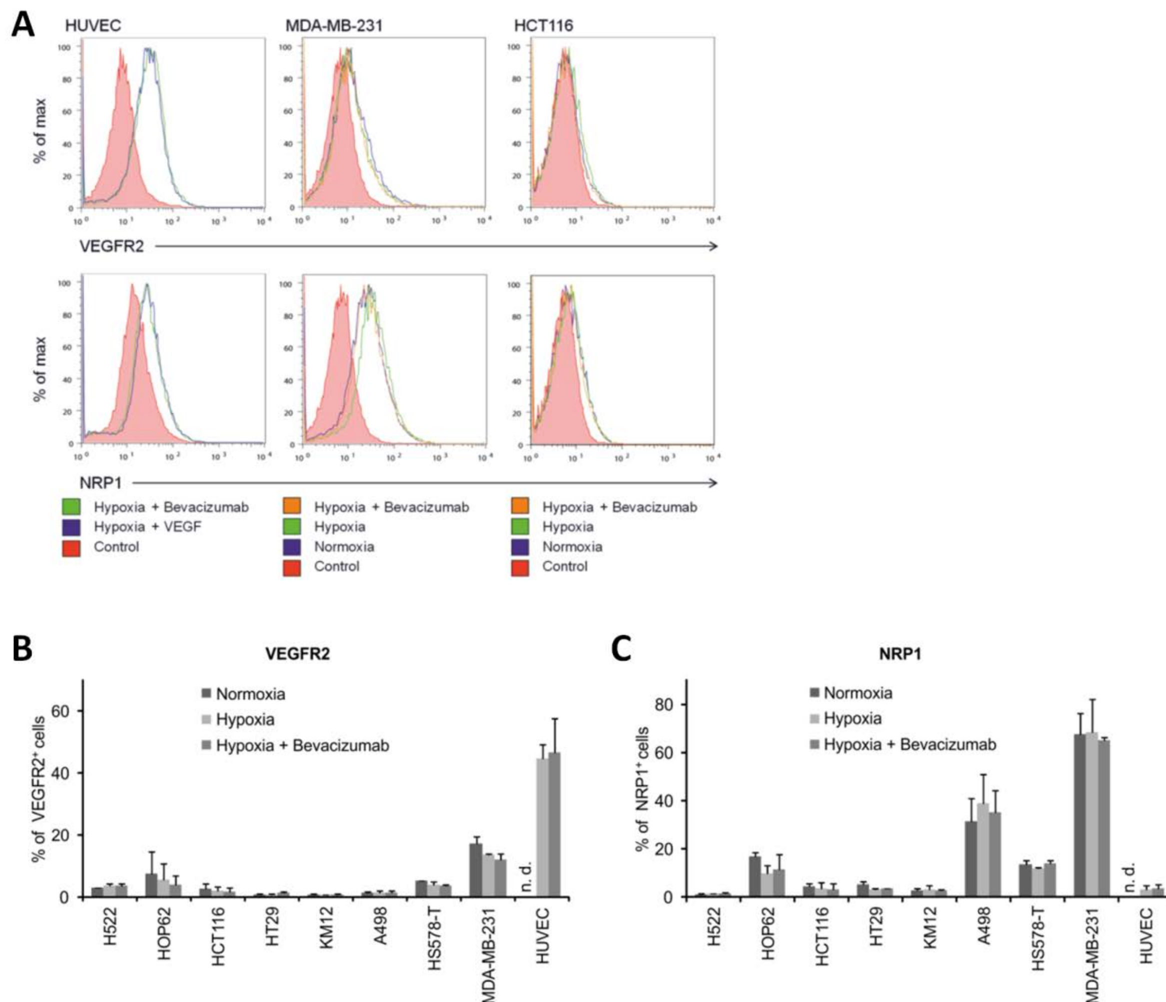


Fig. 6 Cell surface protein expression of VEGFR2 and NRP1.[1] Cell surface protein expression was analyzed by flow cytometry. Unstained cells under normoxic conditions were used as a control. HUVECs, MDA-MB-231 (BC) and HCT-116 (CRC) cells are shown as respective examples (A). Quantification of VEGFR2^{+} (B) and NRP1^{+} (C) cells. n.d. = not determined.

[1]This figure was published similarly in Hein and Graver (2013), Tumor cell response to bevacizumab single agent therapy *in vitro*. Results of RCC and BC cell lines were kindly provided by Shannon Graver and for the sake of completeness additionally presented in this thesis.

Although Neuropilin1 showed expression by western blot analysis in all cell lines investigated, cell surface expression was only evident for the cell lines MDA-MB-231 (BC) and A498 (RCC). HOP62 (NSCLC) and HS-578 T (BC) showed NRP1 cell surface expression to a lower degree with only 10 – 15 % positive cells (Fig. 6C). Cell surface expression of VEGFR2 was even more limited to only one tumor cell line (MDA-MB-231) and the endothelial positive control (HUVECs), while the percentages of positive cells remained below 10 % in the other cell lines investigated (Fig. 6B). In turn, neither hypoxic incubation nor bevacizumab treatment did change protein expression levels on the cell surface of tumor cells (Fig. 6).

6.1.2 Hypoxic induction of VEGF

Activation of the transcription factor HIF-1 under hypoxia leads to a variety of gene expression changes, including induction of *VEGFA* and *GLUT1*. Hence, tumor cells were incubated under normoxia and hypoxia for 24 hours and *VEGFA* and *GLUT1* expression levels were measured by quantitative real-time PCR (Fig. 7).

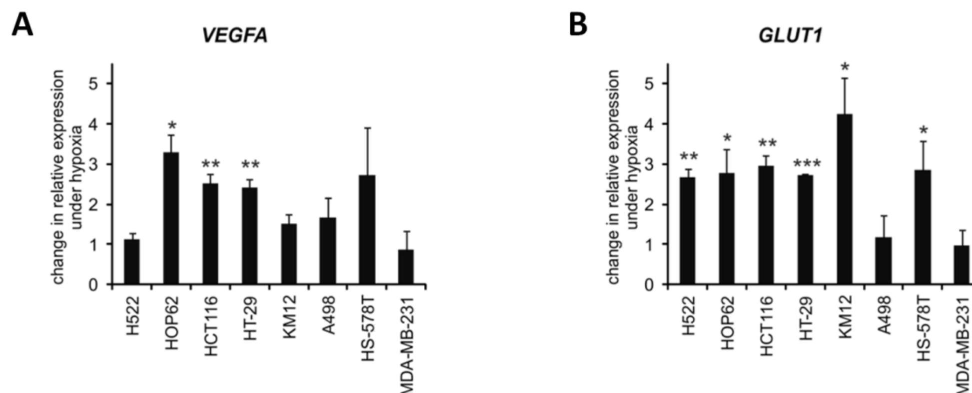


Fig. 7 Hypoxic *VEGFA* and *GLUT1* mRNA induction in tumor cells.[1] Cells were incubated under normoxia and hypoxia for 24 hours and total RNA was extracted. Quantitative real-time PCR was performed with *HPRT* as housekeeping gene. Change in relative expression of *VEGFA* (A) and *GLUT1* (B) is shown under hypoxia compared to normoxic controls. Results are an average of three biological repetitions. p-value ≤ 0.05 = *; p-value ≤ 0.01 = **, p-value ≤ 0.001 = ***.

[1]This figure was published similarly in Hein and Graver (2013), Tumor cell response to bevacizumab single agent therapy *in vitro*. Results of RCC and BC cell lines were kindly provided by Shannon Graver and for the sake of completeness additionally presented in this thesis.

Most of the tumor cell lines showed induction of *VEGFA* and *GLUT1* upon hypoxic incubation, but to variable extent between different tumor cell lines (Fig. 7). *VEGFA* was significantly upregulated in one non-small cell lung cancer cell line (HOP62) and two colorectal cell lines (HCT-116 and HT-29) with up to 3-fold increase (Fig. 7A). *GLUT1* mRNA levels were significantly upregulated in almost all tumor cell lines investigated, up to 4-fold with the exception of A498 (RCC) and MDA-MB-231 (BC), where *GLUT1* remained unregulated after incubation under hypoxia (Fig. 7B). Induction of *VEGFA* and *GLUT1* demonstrates the responsiveness of tumor cell lines to the hypoxic environment and supports the theory of an autocrine or paracrine stimulation of surrounding tumor cells by *VEGFA*.

6.1.3 Regulation of angiogenic pathway molecules upon bevacizumab treatment

Although protein expression of angiogenic receptors like VEGFR1, VEGFR2 and NRP1 was rather limited as measured by western blot and flow cytometry, gene expression analysis was performed to detect possible changes on mRNA levels. Cells were incubated under hypoxia (1 % O_2) and treated with or without bevacizumab for 24 hours. Gene expression analysis was performed by quantitative real-time PCR with *HPRT* as housekeeping gene. Fold changes were calculated from treated versus untreated cells. HUVECs were used as a

control and additionally incubated with rhVEGF to demonstrate activation of the respective receptors (indicated by *) (Fig. 8).

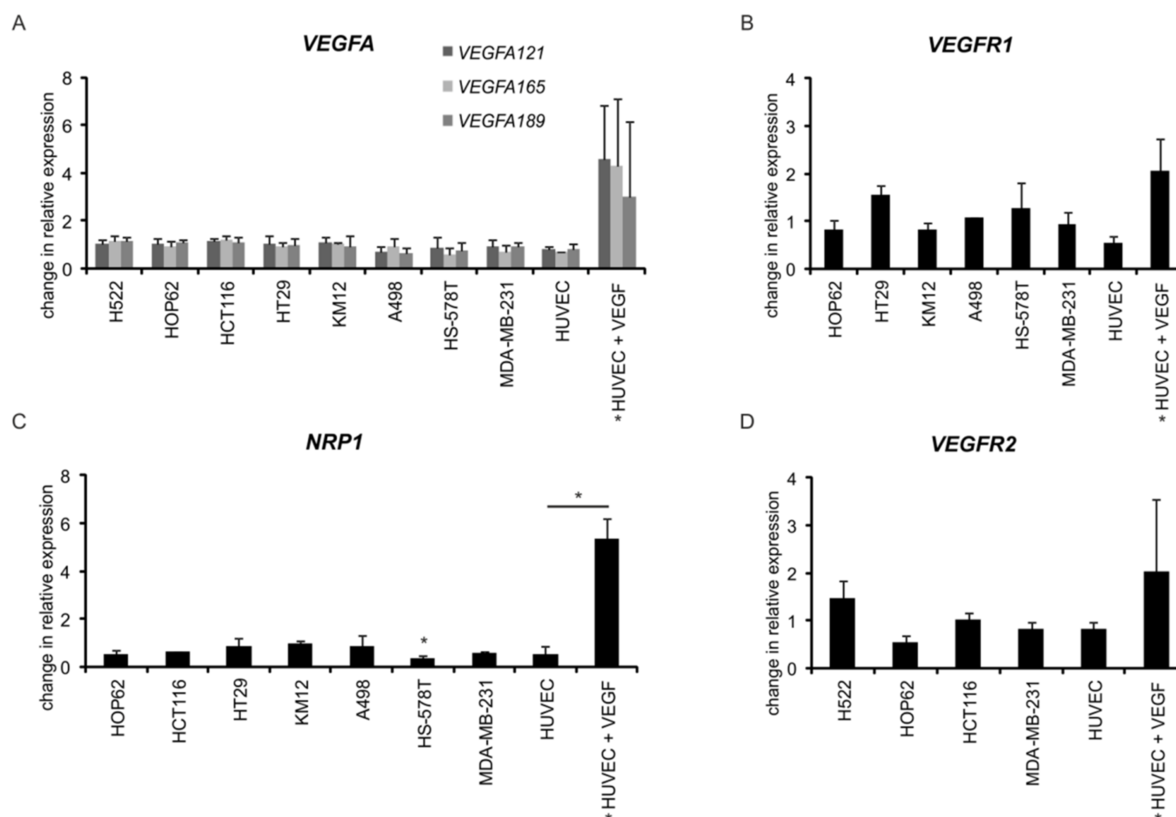


Fig. 8 Gene expression analysis in bevacizumab treated tumor and endothelial cells.[1] Change in relative expression of *VEGFA* isoforms (A), *VEGFR1* (B), *NRP1* (C) and *VEGFR2* (D) in bevacizumab treated cells after 24 hours of hypoxia versus untreated hypoxic cells. Only cell lines with detectable expression are included. p-value ≤ 0.05 = *.
* indicates HUVECs were in addition stimulated with rhVEGF in the absence of bevacizumab and normalized against untreated controls.

[1]This figure was published similarly in Hein and Graver (2013), Tumor cell response to bevacizumab single agent therapy *in vitro*. Results of RCC and BC cell lines were kindly provided by Shannon Graver and for the sake of completeness additionally presented in this thesis.

Different isoforms of *VEGFA* result from alternative splicing (Harper and Bates, 2008) and could be influenced by bevacizumab treatment in a different manner. Thus, $VEGFA_{189}$, $-_{165}$ *and* $-_{121}$ were analyzed with exon specific primers and changes upon bevacizumab treatment were evaluated. Bevacizumab treatment did not change expression of *VEGFA* isoforms in all tumor cell lines investigated as well as in HUVEC controls (Fig. 8A). Addition of rhVEGF led to an increase of *VEGFA* expression in HUVECs, however with quite high standard deviations and therefore not to a significant extent (Fig. 8A). Expression analysis of *VEGFR1* on mRNA level confirmed expression data from protein analysis. Six tumor cell lines as well as the endothelial control (HUVECs) exhibited expression of *VEGFR1*. However, no significant change in expression was obvious after bevacizumab treatment. Addition of rhVEGF showed responsiveness of HUVECs to VEGF stimulation, as *VEGFR1* was upregulated 2-fold (Fig. 8B). *VEGFR2* was only expressed by four tumor cell lines (H522,

HOP62, HCT-116 and MDA-MB-231) and the HUVEC controls, but remained more or less unregulated after bevacizumab exposure (Fig. 8D). In line with the increase of *VEGFR1* in HUVECs upon addition of rhVEGF, also *VEGFR2* expression was increased, however not to a significant extent (Fig. 8D). The VEGF-A co-receptor *Neuropilin1* was expressed by all tumor cell lines with the exception of H522, as already demonstrated by protein analysis (Fig. 8C). In general, a clear trend of downregulation of *Neuropilin1* was observed upon bevacizumab treatment in HOP62, HCT-116, HS-578 T and MDA-MB-231 cells, but only to a significant extent in one breast cancer cell line (HS-578 T). This was in line with HUVEC controls, which also showed a downregulation and an opposing upregulation after addition of rhVEGF, respectively (Fig. 8C).

In summary, gene expression analysis revealed no consistent impact on VEGF-A related genes upon bevacizumab treatment. In contrast, endothelial cells reacted to bevacizumab or rhVEGF treatment as expected. At least, regulation of *Neuropilin1* in HS-578 T cells was comparable to the downregulation seen in endothelial cells.

6.1.4 Effects of bevacizumab on biological functions

Although bevacizumab treatment resulted in rather limited changes in gene and protein expression, downstream biological functions of the VEGF-A pathway like apoptosis, proliferation and migration were evaluated after bevacizumab treatment of tumor cells.

6.1.4.1 Effects of bevacizumab on tumor cell survival

In endothelial cells VEGF-A can act as a survival factor and protect cells from apoptosis. To figure out if bevacizumab can also influence tumor cell survival, cells were treated with bevacizumab and changes in apoptotic levels were investigated. First of all, changes on protein level of cleaved PARP were evaluated after 48 hours of bevacizumab treatment in a hypoxic environment against non-treated controls. Presence and higher amounts of cleaved PARP are associated with an increasing apoptotic cell population. In addition, cells were treated with staurosporine for 24 hours, which is able to induce apoptosis (Fig. 9).

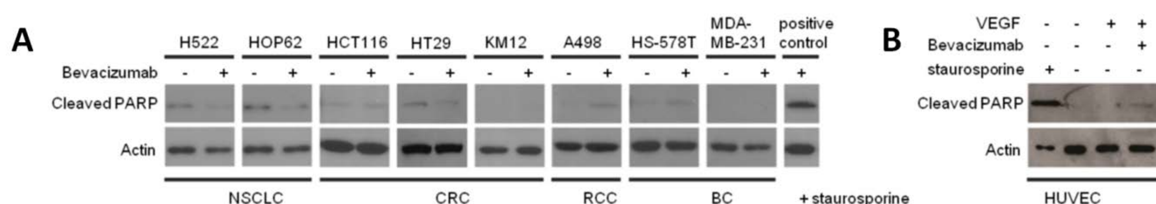


Fig. 9 Tumor cell (A) and endothelial cell (B) survival in bevacizumab treated cells.[1] Levels of apoptosis were determined by western blot analysis using an antibody against cleaved PARP. ß-Actin served as a loading control. As a positive control all cell lines were treated with staurosporine (0.15 µM) for 24 hours to induce apoptosis. An example of KM12 (CRC) and HUVECs is shown.

[1]This figure was published similarly in Hein and Graver (2013), Tumor cell response to bevacizumab single agent therapy *in vitro*. Results of RCC and BC cell lines were kindly provided by Shannon Graver and for the sake of completeness additionally presented in this thesis.

As obvious from Fig. 9, staurosporine was able to induce apoptosis, which is shown for the colorectal cell line KM12 as a representative example. In comparison to the positive control expression level of cleaved PARP was rather low in all cell lines investigated and was more or less unchanged after bevacizumab treatment. Minor changes could be detected in H522 and HOP62 cells with a slight decrease in expression of cleaved PARP, while A498 and HS-578 T cells showed a minor increase in levels of cleaved PARP after bevacizumab treatment (Fig. 9A). In HUVEC cells addition of bevacizumab slightly increased the level of cleaved PARP when compared to the non-treated as well as to the staurosporine treated control (Fig. 9B). Similar results were obtained by flow cytometry, where sub G1 levels were quantified according to propidium iodide positive cell populations (Fig. 10).

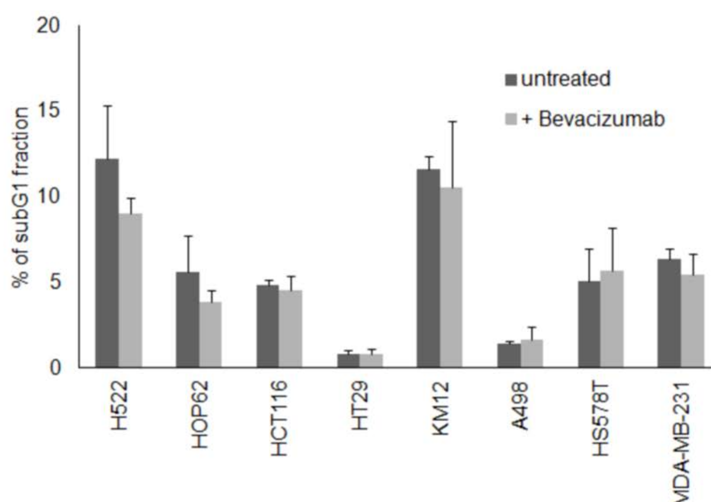


Fig. 10 Tumor cell survival of bevacizumab treated tumor cells analyzed by flow cytometry.[1] Quantification of cellular sub G1 fraction after 48 hours of bevacizumab treatment. Cells were stained with propidium iodide and analyzed by flow cytometry. Averaged data from three experiments are shown.

[1]This figure was published similarly in Hein and Graver (2013), Tumor cell response to bevacizumab single agent therapy *in vitro*. Results of RCC and BC cell lines were kindly provided by Shannon Graver and for the sake of completeness additionally presented in this thesis.

Again the two non-small cell lung cancer cell lines (H522 and HOP62) showed a slight decrease in apoptosis in line with results obtained from western blot analysis, but not to a significant extent. This trend was also evident in two colorectal cell lines, HCT-116 and KM12, but even to a lower degree. Consistent with western blot analysis, HS-578 T and A498 cells reacted to bevacizumab treatment with a slight increase in apoptosis indicated by higher percentages of cells in the sub G1 phase. However, changes in apoptotic levels observed by flow cytometry were not significant at all (Fig. 10).

6.1.4.2 Effects of bevacizumab on tumor cell proliferation

To further evaluate downstream functions of the VEGF-A pathway, proliferation rates of tumor cell lines were investigated. To challenge the system and due to high heterogeneity of different tumor cell lines, the number of cell lines per tumor type was increased (NSCLC: 9,

CRC: 6, RCC: 10, BC: 5) (Fig. 11). Proliferation was investigated for up to 72 hours under hypoxia with or without addition of bevacizumab.

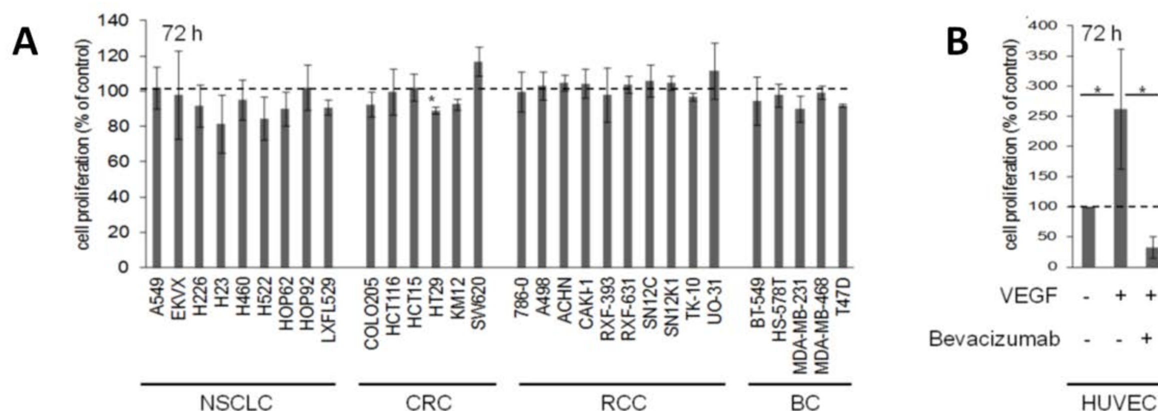


Fig. 11 Tumor (A) and endothelial (B) cell proliferation after bevacizumab treatment.[1]
Proliferation of bevacizumab treated cells as a percentage of control. Tumor cells were cultured under hypoxia and serum starved conditions for 72 hours (A). For comparison HUVECs were stimulated with rhVEGF and treated with bevacizumab (B). p-value ≤ 0.05 = *.

[1]This figure was published similarly in Hein and Graver (2013), Tumor cell response to bevacizumab single agent therapy *in vitro*. Results of RCC and BC cell lines were kindly provided by Shannon Graver and for the sake of completeness additionally presented in this thesis.

HUVECs were used as endothelial controls and in addition stimulated with rhVEGF to show their dependence on VEGF, which led to higher proliferation rates. In contrast, when treated with VEGF and bevacizumab in combination, proliferation was decreased about 77 % compared to untreated controls (Fig. 11B). In general, tumor cells did not show any measureable effect of decreased proliferation rates as shown for HUVECs, with the exception of some colorectal cell lines, e.g. HT29, KM12, HCT-15 (Fig. 11A). To emphasize minor changes observed in some of the colorectal cell lines, growth curves of representative examples under normal growth conditions (10 % FBS) and serum starved conditions (1 % FBS) with or without bevacizumab are shown in Fig. 12. Cell growth of the tumor cell line KM12 was slightly inhibited by bevacizumab after 48 and 72 hours (Fig. 12A), while cell growth of HCT-115 cells was nearly not affected (Fig. 12B). In contrast, both cell lines showed normal proliferation rates under non treated conditions with 10 % FBS (Fig. 12).

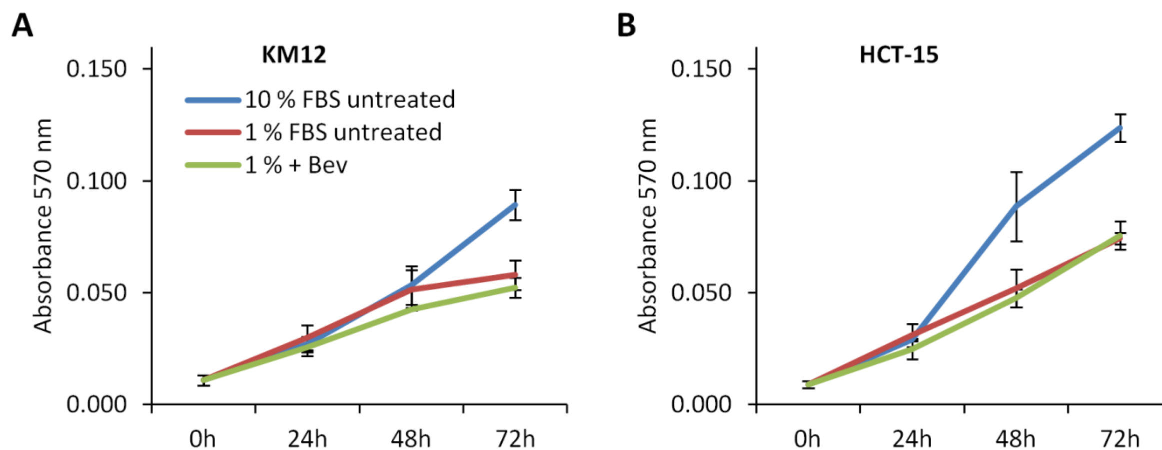


Fig. 12 Cell proliferation rates of colorectal cell lines KM12 and HCT-15. Cell proliferation was assessed for up to 72 hours under hypoxia. Cells were either cultured under standard growth conditions (10 % FBS) or serum starved conditions (1 % FBS) with or without bevacizumab.

6.1.4.3 Effects of bevacizumab on tumor cell migration

As VEGF-A can act as a motility factor in endothelial cells and influence their migratory capabilities, treatment with bevacizumab reduces migration in endothelial cells. Thus, blockade of VEGF-A by bevacizumab could also influence migration of tumor cells that are dependent on VEGF-A. Cell migration was investigated according to the *in vitro* scratch assay (Liang et al., 2007) under hypoxic and serum starved conditions with or without addition of bevacizumab. Dependent on rapid or less motile moving cell lines, migration of tumor cells was examined after six or 24 hours under hypoxia (Fig. 13).

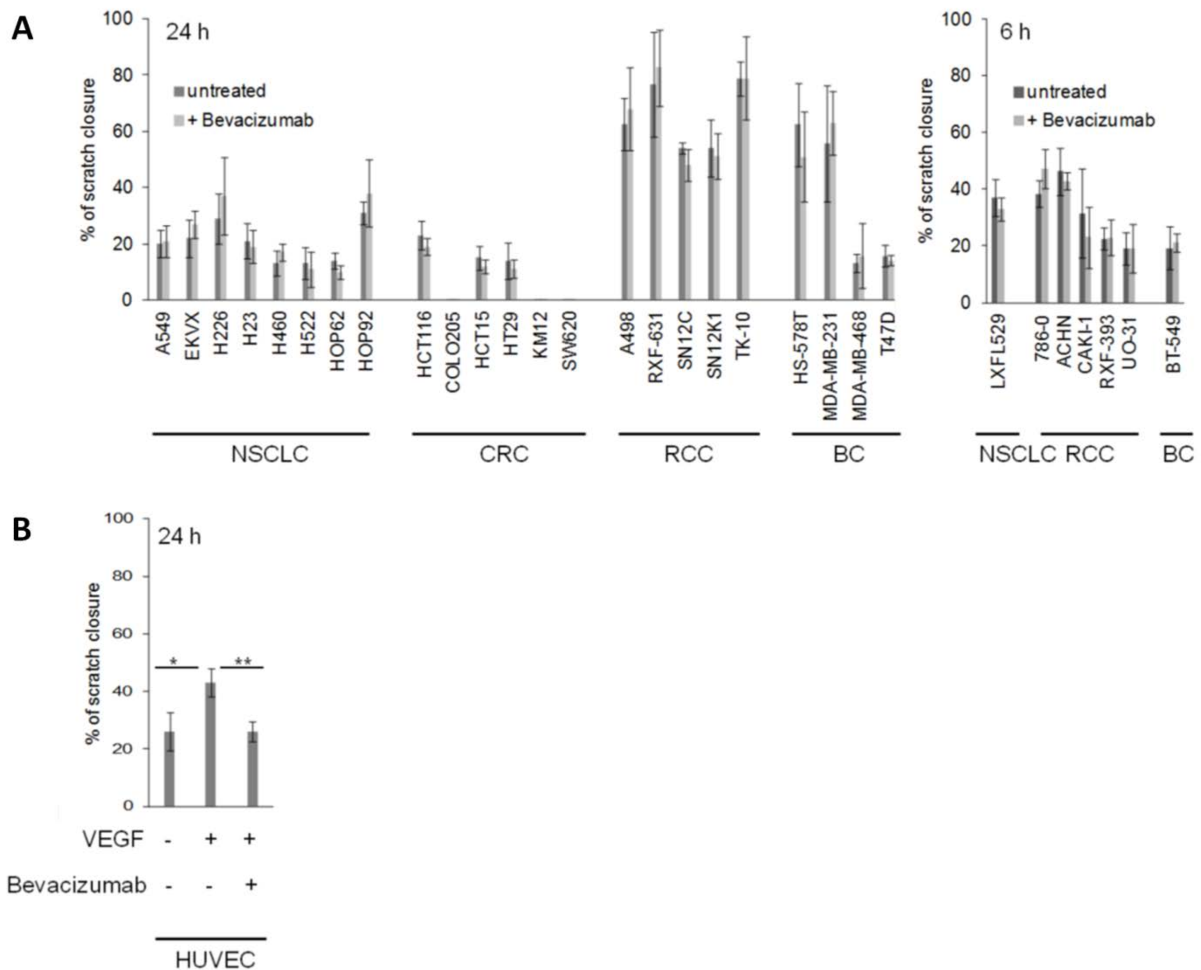


Fig. 13 Tumor cell migration analysis of bevacizumab treated tumor (A) and endothelial cells (B).[1] Migration was assessed under hypoxia after 6 or 24 hours depending on the motility of tumor cells. Endothelial cells were additionally treated with rhVEGF to demonstrate their responsiveness to VEGF. p-value ≤ 0.05 = *; p-value ≤ 0.01 = **.

[1]This figure was published similarly in Hein and Graver (2013), Tumor cell response to bevacizumab single agent therapy *in vitro.* Results of RCC and BC cell lines were kindly provided by Shannon Graver and for the sake of completeness additionally presented in this thesis.

Some colorectal cell lines (COLO205, KM12, SW620) showed absolutely no cell migration, even 24 hours after scratch initiation (Fig. 13A). In contrast, other cell lines (NSCLC: LXFL529, RCC: 786-0, ACHN, CAKI-1, RXF-393, UO-31, BC: BT-549) showed an active motility, which led to a complete closure of the scratch after 24 hours (data not shown). Thus, these cell lines were already investigated after six hours of migration (Fig. 13A). Taken together, tumor cell lines did not show any change in their migratory potential after bevacizumab treatment. In some tumor cell lines slight changes were evident upon bevacizumab treatment, but without being significant (Fig. 13A). In contrast, HUVECs reacted to VEGF addition with a significant increase in migration of cells, which supports the theory of VEGF-A being a chemo-attractant in endothelial cells. With addition of bevacizumab, migration of HUVECs was reduced to the same level as observed for the untreated controls (Fig. 13B).

6.2 *In vitro* differentiation of Hey deficient and Hey re-expressing endothelia to delineate vascular Hey gene functions

As the combined loss of Hey1 and Hey2 *in vivo* leads to a vascular phenotype and results in embryonic lethality (Fischer et al., 2004), an *in vitro* system was established to determine the function of Hey genes in endothelia. Hey1/2 double knockout ES cells were stably transfected with plasmids coding for an antibiotic selection marker driven by an endothelial specific promoter as well as with Hey1/2 doxycycline inducible plasmids and differentiated into endothelial cells. Upon overexpression of Hey1 or Hey2 in embryonic stem cells and ESC-derived endothelial cells, RNA-Seq analysis was performed to identify potential downstream target genes of Hey1 and Hey2.

6.2.1 Generation of stable embryonic stem cell lines for endothelial cell selection

Murine embryonic stem cells can be differentiated into the endothelial lineage by expression of an integrated resistance gene under the control of endothelial specific promoters as published previously (Marchetti et al., 2002). Plasmids expressing a blasticidin resistance gene under the control of endothelial specific promoters, e.g. VE-Cadherin or Tie1, were co-electroporated together with a specific transposase in a ratio of 1:6 into ΔHey1/2 murine embryonic stem cells and E14 Hey wildtype stem cells leading to a transposon based genomic integration. After selection with hygromycin, individual stable clones were picked and grown up separately. Presence of the endothelial specific promoters VE-Cadherin or Tie1 was verified by PCR (Fig. 14A). Subsequently, cells are named ΔHey1/2-(VE-Cad-bsd) and E14-(Tie1-bsd). In addition, genotyping of ES cells was performed by PCR using specific primers to distinguish between Hey1 wildtype and Hey1 deficient cells as described previously (Fischer et al., 2005). Hey1 wildtype cells showed a band at 150 bp, while Hey1 deficient cells show a specific band at 260 bp. Genotyping of ES cells confirmed the Hey1 deficient state of ΔHey1/2-(VE-Cad-bsd) cells (Fig. 14B).

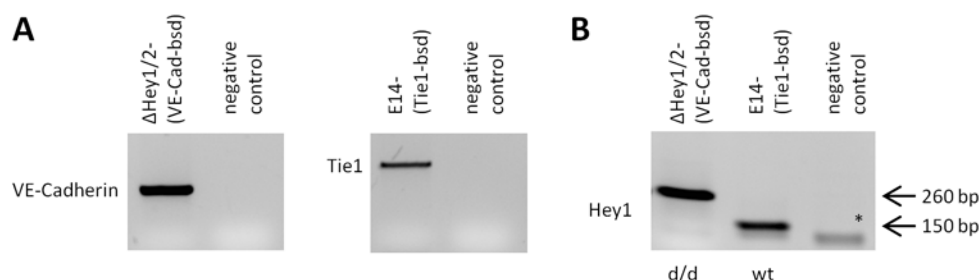


Fig. 14 PCR analysis of stable ES cells expressing endothelial specific selection markers (A) and genotyping of Hey deficient and Hey wildtype ES cells (B). ES cells were harvested and DNA was extracted using the base/neutral solution buffer system. PCR was performed using specific primers for the integrated transgene VE-Cadherin (337 bp) or Tie1 (553 bp) (A). For genotyping of ES cells Hey1 specific primers were used to distinguish between Hey1 wt (150 bp) and Hey1 deleted (260 bp) cells. * indicates primer dimers. For negative controls water was used as template instead of DNA.

The undifferentiated state of ES cells can be determined by high expression of alkaline phosphatase. ∆Hey1/2-(VE-Cad-bsd) and E14-(Tie1-bsd) cells were stained with alkaline phosphatase staining solution and observed for alkaline phosphatase positive cells. As shown in Fig. 15 ES cell colonies were positive for alkaline phosphatase staining, which confirmed their pluripotent state.

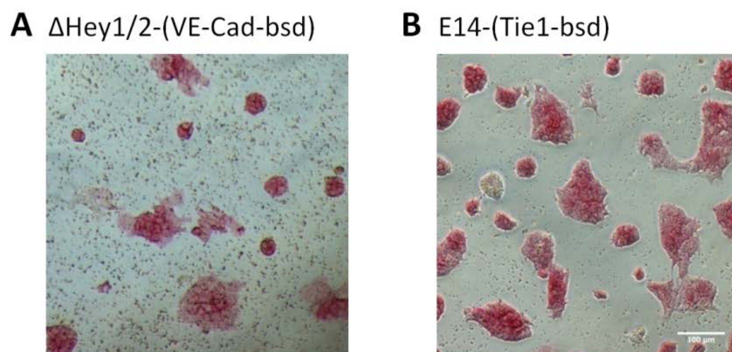


Fig. 15 Alkaline phosphatase staining of ∆Hey1/2-(VE-Cad-bsd) and E14-(Tie1-bsd) cells. Cells were stained with alkaline phosphatase staining solution and a representative picture of alkaline phosphatase positive cells is shown.

6.2.1.1 Establishment of a directed ESC-EC differentiation method

As already mentioned above, murine embryonic stem cells can be directly differentiated into endothelial cells using selection markers driven by an endothelial specific promoter (Marchetti et al., 2002) as well as by the use of chemically defined medium formulations (Blancas et al., 2011). A directed differentiation model for endothelial cells was established by combining selection marker driven enrichment of endothelial cells and the use of chemically defined mediums (Fig. 16). Two chemically defined media formulations supplemented with specific growth factors like VEGF, BMP4 and bFGF were used during differentiation. Cells were differentiated in 2D culture systems for 14 days in the absence of LIF with an induction phase of four days. After four days the culture medium was changed and bFGF was added to the culture dish (Fig. 16). From day seven for E14-(Tie1-bsd) or day eight for ∆Hey1/2-(VE-Cad-bsd) on, selection with blasticidin was started to enrich the culture for endothelial cells and to prevent overgrowth of the culture dish with embryonic stem cells. A time course model of the developed differentiation protocol is shown in Fig. 16.

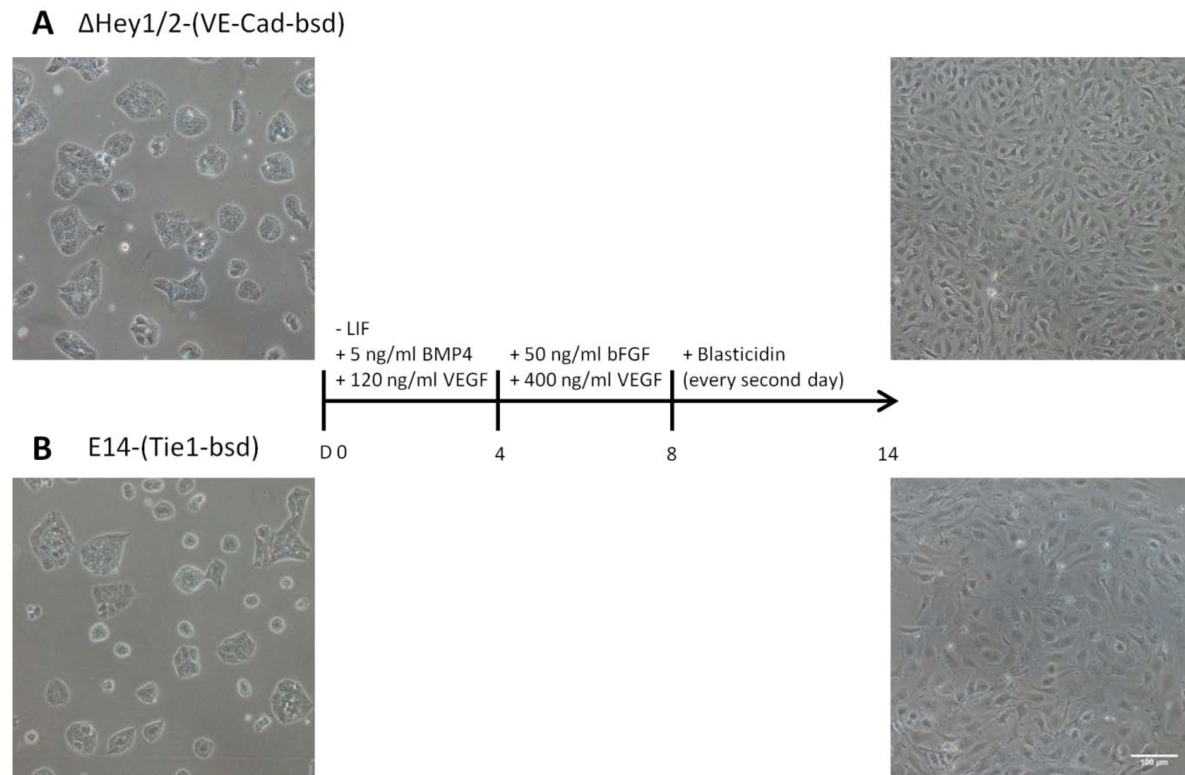


Fig. 16 Schematic model of directed differentiation of embryonic stem cells into endothelial cells. ES cells were differentiated in 2D culture systems for 14 days with an induction phase of four days. After induction a second phase of four days with another specific medium, named LdSK, was following, before selection with blasticidin was started. Selection of E14-(Tie1-bsd) cells was started from day seven, while selection for ∆Hey1/2-(VE-Cad-bsd) cells was started from day eight on.

Time points for starting selection were chosen according to relative mRNA expression levels of VE-Cadherin and Tie1 measured by qRT-PCR during differentiation of ES cells before selection markers were stably integrated into the cells. Expression levels of VE-Cadherin and Tie1 represented as ∆∆ ct values at different time points during differentiation compared to their undifferentiated counterparts are shown in Fig. 17. VE-Cadherin and Tie1 were not detectable in Hey1/2 deficient cells before day eight of differentiation, thus this time point was chosen to start selection with blasticidin (Fig. 17). For E14 ES cells selection with blasticidin was already started on day seven of differentiation because of higher doubling times of E14 ES cells when compared to Hey1/2 deficient ES cells (Fig. 17).

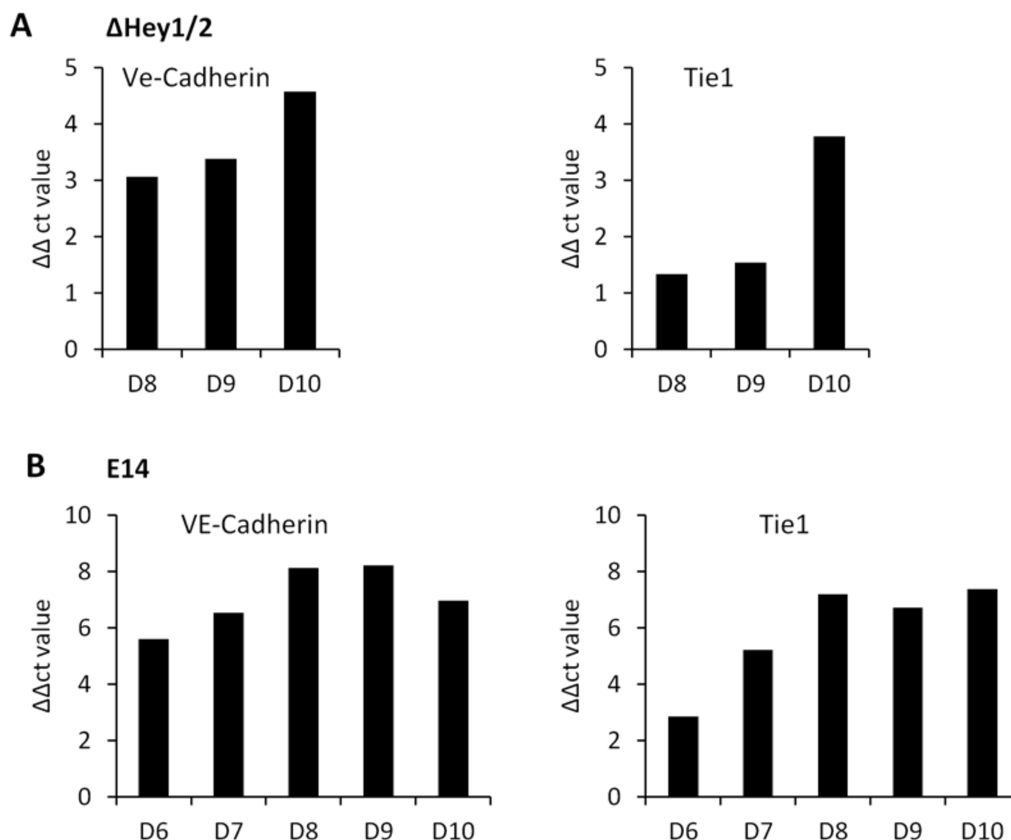


Fig. 17 Relative expression levels of VE-Cadherin and Tie1 during differentiation of ∆Hey1/2 and E14 ES cells. Cells were harvested at the indicated time points and marker expression was analyzed by qRT-PCR. Quantification was done with Hprt as a housekeeping gene and calculated against undifferentiated ES cells, which is represented in ∆∆ ct values.

Respective pictures of ∆Hey1/2-(VE-Cad-bsd) and E14-(Tie1-bsd) cells during differentiation from day eight to 14 are shown in Fig. 18. Both cell lines were able to differentiate into endothelial cells in 2D culture systems to a similar extent and showed typical endothelial cell morphology at day 14 of differentiation. At day eight of differentiation many ES cell like colonies were still present in the culture dish. During differentiation of E14-(Tie1-bsd) cells, ES cell-like colonies were present for a longer time in the culture dish when compared to differentiation of ∆Hey1/2-(VE-Cad-bsd) cells. Therefore, selection with blasticidin was already started one day earlier than for ∆Hey1/2-(VE-Cad-bsd) cells. ES cells started dying with further addition of blasticidin and were removed from the culture dish by medium change. This selection process allowed a selective enrichment of embryonic stem cell derived endothelial cells until day 14 of differentiation (Fig. 18).

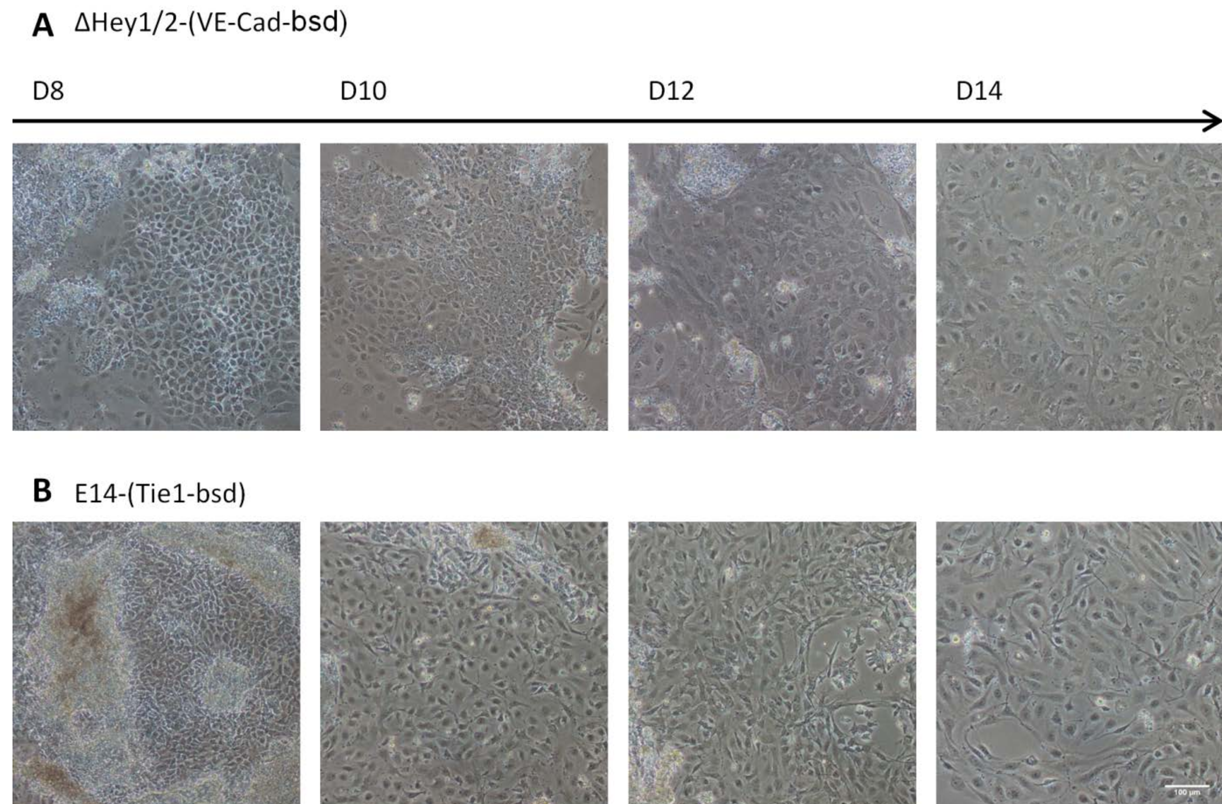


Fig. 18 Differentiation of ΔHey1/2-(VE-Cad-bsd) and E14-(Tie1-bsd) ES cells. Cells were differentiated for 14 days and selected with blasticidin from day seven for E14-(Tie1-bsd) cells and from day eight for ΔHey1/2-(VE-Cad-bsd) cells on. Pictures were taken every second day during the selection phase to observe endothelial specific colonies.

6.2.1.2 Characterization of embryonic stem cell derived endothelial cells

After establishment of a directed differentiation method for endothelial cells, cells were characterized using quantitative real-time PCR, immunofluorescence as well as a functional test. ESC-derived endothelial cells from ΔHey1/2-(VE-Cad-bsd) and E14-(Tie1-bsd) cell lines were harvested after 14 days of differentiation and total RNA was extracted. Endothelial as well as pluripotency marker genes were analyzed using qRT-PCR in comparison to undifferentiated ES cells at day 0 of differentiation (Fig. 19). Endothelial marker genes like VE-Cadherin, Vegfr2, Tie1 and Tie2 were upregulated in both cell lines when compared to their undifferentiated counterparts showing ΔΔ ct values up to 15 cycles. On the other hand, as expected, pluripotency marker genes like Nanog, Oct4 and Sox2 were strongly downregulated showing ΔΔ ct values up to ten cycles compared to undifferentiated ES cells. Taken together, the data demonstrate that both, ΔHey1/2-(VE-Cad-bsd) and E14-(Tie1-bsd) endothelial cells, expressed endothelial marker genes at comparable and high levels, while expression of pluripotency marker genes was strongly reduced (Fig. 19).

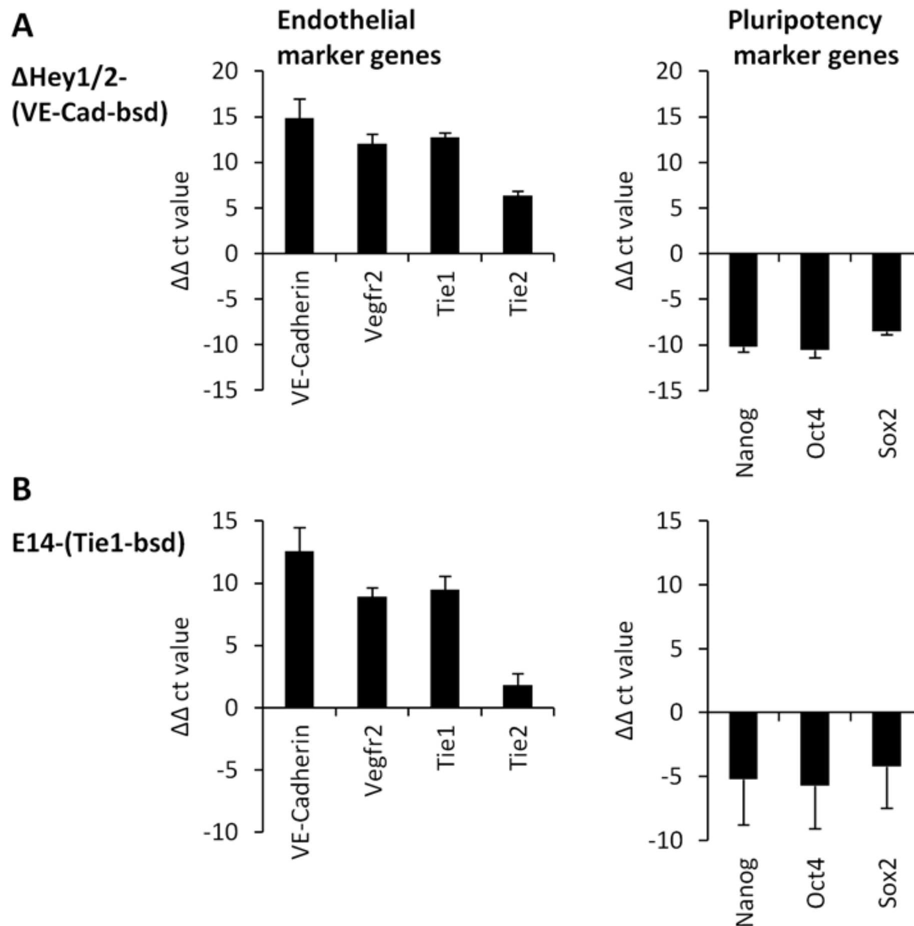


Fig. 19 Relative expression levels of endothelial and pluripotency marker genes of ESC-derived ΔHey1/2-(VE-Cad-bsd) and E14-(Tie1-bsd) endothelial cells. Cells were harvested after 14 days of differentiation and marker gene expression was analyzed by qRT-PCR. Quantification was done with Hprt as a housekeeping gene and calculated against undifferentiated ES cells, which is represented in ΔΔ ct values. Results are an average of three biological repetitions.

To confirm endothelial marker expression seen on mRNA level, protein expression of PECAM-1 and VE-Cadherin was investigated. ESC-derived endothelial cells were fixed on day 14 of differentiation and stained with antibodies against PECAM-1 (CD31) and VE-Cadherin. The immortalized mouse brain endothelioma cell line bEnd5 was used as a positive control (Wagner and Risau, 1994, Rohnelt et al., 1997). Endothelial cells derived from ΔHey1/2-(VE-Cad-bsd) and E14-(Tie1-bsd) embryonic stem cell lines exhibited typical membranous staining of PECAM-1 and/or VE-Cadherin when compared to the positive control bEnd5 (Fig. 20). The respective negative control was only incubated with the primary antibody without the following incubation of the secondary antibody and showed no expression of PECAM-1 and VE-Cadherin on the cell surface. Cell nuclei were co-stained with Hoechst dye (Fig. 20).

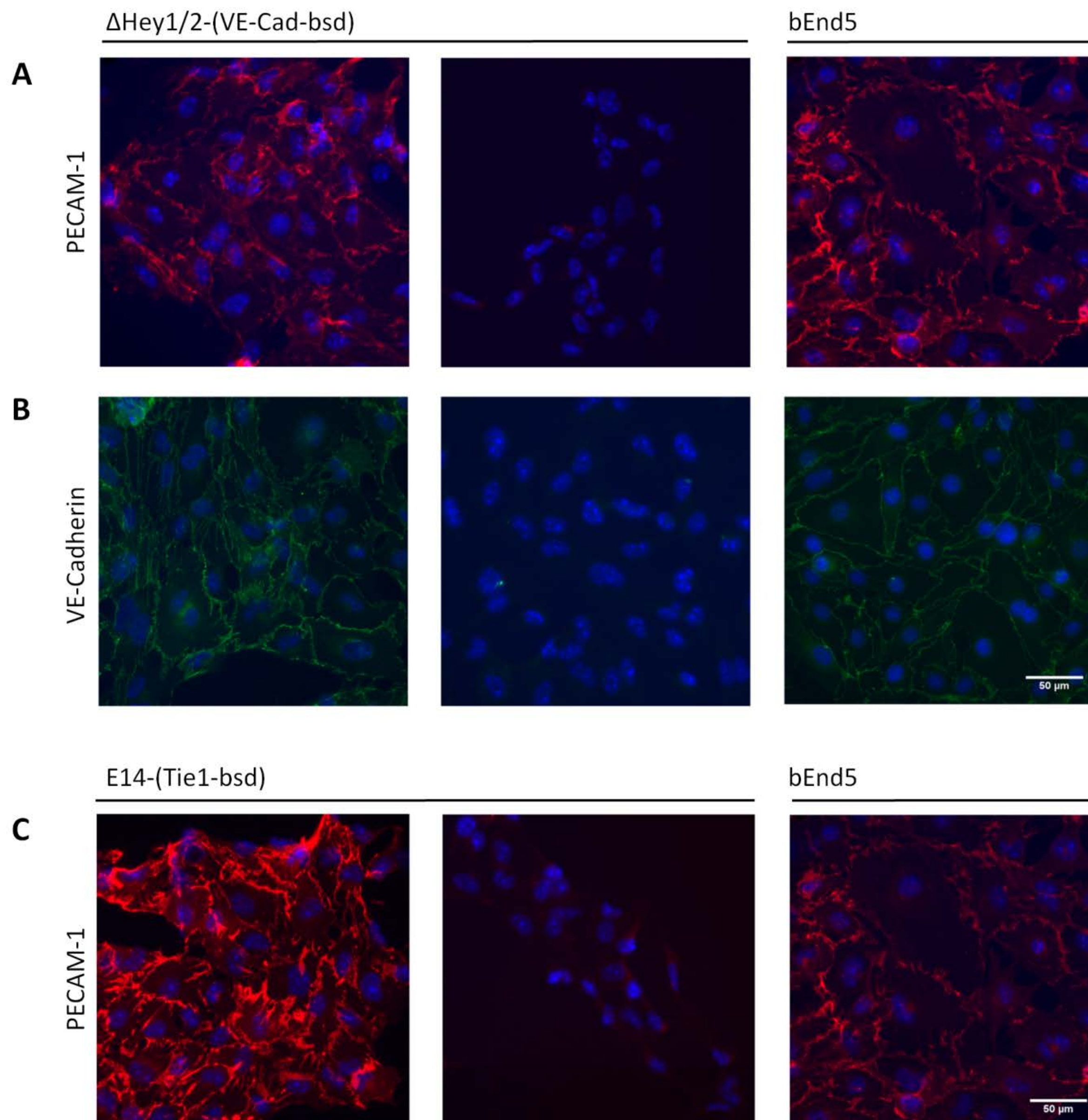


Fig. 20 Expression of PECAM-1 and VE-Cadherin in embryonic stem cell derived endothelial cells. ΔHey1/2-(VE-Cad-bsd) and E14-(Tie1-bsd) embryonic stem cells were differentiated into endothelial cells, fixed on day 14 of differentiation, stained with antibodies to PECAM-1 (A, C) or VE-Cadherin (B) and co-stained with Hoechst dye (A-C).

Functional endothelial cells are able to build capillary like structures when plated on a basement membrane matrix *in vitro* (Arnaoutova and Kleinman, 2010). To assess the biological functionality, ESC-derived endothelial cells were plated in Ldsk culture medium supplemented with 400 ng/ml VEGF and 50 ng/ml bFGF on Matrigel and the formation of capillary like structures was investigated under an inverted cell culture microscope (Fig. 21). Human umbilical vein endothelial cells (HUVECs) were used as a positive control and built tubes as early as three hours after plating on Matrigel (Fig. 21C). ESC-derived endothelial cells did not show any tube formation after plating on Matrigel, even not after longer incubation times on Matrigel (data not shown). As endothelial progenitor cells can be differentiated into mature endothelial cells by fluid shear stress (Obi et al., 2012), adherent

ΔHey1/2-(VE-Cad-bsd) and E14-(Tie1-bsd) ESC-derived endothelial cells were incubated on an orbital shaker to promote maturation. After 72 hours of incubation and shaking, cells were again plated on Matrigel and the formation of tubes was investigated. Capillary like structures were built after six hours, however at a later time point and to a lower extent when compared to HUVECs, which were not exposed to shear stress (Fig. 21A,B).

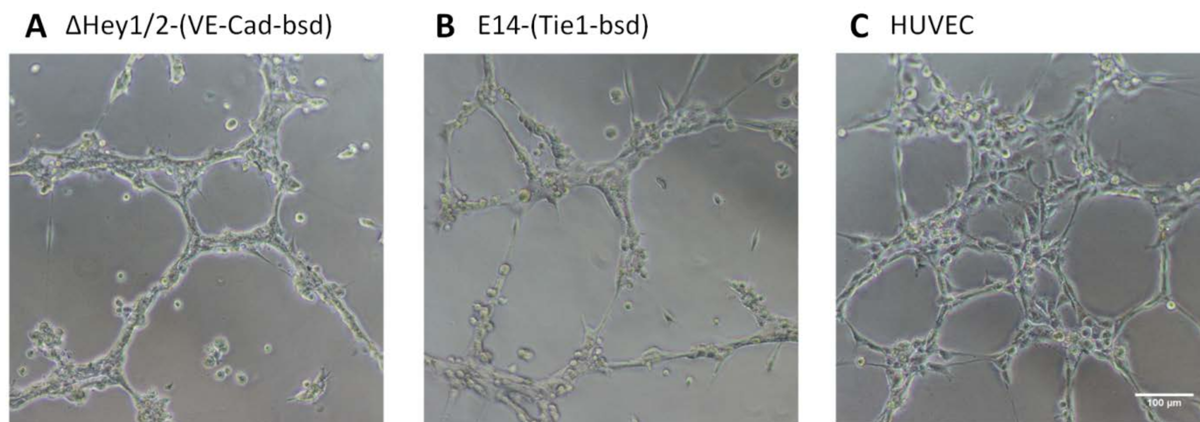


Fig. 21 Tube formation of ΔHey1/2-(VE-Cad-bsd)-, E14-(Tie1-bsd)-derived endothelial cells and HUVECs. 1.5×10^5 cells were seeded in a well of a 96 well plate onto 50 µl gelled Matrigel and investigated for tube formation after three to six hours under an inverted cell culture microscope.

Characterization of embryonic stem cell derived endothelial cells showed that cells lacking Hey1 and Hey2 are still able to differentiate into endothelial cells. Taken together, no differences in endothelial marker gene expression on mRNA level as well as on protein level were evident when compared to Hey1/2 wildtype cells. These data somehow suggested that Hey1 and Hey2 seem not to be essential in this *in vitro* setting. However, the *in vitro* differentiation system was used as a starting point for more detailed characterizations and challenges of endothelial cells. To investigate Hey1 and Hey2 mRNA expression levels during endothelial cell differentiation relative quantification was done in E14-(Tie1-bsd) Hey wildtype cells at different time points during differentiation (Fig. 22). Cells were harvested starting on day seven of differentiation and subsequently every second day from day eight on. Relative Hey1 and Hey2 expression was measured by qRT-PCR and presented as ΔΔ ct values resulting from undifferentiated ES cells at day 0 of differentiation with Hprt as a housekeeping gene. Both, Hey1 and Hey2, are enriched at least more than two cycles compared to the undifferentiated state. Hey1 expression was increased with ongoing differentiation with up to 5.7 cycles at day 14 of differentiation, while Hey2 was less strongly enriched with up to 3.5 cycles (Fig. 22).

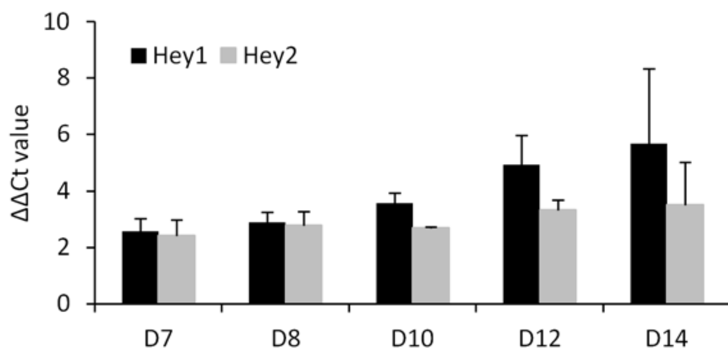


Fig. 22 Relative expression levels of Hey1 and Hey2 during differentiation of E14-(Tie1-bsd) cells. Cells were harvested starting from day seven of differentiation every second day. Hey1 and Hey2 mRNA levels were analyzed by qRT-PCR. Quantification was done with Hprt as a housekeeping gene and calculated against undifferentiated ES cells, which is represented in ΔΔ ct values. Results are an average of three biological repetitions.

6.2.2 Generation of Hey inducible embryonic stem cell lines

To further investigate the impact of Hey1 and Hey2 on endothelial cell differentiation, Hey1 and Hey2 were re-expressed in a Hey deficient background using Hey1/2 deficient cells. Flag-Strep-tagged Hey1 and Flag-tagged Hey2 under the control of a tetracycline dependent promoter were incorporated into ΔHey1/2-(VE-Cad-bsd) embryonic stem cells using the sleeping beauty transposase system as described previously (Mates et al., 2009). Flag-Strep-tagged Hey1 or Flag-tagged Hey2 plasmids were co-transfected together with a transposase (SB100X) in a ratio of 1:1, which led to integration of the transgene to a genomic target site. After selection with puromycin, individual clones were picked and grown up separately. Individual clones were induced with 1 µg/ml doxycycline for 48 hours and harvested for western blot analysis. Induction with doxycycline led to a strong overexpression of Hey1 and Hey2, respectively (Fig. 23). Without addition of doxycycline to the cell culture medium expression of Hey1 and Hey2 was not detectable. Tubulin was used as a loading control (Fig. 23). Subsequently, cells are named ΔHey1/2-(VE-Cad-bsd)-pSB-FS-mHey1 and ΔHey1/2-(VE-Cad-bsd)-pSB-Flag-mHey2.

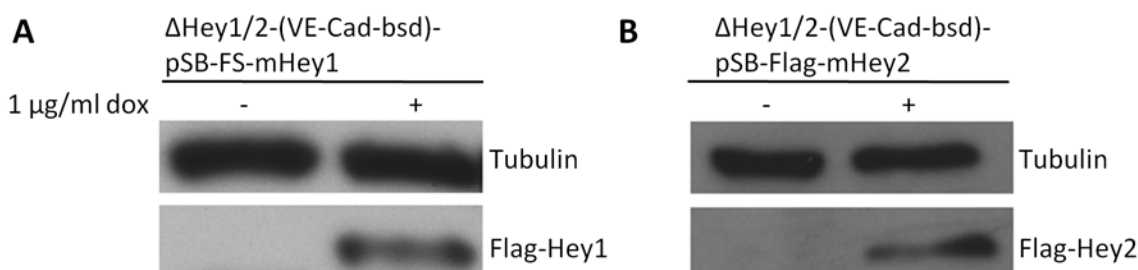
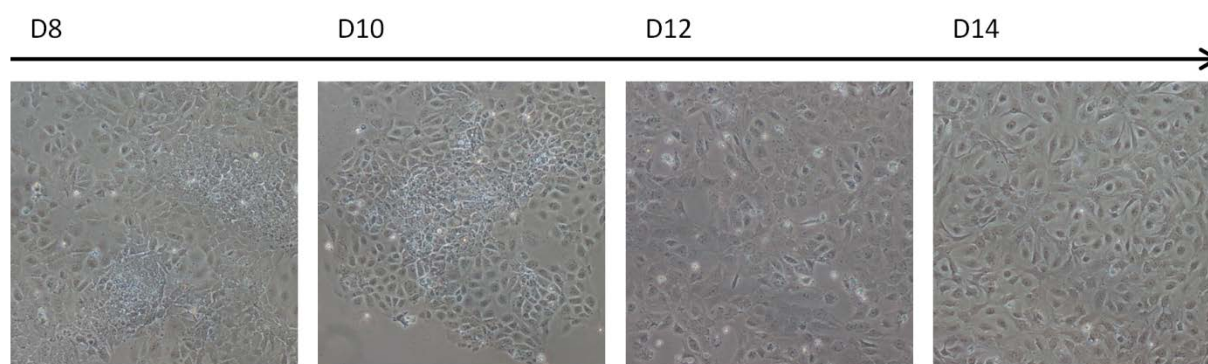


Fig. 23 Western blot analysis of Hey inducible embryonic stem cell lines. ΔHey1/2-(VE-Cad-bsd)-pSB-FS-mHey1 and ΔHey1/2-(VE-Cad-bsd)-pSB-Flag-mHey2 embryonic stem cells were induced with 1 µg/ml doxycycline for 48 hours. Tubulin served as a loading control. α-Flag was diluted 1:2000, α-Tubulin 1:10000.

6.2.2.1 Differentiation and characterization of Hey re-expressing endothelial cells

After stable integration of inducible plasmids containing Hey1 or Hey2, embryonic stem cells were differentiated into endothelial cells as described before (6.2.1.1). Respective pictures of ΔHey1/2-(VE-Cad-bsd)-pSB-FS-mHey1 and ΔHey1/2-(VE-Cad-bsd)-pSB-Flag-mHey2 cells from day eight to 14 during differentiation are shown in Fig. 24. Both cell lines are able to differentiate in 2D culture systems into endothelial cells similarly and show typical endothelial cell morphology at day 14 (Fig. 24).



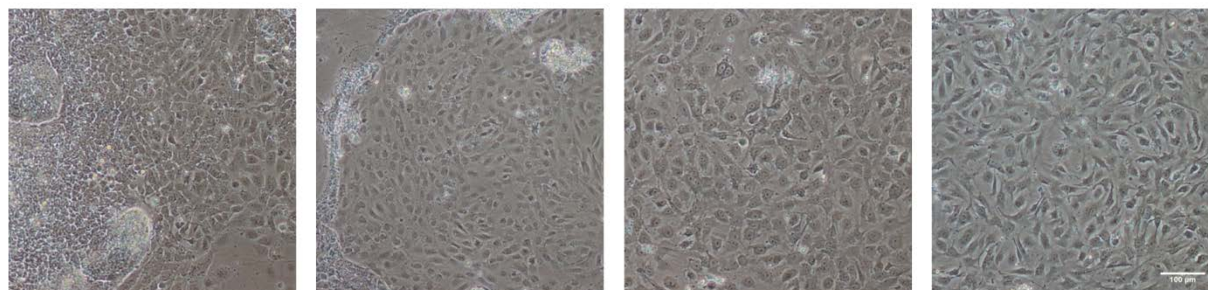


Fig. 24 Differentiation of ΔHey1/2-(VE-Cad-bsd)-pSB-FS-mHey1 and ΔHey1/2-(VE-Cad-bsd)-pSB-Flag-mHey2. Cells were differentiated for 14 days and selected with blastidicidin from day eight on. Pictures were taken every second day during the selection phase to observe endothelial specific colonies.

At day 14 of differentiation embryonic stem cell derived endothelial cells were seeded on a 6 well plate and on the following day doxycycline (1 µg/ml) was added for 48 hours to induce expression of Hey1 and Hey2. Flag-tagged versions of Hey1 or Hey2 are linked via an IRES site to a GFP coding sequence, which is also under the control of the tetracycline dependent promoter. Endothelial cells were investigated for GFP positive cells, which were only present after induction with doxycycline, however to a very low extent, as shown in Fig. 25. These data reflect that Hey1 and Hey2 expression could partially be restored in Hey deficient cells upon addition of doxycycline as demonstrated by GFP expression Fig. 25.

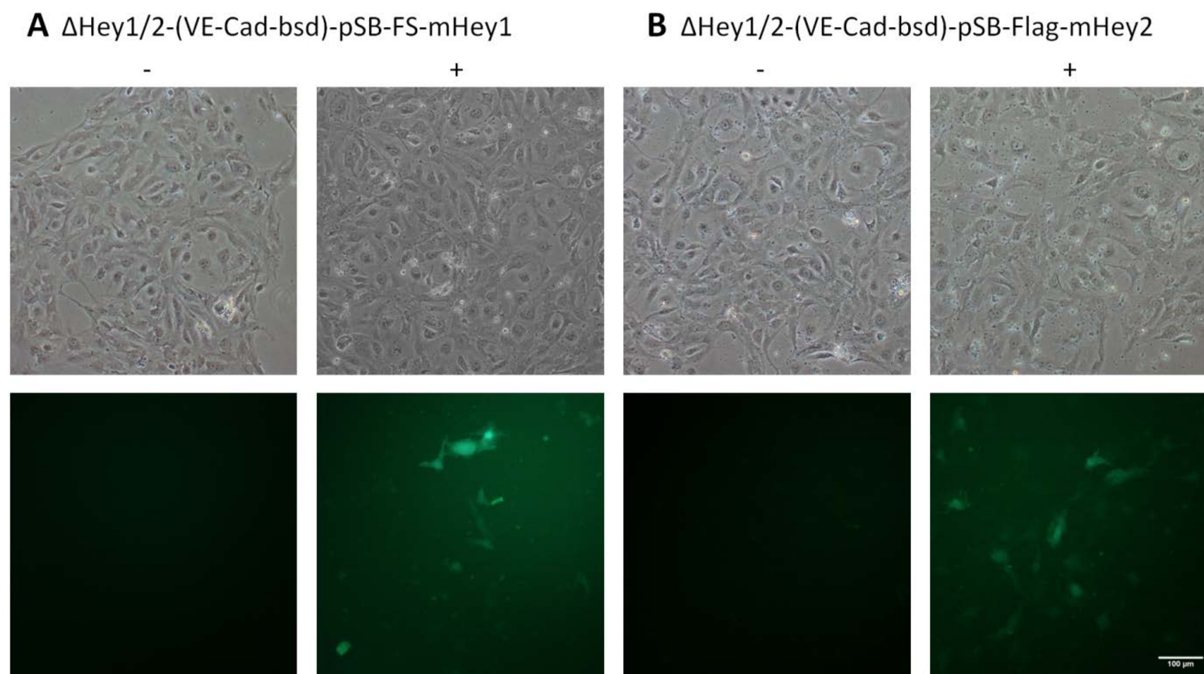


Fig. 25 Induction of ΔHey1/2-(VE-Cad-bsd)-pSB-FS-mHey1 and ΔHey1/2-(VE-Cad-bsd)-pSB-Flag-mHey2 derived endothelial cells. Cells were differentiated for 14 days and selected with blasticidin from day eight on. At day 15 of differentiation cells were induced with 1 µg/ml doxycycline for 48 hours. Pictures in the upper lane were taken with bright-field mode, while pictures in the lower lane represent the corresponding fluorescent picture.

In addition to the analysis of GFP expression levels, protein expression of Hey1 and Hey2 was quantified in embryonic stem cells using Bradford reagent. Cells were induced with two different concentrations of doxycycline, 50 ng/ml and 1000 ng/ml, and harvested after 72 hours of incubation for western blot analysis (Fig. 26). A total amount of 50 µg of protein was separated on a SDS-Page with tubulin serving as a loading control. Subsequently, proteins were transferred to a nitrocellulose membrane and incubated with the respective antibodies. Addition of doxycycline led to a stronger expression of Hey2 compared to Hey1 (Fig. 26). A quite low concentration of doxycycline (50 ng/ml) was already sufficient to induce Hey2 expression, while Hey1 expression was only evident after induction with higher concentrations of doxycycline (1000 ng/ml). These data reflect a stronger overexpression of Hey2 compared to Hey1 in embryonic stem cells upon stimulation with doxycycline, although cells were induced with the same amount of doxycycline (Fig. 26).

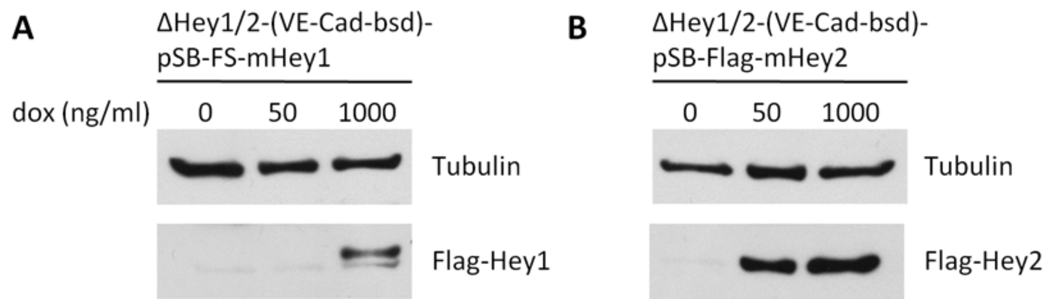


Fig. 26 Western blot analysis of Hey inducible embryonic stem cell lines. ΔHey1/2-(VE-Cad-bsd)-pSB-FS-mHey1 and ΔHey1/2-(VE-Cad-bsd)-pSB-Flag-mHey2 embryonic stem cells were induced with either 50 ng/ml or 1000 ng/ml doxycycline for 72 hours. Tubulin served as a loading control. Protein quantification was done using Bradford reagent and a total amount of 50 µg protein was loaded on a SDS-Page. α-Flag was diluted 1:2000, α-Tubulin 1:10000.

After the establishment of Hey1 and Hey2 overexpressing embryonic stem cell lines, cells were used for further endothelial differentiation experiments to elucidate the role of Hey genes in vascular development. It is known from the literature that Hey expression leads to arteriogenesis and to the development of rather arterial than venous endothelial cells. Therefore, we expected different or opposing regulation of arterial and venous genes in endothelial cells upon Hey overexpression. To this end, ΔHey1/2-(VE-Cad-bsd)-pSB-FS-mHey1 and ΔHey1/2-(VE-Cad-bsd)-pSB-Flag-mHey2 embryonic stem cells were differentiated into endothelial cells and induced with doxycycline (1 µg/ml) for 72 hours. Cells were harvested and gene expression analysis was performed using qRT-PCR (Fig. 27). The fold change was calculated from induced versus non-induced samples but did not show any significant change between arterial (Dll4, EphrinB2) and venous (CoupTFII, CoupTFII alt and EphB4) marker genes. The venous marker gene CoupTFII exists in two isoforms, whereby one isoform lacks a DNA binding domain and is referred as CoupTFII alternative (alt). Arterial genes were slightly induced upon Hey2 overexpression, while the venous genes, CoupTFII and CoupTFII alt, were slightly reduced, however with high standard deviations and thus not to a significant extent (Fig. 27).

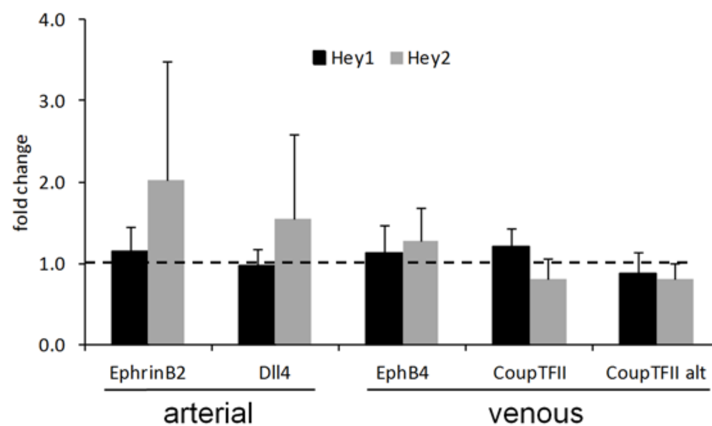


Fig. 27 Relative expression levels of arterial and venous marker genes of ESC-derived endothelial ∆Hey1/2-(VE-Cad-bsd)-pSB-FS-mHey1 and ∆Hey1/2-(VE-Cad-bsd)-pSB-Flag-mHey2 cells. After 14 days of differentiation cells were induced with 1 µg/ml doxycycline for 72 hours. Cells were harvested and gene expression was analyzed by qRT-PCR. Quantification was done with Hprt as a housekeeping gene. The fold change was calculated from doxycycline induced samples against non-induced controls. Results are an average of three biological repetitions.

6.2.3 RNA-Sequencing of Hey inducible cell lines

In order to determine potential vascular Hey1/2 downstream target genes, RNA-Sequencing was performed with ∆Hey1/2-(VE-Cad-bsd)-pSB-FS-mHey1 and ∆Hey1/2-(VE-Cad-bsd)-pSB-Flag-mHey2 embryonic stem cells as well as embryonic stem cell derived endothelial cells that overexpress the respective gene. RNA-Seq analysis revealed that about 90 % of the reads of each sample aligned to the Mus Musculus genome (NCBI37/mm9). For further details see Table 1 in the appendix.

6.2.3.1 RNA-Sequencing of Hey inducible embryonic stem cells and endothelial cells after Hey1/2 overexpression

For RNA-Sequencing of ∆Hey1/2-(VE-Cad-bsd)-pSB-FS-mHey1 and ∆Hey1/2-(VE-Cad-bsd)-pSB-Flag-mHey2 embryonic stem cells and endothelial cells, cells were induced with 1µg/ml doxycycline for 72 hours with the exception of ∆Hey1/2-(VE-Cad-bsd)-pSB-FS-mHey1 endothelial cells, which were only induced for 48 hours. RNA was extracted and cDNA libraries were generated as described in 5.3.5. For reasons of simplification, ∆Hey1/2-(VE-Cad-bsd)-pSB-FS-mHey1 and ∆Hey1/2-(VE-Cad-bsd)-pSB-Flag-mHey2 embryonic stem cells are subsequently named ES Hey1$^+$ and ES Hey2$^+$, while endothelial cells are subsequently named EC Hey1$^+$ and EC Hey2$^+$.

For analysis of raw data, genes with less than ten reads per million (RPM) were excluded from further analysis. Noise expression was calculated from reads mapping to non-exonic regions of the genome and a gene was considered as expressed with a value of ≥ 2.26 reads per kilo base per million (RPKM). This value is ten-fold higher than RPKM values for non-exonic regions of the genome. Analysis of RNA-Sequencing data revealed differences in overexpression intensities of Hey1 and Hey2 in both cell types. Hey1 was elevated 14-times

higher than Hey2 in embryonic stem cells after induction with the same amount of doxycycline (fold change Hey1: 343.17, Hey2: 23.77). Similar results were obtained in endothelial cells, where Hey1 was increased more than 4.7-fold compared to Hey2 (fold change Hey1: 15.27, Hey2: 3.26). RNA-Sequencing showed a stronger increase of Hey1 than Hey2 in both, embryonic stem cells as well as endothelial cells, although protein expression in embryonic stem cells was higher for Hey2 than for Hey1 as shown by western blot analysis (Fig. 26).

To identify Hey target genes the following selection criteria were used: a gene was considered as regulated if the fold change between control and induced sample was ≥ 1.8, while the RPKM value had to be ≥ 2.26 in at least one sample. Genes regulated by either Hey1 or Hey2 were clustered with Cluster 3.0, which resulted in 354 regulated genes in embryonic stem cells and 123 regulated genes in endothelial cells as illustrated by the respective heatmaps in Fig. 28A.

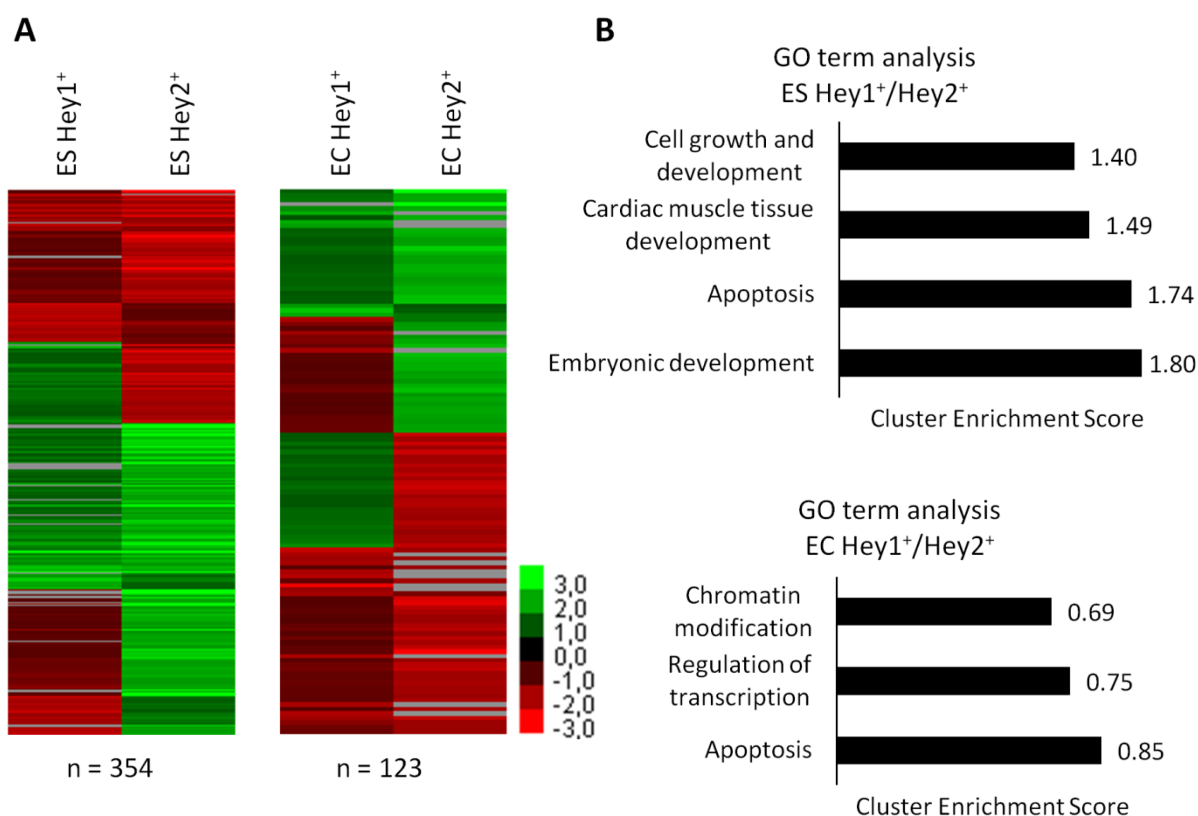


Fig. 28 Heatmap of ES Hey1$^+$/Hey2$^+$ and EC Hey1$^+$/Hey2$^+$ (A) and GO term analysis (B). Clustering was done using Cluster 3.0 with at least one observation with an absolute value ≥ 1.8. GO term analysis was done using DAVID 6.7, while only biological processes were considered. A Cluster Enrichment Score of 1.3 was considered as significant (p-value ≤ 0.05).

Upregulated genes are presented in green colors, while downregulated genes are shown in red colors. No expression of the respective gene is indicated in grey colors. The data reflect that nearly three times more genes were regulated in embryonic stem cells (n = 354) than in endothelial cells (n = 123) upon Hey overexpression (Fig. 28A). The entire list including fold changes of the target genes regulated by Hey1 and Hey2 in ES and endothelial cells is

provided in the appendix in Table 2. Regulated genes in embryonic stem cells and endothelial cells were analyzed for Gene Ontology (GO) terms using DAVID 6.7, while only biological processes were considered. An enrichment score of 1.3 was considered to be significant with a p-value ≤ 0.05. (Fig. 28B). The higher the value of the Cluster Enrichment Score, the higher is the number of regulated genes related to the respective GO term. For regulated genes in endothelial cells the major three GO terms are presented, as no significant enrichment was observed. Most enriched biological processes in endothelial cells were apoptosis, regulation of transcription and chromatin modification, however not to a significant extent. In contrast to endothelial cells, significant enriched biological processes in embryonic stem cells were embryonic development, apoptosis, cardiac muscle tissue development and cell growth as well as embryonic development (Fig. 28B).

6.2.3.2 Comparison of embryonic stem cells and endothelial cells after Hey overexpression by RNA-Sequencing

To further elucidate the role of Hey1 and Hey2 in regulating target genes, comparative analysis between embryonic stem cells and endothelial cells was performed regarding regulated genes by either Hey1 or Hey2. Respective heatmaps and GO term analysis were performed with the same selection criteria as described in section 6.2.3.1 and presented in Fig. 29. Comparison of the number of regulated genes by either Hey1 (n = 106) or Hey2 (n = 382) in embryonic stem cells and endothelial cells revealed that 3.6-fold more genes were regulated by Hey2 than by Hey1, hypothesizing a more important role of Hey2 in target gene regulation (Fig. 29A). The entire list of regulated target genes including the fold changes is provided in Table 3 in the appendix. However, the higher number of genes regulated by Hey2 could also be due to higher protein expression upon induction with doxycycline as shown in Fig. 26. GO term analysis for genes regulated by Hey1 in embryonic stem cells and endothelial cells did not show any significant enrichment, as the number of the Cluster Enrichment Score remained below 1.3. The top three enriched terms upon Hey1 overexpression were cell secretion, cell cycle and apoptosis related processes. In contrast to Hey1, genes regulated by Hey2 revealed five significantly enriched biological processes: Apoptosis, cytoskeleton organization, regulation of transcription, heart development and embryonic development (Fig. 29B). As expected, many developmental processes as well as regulation of transcription were represented, as already shown in a previous study upon Hey1/2 overexpression in HEK-293 cells (Heisig et al., 2012). According to the data derived from RNA-Sequencing in embryonic stem cells and endothelial cells one could hypothesize that Hey2 plays a more important role in the regulation of target genes than Hey1.

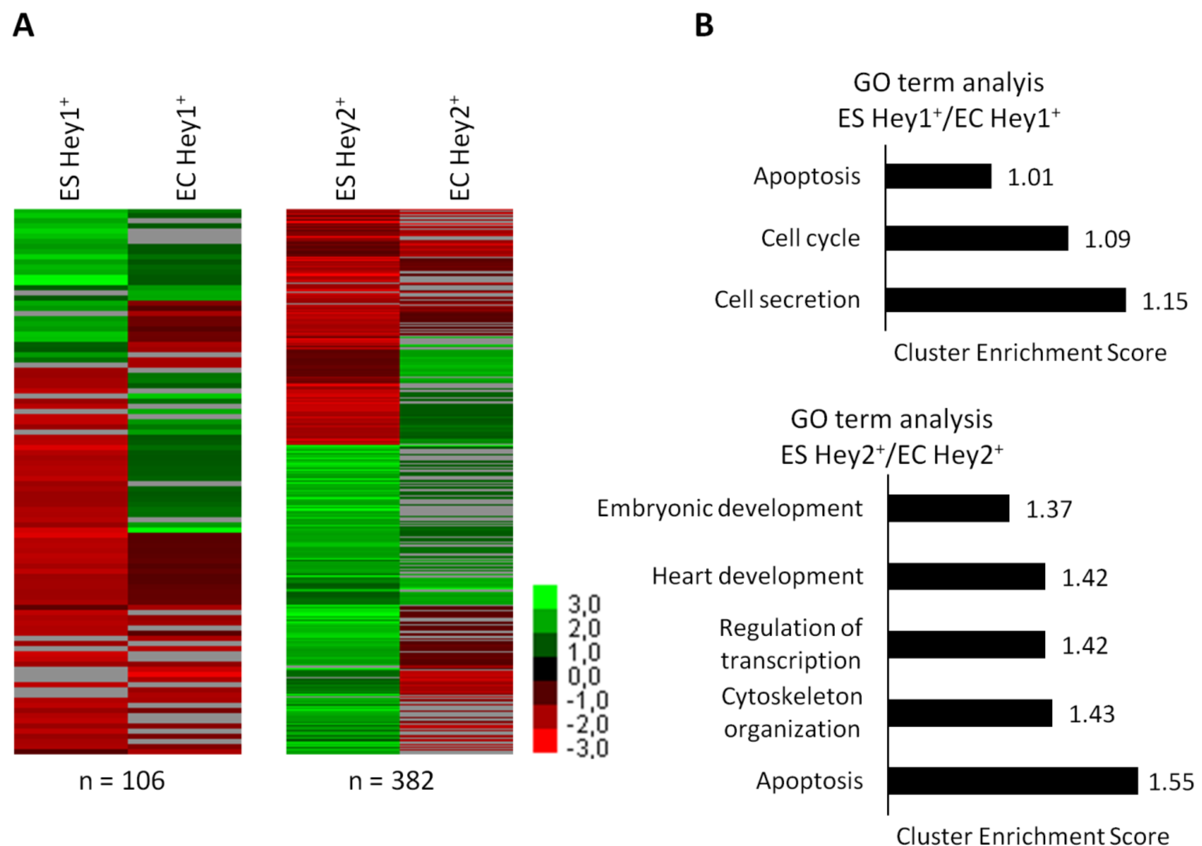


Fig. 29 Heatmap of ES/EC Hey1$^+$ and ES/EC Hey2$^+$ (A) and GO term analysis (B). Clustering was done using Cluster 3.0 with at least one observation with an absolute value ≥ 1.8. GO term analysis was done using DAVID 6.7, while only biological processes were considered. A Cluster Enrichment Score of 1.3 was considered as significant (p-value ≤ 0.05).

6.2.3.3 Target gene validation in embryonic stem cells and endothelial cells

Several Hey regulated genes in embryonic stem cells and endothelial cells were chosen and validated with qRT-PCR. First of all induction, of either Hey1 or Hey2 was verified. Both genes were significantly increased in the respective cell line when compared to the non-induced control. No expression of Hey1 was found in the Hey2 overexpressing cell line and vice versa, as both cell lines were generated on a Hey deficient background of Hey1 and Hey2 (Fig. 30). In embryonic stem cells most of the genes were regulated by both, Hey1 and Hey2. Fold changes for genes, which were upregulated (Smarca2, Krt19, Nptx2, Calcoco2, Icam1, Ptges) or downregulated (Sox1, EG434280, Serpine2, Perp, Fabp3, Skil) by Hey1 and Hey2 could be confirmed very precisely (Fig. 30). Most of the Hey target genes showed a similar regulation by Hey1 and Hey2, which reflects their great redundancy in regulating target genes, as published in a previous study (Heisig et al., 2012). However, there were some genes, which were not regulated by Hey1 and thus regulated by Hey2 alone. Spred3 and Fibronectin1 were upregulated, while Lefty1 and Lefty2 were downregulated by Hey2. No change in regulation was observed for these genes in the corresponding Hey1 overexpressing cell line (Fig. 30).

	A ES Hey1⁺			**B ES Hey2⁺**		
	RNA-Seq	qRT-PCR		RNA-Seq	qRT-PCR	
Hey1	343.17	383.94	***	-	-	
Hey2	-	-		23.77	15.96	**
Smarca2	2.92	2.12	**	3.59	3.99	**
Krt19	2.13	1.70		2.40	2.52	
Nptx2	1.94	2.25	**	2.83	3.31	***
Calcoco2	1.83	1.48		2.13	3.55	**
Icam1	1.73	1.46	*	2.48	2.60	**
Ptges	1.70	1.67	**	2.55	2.41	*
Spred3	1.15	-1.10		2.19	1.92	
Fibronectin1	1.06	1.15		2.46	1.47	**
Sox1	-2.31	-2.68	*	-2.66	-1.56	
EG434280	-2.29	-1.53	*	-2.69	1.06	
Serpine2	-2.13	-1.79	*	-2.50	-2.04	
Perp	-2.18	-1.54		-2.78	-2.86	*
Fabp3	-1.65	-1.55	*	-1.86	-1.66	
Skil	-1.38	-1.56	**	-2.14	-1.98	*
Myl9	-	-1.32		-1.93	1.22	
Lefty1	1.45	1.62		-4.03	-2.37	*
Lefty2	1.36	1.23		-2.22	-1.77	
Actc1	-1.02	-1.40		-5.74	-1.20	
Hspg2	-1.39	-1.12		1.89	-1.02	

Fig. 30 Validation of ES Hey1$^+$ and ES Hey2$^+$ target genes by qRT-PCR. Fold changes of RNA-Seq and qRT-PCR analysis are shown. p-value ≤ 0.05 = *; p-value ≤ 0.01 = **, p-value ≤ 0.001 = ***. Results are an average of three biological repetitions.

In contrast to embryonic stem cells, target gene validation in endothelial cells was rather limited (Fig. 31). However, Hey1 and Hey2 overexpression was confirmed by qRT-PCR as both genes were significantly upregulated. Neither for genes, which were regulated by both, Hey1 and Hey2, (Dnajc4, Rarres1) nor for genes, which were regulated by Hey1 (Cxxc1, Psmb10) or Hey2 (Ddit3, Hdac8, C1d, Esm1, Mier3) alone, regulation could be confirmed by qRT-PCR. According to the data derived from qRT-PCR analysis, target genes of Hey1 and Hey2 in endothelial cells remained more or less unregulated after overexpression of the respective gene (Fig. 31).

	A EC Hey1⁺			B EC Hey2⁺		
	RNA-Seq	qRT-PCR		RNA-Seq	qRT-PCR	
Hey1	15.27	8.55	*	-	-	
Hey2	-	-		3.26	3.68	*
Dnajc4	1.64	-1.13		2.01	1.25	
Rarres1	-1.90	1.10		-2.29	1.70	
Cxxc1	1.89	1.00		-	-	
Psmb10	-2.02	-1.18		1.40	-1.50	
Ddit3	1.01	-1.48		-2.40	-1.14	
Hdac8	1.26	-1.06		-2.08	1.12	
C1d	-1.14	-1.03		-2.02	1.08	
Esm1	1.02	-1.17		-1.97	-1.12	
Mier3	1.10	-1.10	*	1.91	1.11	

Fig. 31 Validation of EC Hey1⁺ and EC Hey2⁺ target genes by qRT-PCR. Fold change of RNA-Seq and qRT-PCR are shown. p-value ≤ 0.05 = *. Results are an average of three biological repetitions.

Limited changes seen in target gene regulation in endothelial cells could be due to the lower overexpression of Hey1 and Hey2 when compared to embryonic stem cells or even could represent random regulations.

7 Discussion

7.1 Impact of bevacizumab treatment on human tumor cell lines *in vitro*

7.1.1 Tumor cell expression of VEGF and VEGF receptors under hypoxia and bevacizumab treatment

It is well known that VEGF receptors are mainly located on the cell surface of endothelial cells and angiogenic signaling is activated through binding of VEGF (Ferrara and Kerbel, 2005). However, it is controversially discussed in the literature if VEGF receptors are also present on tumor cells and whether tumor cells could benefit from VEGF signaling. Autocrine and/or paracrine VEGF signaling loops in tumor cells could facilitate tumor growth and tumor spread by enhanced VEGF signaling (McMahon, 2000). Blocking of VEGF by bevacizumab treatment could therefore exhibit a direct impact on tumor cells. To this end, VEGF receptor expression was analyzed in several human tumor cell lines from four different tumor types. In line with previous studies (Duff et al., 2006, Seto et al., 2006, Ghosh et al., 2008, Soker et al., 1998) expression of VEGFR1, VEGFR2 and NRP1 was confirmed in a variety of human tumor cell lines measured by western blot and flow cytometry analysis. VEGFR1 and NRP1 were expressed by almost all tumor cell lines investigated, while expression of VEGFR2, the major receptor involved in angiogenesis, was limited to four out of nine tumor cell lines. With the exception of one renal cell line (A498), incubation under hypoxia and the addition of bevacizumab did not alter receptor expression. Besides the presence of VEGF receptors on tumor cells, induction of VEGF under hypoxia further supports the hypothesis of a potential autocrine or paracrine stimulation of tumor cells. As summarized by Harris et al. hypoxia leads to a variety of gene expression changes and the production of several growth factors like VEGF through activation of HIF-1 (Harris, 2002). The induction of *VEGFA* and *GLUT1* under hypoxic conditions in this study clearly shows the responsiveness of tumor cell lines to the hypoxic environment, which has been demonstrated for several lung and breast carcinoma cell lines as reported before (Simiantonaki et al., 2008). Besides increased expression of VEGF in tumor cell lines due to hypoxia, secretion of VEGF from tumor cells is also important when evaluating possible autocrine and/or paracrine signaling loops. Thus, in future experiments quantification of secreted VEGF should be determined from culture medium of tumor cells with and without addition of bevacizumab under normoxic and hypoxic conditions, e.g. by VEGF ELISA assays. These experiments would provide insight in the amount of secreted VEGF from tumor cells and could probably be related to receptor activation in tumor cells. VEGF receptor activation in tumor cells is initiated by binding of VEGF to VEGFR2, which leads to autophosphorylation of VEGFR2 and induces downstream signaling. Thus, it would be interesting to investigate the activated form of VEGFR2 in tumor

cells using phospho-specific antibodies against the main autophosphorylation sites, Tyr1175 and Tyr1214, of VEGFR2. Taken together, the presence of at least one receptor in each tumor cell line together with the hypoxic stimulation of VEGF provided the basis for an extended analysis of angiogenic gene expression and biological outputs of VEGF signaling after bevacizumab treatment to further investigate potential autocrine and paracrine functions of tumor cells.

7.1.2 Angiogenic gene expression analysis under hypoxia and bevacizumab treatment

In addition to protein expression analysis, VEGF and VEGFR expression was investigated on mRNA level to detect changes after bevacizumab treatment, which probably may not have been apparent on protein level. First of all, gene expression changes of different isoforms of *VEGFA*, which are generated according to alternative splicing, were investigated. Different isoforms of *VEGFA* are differing in their length and their amino acid content and may also exhibit varying functions (Harper and Bates, 2008). To investigate if bevacizumab treatment influences *VEGFA* expression in an isoform-specific manner, relative expression changes of three different isoforms, $VEGFA_{189}$, $VEGFA_{165}$ and $VEGFA_{121}$, were evaluated after bevacizumab exposure. Overall, none of the tumor cell lines showed any regulation in response to bevacizumab treatment. Upon bevacizumab treatment one could expect a downregulation of VEGF on the one side or an opposing upregulation of VEGF on the other side because tumor cells could react to VEGF depletion with higher production of the growth factor to ensure their survival. However, VEGF expression was confirmed in every single tumor cell line investigated and thus we went on to evaluate tumoral VEGF receptor and co-receptor expression. *VEGFR1* and *NRP1* were present in nearly all tumor cell lines investigated in this study, while *VEGFR2* expression was restricted to four cell lines (H552, HOP62, HCT-116, MDA-MB-231), indicating that *VEGFR2* expression is rather limited in tumor cells when compared to endothelial cells. A lack of expression of VEGFR2 under both, normoxic and hypoxic conditions, has been reported in a previous study with several colorectal, breast and lung carcinoma cell lines, claiming that a functional autocrine loop between VEGF and VEGFR2 is not possible in these cell lines (Simiantonaki et al., 2008). In contrast, our data demonstrate the simultaneous presence of VEGF receptors or co-receptors together with expression of VEGF in the same tumor cell line, which supports the theory of a functional autocrine signaling loop that could be inhibited by bevacizumab. For one breast tumor cell line, HS-578 T, a significant reduction of *NRP1* was evident after bevacizumab treatment under hypoxia, in line with the endothelial control. The reduced expression of *NRP1* upon VEGF depletion in HS-578 T could be due to impaired autocrine VEGF signaling. At least, a change in regulation of *NRP1* due to hypoxia has been reported recently for two other breast carcinoma cell lines, MDA-MB-231 and SUM159, as well as for

HUVECs (Bae et al., 2008), indicating that NRP1 expression is changed in response to hypoxia. However, downregulation of NRP1 in HS-578 T was not seen on protein level, which could be due to alternative regulatory mechanisms or posttranslational modifications. The overall limited detection of autocrine signaling loops in the investigated tumor cell lines might also be due to alternative signaling of angiogenic ligands and receptors, such as VEGFR3 as well as VEGF-C and VEGF-D. VEGF-C and VEGF-D are the major ligands that activate VEGFR3, which is mainly involved in lymphangiogenesis (Takahashi and Shibuya, 2005). Binding of the ligands VEGF-C and VEGF-D is not exclusively restricted to VEGFR3, but can also induce VEGFR2 and NRP2. Activation of VEGFR2 through alternative ligands besides VEGF-A could compensate for VEGF-A depletion by bevacizumab, leading to prolonged angiogenic signaling. It has been reported that VEGF-A depletion by bevacizumab in glioma cell lines led to an induced expression of VEGF-C and VEGF-D, indicating the amplification of other pro-angiogenic molecules, which would lead to escape mechanisms from anti-VEGF therapy (Grau et al., 2011). In addition, it has been shown that binding of other VEGF family members such as VEGF-B and PLGF could activate VEGFR1 and NRP1 independently of VEGF-A, thereby rescuing VEGF signaling, which was blocked by bevacizumab (Takahashi and Shibuya, 2005, Fischer et al., 2008).

7.1.3 Effects of bevacizumab treatment on tumor cell survival

In order to determine already known VEGF-A dependent biological functions, which are impaired after VEGF depletion in endothelial cells (Wang et al., 2004), changes in apoptosis were investigated in tumor cell lines after treatment with bevacizumab. In endothelial cells VEGF-A can act as a survival factor and rescue cells from apoptosis suggesting that VEGF-A can also play a role in tumor cell survival, which in turn could be reduced by bevacizumab treatment. In contrast to endothelial cells, depletion of VEGF-A via bevacizumab did not increase apoptotic levels or change cellular survival in the investigated tumor cells, as measured by the presence of cleaved PARP via protein analysis and by propidium iodide staining via flow cytometry. However, it has been shown in the literature that VEGF and VEGFR1/2 are involved in the survival of tumor cells via autocrine signaling loops (Calvani et al., 2008, Lee et al., 2007). Depletion of VEGF by bevacizumab blocked the survival of certain colorectal cancer cells, which demonstrates their responsiveness to VEGF and its inhibition (Calvani et al., 2008). Loss of VEGF or knockdown of VEGFR1 using siRNAs in breast tumor cell lines led to an increase in apoptotic levels, indicating that expression of VEGF and VEGFR1 are essential for the survival of cancer cells (Lee et al., 2007). Interestingly, decreased survival of breast tumor cells was only dependent on the depletion of VEGFR1, but not on the depletion of VEGFR2 or NRP1, which further highlights the importance of VEGFR1 in regulating tumor cell survival. However, decreased survival of tumor cells was only evident if the endogenous level of VEGFR1 was reduced, but not with

the addition of externally acting proteins like bevacizumab. This might be due to the location of VEGFR1, which is primarily located at nuclear membranes and therefore inaccessible for extracellular ligands (Lee et al., 2007).

7.1.4 Proliferation and migration analysis of human tumor cell lines after bevacizumab treatment

In endothelial cells proliferation and migration are dependent on active VEGF signaling. Active VEGF signaling in tumor cells could also be beneficial for tumor cell proliferation as well as for tumor cell migration and could be reduced by VEGF blocking. To investigate the impact of bevacizumab on tumor cell proliferation and migration, analysis was further extended to a screen including 30 tumor cell lines derived from four tumor types to identify possible cell line specific effects. Experiments were done under hypoxic conditions to induce VEGF-A expression and to mimic the *in vivo* state of tumors. Proliferation rates were determined for up to 72 hours but treatment with bevacizumab did not influence cell proliferation, which remained more or less unchanged in most of the tumor cell lines. The only significant change in proliferation was evident in one colorectal tumor cell line, HT-29, which showed reduced proliferation rates after bevacizumab exposure in line with the endothelial control. Endothelial cells showed a significant reduction in cell proliferation after 72 hours of bevacizumab treatment and an opposing increase in proliferation rates when stimulated with rhVEGF. From the literature it is known, that bevacizumab treatment inhibits endothelial cell proliferation in a dose-dependent manner using a concentration range from 0-500 ng/ml of bevacizumab and that a concentration of 100 ng/ml bevacizumab is sufficient to reduce proliferation of endothelial cells to a significant extent *in vitro* (Wang et al., 2004). However, in this study even higher, clinically relevant concentrations of bevacizumab (250 µg/ml) were used to inhibit VEGF-A, but overall did not influence tumor cell proliferation. A lack of tumor cell response in terms of proliferation changes after bevacizumab treatment *in vitro* has been reported before and even longer exposure of tumor cells to bevacizumab did not significantly impair tumor cell growth (Grau et al., 2011). However, tumor cell lines are responsive to loss of VEGF as demonstrated by VEGF depletion via homologous recombination, resulting in decreased proliferation *in vitro*, but not by VEGF depletion using monoclonal antibodies like bevacizumab (Samuel et al., 2011).

Limited changes in response to bevacizumab treatment were also evident when evaluating cellular migration in tumor cells. Across all investigated tumor cell lines no significant change in tumor cell migration was apparent after bevacizumab treatment, while cell migration in endothelial cells was strongly impaired. In endothelial cells migration is controlled through VEGFR1 signaling, which could additionally be enhanced by the co-expression of Neuropilin1 (Koch et al., 2011), while blocking of VEGFR1 completely abolished cellular migration (Kanno et al., 2000). This emphasizes a critical role of the VEGF/VEGFR1 pathway

in regulating cellular migration in endothelial cells and supposes that tumor cell migration might also be controlled by the VEGF/VEGFR1 signaling axis. Because most of the tumor cell lines investigated exhibited expression of VEGFR1 and NRP1, migration could be influenced upon VEGF depletion by bevacizumab. Although some tumor cell lines expressing NRP1 and high levels of VEGFR1 were highly motile, VEGF blocking via bevacizumab did not influence migratory potential. This raises the possibility that tumor cells might not be exclusively dependent on VEGF alone, but also on other ligands like PLGF and VEGF-B, which can specifically activate VEGFR1 and regulate tumor cell migration. Expression of VEGFR1 as well as the respective ligands VEGF-B and PLGF has been demonstrated in several pancreatic carcinoma cell lines and blocking of VEGFR1 led to reduced tumor cell migration and invasion (Wey et al., 2005). This further supports the involvement of VEGFR1 in regulating tumor cell migration. Tumor cell migration and endothelial permeability are also dependent on the secretion of VEGF and thus tumor cells could contribute to VEGF signaling activation via secretion of VEGF. Indeed, VEGF secretion from tumor cell lines or primary tumors is able to enhance endothelial permeability, which is in turn blocked by the addition of bevacizumab (Prager et al., 2010, Yang et al., 2010). Interestingly, several tumor cell lines showed slightly higher migration rates after bevacizumab treatment when compared to non-treated control cells, however not to a significant extent. Similar results, namely an increase in tumor cell migration, after chronic exposure with bevacizumab for up to three month was apparent by an *in vitro* study by Fan et al. Increased migration and invasion of tumor cells was abrogated by blocking of VEGFR1, which further underlines the essential involvement of VEGFR1 in regulating cellular migration (Fan et al., 2011).

7.1.5 Conclusion

In the 40 years since Judah Folkman first proposed treating angiogenesis as a novel cancer therapy, VEGF-A has been evolved as the major molecule involved in tumor angiogenesis. VEGF-A plays an essential role in generating endothelial cell changes, while blocking of VEGF by bevacizumab has shown direct anti-vascular effects in tumors (Willett et al., 2004). However, less is known about any direct anti-tumoral effects of bevacizumab and whether tumor cells are intrinsically affected by bevacizumab treatment. In this study, VEGF and VEGF receptor expression could be confirmed in a variety of tumor cell lines derived from different tumor types, supporting the theory of an autocrine and/or paracrine VEGF signaling loop. Blockade of VEGF by bevacizumab *in vitro* did not affect tumor cells in a unique manner and the overall measurable effect was rather limited when evaluating downstream outputs of VEGF signaling known from endothelial cells. Besides the limited or even complete lack of response in tumor cells to bevacizumab treatment, tumor cell lines exhibit a very high biological diversity according to their cell morphology differing in cell size and also in their proliferation ability with varying doubling times. This somehow reflects the

heterogeneity of tumor cells *in vivo*, which demonstrates the complexity of cancer therapy and the lack of a unique treatment for curing cancer. Although anti-angiogenic therapy has proven efficacy in patients leading to prolonged survival, further research is needed to elucidate the complex interaction between tumor cells and tumor endothelial cells. Besides VEGF-A, many other angiogenic ligands as well as angiogenic receptors are involved in tumor angiogenesis and also the intensive signaling crosstalk between different cells could contribute to increased tumor growth. For initial experiments *in vitro*, tumor cell lines from the well established NCI-60 panel provided a useful tool to study the direct impact of bevacizumab on tumor cells. However, data derived from the present study showed that tumor cells are not intrinsically affected by bevacizumab single treatment demonstrating that anti-VEGF-A based therapies remain a rather complex process between tumor and endothelial cells.

7.2 *In vitro* differentiation of Hey deficient and Hey re-expressing endothelia to delineate vascular Hey gene functions

7.2.1 Hey deficient endothelia efficiently differentiate *in vitro*

Hey deficient embryonic stem cells were used to study Hey dependent endothelial cell differentiation *in vitro*. Hey deficient embryonic stem cells were generated from blastocysts derived from $Hey1^{fl/fl}/Hey2^{-/-}$ mice. The floxed Hey1 allele was recombined by treatment with a Cre recombinase, which resulted in homozygously deleted embryonic stem cells representing Hey1/2 double-knockout cells (Fischer et al., 2005). These cells are able to differentiate as 3D aggregates, so called embryoid bodies, into various cell types. Analysis of nine-day-old embryoid bodies indicated the formation of endothelial and smooth muscle cells as well as the formation of cardiomyocytes (Fischer et al., 2005). To selectively trigger the differentiation into a specific cell type and to circumvent the formation of several cell types from differentiating ES cells, we made use of a selection based system. A selection marker driven by an endothelial specific promoter was used to achieve a directed differentiation of ES cells into endothelial cells. This method has proven efficacy for the selection of cardiomyocytes (Klug et al., 1996) as well as for the selection of endothelial cells (Marchetti et al., 2002) before. Differentiation into endothelial cells was done with Hey knockout cells as well as E14 embryonic stem cells, which present Hey1/2 wildtype cells and served as a control. Both embryonic stem cell lines were stably transfected with a blasticidin resistance gene under the control of either the VE-Cadherin (Hey knockout cells) or the Tie1 promoter (E14 Hey wildtype cells), which are both vascular endothelial-specific promoters. Selection with blasticidin was started at day seven (E14) or day eight (ΔHey1/2) of differentiation because mRNA levels of VE-Cadherin and Tie1 were highly enriched at this day compared to undifferentiated ES cells, which indicated the formation of endothelial progenitor cells. The selection procedure using blasticidin as a selection agent highly enriched the culture for endothelial cells, while eliminating stem cells from the culture dish, which resulted in a relatively pure endothelial cell population after 14 days. Besides the efficient selection method, high yields of endothelial cells were obtained using chemically defined media formulations with specific growth factors as described in a previous study (Blancas et al., 2011). VEGF and BMP4 were added to the culture medium as both growth factors enhance mesodermal development, in particular haematopoietic and endothelial cell determination, while inhibiting neuronal development (Johansson and Wiles, 1995, Chiang and Wong, 2011). Endothelial cell differentiation *in vitro* is also enhanced by addition of bFGF, which is involved in the survival of endothelial precursor cells and was therefore added to the culture dish after the induction phase and after initial formation of EC progenitor cells (Vittet et al., 1996, Chiang and Wong, 2011). The enhancing effect of EC differentiation due to the

addition of specific growth factors is often also mediated by the use of FBS in cell culture media, as FBS is supplemented with a large number of growth factors. However, the use of FBS in cell culture medium often leads to higher variability according to batch-to-batch variations and to limited reproducibility. To this end, serum-free formulations like KOSR have been explored and used not only for maintenance and establishment of embryonic stem cells (Cheng et al., 2004), but also for the differentiation of endothelial cells (Blancas et al., 2011). Serum-free media was therefore used for the culture of embryonic stem cells as well as for the differentiation into endothelial cells. Taken together, efficiency of endothelial differentiation *in vitro* was not altered between Hey knockout and Hey wildtype cells, which assumes that the loss of Hey1 and Hey2 does not affect the ability of stem cells to differentiate into endothelial cells in this system.

7.2.2 ESC-derived endothelial cells show typical endothelial characteristics

Before starting endothelial differentiation studies with Hey knockout and Hey wildtype ES cells, their pluripotency was investigated. Cells were stained for alkaline phosphatase expression, which is used as a pluripotency marker and confirms the stem cell character of ES cells. Hey knockout and Hey wildtype cells exhibited a similar staining pattern, which indicated that the loss of Hey genes does not impair the stem cell character and thus Hey genes were not involved in the maintenance of the stem cell status. This was also evident from comparative differentiation studies between Hey knockout and Hey wildtype ES cells as the ability of Hey knockout ES cells and Hey wildtype ES cells to differentiate into endothelial cells was not altered between both cell lines (7.2.1). As demonstrated before, Hey deficient cells were also able to differentiate into 3D aggregates and to form several cell types, which further indicates that Hey genes are dispensable for ES cell differentiation (Fischer et al., 2005). Consistent with this, differentiation of cardiomyocytes, endothelial cells and haematopoietic progenitor cells was not disturbed, when Notch signaling was blocked further upstream by loss of RBPkJ (Schroeder et al., 2003). Blocking of Notch signaling in human embryonic stem cells by gamma secretase inhibiton (GSI) even promoted differentiation and induced the formation of mesodermal cells (Jang et al., 2008). In addition to the unaltered differentiation potential of Hey knockout cells, gene expression analysis confirmed the pluripotent character of the investigated stem cell lines. Stem cells exhibited high expression of the pluripoteny marker genes Nanog, Oct4 and Sox2, which were strongly reduced in differentiated endothelial cells. Again, there was no obvious difference in Hey knockout and Hey wildtype cells. Embryonic stem cell derived endothelial cells exhibited typical endothelial characteristics as shown by marker gene expression on mRNA level as well as by immunofluorescent staining. Gene expression analysis revealed a strong induction of endothelial marker genes including receptor tyrosine kinases like Tie1, Tie2 and Vegfr2 as well as the cell adhesion molecule VE-Cadherin in endothelial cells when compared to

embryonic stem cells. Consistent with our findings, upregulation of these endothelial marker genes has been demonstrated before, however in an endothelial differentiation system using embryoid bodies (Vittet et al., 1996). To confirm endothelial marker expression in ESC-derived endothelial cells on protein level, cells were stained for PECAM-1 and VE-Cadherin. VE-Cadherin and PECAM-1 (CD31) are specific endothelial cell surface molecules, which were first described in the early 90s (Newman et al., 1990, Lampugnani et al., 1992). Until today, both proteins are commonly used in immunofluorescent stainings to identify and characterize endothelial cells. VE-Cadherin and PECAM-1 expression was confirmed in ESC-derived endothelial cells as shown by localization at the cell membrane of endothelial cells. Immunofluorescent staining was not altered between Hey knockout and Hey wildtype cells but confirmed the endothelial phenotype of ESC-derived endothelial cells. In order to determine the functionality of endothelial cells, the tube-forming ability is mainly used as a measure of functional endothelium (Arnaoutova et al., 2009). Embryonic stem cell derived endothelial cells were not able to form tubes after plating on Matrigel in contrast to HUVECs, which served as a control. Tube formation of Hey knockout and Hey wildtype endothelial cells only became evident when endothelial cells were incubated on an orbital shaker before seeding them on Matrigel. This observation could be due to the fact that embryonic stem cell derived endothelial cells may lack maturation *in vitro* when compared to primary isolated mouse endothelial cells (McCloskey et al., 2006). Nevertheless, ESC-derived endothelial cells were able to form tube-like structures after incubation on an orbital shaker, which in a way simulated shear stress. It is known from the literature that laminar shear stress induces differentiation of endothelial progenitor cells by activation of VEGFR2 and PI3K/Akt/mTOR signaling, which could contribute to maturation of endothelial cells (Obi et al., 2012). Taken together, ESC-derived endothelial cells exhibited typical endothelial characteristics, but lacked complete functionality *in vitro*.

7.2.3 Re-expression of Hey proteins in Hey1/2 double knockout cells

To investigate Hey protein functions during endothelial cell differentiation Hey1 and Hey2 were re-expressed in Hey knockout ES cells. Therefore, stable embryonic stem cell lines expressing Hey1/2 inducible plasmids were generated. Stable integration was achieved by transfection of the cells using the sleeping beauty transposon system. In this two-component system the gene of interest, in this case Hey1 or Hey2, is positioned between inverted terminal repeats that are recognized by a specific transposase (SB100X). The specific transposase is supplemented in trans and mediates the integration to a genomic target site. This system was chosen as it has been shown to work efficiently in human and mouse embryonic stem cells before (Geurts et al., 2003, Davidson et al., 2009, Mates et al., 2009). Re-expression of Hey proteins was achieved using a doxycycline dependent expression system, which allows Hey protein expression in a reversible and controlled fashion by

addition and withdrawal of doxycycline. The doxycycline dependent gene expression system is based on the *Escherichia coli* tetracycline resistance operon, which was first described for the use in mammalian cells in 1992 (Gossen and Bujard, 1992). In the reverse fashion, the transactivator (rtTA) is composed of the repressor (rtetR) and the herpes simplex virus (HSV) VP16 transcripton activation domain driven by an appropriate promoter, the eukaryotic translation elongation factor 1 alpha (EF1α). In the absence of a tetracycline derivate (doxycycline) the transactivator can not bind to a specific DNA target site and transcriptional activation of the desired gene is suppressed. The addition of doxycycline results in binding of the transactivator to the tet operon (tetO), which leads to transcriptional activation (Gossen et al., 1995). Thus, this system allows the investigation of Hey proteins and their functions in ES cells and during the formation of endothelial cells by switching Hey on or off at defined time-points during differentiation. Differentiation studies in embryonic stem cells using this gene expression system have already been used before and provided a powerful tool to study specific protein functions during ES cell differentiation (Niwa et al., 1998, Era and Witte, 2000). Induction of Hey1 and Hey2 after addition of doxycycline was confirmed in the respective cell line by western blot analysis and whole transcriptome sequencing. Quite strikingly, the induction efficiency of Hey1 and Hey2 was much higher in embryonic stem cells than in ESC-derived endothelial cells. This could be due to the number of read counts for Hey1 and Hey2, which were more than 5-fold higher in ES cells than in endothelial cells when comparing the doxycycline-induced samples between both cell types (Read counts ES Hey1^{+}: 349, EC Hey1^{+}: 66, ES Hey2^{+}: 319, EC Hey2^{+}: 61). These data reflect that the efficiency of tetracycline dependent gene expression can vary between different cell types, although expression was induced using the same concentration of doxycycline. Differences seen in Hey transgene expression between ES and EC cells could be due to promoter silencing during endothelial differentiation or varying promoter strength in different cell types. Expression of the transactivator (rtTA3), which is essential for Hey transgene expression, is driven by the EF1α promoter. The EF1α promoter obviously led to a robust transgene expression in embryonic stem cells and according to the literature should also exhibit solid activity at stage-specific steps during mouse ES cell differentiation (Hong et al., 2007, Wang et al., 2008). However, ESC-derived endothelial cells showed lower Hey transgene expression when compared to ES cells. Further experiments using other promoters e.g. the CMV promoter for transgene control would show, if expression of transgenes could be more efficient and stable in endothelial cells using other promoters driving transgene expression. Transgene expression in endothelial progenitor cells and in HUVECs driven by the CMV promoter has shown higher efficacy than the EF1α promoter (Liu et al., 2006), further supporting the use of other promoters for stable transgene expression in endothelial cells. Furthermore, transgene silencing could also be due to DNA methylation and histone

modifications in the transgene driving promoter. Epigenetic silencing in the genome is often associated with methylation of specific CpG islands on the DNA as well as histone deacetylations, which lead to chromatin changes and transcriptional repression. These mechanisms could also be involved in controlling transgene expression in embryonic stem cells as well as during differentiation of ES cells (Rosenqvist et al., 2002, He et al., 2005).

7.2.4 No significant change in arterial versus venous marker gene expression upon Hey overexpression in ESC-derived endothelial cells

Hey1 and Hey2 are involved in the specification of arterial endothelial cells during embryonic development as demonstrated by analysis of yolk sacs from Hey1 and Hey2 mutant embryos, which revealed a significant downregulation of the arterial specific marker EphrinB2 (Fischer et al., 2004). In contrast, CoupTFII, which has been described as the main transcription factor regulating venous endothelial cell fate determination (You et al., 2005), exhibited a significant upregulation during embryoid body differentiation of Hey deficient ES cells (Diez et al., 2007). Based on these data one would expect to detect differences in arterial versus venous marker gene expression in ESC-derived endothelial cells after Hey overexpression in the *in vitro* differentiation setting used in this study. However, Hey1 and Hey2 overexpression in ESC-derived endothelial cells did not significantly change expression of arterial (EphrinB2, Dll4) and venous (CoupTFII, CoupTFII alt, EphB4) endothelial marker genes as measured by qRT-PCR. The lack of a significant change could be due to the point of time when Hey overexpression was initiated. Hey induction with doxycycline was induced after 14 days of differentiation by the time when endothelial cells were nearly terminally differentiated and specification was probably already determined. Thus, for future experiments endothelial cell differentiation should be investigated in parallel with constant Hey overexpression during the whole time course of differentiation in comparison to Hey deficient cells. Moreover, specification of arterial and venous endothelial cells during embryonic development might not be solely dependent on Hey gene functions alone, but rather remains a complex process involving many other critical factors of different pathways. Several other genes like Sonic Hedgehog and VEGF as well as other transcription factors like Foxc1, Foxc2 and Sox have been implicated in arterial specification (Marcelo et al., 2013, Kume, 2010). Recently, there have been two publications dealing with the molecular mechanisms determining arterial versus venous endothelial cell fate determination using primary freshly isolated arterial (HUAECs) and venous (HUVECs) endothelial cells (Korten et al., 2013, Aranguren et al., 2013). Consistent with the results of this study, overexpression of Hey2 in HUVECs did not influence expression of CoupTFII and other arterial and venous marker genes. However, CoupTFII was able to bind directly to the Hey2 promoter leading to transcriptional repression, which indicates a direct interaction of CoupTFII and Hey2. Transcriptional repression of Hey2 leads to suppression of the arterial program and to

formation of endothelial cells with a venous character, induced by expression of CoupTFII (Korten et al., 2013). Furthermore, overexpression of a combination of eight transcription factors in cultured HUVECs has been required to induce a conversion into an arterial phenotype, while overexpression of Hey2 alone did not reveal strong changes. This diminished the essential role of Hey2 as the current 'golden standard' in arterial cell fate determination (Aranguren et al., 2013).

7.2.5 Whole transcriptome analysis revealed more Hey downstream target genes in embryonic stem cells than in endothelial cells

In order to determine Hey target genes, RNA-Sequencing was performed in Hey overexpressing embryonic stem cells and ESC-derived endothelial cells. Overall, RNA-Seq analysis revealed nearly three-fold more Hey target genes in ES cells than in endothelial cells (ES: n = 354, EC: n = 123). Additionally, regarding regulated genes by either Hey1 or Hey2 in both cell types, more genes were regulated by Hey2 than by Hey1 (Hey1: n = 106, Hey2: n = 382). The identification of a larger number of target genes in embryonic stem cells might be due to varying overexpression intensities of Hey1 and Hey2. Hey overexpression was stronger in ES cells than in endothelial cells as shown by the higher number of read counts for Hey in embryonic stem cells (7.2.3). Although mRNA expression of Hey1 was higher than expression of Hey2, more target genes were regulated by Hey2 than by Hey1, independent of the cell type investigated. This could be a consequence of a stronger protein expression as shown by quantification and western blot analysis. Validation of several target genes in embryonic stem cells by qRT-PCR demonstrated that Hey1 and Hey2 more or less act in a redundant manner, which has been shown for the regulation of target genes of Hey1 and Hey2 in HEK293 cells before (Heisig et al., 2012). Most of the regulated genes in Hey overexpressing ES cells play a functional role during embryonic development and/or are involved in transcriptional regulation. For example, Icam1 and Fibronectin1 were upregulated by Hey and their expression probably also plays a functional role in endothelial cells. Icam1 is a cell surface molecule, which is typically expressed on endothelial cells but its expression is also be found on embryonic stem cells (Frenzel et al., 2009). Fibronectin1 is involved in cell adhesion and thus plays an important role in endothelial cell differentiation, as it is commonly used as a coating agent in differentiation processes *in vitro* (Blancas et al., 2011). Genes downregulated by Hey were for example Sox1, Skil and Lefty2, which all have been implicated in regulation of embryonic development before. Sox1 is a transcriptional regulator involved in the regulation of embryonic development and cell fate determinations. Overexpression of Sox1 in neural progenitor cells leads to neural lineage commitment and promotes neuronal differentiation by several different mechanisms. One of them is related to the direct binding of Sox1 to the Hes1 promoter, which leads to suppression of Notch signaling and Hes1. Hes1 is a potent inhibitor of neurogenesis and thus repression of Hes1

promotes neuronal cell development (Kan et al., 2004). Skil (SKI-like), also known as SnoN, is a member of the SMAD signaling pathway, which regulates cell growth and cell differentiation. Until today, a functional role for Skil has mainly been linked to tumorigenesis, while its role in embryonic development is not fully characterized yet. Recently, it has been reported that Skil is involved in embryonic angiogenesis by regulating interaction of ALK1 and Smad1/5 in endothelial cells. The ALK1 receptor mediates TGF-ß signaling by activating downstream Smad proteins to promote angiogenesis (Zhu et al., 2013). Lefty2, left-right determination factor 2, plays an important role in left-right asymmetry determination of organ systems during development. Lefty proteins function in embryonic stem cells in regulating both, stemness and differentiation, as they can modulate expression of the TGF-ß family member, Nodal. Expression of Nodal is modulated by gene expression changes of Lefty, which results in impaired differentiation or impaired left-right asymmetry (Tabibzadeh and Hemmati-Brivanlou, 2006). Furthermore, expression of Nodal is induced upon active Notch signaling, indicating that the Notch pathway acts upstream of left-right determination in mice (Krebs et al., 2003). However, there were some target genes, which were only regulated by Hey2 and remained unregulated by Hey1 (Spred3, Fibronectin1, Lefty2), indicating a slightly more important role for Hey2 in target gene regulation. This was also evident from RNA-Seq analysis of ESC-derived endothelial cells, as more genes were regulated by Hey2 than by Hey1. However, regulation of target genes in endothelial cells could barely be confirmed by qRT-PCR, which could partially be due to clone-specific regulations of genes. Clonal diversity could reveal distinct gene expression profiles between endothelial cells derived from specific clones and also the ability to differentiate could be altered between different clonal sublines (Martinez et al., 2012). Thus, Hey regulated target genes in ESC-derived endothelial cells could to a certain extent result from clonal artifacts. Varying target gene output between different cells could also be due to increased noise expression of the regulatory protein rtTA3, which controls Hey transgene expression. It has been demonstrated before that increased noise in the transcription of regulatory proteins has led to increased cell-cell variability in target gene regulation and may be an important point to consider when evaluating cellular differentiation processes (Blake et al., 2003). For future experiments several individual ESC-derived endothelial clones should be taken to perform whole transcriptome analysis to identify Hey target genes in endothelial cells. This would then clarify if supposed endothelial Hey target genes could be confirmed in several individual clones or if Hey overexpression in ESC-derived endothelial cells does not influence target gene expression in this *in vitro* setting. However, GO term analysis revealed enrichment of biological processes mainly linked to embryonic development and transcriptional regulation, which is in line with previous Hey overexpression studies in HEK293 cells (Heisig et al., 2012). Furthermore, RNA-Seq analysis from cells harvested at different time points during

endothelial cell differentiation in the presence and absence of Hey proteins would provide insight in stage-specific Hey target genes. In addition, ChIP-sequencing of Hey overexpressing ES cells and ESC-derived endothelial cells could be used to identify direct binding sites for Hey proteins throughout the genome.

7.2.6 Conclusion

Loss of Hey1 and Hey2 leads to impaired vascular development which results in early embryonic lethality *in vivo* (Fischer et al., 2004). Until today, less is known about potential vascular Hey target genes that could be involved in the outcome of the vascular phenotype observed in Hey deficient mice. As Hey proteins mainly act as transcriptional repressors, they could be involved in regulating target genes important for proper vascular network formation. In the present study an *in vitro* differentiation system for endothelial cells was established to identify vascular Hey target genes in embryonic stem cells as well as in endothelial cells. To this end, Hey deficient ES cells were used in comparison to Hey overexpressing ES cells, based on a doxycycline dependent overexpression system.

According to gene expression analysis and immunofluorescent staining as well as tube formation ability, Hey deficient ES cells were able to differentiate into endothelial cells to a similar degree than Hey wildtype cells. This indicates that loss of Hey expression *in vitro* does not influence endothelial cell differentiation ability and thus, Hey deficient ES cells provide a powerful tool to study endothelial cell differentiation. Furthermore, doxycycline dependent Hey protein expression allowed a controlled re-expression of Hey in Hey knockout cells. Whole transcriptome analysis upon Hey overexpression in both cell types revealed many Hey downstream target genes in ES cells and less target genes in ESC-derived endothelial cells. The limited response of Hey proteins in regulating target gene expression in endothelial cells could be due to experimental setups as well as to clonal variations. It furthermore highlights the complexity of target gene regulation, which might not only be dependent on a single transcription factor alone.

8 References

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9 Appendix

9.1 Abbreviations

°C	degree Celsius
µg	microgram
2D	two-dimensional
3D	three-dimensional
alt	alternative
APC	allophycocyanin
BC	breast cancer
bEnd	brain endothelial cells
bFGF	basic fibroblast growth factor
BMP4	bone morphogenetic protein 4
bp	basepairs
BSA	bovine serum albumin
bsd	blasticidin
CADASIL	cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
cDNA	complementary DNA
ChIP	chromatin immunoprecipitaton
cm	centimeter
CMV	cytomegalovirus
CRC	colorectal cancer
Cre	cyclization recombination
ct value	cycle threshold value
D	day of differentiation
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside-triphosphate
dox	doxycyline
EC	endothelial cell
ECGS	endothelial cell growth supplement
EDTA	ethylenediaminetetraacetic acid
EF1α	elongation factor 1 alpha
ELISA	enzyme-linked immunosorbent assay
ES cells	embryonic stem cells

FACS	fluorescent-activated cell sorting
FBS	fetal bovine serum
Fig.	figure
fl	floxed
GFP	green fluorescent protein
GO	Gene Ontology
h	hour
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HSV	herpes simplex virus
HUAEC	human umbilical artery endothelial cell
HUVEC	human umbilical vein endothelial cell
ID	identification
IRES	internal ribosome entry site
kb	kilobase
kDa	kilo Dalton
ko	knockout
KOSR	knockout serum replacement
LDL	low density lipoprotein
LIF	leukemia inhibitory factor
M	molar
mA	milliampere
MEM	Minimum Essential Medium Eagle
mg	milligram
min	minute
ml	milliliter
mM	millimolar
mRNA	messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NEAA	non-essential amino acids
NCI	National Cancer Institute
ng	nanogram
nm	nanometer
NSCLC	non-small cell lung cancer
O_2	oxygen
PAGE	poly acrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction

qRT-PCR	quantitative realtime-PCR
RCC	renal cell carcinoma
rh	recombinant human
RNA	ribonucleic acid
RNA-Seq	ribonucleic acid-sequencing
RPKM	reads per kilo base per million
rpm	reads per million
RPMI	Roswell Park Memorial Institute
rtetR	reverse tetracycline dependent repressor
rtTA3	reverse tetracycline dependent transactivator
s	second
SDS	sodium dodecyl sulfate
siRNA	small interfering RNA
tetO	tet operon
UV	ultraviolet light
V	volt
VEGF	vascular endothelial growth factor
wt	wildtype

9.2 RNA-Seq data

Table 1 Raw reads and aligned reads mm9 genome.

	reads	aligned reads mm9 genome	percentage
ES Hey1 -	6642887	5886045	88.61
ES Hey1 +	6738380	5985985	88.83
ES Hey2 -	11044568	9777373	88.53
ES Hey2 +	6935446	6264752	90.33
EC Hey1 -	29509057	26781653	90.76
EC Hey1 +	31806354	28935172	90.97
EC Hey2 -	4593400	4125723	89.82
EC Hey2 +	5310928	4733459	89.13

Table 2 Target genes in ES Hey1$^+$, ES Hey2$^+$ (A) and EC Hey1$^+$, EC Hey2$^+$ (B). Analysis was performed as described in section 5.3.5 and revealed 354 target genes in ES cells and 123 target genes in endothelial cells. For each target gene Entrez ID, gene name and fold change after Hey1 and Hey2 overexpression are presented. If no number for the fold change is indicated, selection criteria were not fulfilled and the gene was excluded from analysis, n.e. = not expressed.

A

Entrez ID	Gene	fold change	
		ES Hey1$^+$	ES Hey2$^+$
14980		-1.07	1.84
77633		1.31	-1.95
108112		-1.10	1.93
624713		1.62	-2.64
652925		1.08	-2.01
665037		1.72	4.15
751556		1.55	-2.07
100042464		-1.22	3.59
100043040		1.97	1.25
100043813		1.41	-2.30
100049713			-2.06
100169889		-1.20	2.73
100316809		-2.22	1.90
100415787		1.07	-2.38
100499530		-2.22	1.90
100503879		1.17	1.89
100504114		-1.12	2.04
100526469		1.31	-1.97
100526479			1.81
100568459		1.20	1.81
101243624		1.67	-2.35
75394	0610040F04Rik	-1.96	1.75
68618	1110012L19Rik	-1.81	-1.47
66179	1110031I02Rik	-2.15	-2.36
68742	1110032O16Rik	-1.97	1.14
69017	1500031I19Rik	-1.48	2.01
76927	1700021C14Rik	1.17	1.81
66935	1700023B02Rik	-1.96	1.16
69513	1700030C10Rik	-1.38	-1.93
70011	1700030N03Rik		1.87
67105	1700034H14Rik	1.09	-1.97

B

Entrez ID	Gene	fold change	
		EC Hey1$^+$	EC Hey2$^+$
272396		-1.15	-1.81
100043609		-1.05	-2.23
100217431		2.01	
100302648		-1.82	
100503498		-2.05	
100526469		-2.00	
100526479		-2.02	
100628584		-1.95	-1.43
100628588		-1.98	
100628612		-2.80	
100628620		-2.02	
68332	0610010E21Rik	-1.01	-1.96
68347	0610011F06Rik	1.83	1.04
66061	0610012D17Rik	-1.89	
85308	1500005A01Rik	-2.01	
66278	1810013D10Rik	-1.53	1.82
66273	1810020D17Rik	-1.97	
66282	1810029B16Rik	-1.14	-2.01
67892	1810063B05Rik	1.21	1.97
67509	1810063B07Rik	-1.05	-1.99
72429	2010203O07Rik	-1.14	-2.29
72093	2010320M18Rik	-1.01	-2.71
76947	2310030N02Rik	1.35	-1.91
69627	2310031A18Rik	1.15	-2.24
69642	2310046A06Rik	2.35	1.10
75430	3200002M19Rik	-1.19	2.08
69277	3300002I08Rik	1.05	1.92
319518	4930402E16Rik	-1.04	1.96
73863	4930415O20Rik	-2.16	
67651	4930527F14Rik	1.30	-2.40
71532	9030418K01Rik	1.21	-1.85

67524	1700095A21Rik	-1.14	-2.11	330385	9530026P05Rik	1.98	1.38
69186	1810027O10Rik	1.03	-1.89	619321	9530052E02Rik	1.67	-2.29
66291	1810030N24Rik	1.36	2.33	194268	9930104L06Rik	1.02	-2.14
76527	2010004A03Rik	1.50	2.51	381823	Apold1	1.37	-1.94
69871	2010007H12Rik	-1.08	1.84	26879	B3galnt1	-1.26	-2.20
100910	2010209O12Rik	-2.06	-1.04	414069	BC024978	-1.27	-1.85
72093	2010320M18Rik	-1.96	-1.41	14705	Bscl2	1.17	2.06
70080	2210010C17Rik	-2.07	-1.47	57316	C1d	-1.14	-2.02
69698	2310046K01Rik	-1.24	1.82	654318	C530005A16Rik	-1.31	-2.24
71918	2310047A01Rik	-1.08	-3.67	57895	Ccdc126	1.22	-1.88
77032	2610029I01Rik	1.03	-1.85	12615	Cenpa	-1.06	-1.81
73242	2610110G12Rik	-1.11	2.01	56464	Ctsf	1.06	1.86
72481	2610203C22Rik	1.15	-1.85	72865	Cxx1c	1.89	
70435	2610204M08Rik	-1.41	1.93	170716	Cyp4f13	-1.31	-1.83
545428	2610301F02Rik	1.62	1.88	225995	D030056L22Rik	-1.05	1.95
70419	2810408A11Rik	-2.03	-1.01	114585	D17H6S53E	-1.00	2.08
68034	2900009I07Rik	1.05	2.09	231630	D5Ertd40e	-1.25	1.91
73172	3110037I16Rik	-1.87	-1.60	13198	Ddit3	1.01	-2.40
67419	3632451O06Rik	1.63	-2.71	13368	Dffb	-1.10	1.87
432479	4930404N11Rik		1.84	57431	Dnajc4	1.64	2.01
78108	4930414L22Rik	1.06	-2.20	77490	E130218I03Rik	1.10	2.27
67585	4930455J16Rik	-2.29	-1.78	55960	Ebag9	-1.19	-2.06
75060	4930506C21Rik	-1.14	2.12	620393	EG620393	1.13	2.09
74726	4930523O13Rik	-2.24	2.02	72205	Eml2	1.03	2.07
75202	4930546H06Rik	1.64	2.50	71690	Esm1	1.02	-1.97
223626	4930572J05Rik	-2.09	-1.45	71436	Flrt3	-1.03	2.07
78215	4930578N18Rik		1.90	14369	Fzd7	1.12	3.85
70942	4931403E22Rik	3.25	3.04	80860	Ghdc	1.11	2.07
74437	4933402E13Rik		3.84	73690	Glipr1	-1.32	2.27
67531	5730408K05Rik	-1.83	1.36	110304	Glra3	-1.01	1.81
67412	6330407J23Rik	-1.36	2.11	228730	Gm114	-1.47	1.81
231842	6530401C20Rik		2.06	57441	Gmnn	-1.21	1.83
76219	6530401D17Rik	1.61	-1.83	14676	Gna15	1.28	2.46
381062	9030025P20Rik	1.17	1.87	67371	Gtf3c6	-1.03	-1.83
231717	A230106M15Rik	-1.12	-1.86	70315	Hdac8	1.26	-2.08
239559	A4galt	1.70	1.89	15213	Hey1	15.27	n.e.
381272	A630095N17Rik		2.07	15214	Hey2	n.e.	3.26
109202	A930024E05Rik	-2.24	-1.13	15277	Hk2	-1.07	1.90
76491	Abhd14b	-1.02	2.24	15473	Hrsp12	-1.22	-1.95
11433	Acp5	1.40	-2.14	15901	Id1	1.36	1.98
433256	Acsl5	-1.33	-1.98	16336	Insl3	-2.34	-1.88
11464	Actc1	-1.02	-5.74	170771	Khdrbs2	1.26	-2.19
11474	Actn3	-1.83	-1.64	70394	Kptn	-1.19	-1.89
106672	AI413582	-1.76	3.02	17087	Ly96	1.04	-1.91
100165	AI507597	1.06	1.83	26922	Mecr	-1.32	-2.19
11634	Aire	1.57	3.57	17299	Mettl1	-1.10	-1.80
72041	Alkbh4	-1.38	1.81	100034361	Mfap1b	-1.24	1.92
232566	Amn1	-1.67	1.98	17314	Mgmt	1.23	-1.91
69010	Anapc13	1.80	-1.33	218613	Mier3	1.10	1.91
234396	Ankrd41	-1.47	1.89	64660	Mrps24	-1.16	-1.88
12306	Anxa2	1.51	1.91	18030	Nfil3	-1.13	-2.95
27052	Aoah	1.57	2.11	68501	Nsmce2	1.17	2.13
64933	Ap3m2	1.87	2.75	52793	ORF9	-1.04	-2.50
13498	Atn1	1.00	2.14	433287	OTTMUSG00000022109	1.44	-1.97
320871	B230206H07Rik	1.39	2.38	73162	Otud3	1.34	-2.41
319752	B230209E15Rik	1.16	-1.83	76498	Paqr4	-1.00	-1.82
230991	B930041F14Rik	-2.10	-1.09	319455	Pld5	1.01	-1.90
233913	BC017158	-1.92	1.24	217430	Pqlc3	-1.06	1.82
414069	BC024978	1.01	-2.12	77619	Prelid2	2.02	
277154	BC030046	-1.06	2.41	19171	Psmb10	-2.02	1.40

103220	BC030307	-1.40	-1.87
238024	BC032265	-2.09	-1.08
474160	BC033916	-1.07	-2.40
408058	BC048507	2.05	1.21
381066	BC049807	1.51	2.19
230676	BC059842	-1.11	1.96
12159	Bmp4	-1.95	-1.76
101831	C230052I12Rik	-2.47	1.15
97863	C78339	1.91	1.06
76815	Calcoco2	1.83	2.13
12337	Capn5	1.30	2.12
12367	Casp3	1.08	-1.85
12372	Casq1	-1.66	2.92
232664	Ccdc136	-1.28	-2.23
382073	Ccdc84	1.35	1.96
12505	Cd44	-1.51	-3.50
12509	Cd59a	-1.19	2.13
12512	Cd63	-1.65	-1.81
260409	Cdc42ep3	-1.13	-2.02
239096	Cdh24	1.16	1.84
12578	Cdkn2a	2.21	1.38
26365	Ceacam1	1.03	-2.22
231821	Centa1	-1.85	1.32
216859	Centb1	-1.22	2.14
68567	Cgref1	-1.05	-1.97
69065	Chac1	-1.27	-2.22
68119	Cmtm3	1.80	1.20
72042	Cotl1	-1.23	-2.37
12865	Cox7a1	2.50	1.56
12873	Cpa3	-1.86	-1.38
12877	Cpeb1	2.00	1.14
12925	Crip1	1.03	1.94
13032	Ctsc	1.19	1.80
13034	Ctse	-1.73	3.11
435802	Cyp4a30b	-1.18	-2.25
12879	Cys1	1.09	-2.35
320351	D230037D09Rik		-2.51
654822	D330041H03Rik	1.15	1.90
52480	D7Ertd715e	1.27	2.72
228859	D930001I22Rik	-1.51	-1.80
13132	Dab2	-1.28	2.00
240025	Dact2	-1.09	1.82
72185	Dbndd1	-1.54	2.09
13198	Ddit3	-1.08	-1.93
230073	Ddx58	1.60	1.98
209773	Dennd2a	-1.06	-1.83
13346	Des	-1.19	-1.97
107585	Dio3		3.94
227697	Dolk	1.14	-1.86
60364	Donson	-1.84	-1.14
13482	Dpp4	-1.12	-1.94
56405	Dusp14	2.52	1.61
320172	E230016M11Rik	1.01	-1.81
68177	Ebpl	-1.92	-1.16
434197	EG434197	2.35	1.43
434280	EG434280	-2.29	-2.69
627821	EG627821		1.87
665574	EG665574	1.61	1.84
386655	Eid2	1.33	-2.05
100038468	ENSMUSG00000074385	-1.09	-1.83
227746	Rabepk	-1.61	-1.83
218772	Rarb	1.09	2.28
109222	Rarres1	-1.90	-2.29
58175	Rgs20	-1.20	1.90
218215	Rnf144b	1.14	2.12
622404	RP23-195K8.6	1.06	-1.91
101122	Rpusd3	-1.39	-2.29
74648	S100pbp	1.30	-2.44
74729	Setmar	-1.08	1.95
59049	Slc22a17	1.30	1.99
108652	Slc35b3	1.05	1.90
72027	Slc39a4	1.03	-2.10
242259	Slc44a5	-1.04	-1.80
330959	Snapc5	-1.18	-1.91
214616	Spata5l1	1.17	-2.20
67249	Tbc1d19	1.16	1.94
67978	Tctn2	-1.52	2.02
386612	Thoc6	1.13	-1.97
67698	Tmem157	-1.23	-1.98
236792	Tmem32	1.26	2.11
233979	Tpcn2	-1.01	1.85
74019	Traf3ip1	-1.03	1.97
21912	Tspan7	1.03	-1.85
52808	Tspyl2	-1.10	2.25
233276	Tubgcp5	1.27	-1.80
22172	Tyms-ps	-1.88	-1.83
22350	Vil2	-1.24	2.21
22365	Vps45	1.01	2.08
434204	Whdc1	-1.16	-1.90
56220	Zfp386	-1.06	2.15
68040	Zfp593	1.15	1.85
69020	Zfp707	1.21	-2.29
67538	Zswim3	1.10	-1.91

98845	Eps8l2	1.41	2.59
13867	Erbb3	1.19	1.90
14009	Etv1	1.10	1.89
277978	Exoc3l	1.21	2.46
791260	F930017I19Rik	-1.82	-1.15
14077	Fabp3	-1.65	-1.86
327959	Fbxo39		2.31
230903	Fbxo44	1.15	-2.07
14226	Fkbp1b	1.97	1.37
286940	Flnb	-1.04	1.90
226844	Flvcr1	-1.16	2.29
54418	Fmn2	1.06	1.81
14268	Fn1	1.06	2.46
15221	Foxd3	-1.33	2.00
54601	Foxo4	1.07	-1.99
17873	Gadd45b	-2.19	-1.21
14473	Gc		2.90
14620	Gjb3	1.61	2.13
93692	Glrx	1.38	1.85
229599	Gm129	-2.03	1.56
208080	Gm514	-2.25	-1.70
14676	Gna15	-1.22	-2.32
215798	Gpr126	1.10	-2.44
14766	Gpr56		10.24
14804	Grid2	-1.03	1.91
14865	Gstm4	-1.85	1.20
14873	Gsto1	-1.07	1.95
14872	Gstt2	-2.19	-1.10
14964	H2-D1	1.03	1.89
15114	Hap1	1.11	1.98
67666	Hapln3		2.44
171285	Havcr2	-1.02	-1.84
15200	Hbegf	-1.14	-2.09
320473	Heatr5b	-1.09	1.94
69536	Hemk1	-1.02	1.87
15213	Hey1	343.17	n.e.
15214	Hey2	n.e.	23.77
75828	Hormad2	-1.85	1.13
53602	Hpcal1	-1.13	-2.57
101502	Hsd3b7	-1.99	1.39
15530	Hspg2	-1.39	1.89
15894	Icam1	1.73	2.48
50723	Icosl	-1.10	1.81
15903	Id3		2.85
68713	Ifitm1	1.09	1.90
16002	Igf2	-1.90	-2.49
239114	Il17d	-1.47	-2.33
20403	Itsn2	1.30	-1.96
16548	Khk	1.08	-1.80
242721	Klhdc7a	1.56	2.28
70394	Kptn	-1.86	1.02
16668	Krt18	2.02	1.76
16669	Krt19	2.13	2.40
16776	Lama5	-1.22	1.92
214048	Larp2	-2.15	-2.01
13590	Lefty1	1.41	-2.92
320202	Lefty2	1.36	-2.22
232798	Leng8	-1.32	1.98
67803	Limd2	-1.12	-2.37
217708	Lin52	2.18	-1.42

628308	LOC628308	-1.30	1.81
628709	LOC628709	1.64	-2.63
67774	Loh12cr1	-2.09	-1.12
80749	Lrfn1	-1.81	1.24
16971	Lrp1	-1.07	1.84
100604	Lrrc8c	-1.04	-2.02
17035	Lxn	1.55	2.33
26921	Map4k4	-1.21	-2.14
226778	Mark1	-1.10	-1.81
69572	Mfsd3	1.02	1.90
17318	Mid1	-1.12	1.86
214162	Mll1	1.02	1.89
76915	Mnd1	-1.22	-1.83
17888	Myh6		-9.20
98932	Myl9		-1.93
17937	Nab2	-2.27	-1.29
18039	Nefl	1.10	2.26
18073	Nid1	1.11	1.92
77583	Notum	-2.42	-2.45
74091	Npl	-1.18	-2.48
53324	Nptx2	1.94	2.83
106338	Nsun3		2.13
18208	Ntn1	1.02	2.17
229228	Nudt6		2.02
77595	Nup210l	1.17	-2.27
75475	Oplah	1.06	1.93
14539	Opn1mw	-1.07	-2.02
52793	ORF9	1.13	-2.24
217066	OTTMUSG00000001305	-1.98	1.78
627585	OTTMUSG00000010105	2.27	1.08
93737	Pard6g	-1.29	-2.57
30052	Pcsk1n	1.31	-1.89
18574	Pde1b	-1.23	2.29
18616	Peg3	1.21	2.31
64058	Perp	-2.18	-2.78
69129	Pex11c		2.03
18639	Pfkfb1	-1.02	2.46
66268	Pigyl	1.11	2.01
18715	Pim2	1.26	1.97
330890	Piwil4	-2.11	-2.42
85031	Pla1a	-1.02	3.33
329502	Pla2g4e	1.57	-2.24
18793	Plaur	-1.06	1.88
72469	Plcd3	-1.86	1.38
269608	Plekhg5	1.32	2.10
84094	Plvap	-1.31	1.81
67448	Plxdc2	-1.29	-2.54
110312	Pmch	1.31	-1.98
99011	Pomt1	-1.25	1.84
18997	Pou4f2	1.23	2.11
67905	Ppm1m	-1.53	-1.95
381813	Prmt8	-1.73	-3.27
65116	Prrg2	-2.64	-1.03
64292	Ptges	1.70	2.55
19228	Pthr1	-2.25	
19245	Ptp4a3	-1.38	-1.84
329384	Ptrh1	-1.64	-2.34
19286	Pts	-2.61	1.46
226422	Rab7l1	-1.15	2.14
67286	Rabl5	1.34	1.80

56089	Ramp3	1.45	3.19
223864	Rapgef3	-1.03	1.83
68895	Rasl11a	1.57	2.14
54354	Rassf5	1.50	2.29
26611	Rcn2	-1.14	-1.89
226594	Rcsd1		2.82
13650	Rhbdf1	-1.37	1.92
242662	Rims3	-2.13	-1.38
56532	Ripk3	-2.09	-1.29
11858	Rnd2	-1.22	-2.17
19881	Rom1	2.38	2.18
26564	Ror2	-1.06	1.81
57294	Rps27	1.41	-2.30
56367	Scoc	1.42	-2.34
69938	Scrn1	-2.03	-1.48
20720	Serpine2	-2.13	-2.50
53609	Sfrs16	-1.05	2.00
20401	Sh3bp1	1.25	2.66
20482	Skil	-1.38	-2.14
237831	Slc13a5		5.31
71781	Slc16a14	2.28	2.10
110877	Slc18a1	-1.05	-2.41
55963	Slc1a4	-1.15	-1.90
67554	Slc25a30	1.06	1.81
71279	Slc29a3	1.15	2.23
72027	Slc39a4	1.53	2.10
20532	Slc3a1	-2.16	-1.10
56774	Slc6a14	1.26	-1.88
240332	Slc6a7	1.97	3.35
11989	Slc7a3	-1.22	-1.89
24059	Slco2a1	1.07	2.30
67155	Smarca2	2.92	3.59
104367	Snora65	1.28	2.63
20664	Sox1	-2.31	-2.66
20668	Sox13	-1.84	-1.60
101809	Spred3	1.15	2.19
20446	St6galnac2	-1.06	1.97
29819	Stau2	-1.06	-1.91
20907	Stx1a	1.16	2.00
20909	Stx4a	-2.12	1.16
57429	Sult5a1	1.83	3.53
68760	Synpo2l		3.07
21407	Tcf15	-1.52	-2.17
226896	Tcfap2d	1.09	-1.88
21679	Tead4	1.33	1.96
21752	Tert	-1.14	2.00
21803	Tgfb1	1.55	1.85
69876	Thap3	-1.02	1.83
66231	Thoc7	1.80	-1.54
30058	Timm8a1	1.15	-1.84
101883	Tmem149	-1.39	1.83
224019	Tmem191c	1.45	1.80
56277	Tmem45a	1.35	-2.25
230657	Tmem69	1.24	-1.91
75002	Tmprss12	-2.24	1.41
67971	Tppp3	1.43	-1.80
59005	Trappc2l	1.01	-2.09
22041	Trf	1.25	1.84
22044	Trh	-1.12	-2.07
66597	Trim13	-1.64	-1.93

235631	Tsp50	1.25	2.55
235330	Ttc12	-1.08	2.50
237930	Ttll6	-1.07	2.61
22147	Tuba3b	1.59	-1.82
637908	Vmn2r53	1.20	-1.88
320808	Wdr22	-1.75	-1.93
76646	Wdr38	-1.06	-2.42
68980	Wdr53	-1.81	1.41
103784	Wdr92	1.03	-1.82
434204	Whdc1	-1.03	2.39
73750	Whrn	-1.82	-1.17
320916	Wscd2	1.07	2.40
67057	Yaf2	-1.29	-1.96
235320	Zbtb16	2.04	1.90
22724	Zbtb7b	1.21	1.93
207259	Zbtb7c	-1.22	1.92
67106	Zbtb8os	-1.17	-1.88
230738	Zc3h12a	-2.07	1.28
71164	Zdhhc11	-1.83	-1.24
239102	Zfhx2	-1.27	1.95
69234	Zfp688	-1.02	-2.23
218441	Zfyve16	1.12	1.86
65100	Zic5	-2.22	1.04
665902	Zscan4f	1.38	2.59

Table 3 Target genes in ES Hey1⁺, EC Hey1⁺ (A) and ES Hey2⁺, EC Hey2⁺ (B). Analysis was performed as described in section 5.3.5 and revealed 106 Hey1 target genes and 382 Hey2 target genes. For each target gene Entrez ID, gene name and fold change after Hey1 and Hey2 overexpression are presented. If no number for the fold change is indicated, selection criteria were not fulfilled and the gene was excluded from analysis.

A

Entrez ID	Gene	fold change	
		ES Hey1⁺	EC Hey1⁺
100043040		1.97	-1.31
100217431			2.01
100302648			-1.82
100316809		-2.22	
100499530		-2.22	
100503498			-2.05
100526469		1.31	-2.00
100526479			-2.02
100628584			-1.95
100628588			-1.98
100628612			-2.80
100628620			-2.02
68347	0610011F06Rik	1.12	1.83
66061	0610012D17Rik	1.05	-1.89
75394	0610040F04Rik	-1.96	1.43
68618	1110012L19Rik	-1.81	-1.42
66179	1110031I02Rik	-2.15	-1.91
68742	1110032O16Rik	-1.97	1.14
85308	1500005A01Rik	-1.02	-2.01
66935	1700023B02Rik	-1.96	1.02
66273	1810020D17Rik	-1.13	-1.97
100910	2010209O12Rik	-2.06	1.04
72093	2010320M18Rik	-1.96	-1.01
70080	2210010C17Rik	-2.07	
69642	2310046A06Rik		2.35
70419	2810408A11Rik	-2.03	-1.73

B

Entrez ID	Gene	fold change	
		ES Hey2⁺	EC Hey2⁺
14980		1.84	1.14
77633		-1.95	1.21
108112		1.93	
272396		-1.61	-1.81
624713		-2.64	1.54
652925		-2.01	
665037		4.15	-1.01
751556		-2.07	1.06
100042464		3.59	
100043609			-2.23
100043813		-2.30	1.09
100049713		-2.06	
100169889		2.73	-1.06
100316809		1.90	
100415787		-2.38	1.74
100499530		1.90	
100503879		1.89	
100504114		2.04	
100526469		-1.97	
100526479		1.81	
100568459		1.81	-1.23
101243624		-2.35	
68332	0610010E21Rik	-1.58	-1.96
66179	1110031I02Rik	-2.36	-1.38
69017	1500031I19Rik	2.01	
76927	1700021C14Rik	1.81	

73172	3110037I16Rik	-1.87	-1.17	69513	1700030C10Rik	-1.93	-1.05
73863	4930415O20Rik		-2.16	70011	1700030N03Rik	1.87	
67585	4930455J16Rik	-2.29	1.78	67105	1700034H14Rik	-1.97	-1.50
74726	4930523O13Rik	-2.24	-1.15	67524	1700095A21Rik	-2.11	
223626	4930572J05Rik	-2.09	-1.98	66278	1810013D10Rik	1.23	1.82
70942	4931403E22Rik	3.25	1.05	69186	1810027O10Rik	-1.89	-1.34
67531	5730408K05Rik	-1.83	-1.62	66282	1810029B16Rik	1.44	-2.01
330385	9530026P05Rik	1.07	1.98	66291	1810030N24Rik	2.33	
109202	A930024E05Rik	-2.24	-1.08	67892	1810063B05Rik	-1.09	1.97
11474	Actn3	-1.83	1.34	67509	1810063B07Rik	-1.15	-1.99
69010	Anapc13	1.80	-1.28	76527	2010004A03Rik	2.51	
230991	B930041F14Rik	-2.10	1.08	69871	2010007H12Rik	1.84	-1.01
233913	BC017158	-1.92	-1.05	72429	2010203O07Rik	1.23	-2.29
238024	BC032265	-2.09	1.03	72093	2010320M18Rik	-1.41	-2.71
408058	BC048507	2.05	1.11	76947	2310030N02Rik	1.30	-1.91
12159	Bmp4	-1.95	1.08	69627	2310031A18Rik		-2.24
101831	C230052I12Rik	-2.47	3.20	69698	2310046K01Rik	1.82	
97863	C78339	1.91	1.01	71918	2310047A01Rik	-3.67	-1.01
76815	Calcoco2	1.83		77032	2610029I01Rik	-1.85	-1.35
12578	Cdkn2a	2.21	1.05	73242	2610110G12Rik	2.01	1.43
231821	Centa1	-1.85		72481	2610203C22Rik	-1.85	-1.19
68119	Cmtm3	1.80	-1.01	70435	2610204M08Rik	1.93	1.02
12865	Cox7a1	2.50		545428	2610301F02Rik	1.88	
12873	Cpa3	-1.86	-1.71	68034	2900009I07Rik	2.09	-1.09
12877	Cpeb1	2.00	1.03	75430	3200002M19Rik	1.01	2.08
72865	Cxx1c		1.89	69277	3300002I08Rik	1.03	1.92
60364	Donson	-1.84	1.38	67419	3632451O06Rik	-2.71	-1.15
56405	Dusp14	2.52	1.33	319518	4930402E16Rik	1.34	1.96
68177	Ebpl	-1.92	-1.14	432479	4930404N11Rik	1.84	
434197	EG434197	2.35	-1.32	78108	4930414L22Rik	-2.20	1.04
434280	EG434280	-2.29		75060	4930506C21Rik	2.12	
791260	F930017I19Rik	-1.82	1.41	74726	4930523O13Rik	2.02	
14226	Fkbp1b	1.97		67651	4930527F14Rik	-1.37	-2.40
17873	Gadd45b	-2.19	1.08	75202	4930546H06Rik	2.50	1.10
229599	Gm129	-2.03	-1.54	78215	4930578N18Rik	1.90	
208080	Gm514	-2.25		70942	4931403E22Rik	3.04	-1.12
14865	Gstm4	-1.85		74437	4933402E13Rik	3.84	
14872	Gstt2	-2.19		67412	6330407J23Rik	2.11	
15213	Hey1	343.17	15.27	231842	6530401C20Rik	2.06	
75828	Hormad2	-1.85		76219	6530401D17Rik	-1.83	
101502	Hsd3b7	-1.99	-1.22	381062	9030025P20Rik	1.87	-1.06
16336	Insl3		-2.34	71532	9030418K01Rik		-1.85
70394	Kptn	-1.86	-1.19	619321	9530052E02Rik	1.68	-2.29
16668	Krt18	2.02	-1.56	194268	9930104L06Rik	1.20	-2.14
16669	Krt19	2.13	1.43	231717	A230106M15Rik	-1.86	-1.42
214048	Larp2	-2.15	1.09	239559	A4galt	1.89	
217708	Lin52	2.18	1.21	381272	A630095N17Rik	2.07	
67774	Loh12cr1	-2.09	-1.05	76491	Abhd14b	2.24	-1.27
80749	Lrfn1	-1.81	1.85	11433	Acp5	-2.14	
17937	Nab2	-2.27	1.18	433256	Acsl5	-1.98	1.01
77583	Notum	-2.42		11464	Actc1	-5.74	
53324	Nptx2	1.94		106672	AI413582	3.02	-1.10
217066	OTTMUSG00000001305	-1.98		100165	AI507597	1.83	
627585	OTTMUSG00000010105	2.27	-1.28	11634	Aire	3.57	
64058	Perp	-2.18	-1.09	72041	Alkbh4	1.81	-1.09
330890	Piwil4	-2.11		232566	Amn1	1.98	1.06
72469	Plcd3	-1.86	-1.02	234396	Ankrd41	1.89	
77619	Prelid2		2.02	12306	Anxa2	1.91	1.11
65116	Prrg2	-2.64	-1.06	27052	Aoah	2.11	
19171	Psmb10	1.12	-2.02	64933	Ap3m2	2.75	-1.05

19228	Pthr1	-2.25	
19286	Pts	-2.61	1.29
242662	Rims3	-2.13	
56532	Ripk3	-2.09	-1.08
19881	Rom1	2.38	1.08
69938	Scrn1	-2.03	1.30
20720	Serpine2	-2.13	1.04
71781	Slc16a14	2.28	
20532	Slc3a1	-2.16	-1.15
67155	Smarca2	2.92	1.03
20664	Sox1	-2.31	
20668	Sox13	-1.84	1.19
20909	Stx4a	-2.12	-1.05
57429	Sult5a1	1.83	-1.04
66231	Thoc7	1.80	1.05
75002	Tmprss12	-2.24	
68980	Wdr53	-1.81	-1.44
73750	Whrn	-1.82	1.05
230738	Zc3h12a	-2.07	1.33
71164	Zdhhc11	-1.83	1.09
65100	Zic5	-2.22	

381823	Apold1		-1.94
13498	Atn1	2.14	-1.01
320871	B230206H07Rik	2.38	
319752	B230209E15Rik	-1.83	1.16
26879	B3galnt1	-1.61	-2.20
414069	BC024978	-2.12	-1.85
277154	BC030046	2.41	1.02
103220	BC030307	-1.87	
474160	BC033916	-2.40	
381066	BC049807	2.19	-1.15
230676	BC059842	1.96	1.26
14705	Bscl2	-1.06	2.06
57316	C1d	1.01	-2.02
654318	C530005A16Rik	1.05	-2.24
76815	Calcoco2	2.13	
12337	Capn5	2.12	
12367	Casp3	-1.85	1.16
12372	Casq1	2.92	-1.67
57895	Ccdc126	-1.03	-1.88
232664	Ccdc136	-2.23	
382073	Ccdc84	1.96	1.33
12505	Cd44	-3.50	-1.07
12509	Cd59a	2.13	1.02
12512	Cd63	-1.81	1.09
260409	Cdc42ep3	-2.02	-1.12
239096	Cdh24	1.84	-1.01
26365	Ceacam1	-2.22	
12615	Cenpa	1.08	-1.81
216859	Centb1	2.14	
68567	Cgref1	-1.97	-1.35
69065	Chac1	-2.22	
72042	Cotl1	-2.37	1.05
12925	Crip1	1.94	
13032	Ctsc	1.80	
13034	Ctse	3.11	
56464	Ctsf	-1.12	1.86
435802	Cyp4a30b	-2.25	
170716	Cyp4f13	1.31	-1.83
12879	Cys1	-2.35	
225995	D030056L22Rik	-1.10	1.95
114585	D17H6S53E	-1.47	2.08
320351	D230037D09Rik	-2.51	-1.32
654822	D330041H03Rik	1.90	1.19
231630	D5Ertd40e	-1.19	1.91
52480	D7Ertd715e	2.72	
228859	D930001I22Rik	-1.80	1.05
13132	Dab2	2.00	1.14
240025	Dact2	1.82	
72185	Dbndd1	2.09	-1.02
13198	Ddit3	-1.93	-2.40
230073	Ddx58	1.98	1.27
209773	Dennd2a	-1.83	-1.13
13346	Des	-1.97	
13368	Dffb	-1.13	1.87
107585	Dio3	3.94	
57431	Dnajc4	1.12	2.01
227697	Dolk	-1.86	1.21
13482	Dpp4	-1.94	
77490	E130218I03Rik	1.15	2.27
320172	E230016M11Rik	-1.81	1.03

55960	Ebag9	1.34	-2.06
434280	EG434280	-2.69	
620393	EG620393	1.19	2.09
627821	EG627821	1.87	
665574	EG665574	1.84	
386655	Eid2	-2.05	-1.06
72205	Eml2	1.34	2.07
100038468	ENSMUSG00000074385	-1.83	
98845	Eps8l2	2.59	
13867	Erbb3	1.90	
71690	Esm1		-1.97
14009	Etv1	1.89	1.29
277978	Exoc3l	2.46	-1.15
14077	Fabp3	-1.86	
327959	Fbxo39	2.31	
230903	Fbxo44	-2.07	
286940	Flnb	1.90	1.05
71436	Flrt3	1.23	2.07
226844	Flvcr1	2.29	1.03
54418	Fmn2	1.81	
14268	Fn1	2.46	1.07
15221	Foxd3	2.00	
54601	Foxo4	-1.99	-1.06
14369	Fzd7	1.36	3.85
14473	Gc	2.90	
80860	Ghdc	-1.13	2.07
14620	Gjb3	2.13	
73690	Glipr1	-1.06	2.27
110304	Glra3	1.03	1.81
93692	Glrx	1.85	1.13
228730	Gm114	-1.29	1.81
57441	Gmnn	-1.25	1.83
14676	Gna15	-2.32	2.46
215798	Gpr126	-2.44	1.04
14766	Gpr56	10.24	
14804	Grid2	1.91	1.36
14873	Gsto1	1.95	1.02
67371	Gtf3c6	-1.07	-1.83
14964	H2-D1	1.89	1.34
15114	Hap1	1.98	
67666	Hapln3	2.44	
171285	Havcr2	-1.84	
15200	Hbegf	-2.09	-1.28
70315	Hdac8	-1.01	-2.08
320473	Heatr5b	1.94	1.11
69536	Hemk1	1.87	
15214	Hey2	23.77	3.26
15277	Hk2	1.22	1.90
53602	Hpcal1	-2.57	1.03
15473	Hrsp12	-1.52	-1.95
15530	Hspg2	1.89	1.12
15894	Icam1	2.48	1.13
50723	Icosl	1.81	
15901	Id1	-1.09	1.98
15903	Id3	2.85	-1.06
68713	Ifitm1	1.90	
16002	Igf2	-2.49	-1.01
239114	Il17d	-2.33	
16336	Insl3	1.45	-1.88
20403	Itsn2	-1.96	-1.02

170771	Khdrbs2	-1.34	-2.19
16548	Khk	-1.80	-1.38
242721	Klhdc7a	2.28	
70394	Kptn	1.02	-1.89
16669	Krt19	2.40	
16776	Lama5	1.92	1.43
214048	Larp2	-2.01	
13590	Lefty1	-2.92	
320202	Lefty2	-2.22	
232798	Leng8	1.98	1.01
67803	Limd2	-2.37	1.03
628308	LOC628308	1.81	-1.35
628709	LOC628709	-2.63	
16971	Lrp1	1.84	
100604	Lrrc8c	-2.02	-1.01
17035	Lxn	2.33	-1.00
17087	Ly96		-1.91
26921	Map4k4	-2.14	-1.09
226778	Mark1	-1.81	
26922	Mecr	1.24	-2.19
17299	Mettl1	1.12	-1.80
100034361	Mfap1b	-1.08	1.92
69572	Mfsd3	1.90	
17314	Mgmt	-1.36	-1.91
17318	Mid1	1.86	1.19
218613	Mier3	1.07	1.91
214162	Mll1	1.89	-1.02
76915	Mnd1	-1.83	
64660	Mrps24	-1.04	-1.88
17888	Myh6	-9.20	
98932	Myl9	-1.93	1.10
18039	Nefl	2.26	-1.31
18030	Nfil3	-1.24	-2.95
18073	Nid1	1.92	1.13
77583	Notum	-2.45	
74091	Npl	-2.48	1.77
53324	Nptx2	2.83	
68501	Nsmce2	-1.23	2.13
106338	Nsun3	2.13	
18208	Ntn1	2.17	-1.53
229228	Nudt6	2.02	
77595	Nup210l	-2.27	1.42
75475	Oplah	1.93	
14539	Opn1mw	-2.02	1.05
52793	ORF9	-2.24	-2.50
433287	OTTMUSG00000022109	1.35	-1.97
73162	Otud3	1.07	-2.41
76498	Paqr4	1.15	-1.82
93737	Pard6g	-2.57	1.34
30052	Pcsk1n	-1.89	
18574	Pde1b	2.29	
18616	Peg3	2.31	-1.19
64058	Perp	-2.78	
69129	Pex11c	2.03	-1.15
18639	Pfkfb1	2.46	
66268	Pigyl	2.01	-1.62
18715	Pim2	1.97	-1.15
85031	Pla1a	3.33	
329502	Pla2g4e	-2.24	-1.59
18793	Plaur	1.88	-1.24

319455	Pld5	-1.48	-1.90
269608	Plekhg5	2.10	-1.03
84094	Plvap	1.81	-1.26
67448	Plxdc2	-2.54	1.17
110312	Pmch	-1.98	
99011	Pomt1	1.84	1.46
18997	Pou4f2	2.11	
67905	Ppm1m	-1.95	1.05
217430	Pqlc3	1.83	1.82
381813	Prmt8	-3.27	
64292	Ptges	2.55	
19245	Ptp4a3	-1.84	-1.21
329384	Ptrh1	-2.34	-1.10
226422	Rab7l1	2.14	1.31
227746	Rabepk	1.16	-1.83
67286	Rabl5	1.80	1.33
56089	Ramp3	3.19	
223864	Rapgef3	1.83	-1.13
218772	Rarb	-1.24	2.28
109222	Rarres1		-2.29
68895	Rasl11a	2.14	
54354	Rassf5	2.29	1.40
26611	Rcn2	-1.89	-1.15
226594	Rcsd1	2.82	-1.03
58175	Rgs20	-1.08	1.90
13650	Rhbdf1	1.92	1.00
11858	Rnd2	-2.17	
218215	Rnf144b	1.56	2.12
26564	Ror2	1.81	
622404	RP23-195K8.6	1.68	-1.91
57294	Rps27	-2.30	1.09
101122	Rpusd3	1.26	-2.29
74648	S100pbp	1.08	-2.44
56367	Scoc	-2.34	1.03
20720	Serpine2	-2.50	1.17
74729	Setmar	-1.37	1.95
53609	Sfrs16	2.00	-1.03
20401	Sh3bp1	2.66	
20482	Skil	-2.14	-1.02
237831	Slc13a5	5.31	
71781	Slc16a14	2.10	
110877	Slc18a1	-2.41	
55963	Slc1a4	-1.90	1.18
59049	Slc22a17	1.52	1.99
67554	Slc25a30	1.81	1.30
71279	Slc29a3	2.23	1.14
108652	Slc35b3	1.07	1.90
72027	Slc39a4	2.10	-2.10
242259	Slc44a5	-1.00	-1.80
56774	Slc6a14	-1.88	
240332	Slc6a7	3.35	
11989	Slc7a3	-1.89	
24059	Slco2a1	2.30	
67155	Smarca2	3.59	1.26
330959	Snapc5	-1.15	-1.91
104367	Snora65	2.63	
20664	Sox1	-2.66	
214616	Spata5l1	1.79	-2.20
101809	Spred3	2.19	-1.16
20446	St6galnac2	1.97	

29819	Stau2	-1.91	1.28
20907	Stx1a	2.00	1.11
57429	Sult5a1	3.53	
68760	Synpo2l	3.07	
67249	Tbc1d19	-1.18	1.94
21407	Tcf15	-2.17	
226896	Tcfap2d	-1.88	-1.34
67978	Tctn2	-1.10	2.02
21679	Tead4	1.96	1.13
21752	Tert	2.00	
21803	Tgfb1	1.85	1.08
69876	Thap3	1.83	-1.10
386612	Thoc6	-1.03	-1.97
30058	Timm8a1	-1.84	
101883	Tmem149	1.83	
67698	Tmem157	-1.35	-1.98
224019	Tmem191c	1.80	
236792	Tmem32	-1.20	2.11
56277	Tmem45a	-2.25	
230657	Tmem69	-1.91	1.51
233979	Tpcn2	1.24	1.85
67971	Tppp3	-1.80	
74019	Traf3ip1	-1.05	1.97
59005	Trappc2l	-2.09	1.06
22041	Trf	1.84	
22044	Trh	-2.07	
66597	Trim13	-1.93	
235631	Tsp50	2.55	
21912	Tspan7	-1.25	-1.85
52808	Tspyl2	-1.19	2.25
235330	Ttc12	2.50	1.07
237930	Ttll6	2.61	
22147	Tuba3b	-1.82	
233276	Tubgcp5	1.27	-1.80
22172	Tyms-ps	-1.03	-1.83
22350	Vil2	-1.01	2.21
637908	Vmn2r53	-1.88	
22365	Vps45	1.14	2.08
320808	Wdr22	-1.93	1.00
76646	Wdr38	-2.42	-1.76
103784	Wdr92	-1.82	-1.10
434204	Whdc1	2.39	-1.90
320916	Wscd2	2.40	
67057	Yaf2	-1.96	1.02
235320	Zbtb16	1.90	
22724	Zbtb7b	1.93	1.09
207259	Zbtb7c	1.92	
67106	Zbtb8os	-1.88	1.08
239102	Zfhx2	1.95	-1.16
56220	Zfp386	1.08	2.15
68040	Zfp593	-1.19	1.85
69234	Zfp688	-2.23	-1.08
69020	Zfp707	1.56	-2.29
218441	Zfyve16	1.86	-1.11
665902	Zscan4f	2.59	
67538	Zswim3	-1.09	-1.91

9.3 Publications

Hein M, Graver S, (2013). Tumor cell response to bevacizumab single agent therapy *in vitro*. *Cancer Cell International* 2013 13:94.

9.4 Oral Presentations and Posters

06/2012 **17th International Vascular Biology Meeting, Wiesbaden**

Poster: *In vitro* differentiation of Hey deficient endothelia to delineate vascular Hey gene functions

06/2012 **Joint Symposium of the Collaborative Research Center (SFB) 688, the Rudolf Virchow Center and the Comprehensive Heart Failure Center, "Cell-cell interactions in cardiovascular damage and healing", Würzburg**

Poster: *In vitro* differentiation of Hey deficient endothelia to delineate vascular Hey gene functions

10/2012 **7th International Symposium organized by the Students of the Graduate School of Life Sciences, EPOS, Everything's Part Of Science**

Poster: *In vitro* differentiation of Hey deficient endothelia to delineate vascular Hey gene functions

10/2013 **8th International Symposium organized by the Students of the Graduate School of Life Sciences, SCI, Scientific Crosstalk, Würzburg**

Talk: *In vitro* differentiation of Hey deficient endothelia to identify vascular Hey target genes

9.5 Curriculum vitae

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Ohne euch wäre das Gelingen dieser Arbeit nicht möglich gewesen! **Dankeschön!**

9.7 Affidavit

Affidavit

I hereby confirm that my thesis entitled **Functional analysis of angiogenic factors in tumor cells and endothelia** is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore I confirm that the thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Place, Date   Signature

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation **Funktionelle Analyse angiogener Faktoren in Tumorzellen und Endothelien**, eigenständig, d.h. insbesondere selbstständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen, als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Ort, Datum   Unterschrift