Aus der Medizinischen Klinik und Poliklinik I Großhadern

der Ludwig-Maximilians-Universität München

Direktor: Prof. Dr. med. G. Steinbeck

Regulation of Dickkopf-1 in hypoxic endothelial activation

(Regulation von Dickkopf-1 bei hypoxischer endothelialer Aktivierung)

Dissertation

zum Erwerb des Doktorgrades der Medizin

an der Medizinischen Fakultät der

Ludwig-Maximilians-Universität zu München

vorgelegt von

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aus

München

2008

Mit Genehmigung der Medizinischen Fakultät

der Universität München

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Tag der mündlichen Prüfung:	10.07.2008

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2. Introduction

In the United States and Europe cardiovascular disease, including stroke is the major cause of death and morbidity. The common pathological substrate forming the basis of coronary heart disease and ischemic stroke is dysfunction of the endothelium leading to atherosclerosis. Atherosclerotic narrowed or blocked arteries impair blood flow and cause tissue ischemia. Manifest ischemic stroke and myocardial infarction usually originate from rupture of atherosclerotic plaques and consequent thrombotic occlusion of arteries. The molecular mechanisms underlying myocardial infarction, stroke and atherosclerosis are very complex and a large variety of molecules and signaling processes is needed to promote the inflammatory response and wound healing (Ross, 1999; Davignon & Ganz, 2004; Hansson, 2005; Frangogiannis et al., 2002; Zheng & Yenari, 2004). Another major issue in ischemic tissues is neovascularization. Sprouting of new vessels is observed after infarction of the brain and the heart as is the formation of collaterals in chronic ischemia. Since common therapeutic strategies to restore blood flow in tissue affected by ischemia such as implantation of bypass grafts or angioplasty/stenting have their mechanical limitations, new therapeutic approaches to rescue ischemic tissue by inducing vessel growth to ameliorate angina symptoms and reduce loss of functional tissue have attracted much attention. To apply a specific therapy, molecular mechanisms of neovascularization involving a large variety of factors and signaling pathways need to be closely understood (Greenberg & Jin, 2005; Krupinski et al., 1994; Nelissen-Vrancken et al., 1996; Toyota et al., 2004; Schaper & Ito, 1996).

2.1. Angiogenesis, arteriogenesis and vasculogenesis

Blood vessel growth, generally referred to as neovascularization, is distinguished in three different processes that participate in the formation of the vascular system (Simons, 2005).

(1) Angiogenesis is mainly characterized as sprouting of new vessels out of pre-existing capillaries. One of the most important stimuli for angiogenesis is hypoxia, which strongly regulates angiogenesis via hypoxia inducible factor 1-α (HIF-1α) (Simons, 2005; Carmeliet, 2003).

(2) Arteriogenesis is triggered by local alterations in shear-stress in the vicinity of arterial occlusion, but mainly occurs outside areas of ischemia. It is currently debated whether arteriogenesis describes a process of de novo forming collaterals that are stabilized by mural cells such as smooth muscle cells or the remodeling of pre-existing arterioles into larger conductance arteries (Simons 2005; Carmeliet 2000; Carmeliet, 2003; Schaper & Scholz, 2003).

(3) Vasculogenesis occurs during embryogenesis and describes a process of local formation of a primitive vascular network by differentiation of immature endothelial progenitor cells (angioblasts) into endothelial cells that form a primitive vascular network. Lately, it could be shown that endothelial progenitor cells (EPCs) derived from the bone marrow also circulate postnatally in the peripheral blood and contribute to postnatal neovascularization (postnatal vasculogenesis) (Carmeliet, 2000; Luttun & Carmeliet, 2003; Asahara et al., 1999).

2.2. Angiogenesis and vasculogenesis in ischemic cardiovascular disease

Angiogenesis can be found in the infarcted area after myocardial infarction and stroke.

In ischemic stroke angiogenesis is observed in the penumbra, the border zone of the infarcted area. In contrast to the core of the infarcted area, blood supply in the penumbra is diminished but not absent. Improvement of blood flow in the penumbra might have an impact on cell survival and limitation of tissue damage. It could be shown that vascular endothelial growth factor (VEGF)-induced angiogenesis after stroke in rats was beneficial regarding the

neurological deficit (Krupinski et al., 1994; Greenberg & Jin, 2005; Zhang et al., 2000). Similar to ischemic stroke, angiogenesis is also found in the border zone of myocardial infarction. In the acute phase of infarction, pre-existing collateral vessels limit the infarct size and increase the blood flow to the injured area. The chronic phase is characterized by the appearance of newly formed vessels in the area of infarction that are a critical step to the final restoration of blood flow (Nelissen-Vrancken et al., 1996; Cleutjens et al., 1999).

Therapeutic administration of pro-angiogenic factors such as VEGF and fibroblast growth factor (FGF) to induce vessel growth by angiogenesis has been given much thought lately (Simons & Ware, 2003; Toyota et al., 2004). Apart from that, postnatal vasculogenesis is another major issue in the therapeutic attempt to rescue ischemic tissue. In embryonic vasculogenesis, EPCs are assembled to form a primitive vascular network. Postnatally, EPCs are also found to circulate in the peripheral blood and have the ability to proliferate and differentiate into mature endothelial cells. It has been shown that EPCs may contribute to neovascularization in ischemic tissue consistent with postnatal vasculogenesis (Asahara et al., 1997; Takahashi et al., 1999; Asahara et al., 1999; Hristov et al., 2003; Dzau et al., 2005).

Embryonic endothelial progenitor cells (eEPCs) were first isolated by Hatzopoulos et al., (1998) from murine embryos at E7.5. These cells appear in early embryonic vascular development and show the characteristic properties of endothelial progenitors as well as unlimited stem-cell like growth. In vitro, eEPCs retain their progenitor properties and have the potential to differentiate under the influence of retinoic acid and cyclic adenosine monophosphate (cAMP) showing a more typical gene expression profile and morphology for endothelial cells. It has been shown, that eEPCs contribute to neovascularization in tumors and preferentially home to hypoxic metastases (Vajkoczy et al., 2003; Wei et al., 2004). Recent studies show that eEPCs increase neovascularization and improve tissue recovery in a model of chronic hind limb ischemia in rabbits as well as in a model of myocardial ischemia in mice. It

should be noted that administration of eEPCs to sites of ischemia seems to induce angiogenesis, possibly through a broad range of eEPC-secreted factors (Kupatt et al., 2005). Therefore, eEPCs are a powerful model to study regulation of vascular development and factors involved in the formation of vessels.

2.3. Regulation of angiogenesis and vasculogenesis by hypoxia and the role of VEGF

Among other metabolic conditions that promote angiogenesis, such as acidosis and hypoglycaemia, hypoxia is an important stimulus for angiogenesis (Emanueli & Madeddu, 2001). It has been hypothesized that alterations in oxygen availability in tissues is sensed by the HIF hydroxylase system. Low oxygen availability leads to the activation of HIF-1α, a transcription factor that is known to act as a key regulator of oxygen homeostasis at the transcriptional level (together with HIF-1ß). Most importantly among the genes induced by HIF-1α is *VEGF*, which is thus regulated by hypoxia (Pugh & Ratcliffe, 2003; Strieter, 2005).

VEGF is one of the most important growth factors in angiogenesis. Six members belong to the *VEGF* gene family: *VEGF-A – VEGF-E* and *placental growth factor (PLGF)*. VEGF-A (usually referred to as VEGF) has been shown to be involved, beside other factors, in several of the steps leading to the formation of new vessels. VEGF for example increases vascular permeability and promotes endothelial cell growth as well as endothelial cell survival (Ferrara et al., 2003; Carmeliet, 2000).

Besides angiogenesis, hypoxia also seems to be a stimulus for postnatal vasculogenesis. It could be shown that the number of postnatal circulating EPCs was augmented in animal models of tissue ischemia which was accompanied by increased neovascularization (Takahashi et al., 1999).

2.4. Canonical Wnt signaling and inhibition of Wnt signaling by Dkk-1

Wnt signaling is well established in development and disease, especially cancer (Cadigan & Nusse, 1997; Polakis, 2000). However, an additional role of Wnt signaling in cardiovascular disease has only recently begun to take shape. Components of the Wnt signaling pathway have been found to be involved in wound healing after myocardial infarction and neovascularization processes (van Gijn et al., 2002). Interestingly, several genes that are known to participate in angiogenesis have been shown to be Wnt target genes. An increasing body of evidence leads to the suggestion, that Wnt signaling is one of the pathways involved in the formation and remodeling of blood vessels (Goodwin & D´Amore, 2002).

Wnt signaling is initiated by binding of Wnt proteins to frizzled (Fz) receptors located in the cell membrane (Bhanot et al., 1996). Wnt proteins are a family of secreted glycoproteins, which represent a major group of developmental regulators (Cadigan & Nusse, 1997; Logan & Nusse, 2004). In mammals, 19 different Wnt homologs and 10 different Fz receptors have been identified (for updates on Wnt signaling see: http: //www.stanford.edu/~rnusse/wntwindow.html). Different Wnt proteins seem to preferably activate distinct pathways, i.e., the Wnt/ß-catenin pathway or the Wnt/Ca^{2+} pathway that are involved in lineage specification and body axis formation in early embryos (Kühl et al., 2000; Miller et al., 1999; Wharton, 2003). The third pathway is the Planar Cell Polarity/Convergent Extension pathway (PCP/CE), which mainly promotes tissue polarity and convergent extension movements. Disheveled (Dvl), a protein downstream from the Fz receptors is supposed to be the node between Wnt/ß-catenin and PCP signaling and seems to be able to discriminate among the different pathways by distinct interactions of its protein domains (Boutros et al., 1998; Wharton, 2003; Povelones & Nusse, 2002). The Wnt/ß-catenin signaling pathway is referred to as the canonical Wnt pathway, whereas Wnt/Ca^{2+} and PCP signaling pathways are termed noncanonical Wnt signaling pathways (Fig.1).

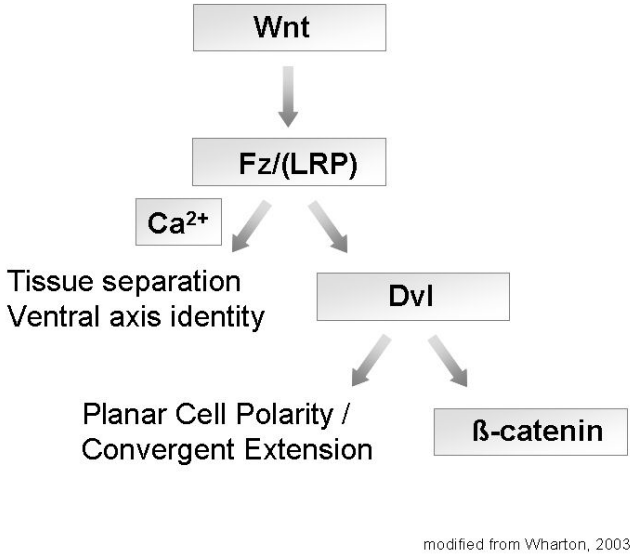


Figure 1 The Wnt protein activates three different pathways. By binding to Fz receptors, either the Wnt/Ca^{2+} pathway (left) that promotes tissue separation and ventral axis identity is activated or signals are transmitted through Dvl. Dvl is thought to be able to distinguish signals and promote signaling through the planar cell polarity/ convergent extension pathway or the Wnt/ß-catenin pathway that leads to ß-catenin accumulation and transcription of target genes. LRP is believed to function specifically in Wnt/ß-catenin signaling.

In cardiovascular disease, however, most insight has been gained on components and targets of the canonical Wnt pathway. There also might be a role for noncanonical Wnt signaling pathways in cardiovascular disease and in the vasculature, but this has not been closely investigated (Goodwin & D´Amore, 2002). Further focus of this work will therefore be on the canonical Wnt signaling pathway.

Canonical Wnt signaling is thought to be activated by binding of Wnt proteins to Fz receptors in the presence of the co-receptor low density lipoprotein (LDL) receptor-related protein 6 (LRP6) and apparently also LRP5, which shares 71% amino-acid identity with LRP6 (Tamai et al., 2000; Wehrli et al., 2000; Pinson et al., 2000; Mao et al., 2001). The exact nature of activation of Wnt signaling and transduction of the signal to the cell has only been poorly understood and is currently controversial discussed. It has been proposed that the formation of a ternary complex consisting of the Wnt protein, the Fz receptor and the co-receptor LRP 5/6 and

the association of this complex with intracellular Axin is necessary for transduction of the signal (Tamai et al., 2004; Mao et al., 2001; Tolwinski & Wieschaus, 2004). In this process Dvl seems to be required in relocating Axin to the plasma membrane (Cliffe et al., 2003).

Active Wnt signaling leads to the release of ß-catenin from a cytosolic complex, consisting mainly of the scaffolding protein Axin, the tumor suppressor protein adenomatous polyposis coli (APC), the glycogen synthetase kinase-3ß (GSK-3ß), the casein kinase I (CKI) and ß-catenin. In the absence of Wnt signaling, ß-catenin is phosphorylated by GSK-3ß and CKI which results in the degradation of ß-catenin. Positive Wnt signaling blocks phosphorylation of ß-catenin, which is subsequently not degraded. This process leads to accumulation and stabilization of cytosolic ß-catenin, which is now able to translocate to the nucleus. The presence of ß-catenin in the nucleus and its interactions with the T-cell-specific transcription factor (Tcf)/ lymphoid enhancer-binding factor (Lef) DNA-binding proteins mediate the transcription of Wnt-signaling-depending target genes (Logan & Nusse, 2004; Polakis, 2002; Clevers & van de Wetering, 1997; Behrens et al, 1996; Molenaar et al., 1996).

Wnt signaling is regulated by a number of extracellular inhibitors. Among the many factors that inhibit Wnt signaling are the secreted frizzled related proteins (sFRPs), Wnt inhibitory factor (WIF) and the *Dickkopf (Dkk)* gene family, that comprises three members in mice (*Dkk-1, Dkk-2 and Dkk-3*) and four members in humans (*DKK-1, DKK-2, DKK-3* and *DKK-4*). It should be noted, however, that distinct Wnt proteins that preferably activate noncanonical Wnt signaling are also able to inhibit canonical Wnt signaling (Kawano & Krypta, 2003) (Fig.2).

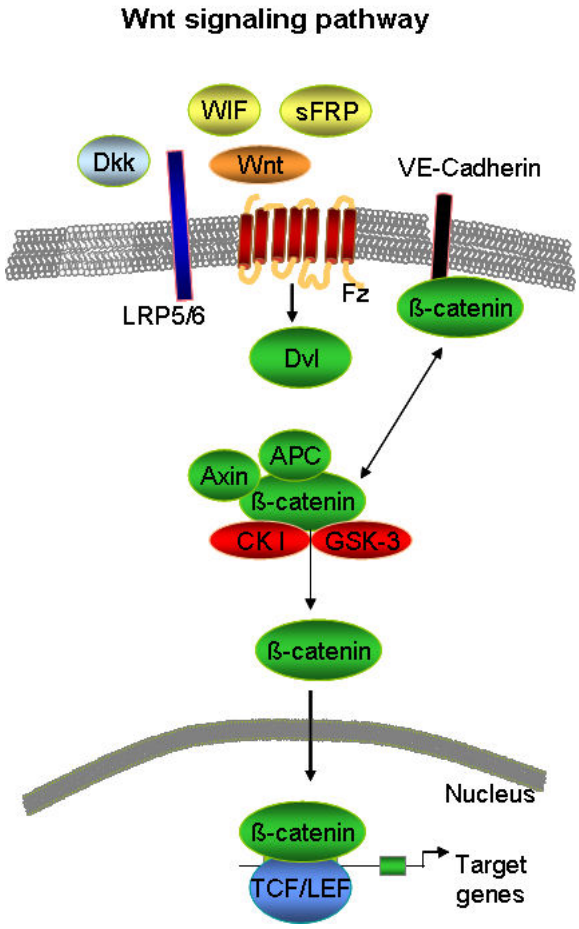
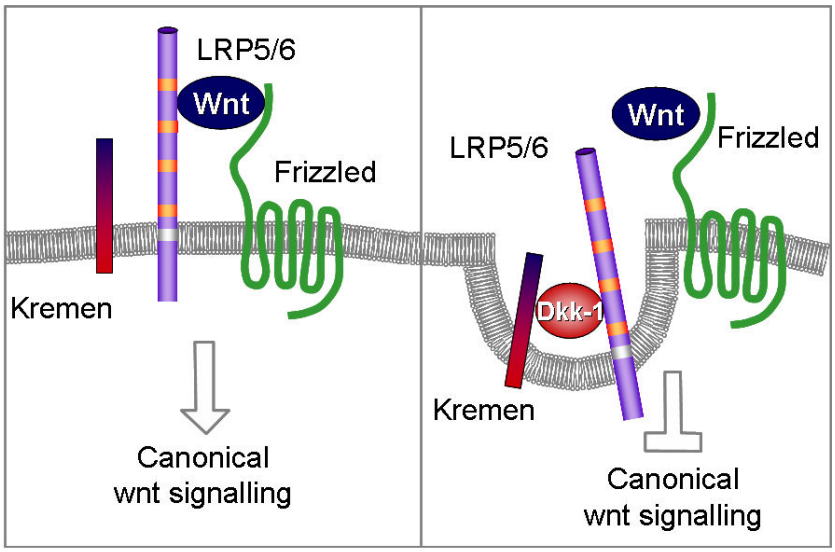


Figure 2 Simplified schematic drawing of the canonical Wnt signaling pathway. Wnt signaling is initiated by binding of the Wnt protein to the Fz receptor and the co-receptor LRP5/6. The signal is passed via Dvl and the cytosolic complex consisting of Axin, APC, CK I, GSK-3ß and ß-catenin. In the case of active signaling phosphorylation of ß-catenin by CK I and GSK-3ß is inhibited which leads to a release of ß-catenin from the complex. ß-catenin accumulates in the cytoplasm and translocates to the nucleus where it interacts with transcription factors (Tcf/Lef family) thus promoting the transcription of target genes. Apart from its role in Wnt signaling ß-catenin plays also a role in cell adhesion. ß-catenin links cadherins in the cell membrane, for example VE-cadherin in endothelial cells, to the actin cytoskeleton.
Wnt signaling is inhibited by different extracellular antagonists (Dkk, sFRP, WIF) that interfere directly or indirectly with the Wnt protein. In the absence of Wnt signaling ß-catenin is phosphorylated by GSK-3ß or CKI and subsequently degraded.

One of the best characterized Wnt inhibitors is Dkk-1. Dkk-1, the first member of the Dkk gene family, was described in 1998. It was shown to be a potent Wnt antagonist and is required for head formation in Xenopus laevis and mice (Glinka et al., 1998; Mukhopadhyay et al., 2001). Dkk-1 inhibits Wnt signaling by directly interacting with LRP6. It has been shown, that LRP6 is a high-affinity receptor for Dkk-1 and Dkk-2, independent of Fz. It is thought that Dkk-1

blocks Wnt/ß-catenin signaling by disrupting the Fz-LRP6 association by binding to distinct domains of LRP that are not required for Wnt/Fz interaction (Mao et al., 2001; Bafico et al., 2001; Semenov et al., 2001; Nusse, 2001). The presence of the co-receptor LRP appears to specify Wnt signaling to the Wnt/ß-catenin pathway, because LRP5/6 could not be shown to act in other Wnt signaling pathways (Wehrli et al., 2000). This points to an important role for Dkk-1 as a specific inhibitor of canonical Wnt/ß-catenin signaling (Semenov et al., 2001; Zorn, 2001). In contrast, other Wnt antagonists are believed to bind directly to the Wnt protein thus inhibiting the binding of Wnts to the Wnt receptor complex. Based on this idea, these antagonists are thought to inhibit both, canonical and noncanonical pathways (Kawano and Kypta, 2003).

Another component of Dkk action is the presence of yet another transmembrane receptor family called Kremen receptors (Krm). Krm-1 and Krm-2 are high-affinity Dkk-1 and Dkk-2 receptors. In the case of Dkk-1, formation of a ternary complex consisting of Krm, Dkk-1 and LRP6 leads to rapid endocytosis and thus removal of the Wnt receptor LRP from the plasma membrane. This leads to inhibition of wnt signaling through absence of the co-receptor for Wnt signaling (Mao et al., 2002; Rothbächer & Lemaire, 2002) (Fig.3).



modified from Mao et al., 2002

Figure 3 Dkk-1 exerts its inhibitory function on canonical Wnt signaling through two membrane receptors, LRP 5/6 and Kremen. Binding of Dkk-1 to LRP5/6 and Kremen leads to endocytosis of the ternary complex. Thus, the co-receptor LRP 5/6, which is required for positive Wnt signaling, is removed from the cell surface resulting in inhibition of canonical Wnt signaling.

Dkk-1 expression seems to be regulated by a negative feedback loop in Wnt signaling. Recently, it could be shown that human Dkk-1 is a target gene of ß-catenin/TCF and that active canonical Wnt signaling induced Dkk-1 transcription (Niida et al., 2004; González-Sancho et al., 2005).

2.5. Wnt target genes

A role for Wnt signaling in cardiovascular disease and the vasculature has only recently been recognized. Therefore, relatively few studies are currently available that address the connections between downstream components of the Wnt signaling pathway, Wnt target genes and their function in cardiovascular disease and the vasculature. However, among the target genes of Wnt signaling are several genes that are known to have a function in the vasculature and angiogenesis.

Some of the matrix metalloproteinases (MMPs) that are thought to play a role in angiogenesis are Wnt targets. By degrading matrix molecules, MMPs help to loosen periendothelial support that allows endothelial cells to emigrate from their original site to form new vessels (Carmeliet, 2000; Davis & Senger, 2005). MMP-7 was shown to induce proliferation of endothelial cells in vitro. In vascular smooth muscle cells, fibronectin, an extracellular matrix protein, could be found to promote proliferation. Cyclin D1 is a cell cycle factor that responded to Wnt signaling in vascular smooth muscle cells. C-myc and cyclooxygenase-2(Cox-2), other targets of Wnt signaling, are able to induce endothelial cell migration and angiogenesis in vivo (Goodwin & D´Amore, 2002).

Most prominently among the Wnt target genes involved in angiogenesis is *VEGF*. *VEGF* could be shown to be up-regulated by Wnt signaling/ß-catenin in colon cancer cells and skeletal myocytes (Zhang et al., 2001; Easwaran et al., 2003; Kim et al., 2006). VEGF is known to play a crucial role in endothelial cell proliferation, migration and survival as well as in physiological and pathological angiogenesis (Ferrara et al., 2003).

2.6. Wnt signaling in cardiovascular disease and the vasculature

Recently, a role of Wnt/ß-catenin signaling in cardiovascular diseases has been emphasized. Components and inhibitors of the Wnt signaling pathway have been shown to be involved in wound healing after myocardial infarction (e.g. Fz2, Dvl1, ß-catenin, FrzA/sFRP1) (van Gijn et al., 2002; van Gijn et al., 1997; Blankesteijn et al., 1999; Blankesteijn et al., 1997; Chen et al., 2004; Barandon et al., 2003), in cardiac hypertrophy (e.g. GSK-3ß, ß-catenin)(Hardt & Sadoshima, 2002; Haq et al., 2000; Masuelli et al., 2003), heart failure (e.g. sFRPs) (Schumann et al., 2000) and arterial injury (e.g. FrzB, Fz1, Fz2) (Mao et al., 2000) .

Similarly, in the vasculature, many components of the Wnt signaling pathway are found. In vitro, vascular cells like endothelial cells and smooth muscle cells express several Wnts and Fzs. Additionally, it could be shown that endothelial cells proliferate when stimulated by canonical Wnts, whereas noncanonical Wnts have no such effect (Wright et al., 1999; Goodwin & D´Amore, 2002).

There is also evidence for a role of Wnt/ß-catenin signaling in angiogenesis. ß-catenin is the center of the canonical Wnt signaling cascade and is thought to have a dual role. Firstly, ß-catenin controls gene transcription and cell proliferation. Secondly, ß-catenin participates in the regulation of cell adhesion by connecting cadherins through α-catenin to the actin cytoskeleton (Nelson & Nusse, 2004). These two major features of ß-catenin link it to angiogenesis since angiogenesis depends on proliferation and migration of endothelial cells, which in turn requires the disruption of cell-cell contacts. These considerations concerning the role of ß-catenin in angiogenesis are supported in recent studies. GSK-3ß and the GSK-3ß/ß-catenin axis were shown to regulate angiogenesis in endothelial cells. ß-catenin promoted angiogenesis in endothelial cells by activating VEGF (Kim et al., 2002; Skurk et al., 2005).

In early angiogenesis, endothelial cell contacts need to loosen in order to allow the cells to migrate into newly formed vessels. In endothelial adhesion, vascular endothelial (VE)-cadherin at adherens junctions is connected to the actin cytoskeleton through ß-catenin (among other factors) (Liebner et al., 2006). VEGF is able to induce tyrosine phosphorylation of ß-catenin in endothelial cells which is accompanied by reorganization of adherens junctions that contain ß-catenin and endothelial barrier dysfunction. It could be shown that 3 hrs after induction of angiogenesis by VEGF, ß-catenin and vascular endothelial (VE)-cadherin were lost from regions of endothelial cell-cell contacts consistent with loosened cell contacts (Cohen et al., 1999; Wright et al., 2002). These observations show a close link between ß-catenin and VEGF.

On one hand, *VEGF* is a target gene of Wnt/ß-catenin signaling and on the other hand ß-catenin in cell adhesion sites is regulated by VEGF mediated phosphorylation in endothelial cells.

In vivo, ß-catenin could also be shown to be involved in angiogenesis. Accumulation of cytosolic and nuclear ß-catenin was observed in proliferating vessels of rat N-ethyl-N-nitrosurea-induced gliomas, in proliferating vascular cells of glioblastoma multiforme, medulloblastoma and other central nervous system tumors (Yano et al., 2000a; Yano et al., 2000b; Eberhart et al., 2000). In contrast to that, adult vessels, i.e. quiescent vessels, show no significant levels of cytosolic and nuclear ß-catenin (Goodwin & D´Amore, 2002).

An animal model of hind limb ischemia revealed an increase of ß-catenin expression in the ischemic tissue. Overexpression of ß-catenin in the ischemic tissue led to increased angiogenesis and restoration of blood flow (Kim et al., 2006). During neovascularization after myocardial infarction, ß-catenin and APC are found in the cytoplasm of vascular endothelial cells in newly formed vessels. Dvl-1 expression is also observed in endothelial cells of larger arteries in the infarcted area implying a role of Wnt/ß-catenin signaling in neovascularization (Blankesteijn et al., 2000).

2.7. Wnt antagonism and angiogenesis

There is evidence that positive Wnt/ß-catenin signaling can promote angiogenesis. In contrast to that, inhibition of Wnt signaling is thought to be associated with vessel stability or vessel regression (Goodwin and D´Amore, 2002). However, the role of Wnt inhibition in angiogenesis and cardiovascular disease is not yet clear. Nevertheless, there are clues that point to a role of Dkk-1 in the vasculature.

Maintenance and formation of vessels depend on a close interplay between endothelial survival and apoptosis. Activated endothelial cells proliferate and migrate to form new vessels. After the

formation of new vessels is completed, endothelial cells become quiescent and survive for a long time. One of the major factors in endothelial survival is VEGF. It has been shown that endothelial survival, mediated by VEGF, is linked to the interaction of VEGF-receptor2 (VEGFR2), ß-catenin and VE-cadherin. Endothelial survival is in contrast to endothelial apoptosis, which occurs in vascular regression (Carmeliet, 2000; Carmeliet et al., 1999; Dimmeler & Zeiher, 2000). Dkk-1 was shown to possess pro-apoptotic properties and a tumor suppressing function in tumor cell lines (Wang et al., 2000; Shou et al., 2002). Wnt signaling is not only implicated in endothelial cell proliferation required in angiogenesis, but also in neoplastic cell proliferation. Wnts themselves have been described to be proto-oncogenes. Mis-regulation of Wnt signaling and mutations of distinct Wnt components lead to cancer (Polakis, 2000; Peifer & Polakis, 2000). It could be demonstrated that Wnt1 promoted cell survival and mediated anti-apoptotic signaling through ß-catenin activation (Chen et al., 2001). Wnt-induced cell proliferation could be shown to be inhibited by Dkk-1 (Fedi et al., 1999).

In the induction of heart formation in vertebrates, Wnt antagonists such as Dkk-1 and Crescent have an important role (Schneider & Mercola, 2001; Marvin et al., 2001; Harvey, 2002; Olson, 2001). Interestingly, Wnt/ß-catenin signaling was involved in proliferation of endocardial cells that represent a special endothelial layer of the inner heart. Wnt signaling participated in the formation of endocardial cushions and helped regulating cardiac valve formation. In these experiments, Dkk-1 was able to block endocardial cushion formation pointing to a possible role for Dkk-1 in blocking endothelial cell proliferation (Hurlstone et al., 2003). In mouse embryogenesis, Dkk-1 is spatially expressed in the heart (bulbis cordis, endocardium, septum transversum, endocardial cushion) and big vessels such as aorta (Monaghan et al., 1999). There are no accounts, however, concerning the expression of Dkk-1 in adult vessels and its role in angiogenesis.

2.8. Project of the thesis

A growing body of evidence supports the hypothesis that active canonical Wnt signaling is one of the pathways promoting angiogenesis. In recent work, it has been emphasized that ß-catenin, the central component of canonical Wnt signaling, plays an important role in promoting endothelial cell proliferation, endothelial cell migration and angiogenesis. In contrast, inhibition of Wnt signaling is thought to be associated with vessel stability and vessel regression. Dickkopf-1 (Dkk-1) is a well-characterized and potent inhibitor of Wnt signaling which is thought to specifically antagonize canonical Wnt signaling. However, there is little known about Dkk-1 and its role in the adult vasculature and endothelial cells. In the present work, we addressed the following questions:

1) What is the role of Dkk-1 in endothelial cells?

   a) Is Dkk-1 expressed in progenitor and mature endothelial cells?

   b) Does the expression of Dkk-1 change when cells are activated or when cells are challenged with a strong angiogenic stimulus such as hypoxia?

   c) Does Dkk-1 overexpression in endothelial cells have an impact on the expression of factors associated with promotion of angiogenesis such as ß-catenin and VEGF?

2) Is Dkk-1 expressed in vessels in vivo and, is it found in the endothelium?

3) What expression pattern of Dkk-1 is observed in a model of ischemic stroke in mice especially in the penumbra? Is ß-catenin expressed in the penumbra parallel to the observations made in myocardial infarction (Blankesteijn et al., 2000)?

3. Materials and Methods

3.1. Materials

3.1.1. Instruments

Item	Company, Type
Bacterial incubator	Heraeus, B 6200
Bacterial shaker	New Brunswick Scientific, innova 4330
Cell counting chamber	GLW
Cell culture incubator	Forma Scientific, CO_2 water jacketed incubator series II
Culture Hood	Clean Air
Digital camera	Zeiss Axiovert 200M digital camera, Carl Zeiss
Electrophoresis power supply	GibcoBRL PS 305, GIBCO invitrogen
Electrophoresis unit	Wide Mini-Sub Cell RT electrophoresis system, BIO-RAD
Fine scale	SBC 21, Scale Tec
Freezers	-20 °C (Liebherr); -80 °C (Revco)
Heat block	Thermomixer comfort 1.5 ml, eppendorf
Mega centrifuge	J-6B, Beckman; Megafuge 1.0, Heraeus; RC 5B plus, Sorval
Microscope	Eclipse TE300, Nikon; Carl Zeiss
Microscope slides and cover glasses	SuperFrost Plus, Menzel and microscope cover glasses, Menzel
Mini centrifuge	Mikro 20, Hettich
Parafilm M	American National Can

Pap Pen (for immunostaining)	SCI Science Services
pH meter	Microprocessor, WTW
Photo software	Openlab, Photoshop
Pipettes	2 µl, 20 µl, 200 µl, 1000 µl pipetman, Gilson; Multipette plus, Eppendorf; Pipetboy acu, IBS Integra Biosciences
Pipette tips	disposable graduated filter tips, DNase, RNase, pyrogen free, starlab; Combitips plus 0,2 ml, Eppendorf
Plastic pipettes	2ml, 5ml, 25ml, 50ml stripette, Corning
Polaroid camera	Mitsubishi electric
Refrigerator	4°C (Liebherr)
Shakers	Unimax 2010, Heidolph
Scale	BP2100S, BP310S, Sartorius
Software for primer selection	MacVector
Spectrophotometer	Biophotometer 6131, eppendorf
Thermal cycler	PTC-100 Peltier, MJ Research
Tissue cutter	Jung Frigocut 2800E Cryostat, Leica
Tubes	Blue Max 50 ml Polypropylene Tube, Falcon; 14 ml Polypropylene Round-Bottom Tube 17 x 100 mm style Nonpyrogenic, Falcon; Thermo Tube TM PCR Tubes 0.2 ml; peqlab biotechnologie; 1.5 ml safe lock tubes, eppendorf
Vortex	JK MS2 Minishaker, IKA
Water bath	C20CS edition2000, Lauda

3.1.2. Reagents and general materials

Items	Company
1 kb DNA ladder	Invitrogen
100 bp DNA ladder	New England Biolabs
2-mercaptoethanol	Sigma
Acetone	Merck
Agarose, electrophoresis grade	invitrogen
all-trans retinoic acid	Sigma
Bacto-Agar	Roth
Bacto-tryptone	DIFCO
BamHI (20 000U/ml)	New England Biolabs
Boric acid	Sigma
Bovine serum albumin (BSA)	ICN Biomedicals
Bromphenolblue	Sigma
BSA purified (100 x; 10mg/ml; for restriction digest)	New England Biolabs
Calciumchloride ($CaCl_2$)	Sigma
dibutyryl cyclic AMP	Sigma
Dimethylsulfoxide (DMSO)	Sigma
DNase (from RNeasy Mini Kit)	Qiagen
dNTP (20 mM; 500µl)	Amersham
EcoRI buffer	New England Biolabs
EcoRI (20 000U/ml)	New England Biolabs

Ethylenediaminetetraacetic acid (EDTA)	Sigma
Ethanol	Merck
Ethidiumbromide	Merck
Gelatine (porcine skin)	Sigma
Glycerine	Roth
Isopropanol	Merck
Kanamycin	Sigma
Ligase buffer for T4 Ligase	New England Biolabs
Magnesiumchloride ($MgCl_2$)	Merck
Mo-MLV Reverse Transcriptase (200U/µl)	GIBCO Invitrogen
Oligo (dt)15 Primer 20µg 500µg/ml	Promega
Potassiumacetate	Merck
Potassiumchloride (KCl)	Sigma
Potassiumdihydrophosphate (KH_2PO_4)	Merck
RNase A (from EndoFree Plasmid Maxi Kit)	Qiagen
Rnase	Roche
Rnasin RNase inhibitor (2500U; 40U/µl)	Promega
Saccharose	Sigma
Sodiumchloride (NaCl)	Sigma

Sodiumdihydrophosphate (NaH_2PO_4)	Merck
Sodiumdodecylsulphate (SDS)	Merck
Sodiumhydroxide (NaOH)	Sigma
T4 DNA Ligase	New England Biolabs
Taq DNA Polymerase (5 U/µl)	Promega
TissueTek tissue embedding medium	Sakura Finetek
Tris-(hydroxymethyl)-aminomethane (Tris)	Roth
Trypsin-EDTA	GIBCO Invitrogen
Tween 20	Merck
Vectashield mounting medium	Vector laboratories
Vent DNA Polymerase (2000U/ml)	New England BioLabs
Xylencyanol	Sigma
Yeast extract	Life Technologies

3.1.3. Cell lines and bacteria strains

Cell line	**Reference**
HUVECs	Human umbilical vein endothelial cells, Cambrex
eEPCs	Mouse embryonic endothelial progenitor cells, Hatzopoulos et al., 1998
DH5α	E.coli bacterial cells, GIBCO Invitrogen

3.1.4. Cell culture media

3.1.4.1 eEPC medium

DMEM EPC day 7.5 Medium (end volume 500ml)

Medium contained 77% (385 ml) of DULBECCO´S MEM with 25 mM HEPES, 20% (100 ml) of Fetal Bovine Serum, 3.5 µl per 500 ml of 2-mercaptoethanol, 1% (5 ml) of 200 mM L-glutamine (100x), 1% (5 ml) of Penicillin/Streptomycin (10.000 units/ml Penicillin, 10 mg/ml Streptomycin) and 1% (5 ml) of non-essential amino acids MEM.

3.1.4.2 HUVECs medium

For HUVECs EBM®-2 (Cambrex) was used (end volume 500ml). Medium contains basal medium for human endothelial cells without bovine brain extract (BBE) and a final FBS (fetal bovine serum) concentration of 2%. The medium further contains 0.04% Hydrocortisone, 0.4% hFGF, 0.1% hEGF, 0.1% GA-1000 (Gentamicin, Amphotericin-B), 0.1% VEGF, 0.1% R^3-IGF-1, 0.1% Ascorbic Acid, 0.1% Heparin (all purchased from Cambrex).

3.1.4.3 LB (Luria-Bertani) medium

1% Bacto-tryptone

1% NaCl

0.5% Bacto-yeast extract

10 g Bacto-tryptone, 5 g Bacto-yeast extract and 10 g NaCl were dissolved in 800 ml distilled water. pH was adjusted to 7.0 with NaOH and volume was adjusted to 1 liter with distilled water. To sterilize, autoclaving was performed. For LB plates, 15 g Bacto-agar was added.

3.1.5. Cell culture materials

Item	Company
Lipofectamine 2000 TM	Life Technologies, Inc.,
Cell culture dishes	NUNC
Cell scraper	Sarstedt
DMEM with 25mM HEPES	GIBCO Invitrogen
Fetal bovine serum (FBS)	GIBCO Invitrogen
L-glutamine	GIBCO Invitrogen
Non-essential amino acids MEM	GIBCO Invitrogen
Penicillin/Streptomycin	GIBCO Invitrogen
EBM-2 BulletKit	Cambrex

3.1.6. Primary antibodies

Anti-Dkk-1 antibody

Anti-human DKK-1 Antibody 0.1 mg/ml; human specific goat IgG; R&D systems

Anti-ß-catenin antibody

Anti-ß-catenin developed in Rabbit Delipidized, whole Antiserum; 56 mg/ml; Sigma

Anti-neuronal nuclei (NeuN) antibody

Mouse Anti-neuronal nuclei (NeuN) monoclonal antibody 1 mg/ml reacts with human, mouse, rat, ferret, chick and salamander; Chemikon

Anti-glial fibrillary acidic protein (GFAP) antibody

Mouse Ant-glial fibrillary acidic protein (GFAP) monoclonal antibody reacts with human, pig and rat; Chemikon

Anti-CD31 (PECAM-1) antibody

Purified rat anti-mouse CD31 (PECAM-1) Monoclonal antibody; BD Pharmingen

Anti-actin antibody

Actin, Smooth Muscle Specific (ab-2), mouse monoclonal antibody; Oncogene

3.1.7. Secondary antibodies

Anti -rat

Cy TM-conjugated AffiniPure Donkey Anti-Rat IgG (H+L) 1.5 mg/ml; Dianova

Fluorescein (FITC)-conjugated AffiniPure Donkey Anti-Rat IgG (H+L) 1.5 mg/ml; Dianova

Alexa Fluor® 488 goat anti-rat IgG (H+L) 2 mg/ml; Molecular Probes MoBiTec

Anti-goat

Cy TM-conjugated AffiniPure Donkey Anti-Goat IgG (H+L) 1.5 mg/ml; Dianova

Fluorescein (FITC)-conjugated AffiniPure Donkey Anti-Goat IgG (H+L) 1.5 mg/ml; Dianova

Alexa Fluor® 568 donkey anti-goat IgG (H+L) 2 mg/ml; Molecular Probes MoBiTec

Anti-rabbit

Cy TM-conjugated AffiniPure Donkey Anti-Rabbit IgG (H+L) 1.5 mg/ml; Dianova

Fluorescein (FITC)-conjugated AffiniPure Goat Anti-Rabbit IgG (H+L) 1.5 mg/ml; Dianova

Alexa Fluor® 488 goat anti-rabbit IgG (H+L) 2 mg/ml; Molecular Probes MoBiTec

Alexa Fluor® 568 goat anti-rabbit IgG (H+L) 2 mg/ml; Molecular Probes MoBiTec

Anti-mouse

Fluorescein (FITC)-conjugated AffiniPure Donkey Anti-Mouse IgG, Fc fragment specific 1.4 mg/ml; Dianova

Alexa Fluor® 488 goat anti-mouse IgG (H+L) 2 mg/ml; Molecular Probes MoBiTec

3.1.8. RT-PCR Primer

Mouse primer

Dkk-1	CAACTACCAGCCCTACCCTTGCG (forward 5´to 3´)
	CAGACGGAGCCTTCTTGTCCTTTG (reverse 3`to 5`)
ß-catenin	CGAAGGGTGTACTGGAGCTCTC
	GTCCAGTCCAAGATCTGCAGTCTC
Vegf	GGATCCATGAACTTTCTGCT
	GGGTGCACTGGACCCTGGCT

ß-actin	CTACGAGGGCTATGCTCTCCC
	CCGGACTCATCGTACTCCTGC

Human primer

DKK-1	AGACCATTGACAACTACCAGCCGTA
	GTTCTTCTGGAATACCCATCCAAGG
ß-catenin	GCTTAGCTGAGCTTAGATGATAG
	TGATGATTGCTCATCATGATAGTA
VEGF	CAAGTGGTCCCAGGCTGCACCC
	CCCTGAGGAGGCTCCTTCCTGCC
GAPDH	AGAACATCATCCCTGCCTCTACTG
	TGTCGCTGTTGAAGTCAGAGGAGA
LRP 6	ATCATGTCATGCTACTCATCGAT
	GCATACTGCTATGGCTATCTGC

Human/Mouse primer

Aldolase	AGCTGTCTGACATCGCTCACCG
	CACATACTGGCAGCGCTTCAAG

All primers were synthesized by MWG-Biotech.

3.1.9. Cloning primers for human Dkk-1

CGGGATCcttctgagatgatggctctgggcgc	forward primer + BamHI
GGAATTCggatagctggtttagtgtctctgac	reverse primer + EcoRI

(Restriction sites are indicated in capital letters)

Cloning primers were synthesized by MWG-Biotech.

3.1.10. Kits

RNeasy® Mini Kit (for RNA isolation)	Qiagen
RNase-Free DNase Set	Qiagen
QIAquick® PCR purification Kit	Qiagen
Qiagen®EndoFree Plasmid Maxi Kit	Qiagen
M.O.M kit TM for detecting mouse primary antibodies on mouse tissue	Vector Laboratories, Inc.

3.1.11. Plasmids

pBK-CMV phagemid 4518bp	Stratagene
pBK-CMV-DKK-1	created in this work

3.1.12. Solutions

Blocking buffer (for antibody staining)

1.5% BSA in 1x PBS

Buffer NX 10x (for reverse transcription)

2M KCl,

1M Tris-Cl pH 8.4,

1M $MgCl_2$,

3% Tween 20

Buffer 10x (for RT-PCR)

0.1 M Tris-Cl pH 8.4,

0.5 M KCl,

15 mM $MgCl_2$

10 x DNA gel loading buffer

40% (w/v) saccharose

0.25% bromphenolblue

0.25% xylencyanol

Phosphate-Buffered Saline (PBS) 20x

136 mM NaCl,

2.6 mM KCl,

10 mM NaH_2PO_4,

1.5 mM KH_2PO_4, pH 7.4

10x Tris-Borate-EDTA (TBE)

108 g Tris base

55 g boric acid

20 ml 0.5 M EDTA, pH 8.0

Volume was adjusted to 1l with H_2O.

Buffer P1 (resuspension buffer)

50 mM Tris-Cl, pH 8.0

10 mM EDTA

100 µg/ml RNase A

Buffer P2 (lysis buffer)

200 mM NaOH

1% SDS (w/v)

Buffer P3 (neutralization buffer)

3.0 M potassium acetate, pH 5.5

Buffers included in Qiagen Kits

RLT	included in RNeasy Mini Kit (Qiagen)
RW1	included in RNeasy Mini Kit (Qiagen)
RPE	included in RNeasy Mini Kit (Qiagen)

RDD	included in RNase-Free DNase Set (Qiagen)
ER	included in EndoFree Plasmid Maxi Kit (Qiagen)
QBT	included in EndoFree Plasmid Maxi Kit (Qiagen)
QC	included in EndoFree Plasmid Maxi Kit (Qiagen)
QN	included in EndoFree Plasmid Maxi Kit (Qiagen)
TE	included in EndoFree Plasmid Maxi Kit (Qiagen)
PB	included in QIAquick PCR purification Kit (Qiagen)
PE	included in QIAquick PCR purification Kit (Qiagen)
EB	included in QIAquick PCR purification Kit (Qiagen)

3.2. Methods

3.2.1. Cell culture and bacterial cultures

3.2.1.1 Maintenance of cells in culture

All cells were incubated and maintained at 37°C, 5% CO_2 and 21% O_2. Culturing of the cells was carried out on NUNCLON plates that were 6 cm or 10 cm in diameter. For better attachment of eEPCs to the plates, the plates were incubated with 4 ml or 12 ml of 0.1 % gelatin for at least 20 minutes at 37°C before use.

Embryonic EPCs and HUVECs were maintained in DMEM EPC day 7.5 Medium and HUVECs medium (EBM-2), respectively. When the cell layer on a plate reached about 90% confluency, the cells were passaged in a ratio appropriate for each cell type. Tissue culture work was performed in a sterile tissue culture bank. All materials used in the culture bank were sterilized with 70% ethanol or UV light for 15 minutes to avoid contamination.

3.2.1.2 Passaging of cells

To split cells that had reached about 90% confluency, the cell layer was washed with 6 ml 1x PBS. The cells were then incubated 5-7 minutes with 1x Trypsin-EDTA to detach them from the plates. Treatment with Trypsin was stopped with 5 ml Medium. The cell suspension was transferred from the plates to a sterile tube and then spun at 1200 rpm for 5 minutes. After spinning, the supernatant was removed and cells were resuspended in fresh medium and placed in new plates.

3.2.1.3 Differentiation of eEPCs in vitro by cAMP and retinoic acid

eEPCs maintained in culture as described above, were differentiated by adding 1 µM all-trans retinoic acid (RA) and 0.5 mM dibutyryl cyclic AMP (cAMP). eEPCs were treated with RA and cAMP for 4 days. The changes in cell morphology were monitored by microscopy.

3.2.1.4 Stimulation of eEPCs and HUVECs by hypoxia

eEPCs and HUVECs were cultured under normoxic (21% O_2) and hypoxic (2% O_2) conditions as described before (Wei et al., 2004). Successful stimulation of eEPCs and HUVECs was monitored by an induction of VEGF expression. VEGF mRNA was detected by RT-PCR using Aldolase as a control.

3.2.1.5 Lipofectamine transfection

For transfection of HUVECs, cells in passage 4 or 5 with a confluency of 80 – 90% were used. HUVECs were grown in their normal growth medium containing EBM-2 basal medium, growth factors and gentamycin (see above). For transfection, a special medium containing EBM-2, growth factors, but no serum and antibiotics, was prepared. For each culture dish with HUVECs to be transfected, 8 µg of DNA was diluted in 1.5 ml serum and antibiotic free EBM-2 medium. 16 µl of lipofectamine was diluted in 1.5 ml of serum and antibiotic free EBM-2 medium and added to the diluted DNA. Incubation was allowed for 30 minutes at room temperature to form DNA-lipofectamine complexes for transfection. The DNA-lipofectamine mixture was then added to the cells. Cells were incubated at 37 °C for 3 hrs and transfection was then stopped by adding normal growth medium to the plates. Cells were lysed 24 hrs after the start of transfection for molecular analysis.

3.2.1.6 Lysis of cells

Cells growing in monolayers on cell-culture plates were lysed directly in the culture dishes. Medium was removed and the cells were washed twice with 1x PBS. Depending on the cell number, RLT Lysis buffer plus 2-Mercaptoethanol was added to the plates and incubated for 5 minutes (5×10^6 cells in 6 cm plates 350 µl RLT; 5×10^6 – 1×10^7 cells in 10 cm plates, 600 µl RLT). Cell lysate was collected using a rubber police-man and transferred to 1.5 ml tubes. At this point lysates could be kept at -20 °C frozen for storage.

3.2.1.7 Competent bacteria

Highly competent E.coli are needed for transforming ligation products. In E. coli, treatment with ice-cold $CaCl_2$ creates a transient state of "competency", during which, they can take up DNA.

From a single colony of E. coli (DH5α) on a petri dish an overnight preculture was started in 2 ml LB media by incubating the culture at 37°C and shaking for oxygenation. On the next day, 1 ml of the preculture was inoculated in 100 ml fresh media and the culture was grown at 37°C until the OD at the wavelength of 600 nm reached 0.2 to 0.4. The culture was cooled on ice for at least 15 min. The following steps were carried out at 4°C in pre-cooled sterile tubes. Cells were spun for 5 minutes at 5000 rpm and supernatant was discarded. Bacterial pellets were carefully resuspended in a small volume of ice-cold 100 mM $CaCl_2$. The suspension was diluted with the $CaCl_2$ solution to a final volume of 30-40 ml and left on ice for 25 min with occasional shaking. Cells were spun down as before, supernatant was discarded and pellets were resuspended in 5 ml glycerol/$CaCl_2$. The resulting suspension was aliquoted in 100 to 400 µl and stored at –70°C.

3.2.1.8 Transformation of competent bacteria

100 µl of competent E. coli (DH5α) in 1.5 ml tubes were thawed on ice and mixed with approximately 40 ng of ligated DNA. This mixture was kept on ice for 30 minutes and then heat-shocked for 90 seconds at 42 °C in a thermomixer. The bacteria were put on ice for another 2 minutes and 900 µl of antibiotic-free LB-medium was added to the tubes. Bacteria cultures were then incubated at 37 °C and 1200 rpm in the thermomixer for 30 - 60 minutes. Tubes containing the bacteria cultures were centrifuged for 20 seconds and supernatant was

decanted. After resuspension of the pellet, bacteria were streaked on the plates. Selection of transformed bacteria was performed by using antibiotic containing (50 µg/ml kanamycin) agar plates. Only bacteria that have taken up the right plasmid containing the antibiotic kanamycin resistance cassette can grow and form colonies on kanamycin containing plates. Some colonies growing on the plates were picked for expansion in LB medium and subsequent DNA preparation.

3.2.2. RNA techniques

3.2.2.1 Total RNA isolation (RNeasy® method; RNeasy Mini Kit, Qiagen)

Lysates from cells (see 3.2.1.6) were transferred onto a QIAshredder spin column placed in a 2 ml collection tube and centrifuged for 2 minutes at maximum speed to be homogenized. One volume (350 µl or 600 µl, respectively) of 70% ethanol was added to the homogenized lysate and mixed well by pipetting to adjust the binding conditions. Up to 700 µl of the sample was transferred to the RNeasy spin column where the RNA could adsorb to the membrane. The spin column was placed in a 2 ml tube and centrifuged for 1 minute at 10 000 rpm. Flow-through was discarded. For washing 700 µl buffer RW1 was added to the column and centrifuged for another 1 minute at 10 000 rpm. To degrade genomic DNA, 80 µl of DNAse incubation mix (10 µl DNase I stock solution plus 70 µl RDD buffer) was applied directly onto the spin column membrane. Incubation with DNAse was allowed for approximately 15 minutes at room temperature. After treatment with DNAse, the column was washed with 350 µl buffer RW1 for 1 minute at 10 000 rpm and flow-through was discarded. The column was placed in a new 2 ml collection tube. 500 µl RPE buffer was applied to the column, the sample was centrifuged for 1 minute at maximum speed and the flow-through was discarded. To wash the silica-gel

membrane, 500 µl RPE buffer was applied to the column and centrifuged for 3 minutes at maximum speed. After discarding the flow-through, the column was again centrifuged for 2 minutes at full speed to remove residual liquid left from the last washing step. The RNeasy column was transferred into a new tube and 50 µl of RNase free water was pipetted directly onto the spin column membrane. After 10 minutes, the column was centrifuged at 10,000 rpm for 2 minutes to elute the RNA. RNA was immediately put on ice. For long term storage, isolated RNA was kept at -80°C.

3.2.2.2 Measurement of RNA concentration

RNA concentrations were measured by using a UV spectrophotometer at a wavelength of 260 nm. Absorption of 1.0 at 260 nm corresponds to ~40 µg/ml of RNA.

3.2.2.3 Reverse Transcriptase (RT) reaction

Reverse Transcription (RT) was used to synthesize cDNA for RT-PCR. Reverse transcriptases with RNA-dependent DNA polymerase activity synthesize complementary DNA (cDNA). Isolated RNA served as a template for Polymerase Chain Reactions (PCR).

For the RT-reaction, 3 µg of RNA was diluted in ddH_2O up to a total volume of 15 µl. 3.75 µl of oligo-dT primer was added to the tubes. In order to open up secondary structures of the RNA and to make poly A tails of mRNA accessible for binding to oligo-dT primers, the mixture was incubated at 65°C for 5 minutes. To avoid refolding of the RNA, tubes were immediately put on ice. To start the RT-reaction, 11.25 µl of RT-Mix was pipetted into the tubes and the mixture was incubated at 37°C for 55 minutes.

RT-Mix contained:

4.5 µl NX buffer

1.5 µl dNTPs

3.0 µl 2-mercaptoethanol

0.75 µl RNasin

1.5 µl Mo-MLV Reverse Transcriptase

11.25 µl

To stop the enzymatic reaction, the mixture was incubated at 95°C for 5 minutes. Finally, 270 µl of ddH_20 was added to the tubes. The final volume was 300 µl and cDNA had a concentration of approximately 10 ng/µl.

3.2.3. DNA techniques

3.2.3.1 Design of oligonucleotide primers for RT-PCR

RT-PCR primers for mouse and human *Dkk-1* were designed based on the corresponding mouse and human gene sequences, respectively (mouse *Dkk-1* Acc.no. AF 030433, www.ncbi.nlm.nih.gov, human *DKK-1* Acc.no. AF 127563, www.ncbi.nlm.nih.gov). For selecting RT-PCR primer pairs, the computer program MacVector was used. This software program helps to analyze the secondary structure (e.g. hairpins) and annealing temperatures of PCR primers. It was also used to compare the homology between the target DNA and sequenced DNA. Characteristics such as the isoelectric point (IP), molecular weight (MW) were also analyzed. Additionally, BLAST (Basic Local Alignment Search Tool) analysis of the different primer pairs using the NCBInr Database (National Center for Biotechnology

Information, www.ncbi.nlm.nih.gov/BLAST/) was carried out to ensure that the newly designed primer pairs were gene-specific.

Oligonucleotid primers were commercially synthesized and delivered in a lyophilised form. All primers were dissolved in sterile water to obtain a final concentration of 100 pmol/µl.

3.2.3.2 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Gene expression was determined by means of RT-PCR using gene-specific primers and cDNA as a template (analytical amplification). For the amplification of long DNA fragments for cloning purposes, the Vent DNA Polymerase with a high proof-reading cabability was used.

The Master Mix for amplification contained:

12.68 µl H_2O

1.0 µl DMSO

0.2 µl dNTP

2.0 µl 10x buffer

2.0 µl primer pair (each 5 µM)

0.12 µl DNA Polymerase

18.00 µl

18 µl of the Master Mix was added to 2 µl cDNA (10 ng/µl).

The PCR amplification was performed according to the following protocol by varying the annealing temperatures and the elongation times:

Step 1 Initial denaturation: 94°C for 1 minute

Step 2 Denaturation: 94°C for 30 seconds

Step 3 Annealing: 60-65°C for 1 minute

Step 4 Elongation: 72°C for 1 minute

Step 5 back to step 2

Step 6 Final elongation: 72°C for 5 minutes

Step 7 keep samples at 4° C

3.2.3.3 Agaraose gel electrophoresis of DNA

The separation of DNA fragments according to their size was performed by gel electrophoresis on 1.5% agarose gels. For the gel, 1.5 g agarose was added to 100 ml 1x TBE buffer. The mixture was boiled in the microwave to solubilize the agarose and then poured into the gel tray. The gel was left for 30 minutes to polymerize before it was used for electrophoresis. Electrophoresis was carried out in gel chambers in 1x TBE electrophoresis buffer. DNA gel loading buffer was added to the DNA samples and these were loaded onto the gel; a well was loaded with a 100 bp or 1kb ladder to determine the size of the DNA fragments. Gels were run at 60 – 90 V. After the separation of DNA fragments was completed, gels were stained in a 0.02% ethidium bromide solution for 10 minutes. The gels were photographed under UV light using a Polaroid camera.

3.2.4. Cloning into plasmid vectors

3.2.4.1 Construction of Dkk-1 gene expression vector

The full-length human *DKK-1* cDNA was amplified by PCR and cloned into the multiple cloning site (MCS) of the pBK-CMV vector. The primers were designed based on the human *DKK-1* sequence (NCBI Acc. no. AF 127563 www.ebi.ac.uk). The sense primer was designed to include the start codon and the antisense primer included the stop codon, respectively. Both primers contained a restriction enzyme recognition sequence, that was not present elsewhere in the gene, but was present in the MCS of the vector. To verify this, a restriction summary and

the vector map were compared (Restriction summary: The sequence manipulation suite www.bioinformatics.org/sms/). Oligonucleotide primers were commercially synthesized and dissolved in sterile water to obtain a 100 pmol/µl solution. Primers were tested by RT-PCR. PCR products were run on an electrophoresis gel and compared to a 1 kb ladder to ensure, that the PCR products had the correct sizes. PCR products were then digested with the appropriate restriction enzymes and thereafter purified using the QIAquick PCR purification Kit to remove enzymes and other agents. The insert was then ligated into the expression vector, which had been digested with the same enzymes as the PCR product, by using T4 DNA ligase. The products of ligation were then transformed in E. coli, which were plated on appropriate selection agar plates. Single transformed colonies were expanded in bacterial cultures for Mini-preparation. The resulting purified DNA was used for restriction digestion analysis. For sequencing of the plasmid and transfection into cells, Maxi-Preparation was performed, to obtain sufficient amounts of plasmid DNA.

3.2.4.2 Purification of DNA (QIAquick PCR purification kit)

During the process of purification, small molecules and proteins pass through a column while DNA adsorbes to the silica-membrane of the column. Finally, pure DNA is eluted with Tris buffer or H_2O. To 1 volume of the PCR sample 5 volumes of buffer PB were added. The sample was applied to the QIAquick spin column sitting in a 2 ml collection tube and centrifuged for 30-60 seconds at 13 000 rpm. Flow-through was discarded and 0.75 ml buffer PE was pipetted to the column and centrifuged for 30-60 seconds. Flow-through was again discarded and the column and collection tube were centrifuged for another 60 seconds to remove residual liquids. To elute the DNA, the column was placed in a new 1.5 ml microcentrifuge tube, 30 µl of buffer EB was pipetted onto the center of the QIAquick

membrane. The column was centrifuged for 60 seconds and flow through was not discarded. Another 30 µl of buffer EB was added to the center of the column and centrifuged for 60 seconds. Purified DNA was kept at -20°C.

3.2.4.3 Digestion of DNA with restriction endonucleases

Within double strand DNA, particular restriction endonucleases recognize and cleave usually 4-6 base pairs specific sequences creating, cohesive ends. The restriction digest was performed according to the manufacturer's recommendations for buffer systems and reaction conditions. 1U of enzyme is needed per 1 µg of DNA to complete digestion in one hour. To ensure complete digestion, usually 10 fold excess units were used. Incubation of the digestion mix was allowed for 5 hours at 37 °C. The completion of the reaction was monitored by agarose gel electrophoresis.

3.2.4.4 Cohesive-end ligation

This procedure was performed to ligate DNA inserts with a vector plasmid to create new plasmids. DNA fragments were prepared for ligation by restriction digest and following purification as described above. The ligation reaction was set up as follows:

50 ng vector DNA

X ng insert DNA

1 µl T4 DNA ligase

2 µl ligation buffer

ddH_20 to 20 µl

Vector and insert were added at a molar ratio of 1:10. The final volume of the ligation mix was 20 µl. Incubation was allowed for 2 hrs at 16 °C. Finally, the mixture was heated at 65°C for 10 minutes to inactivate the enzyme.

3.2.4.5 Mini-preparation of plasmid DNA

Small scale plasmid preparation was used to check if single bacterial colonies carried a certain plasmid. Overnight cultures were grown at 37 °C with shaking at 190 rpm in 2 ml of LB-medium containing 50 µg/ml of kanamycin. To start Mini-preparation and harvest cells, cultures were transferred to 1.5 ml tubes and centrifuged for 20 sec at 13 000 rpm. Supernatant was removed and 100 µl of buffer 1 (resupension buffer) was added to the tubes and mixed well. 100 µl of buffer 2 (lysis buffer) was pipetted into the tube, gently mixed and incubated for 5 minutes at room temperature. Next, 100 µl of buffer 3 (neutralization buffer) was added to the tube and mixed by gently shaking the tube. After incubating on ice for 3 minutes, tubes were centrifuged for 5 minutes at 13 000 rpm. Supernatant was transferred to new 1.5 ml tubes and mixed with 600 µl of ice cool 100% ethanol for precipitation. Tubes were kept at -80 °C for 10 minutes and then again centrifuged for 5 minutes at 13 000 rpm. Ethanol was removed from the tube avoiding the pellet. The pellet was air-dried for 10 minutes and then resuspended in 40 µl of ddH_2O.

3.2.4.6 Maxi-preparation of plasmid DNA

Maxi preparation of plasmid DNA was carried out with the endotoxin-free EndoFree Plasmid Maxi Kit (Qiagen). This special kit minimizes the amount of contaminating bacterial endotoxin during DNA isolation. Because endotoxin is known to affect eukaryotic cells and transfection

efficiency, removal of endotoxin is critical before DNA is introduced in cells. The general procedure of plasmid purification is based on a modified alkaline lysis method.

Overnight bacterial cultures were started from Mini-preparation cultures containing the plasmids. Cultures were grown in 50 ml LB-medium containing 50 µg/ml of kanamycin at 37°C with shaking at 190 rpm. Bacterial cells were pelleted at 6 000 rpm and 4 °C for 15 minutes. For lysis of the cells, the pellet was resuspended in 10 ml of buffer P1 (resuspension buffer). Buffer P2 (lysis buffer) (10 ml) was added and mixed by gently inverting the tube. Incubation of P2 was allowed for 5 minutes at room temperature. Meanwhile the QIAfilter cartridge was prepared. 10 ml of chilled (4 °C) buffer P3 (neutralization buffer) was added to the lysate and mixed gently. The lysate was poured into the QIAfilter cartridge and left for 10 minutes to allow precipitate to float to the top of the solution. Filtration was started by inserting the plunger into the cartridge. The cell lysate was filtered into a 50 ml collection tube. 2.5 ml of buffer ER was added to the filtrate, gently mixed and incubated on ice for 30 minutes. The filtered lysate was applied to a QIAGEN-tip 500 equilibrated with 10 ml QBT buffer. The lysate entered the resin by gravitiy flow. The QIAGEN-tip was washed twice with 30 ml of buffer QC. DNA was eluted with 15 ml of buffer QN. Following this, DNA was precipitated by applying 0.7 volumes (10.5 ml) of isopropanol to the eluted DNA and centrifuged at 4000 rpm for 30 minutes at 4 °C. The DNA pellet was washed twice with 70 %ethanol (96-100% ethanol in endotoxin-free water) for 10 minutes at 4 000 rpm. The supernatant was carefully removed and the pellet was air-dried for 5-10 minutes. The DNA pellet was dissolved in a suitable volume of buffer TE and DNA concentration was determined.

3.2.4.7 Measurement of DNA concentration

DNA concentrations were measured using a spectrophotometer at a wavelength of 260 nm. An absorption value of 1.0 corresponds to ~ 50 µg/ml of DNA.

3.2.4.8 Verification of plasmid DNA

Identity of the plasmid DNA was verified by restriction endonuclease digestion. If plasmid and insert were separated correctly by the restriction digest and had the right sizes in an analytical gel electrophoresis, plasmid DNA was sequenced. DNA sequencing was performed by Sequiserve, Vaterstetten, Germany.

3.2.5. Histology

3.2.5.1 Tissue sectioning

Mouse tissues were embedded in OCT Tissue Tek for cryopreservation. Embedded tissues were stored at –80°C for further work. Tissue sectioning was carried out with a JUNG FRIGOCUT 2800E Cryostat. All sections had a thickness of 10 µm. After sectioning, sections were air-dried for 30 minutes, fixed in ice cool acetone for 20 minutes at – 20 °C and again air dried for 1- 2 hrs at room temperature. Finally, sections were stored in dry boxes at - 20 °C for further work.

3.2.5.2 Immunofluorescence

To detect proteins in tissue sections, immunofluorescence was used. Immunofluorescence is based on the binding of a primary monoclonal or polyclonal antibody to its respective antigen. Secondary antibodies, conjugated with a fluorescence dye, bind to the primary antibodies. The staining of the sections is observed by fluorescence microscopy.

For antibody staining of cryopreserved tissue sections, sections were thawed at room temperature for 5 to 10 minutes and washed for 10 minutes in 1x PBS. Sections were surrounded with a PapPen and washed 5 times with 1x PBS for 10 min. Following this, sections were blocked for 1 hour with blocking buffer to ensure that no unspecific antibody binding to the tissue sections occurred. Primary antibodies in their respective dilutions were pipetted onto sections and incubated O/N at 4°C. The next day, sections were washed 5 times with 1x PBS and incubated with the secondary antibody for 1 hour at room temperature. In a last step, the sections were washed 5 times with 1x PBS, air-dried for 5 minutes and mounted with VECTASHIELD Mounting Medium.

For detection of mouse primary antibodies (NeuN, GFAP and Actin) on mouse tissues, sections were treated with M.O.M kit TM BASIC according to the manufacturer's recommendations.

3.2.5.3 Microscopy and fluorescence microscopy

For microscopy and fluorescence microscopy the NIKON Eclipse TE300 Microscope was used. The fluorescence colour was observed using a NIKON Super High Pressure Mercury Lamp at appropriate wavelengths through the appropriate filters. Sections were photographed using a Zeiss Axiovert 200M digital camera. Pictures were processed using photo software (openlab®, photoshop®).

4. Results

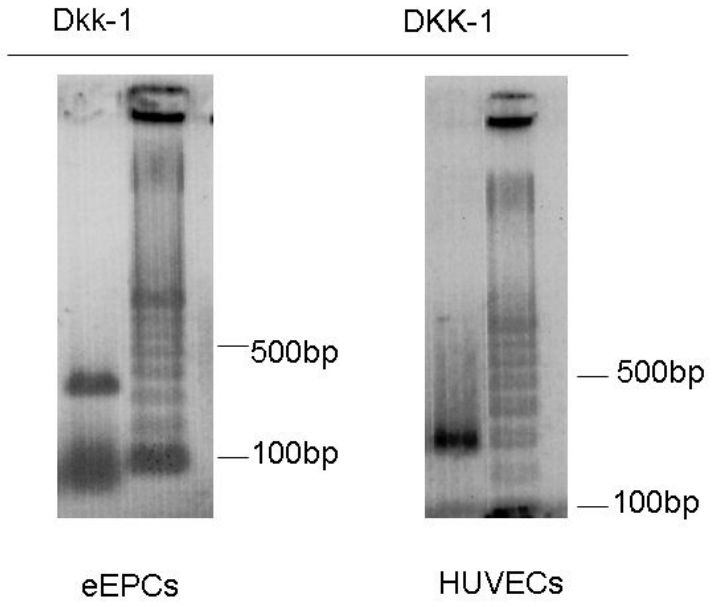
4.1. Expression of Dkk-1 and ß-catenin in endothelial cells in-vitro

To investigate the expression of *Dkk-1* in endothelial cells, two different cell lines, mouse embryonic endothelial progenitor cells (eEPCs) and human umbilical vein endothelial cells (HUVECs), were used. eEPCs represent immature endothelial progenitor cells, which have retained their ability to proliferate and give rise to functional progeny, whereas HUVECs are considered to be differentiated, mature endothelial cells. Both cell types were cultured according to standard procedures. For expression analysis, cells were lysed, RNA was extracted, purified and used as a template to generate cDNA by reverse transcription. The expression of *Dkk-1* and *ß-catenin* was then determined by RT-PCR. For that purpose, RT-PCR primers were designed for the mouse and the human *Dkk-1* and *ß-catenin* genes.

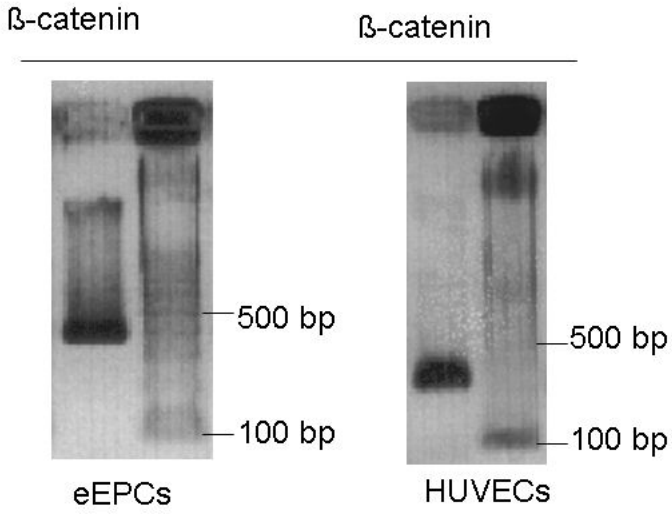
Fig. 4A shows that *Dkk-1* is strongly expressed in both eEPCs and HUVECs. The detected products from the RT-PCR correspond to the calculated size of 348 bp and 292 bp for the mouse and human primer products, respectively. It is further shown in Fig. 4B, that *ß-catenin* is also strongly expressed in eEPCs and HUVECs. The respective primer products of 387 bp and 312 bp for the mouse and human *ß-catenin* gene correspond to the estimated sizes.

The expression of *Dkk-1* in eEPCs was dependent on the time cells were kept in culture. As demonstrated by Fig. 5, there was an up-regulation of *Dkk-1* expression after 24 hrs of culturing in comparison to 6 hrs as cells begin to reach confluency.

If not indicated otherwise, cells were lysed after 24 hrs for molecular analysis.



A



B

Figure 4 (A) *Dkk-1* and (B) *ß-catenin* are strongly expressed in eEPCs and HUVECs. Gene expression was determined by RT-PCR analysis.

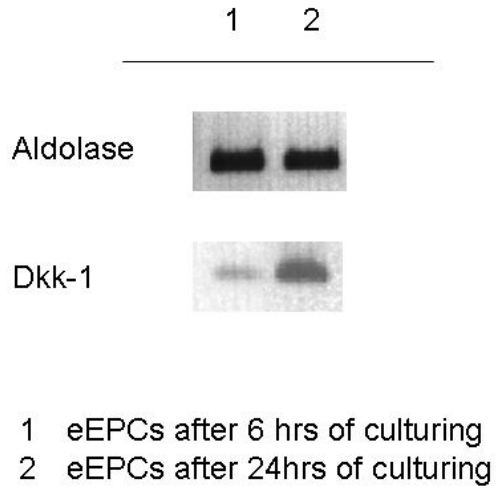


Figure 5 *Dkk-1* expression increases in a time-dependent manner in cultured eEPCs. RT-PCR analysis of *Dkk-1* after 6 and 24 hrs of culturing. Aldolase serves as a positive control to monitor comparable RNA amounts and quality among samples.

4.2. Expression of Dkk-1 in activated endothelial cells

It has been shown above, that *Dkk-1* is expressed in eEPCs and HUVECs. To investigate a possible role of *Dkk-1* in neovascularization processes, we used the model of endothelial progenitor cell differentiation via cAMP and cell culture under hypoxia as potent angiogenic stimuli. Our first model used the special properties that eEPCs show in-vitro. eEPCs, which were first isolated and characterized by Hatzopoulos and co-workers (1998), represent immature endothelial progenitors retaining their ability to differentiate into more mature cells in vitro when stimulated with cAMP plus retinoic acid (RA/cAMP). The differentiation under the influence of cAMP represents an activation process that leads to a more typical gene expression profile and morphology of endothelial cells (Hatzopoulos et al, 1998).

Therefore, we investigated the expression of *Dkk-1* in eEPCs activated by RA/cAMP and compared the expression levels with untreated eEPCs. The concentrations of the differentiation activators all-trans retinoic acid and cAMP (dibutyryl cAMP) were 1 µM and 0.5 mM, respectively, and the incubation time was 4 days. Fig. 6 shows the RT-PCR results for *Dkk-1*. It can be seen that *Dkk-1* was strongly down-regulated in activated eEPCs. RT-PCR analysis of the house-keeping gene Aldolase, which was used as a control, did not change upon stimulation by RA/cAMP.

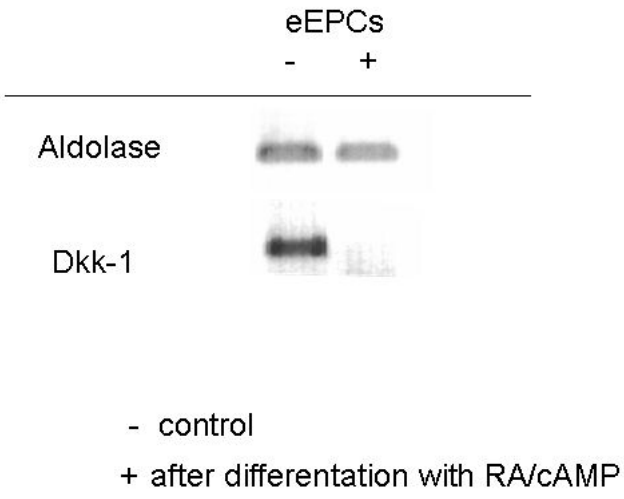


Figure 6 Treatment with RA/cAMP leads to eEPC activation and differentiation. Upon activation of eEPCs by RA/cAMP *Dkk-1* is down-regulated. RT-PCR analysis of *Dkk-1* in activated embryonic endothelial cells and untreated controls. Aldolase was used as a control.

Our second model used hypoxia as a strong angiogenic stimulus in eEPCs and HUVECs. Hypoxic conditions were induced as described previously (Wei et al., 2004) by incubating eEPCs and HUVECs in normal medium for 6 and 24 hrs in 2% O_2. eEPCs are known to participate in tumor neovascularization in hypoxic metastases, which is accompanied by an

increase of *VEGF* expression in recruited eEPCs and tumor cells (Wei et al., 2004). In the following experiments, cDNA of eEPCs, that had been exposed to hypoxia for 6 and 24 hrs were used for analysis of *Dkk-1* expression. eEPCs that had been cultured under normoxic (21% O_2) conditions for the same periods of time were used for comparison. Fig. 7 demonstrates that the expression of *Dkk-1* is down-regulated after 6 hrs and is completely suppressed after 24 hrs of hypoxia. Similar results were obtained in two other independent experiments. To verify that eEPCs were indeed activated by hypoxia, the expression of *VEGF* was investigated under the same conditions by RT-PCR analysis. As expected, Fig. 7 shows an up-regulation of *VEGF* expression in eEPCs submitted to hypoxia. As a control, the expression of the house-keeping gene *Aldolase* was determined, and Fig. 7 shows that the expression of *Aldolase* remained unchanged under hypoxic conditions.

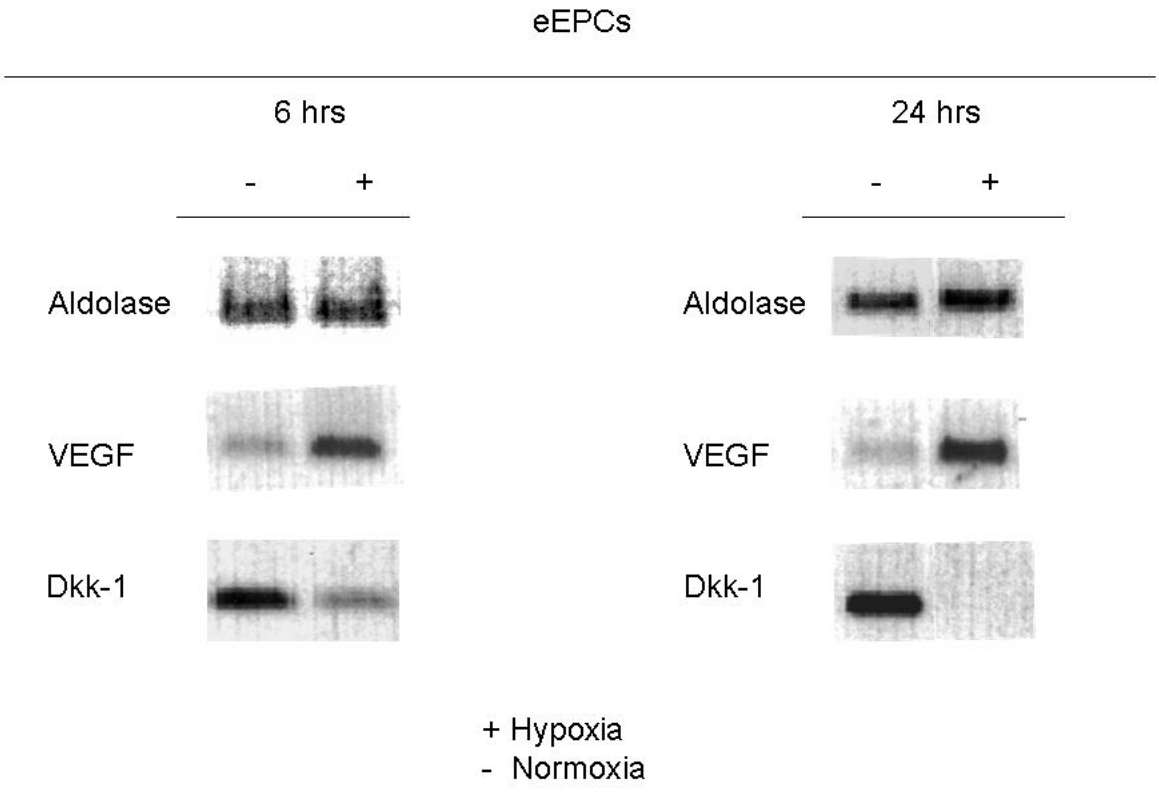


Figure 7 *Dkk-1* expression is down-regulated by hypoxia in eEPCs.
RT-PCR analysis of *Dkk-1* expression in eEPCs submitted to hypoxia for 6 hrs and 24 hrs respectively. *VEGF* was used as a positive control, *Aldolase* as a quality control.

Similar experiments as described above for eEPCs were carried out in HUVECs, which in contrast to eEPCs represent a fully mature endothelial cell line. In order to follow the time course of hypoxia-induced activation more closely, the expression of *DKK-1* and VEGF was determined after 2, 6, 18, 24 and 36 hrs. HUVECs that were cultured for the same periods of time under normoxic conditions served as controls. After the times indicated, non-activated and activated HUVECs were lysed, RNA was extracted and transcribed into cDNA by reverse transcription for RT-PCR analysis.

As shown before with eEPCs, up-regulation of *VEGF* in HUVECs confirmed the hypoxia-induced activation of this cell line (Fig.8). It is further shown in Fig.8, that the expression of *DKK-1* is suppressed by hypoxia, with the strongest inhibitory effect on *Dkk-1* expression after 18 hrs. This finding was confirmed in two other independent experiments. In these experiments, *GAPDH* served as the house-keeping gene, and Fig. 8 illustrates that the respective PCR products were not affected by hypoxia. It has been demonstrated by Skurk and coworkers (2005) that ß-catenin, a central component of the Wnt signaling pathway, is involved in the activation of *VEGF* expression in HUVECs. We therefore investigated, whether the enhanced expression of *VEGF* by hypoxia was also associated with an augmented *ß-catenin* expression. However, it is shown by the time-dependent RT-PCR analysis in Fig. 8, that there was no induction of *ß-catenin* expression after 2 to 36 hrs of hypoxia.

In summary, the results show that *Dkk-1* is expressed in endothelial cells in-vitro and is down-regulated in two models of endothelial cell activation.

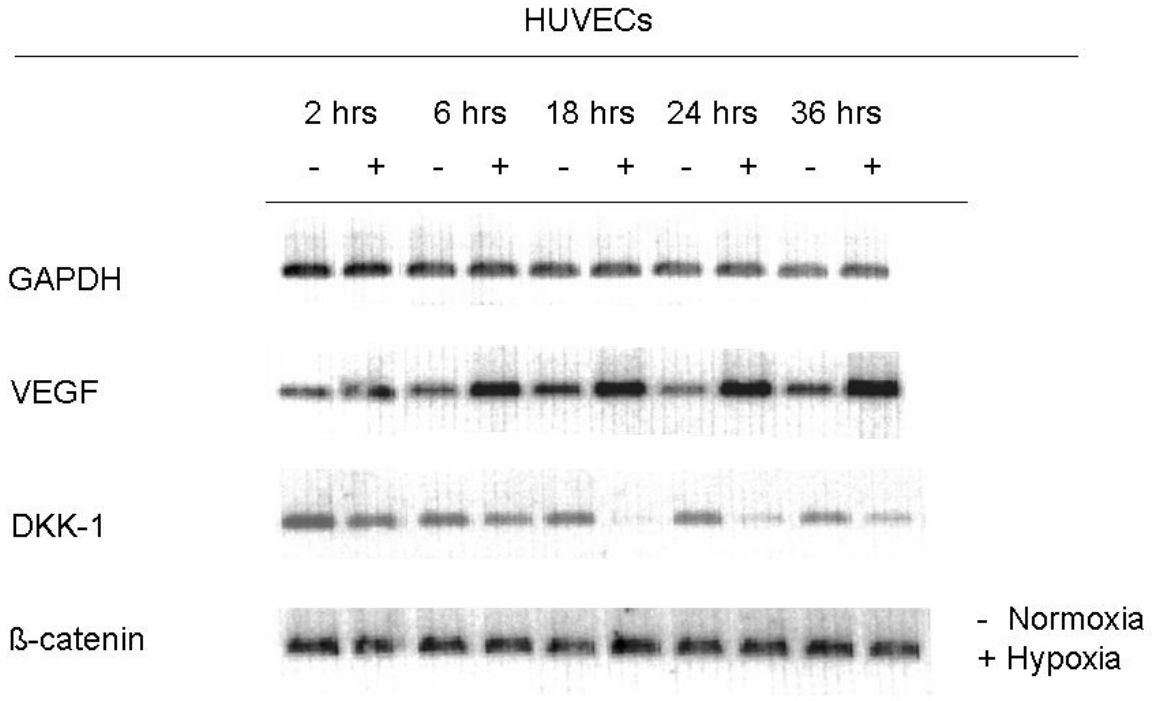


Figure 8 *Dkk-1* is down-regulated by hypoxia in HUVECs whereas *ß-catenin* expression remains unchanged. RT-PCR analyis of *Dkk-1* and *ß-catenin* expression in HUVECs stimulated by hypoxia for 2, 6, 18, 24 and 36 hrs. *VEGF* served as a positive control, *GAPDH* was used as a quality control.

4.3. Cloning of the Dkk-1 CMV construct

The results of Figs. 7 and 8 demonstrate that *Dkk-1* and *VEGF* show a reciprocal expression pattern upon activation by hypoxia. This prompted us to investigate whether *DKK-1* overexpression had an effect on *VEGF* expression and other components of the Wnt signaling pathway in endothelial cells.

To overexpress *DKK-1*, the gene was cloned into an expression vector carrying the strong CMV enhancer/promoter transcriptional elements. Cloning primers were designed that included the whole protein coding sequence of *DKK-1* (DKK-1 full length). A forward primer was chosen that included the start codon and a reverse primer that included the stop codon. In Fig. 9 the whole *DKK-1* sequence is shown. Forward primer, reverse primer, start codon and stop codon are highlighted.

Human Dickkopf-1

```
   1 ggccattacc aatcgcgaaa ccccgcaggg ggctcccgga ccctgactct gcagccgaac
  61 cggcacggtt tcctggggac ccaggcttgc aaagtgacgg tcattttctc cttctttctc
 121 cctcttgagt ccttctgaga tgatggctct gggcgcagcg ggagctaccc gggtctttgt
 181 cgcgatggta gcggcggctc tcggcggcca ccctctgctg ggagtgagcg ccaccttgaa
 241 ctcggttctc aattccaacg ctatcaagaa cctgccccca ccgctgggcg gcgctgcggg
 301 gcacccaggc tctgcagtca gcgccgcgcc gggaatcctg tacccgggcg ggaataagta
 361 ccagaccatt gacaactacc agccgtaccc gtgcgcagag gacgaggagt gcggcactga
 421 tgagtactgc gctagtccca cccgcggagg ggacgcgggc gtgcaaatct gtctcgcctg
 481 caggaagcgc cgaaaacgct gcatgcgtca cgctatgtgc tgccccggga attactgcaa
 541 aaatggaata tgtgtgtctt ctgatcaaaa tcatttccga ggagaaattg aggaaaccat
 601 cactgaaagc tttggtaatg atcatagcac cttggatggg tattccagaa gaaccacctt
 661 gtcttcaaaa atgtatcaca ccaaaggaca agaaggttct gtttgtctcc ggtcatcaga
 721 ctgtgcctca ggattgtgtt gtgctagaca cttctggtcc aagatctgta aacctgtcct
 781 gaaagaaggt caagtgtgta ccaagcatag gagaaaaggc tctcatggac tagaaatatt
 841 ccagcgttgt tactgtggag aaggtctgtc ttgccggata cagaaagatc accatcaagc
 901 cagtaattct tctaggcttc acacttgtca gagacactaa accagctatc caaatgcagt
 961 gaactccttt tatataatag atgctatgaa aacctttat gaccttcatc aactcaatcc
1021 taaggatata caagttctgt ggtttcagtt aagcattcca ataacacctt ccaaaaacct
1081 ggagtgtaag agctttgttt ctttatggaa ctcccctgtg attgcagtaa attactgtat
1141 tgtaaattct cagtgtggca cttacctgta aatgcaatga aacttttaat tatttttcta
1201 aaggtgctgc actgcctatt tttcctcttg ttatgtaaat ttttgtacac attgattgtt
1261 atcttgactg acaaatattc tatattgaac tgaagtaaat catttcagct tatagttctt
1321 aaaagcataa ccctttaccc catttaattc tagagtctag aacgcaagga tctcttggaa
1381 tgacaaatga taggtaccta aaatgtaaca tgaaaatact agcttatttt ctgaaatgta
1441 ctatcttaat gcttaaatta tatttccctt taggctgtga tagtttttga aataaaattt
1501 aacatttaat atcatgaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaa
```

Figure 9 Gene sequence of the human *DKK-1* gene (Acc. No. AF 127563; www.ncbi.nlm.nih.gov). Start and stop codon are highlighted in red. Forward and reverse primers include the start and the stop codon, respectively, to be able to clone the whole protein coding sequence of *DKK-1* (DKK-1 full length) into the plasmid vector. Primers are highlighted in grey.

To ligate the *DKK-1* gene product into the expression vector, the restriction sites for BamHI and EcoRI were added to the forward and reverse primers, respectively. The primers were then synthesized by MWG. After synthesis, the cloning primers were tested using cDNA prepared from HUVEC RNA. Fig. 10 shows the electrophoresis gel of the primer product which corresponds to the calculated product size of 796 bp.

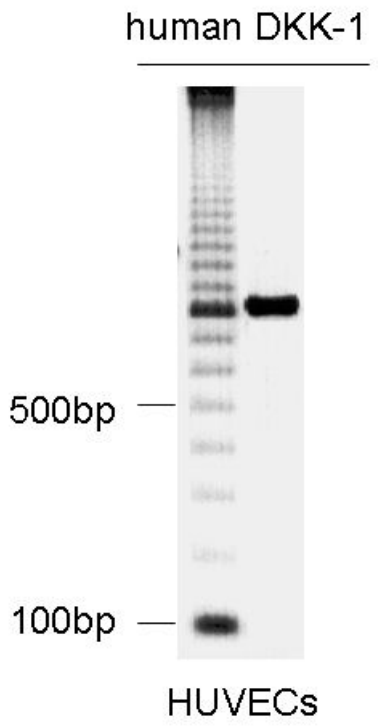


Figure 10 RT-PCR using the DKK-1 cloning primers in HUVECs. The PCR product (DKK-1 full length) matches the calculated product size of 796 bp.

Following amplification, several PCRs containing the DKK-1 full length product were pooled to increase the amount of DNA. This pooled DNA was purified, digested with the appropriate restriction enzymes (BamHI and EcoRI) and finally ligated into the CMV-expression vector (pBK-CMV, Stratagene). The ligation product was transformed into competent E. coli. The

transformed bacteria were transferred to LB-medium and amplified on culture plates containing kanamycin for selection. After Mini plasmid DNA preparation, plasmids were digested with EcoRI and BamHI and gel-electrophoresis was carried out. In two of eight clones from the Mini-preparation, the *DKK-1* insert could be detected with the correct size of 796 bp. From the Mini-preparation cultures that contained the correct inserts, Maxi- preparations were carried out to increase the yield of DNA. Commercial sequencing (Sequiserve, Vaterstetten) confirmed the correct sequence for *DKK-1* in the pBK-CMV-vector. Fig. 11 shows the DKK-1 construct (pBK-CMV-DKK-1).

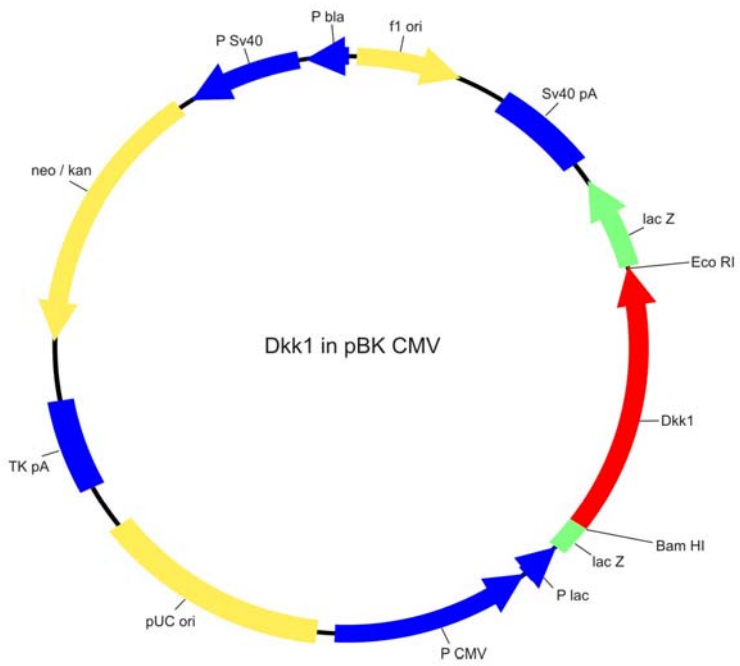


Figure 11 DKK-1 construct (pBk-CMV-DKK1)

4.4. Influence of DKK-1 overexpression in HUVECs on VEGF and components of Wnt signaling

HUVECs were transfected with the pBK-CMV-DKK-1 expression vector and the expression of *VEGF* (a target gene of Wnt signaling) and important components of the Wnt signaling pathway such as *LRP6, ß-catenin* and *DVL* was investigated.

To transfect HUVECs with the pBK-CMV-DKK-1 construct, three plates of HUVECs in culture (passage 4, 80-90% confluency) were prepared. One plate was left untransfected and the second was transfected with an empty pBK-CMV-vector as control. HUVECs on the third plate were transfected with 8µg/µl DNA of the pBK-CMV-DKK-1 construct. Transfection was carried out with lipofectamine. After 3hrs, the transfection was stopped by adding fresh medium. The cells were left overnight in culture and were lysed 24 hrs after stopping the transfection. Using an RNA isolation kit (Qiagen), the RNA was extracted from the cells and reversely transcribed into cDNA for RT-PCR analysis. As shown in Fig. 12, transfection with the DKK1-CMV-construct increased *DKK-1* expression in comparison to the control transfection with the empty pBK-CMV-vector. It is further shown, that overexpression of *Dkk-1* had no effect on the expression of the Wnt co-receptor *LRP6* and the central component of the Wnt signalling pathway *ß-catenin*. There was also no effect on the expression of *DVL* (data not shown). The expression of *VEGF* however was strongly inhibited in the *DKK-1* overexpressing cells. These results were confirmed in two further independent experiments. In addition, Fig. 12 shows that the expression of the house-keeping gene *Aldolase*, which served as a control, was not affected in the *DKK-1* overexpressing cells.

Taken together, the results demonstrate that *DKK-1* overexpression directly decreased *VEGF* expression, but had no influence on the RNA expression of the analysed Wnt signaling pathway components.

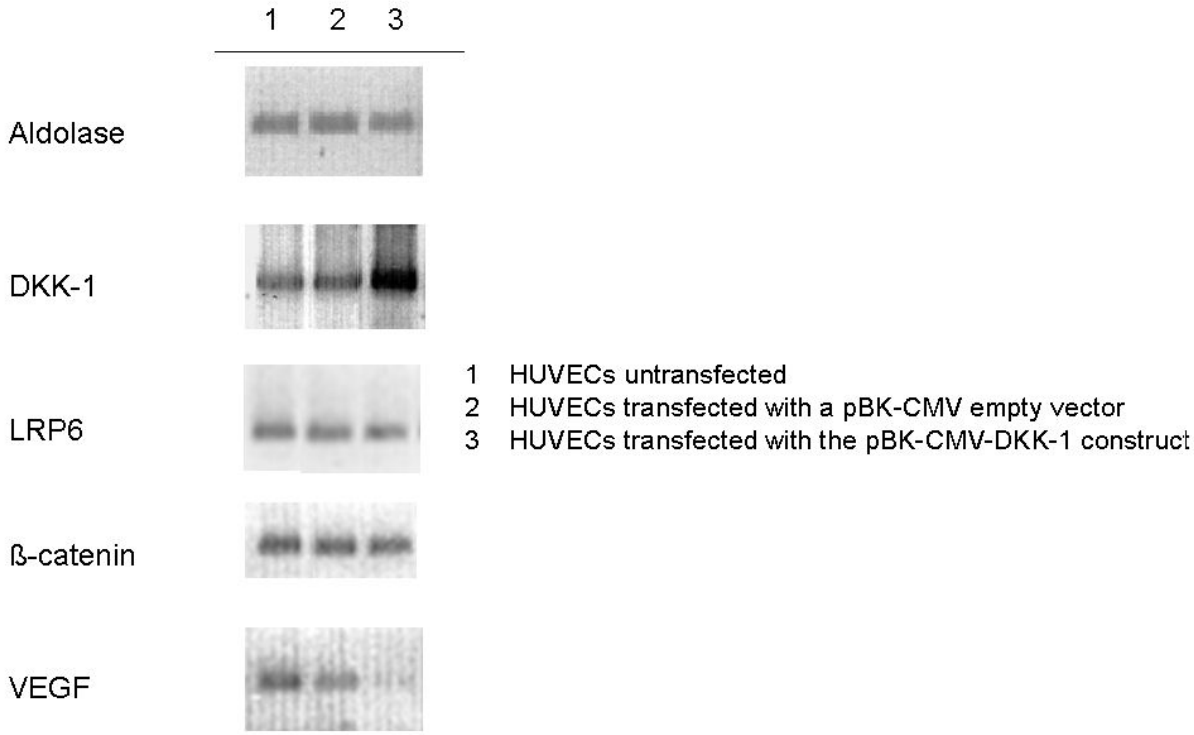


Figure 12 *DKK-1* overexpression down-regulates *VEGF* expression in HUVECs, whereas components of the Wnt signaling pathway such as *LRP6* and *ß-catenin* remain unchanged. RT-PCR analysis using gene specific primers reveals that that *DKK-1* expression is increased by transfection of the pBK-CMV-DKK-1 construct in comparison to the controls that were untransfected or transfected with an empty vector. *Aldolase* was used as a control.

4.5. Expression of Dkk-1 and ß-catenin in vessels and adult mouse tissues in-vivo

In a next step, we investigated, whether *Dkk-1* was also expressed in vessels in vivo. For that purpose, the thoracic and abdominal aorta, the carotid artery and the iliac arteries were isolated from mice and pooled. From these arteries, RNA was transcribed into cDNA for RT-PCR

analysis. As shown in Fig. 13, there was strong expression of the *Dkk-1* gene in these blood vessels. *Aldolase* was used as a control.

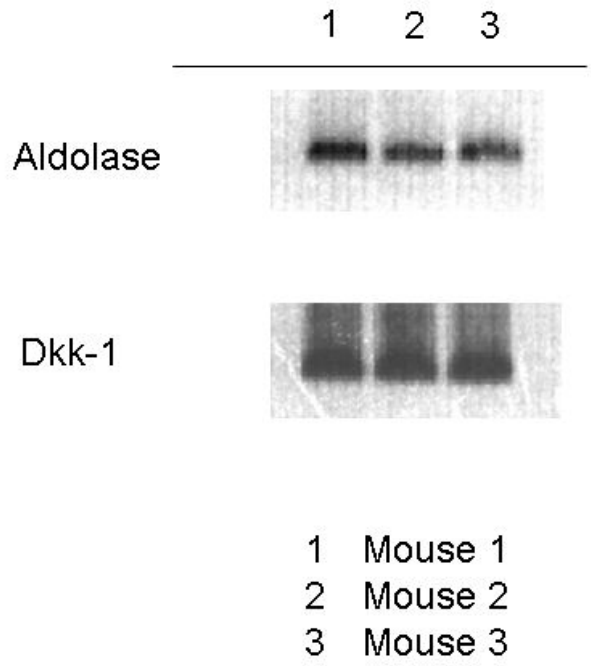


Figure 13 *Dkk-1* is strongly expressed in mouse arteries. RT-PCR analysis of *Dkk-1* using RNA isolated from big mouse arteries (aorta, iliac arteries and carotid arteries) that were pooled before RNA extraction was performed. Results are shown for three independent animals. *Aldolase* serves as a control.

Since *Dkk-1* was expressed in endothelial cells in-vitro, we investigated the localization of Dkk-1 in the mouse aorta. Sections of mouse aorta were prepared and stained with an antibody directed against Dkk-1. Fig. 14 shows that the corresponding immunofluorescence signal for Dkk-1 is located predominantly in the endothelial layer of the aorta (Fig. 14A). To further validate the endothelial localization, we performed double staining for Dkk-1 with the endothelial marker platelet endothelial cell adhesion molecule-1 (PECAM-1). The results in

Fig. 14B confirm that Dkk-1 and PECAM-1 co-localize in the endothelium. It is further shown that Dkk-1 expression is predominant in the endothelium and only weakly expressed in the smooth muscle layer of the aorta.

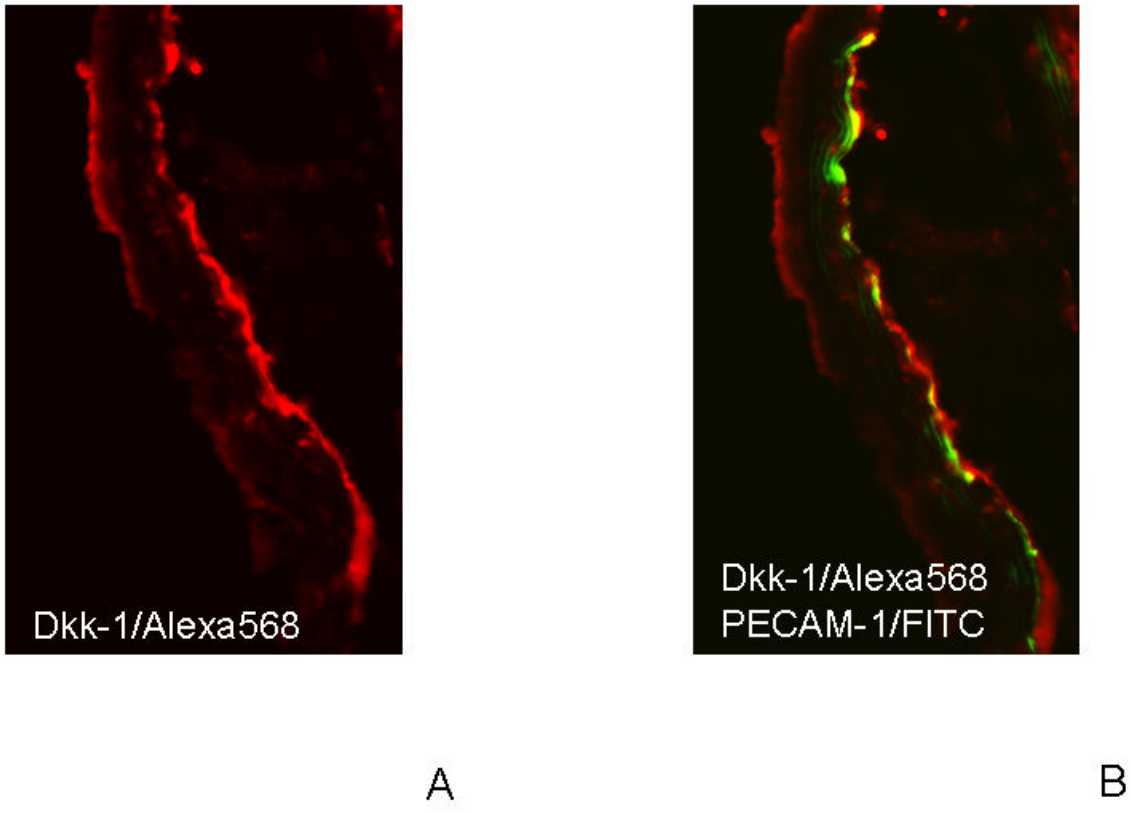


Figure 14 Dkk-1 is expressed in the endothelium in vivo.
Immunofluorescence staining of mouse aorta (magnification 20x). (A) Dkk-1 is predominantly found in the endothelium. (B) Co-staining with the endothelial marker PECAM-1 (CD 31, in green) confirms a predominant endothelial expression of Dkk-1 (red color).

In order to assess the expression of ß-catenin in blood vessels, mouse heart tissue sections from the left ventricle were stained with an antibody directed against ß-catenin. Fig. 15 shows the corresponding immunofluorescence signal from a coronary artery of the epicardium, indicating that ß-catenin expression is evenly distributed across the coronary vessel wall. Note that the

neighbouring coronary vein shows no ß-catenin staining. In addition to the expression in coronary vessels, ß-catenin is found in intercalated discs throughout the myocardium (Fig.15 insert).

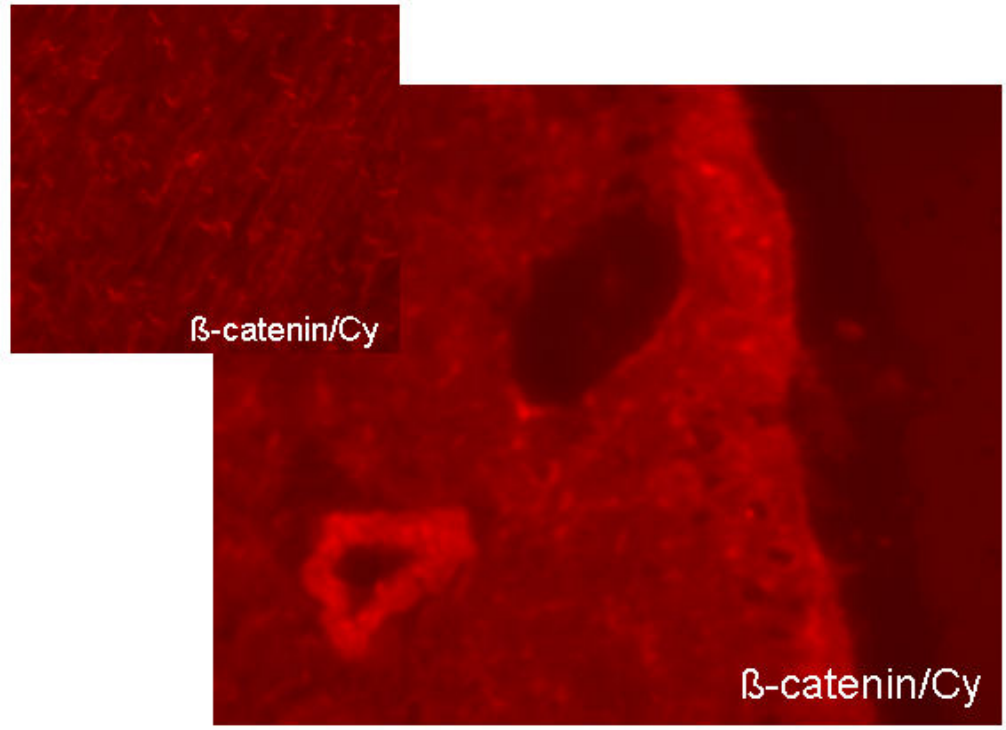


Figure 15 ß-catenin is expressed in coronary vessels of the myocardium. Immunofluorescence staining of the mouse myocardium (magnification 20x). The picture was taken of the epicardial area of the left ventricle showing a coronary artery and a coronary vein. Immunofluorescence staining against ß-catenin shows that expression is predominantly found in the smooth muscle layer. Additionally, ß-catenin is found in the intercalated discs of the myocardium (top left insert).

To evaluate the distribution of *Dkk-1* and *ß-catenin* in adult tissues, we analyzed different mouse tissues by RT-PCR. For that purpose, RNA isolated from various mouse organs was transcribed into cDNA. Fig. 16 shows that *Dkk-1* is expressed in distinct tissues with a

comparatively stronger expression in adipose tissue and skeletal muscle. There was also a positive signal in mouse brain. *ß-catenin* is equally expressed in all mouse organs tested showing only a weaker expression in spleen tissue. *ß-actin* was used as a control.

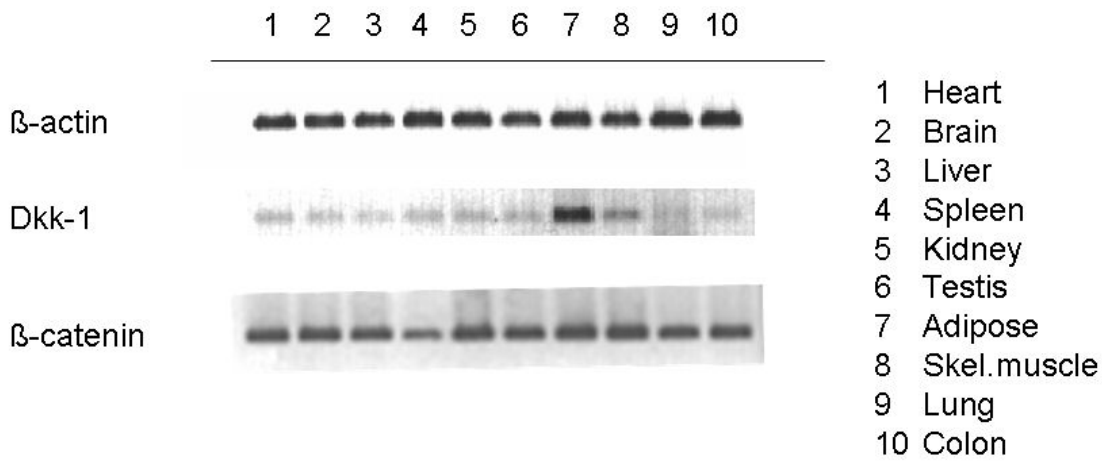
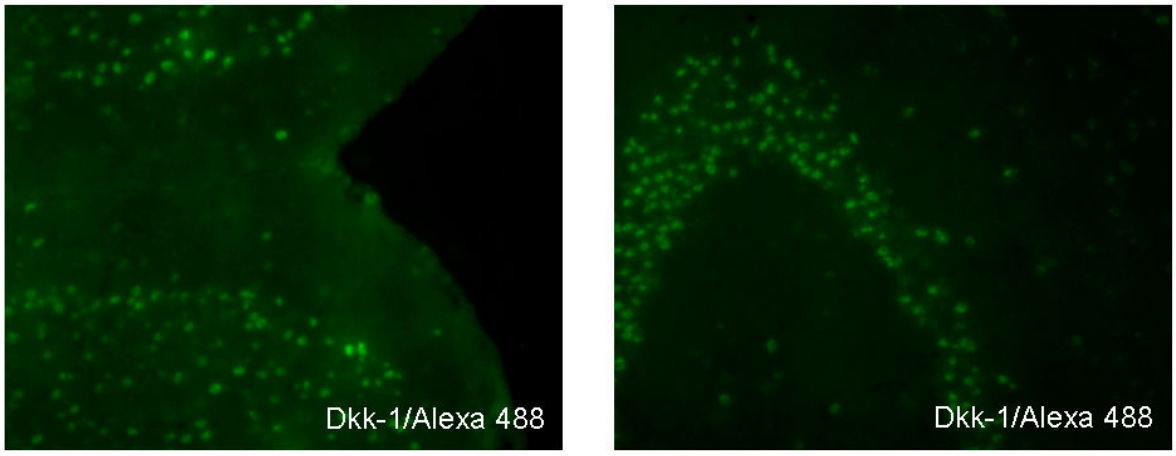


Figure 16 *Dkk-1* and *ß-catenin* are expressed in adult mouse tissues. RT-PCR analysis of *Dkk-1* and *ß-catenin* expression in various adult mouse organs. *Dkk-1* is weakly expressed in all organs tested except for lung tissue with comparatively stronger expression in adipose tissue and skeletal muscle. *ß-catenin* is equally expressed in all organs with comparatively weaker expression in spleen tissue. *ß-actin* served as control.

4.6. Expression of Dkk-1 and ß-catenin in normal and ischemic mouse brain

The in-vitro experiments have shown that *Dkk-1* is down-regulated in endothelial cells submitted to hypoxia. This effect is accompanied by an increase in *VEGF* but no change in *ß-catenin* expression. We have further shown that in-vivo *Dkk-1* and *ß-catenin* are expressed in big arteries of the mouse (Fig. 13-15) and in various adult mouse tissues, including mouse brain (Fig. 16). In a next step we used a mouse model of stroke to evaluate a possible role of Dkk-1 and ß-catenin in vivo in brain ischemia/hypoxia. Expression patterns of Dkk-1 and ß-catenin in the infarcted area were directly compared to the unaffected contralateral hemisphere.

Frontal sections of the mouse brain were prepared and stained with antibodies directed against Dkk-1. Fig. 17 shows sections of the cortex (A), the hippocampus (B) and the subcortical areas of the mouse brain (C-E). To our surprise, we found that in these areas Dkk-1 was specifically expressed in neurons but not in blood vessels. Double-staining with the specific neuronal marker neuron specific nuclear protein (NeuN) shows that the signals for Dkk-1 and NeuN co-localize (D). To further specify the neuronal expression of Dkk-1, co-staining of Dkk-1 and a marker for astrocytes glial fibrillary acidic protein (GFAP) was performed. As can be seen in Fig. 17 E, there was no co-localization of Dkk-1 and GFAP, indicating that astrocytes and probably other glial cells do not express Dkk-1.



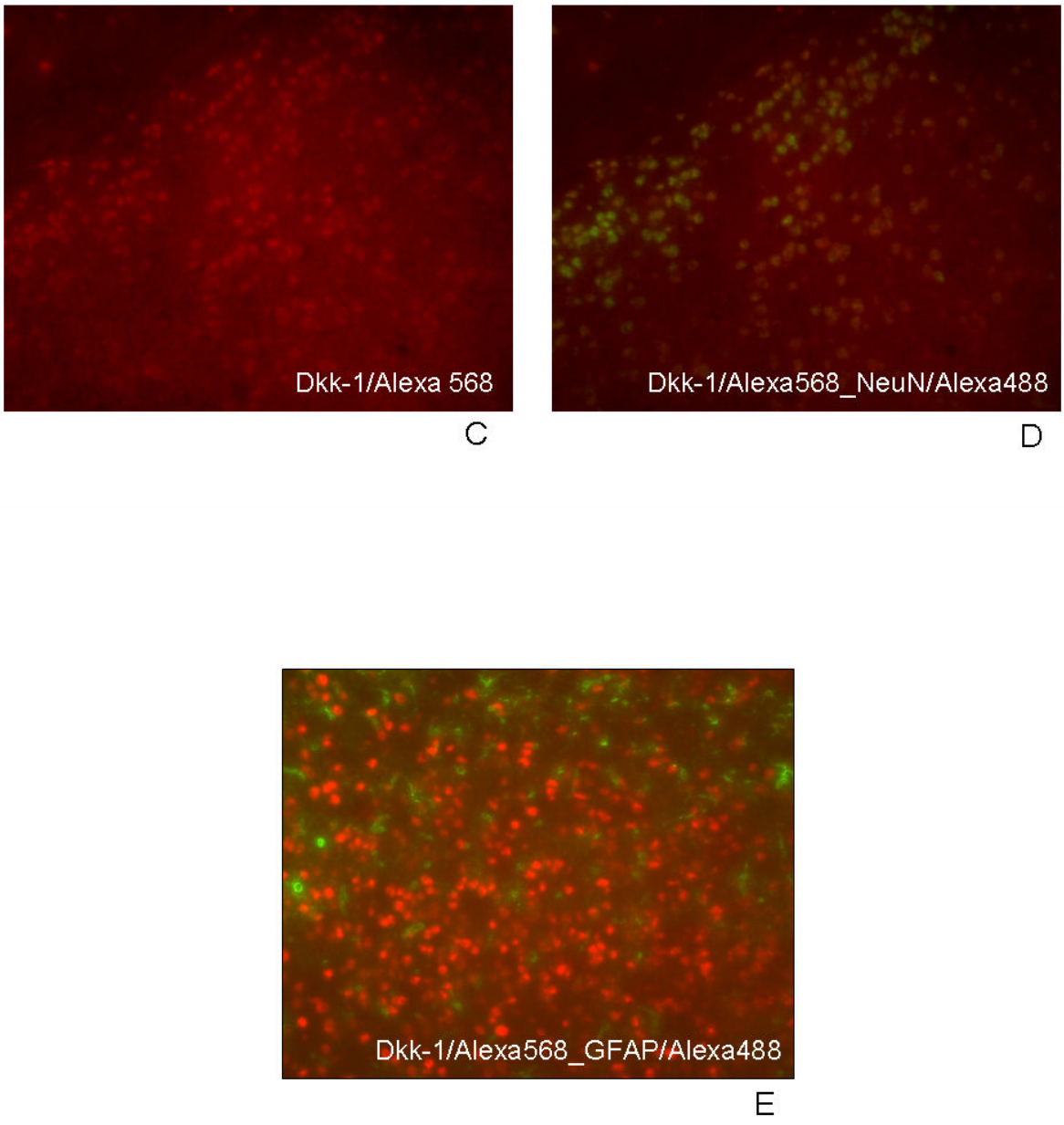


Figure 17 Dkk-1 is expressed in neurons of the mouse brain.
Immunofluorescence staining against Dkk-1 on frontal sections of mouse wild-type brains (magnification 20x). (A) Dkk-1 is expressed in neurons of the cortex, (B) the hippocampus and (C, D) subcortical areas. (D) Co-staining with the neuronal marker NeuN shows co-localization of NeuN (green) and Dkk-1 signals (red color). (E) Dkk-1 (red color) and GFAP (green) co-staining shows that Dkk-1 is not expressed in astrocytes.

In another set of experiments, ß-catenin expression in normal wildtype brain tissue was evaluated. Directing special attention to the vessels, frontal sections of the brain hemispheres were viewed after immunofluorescence staining with ß-catenin antibodies. In contrast to Dkk-1, ß-catenin was expressed in medium-sized vessels of the brain, but not in neurons or glial cells (Fig. 18).

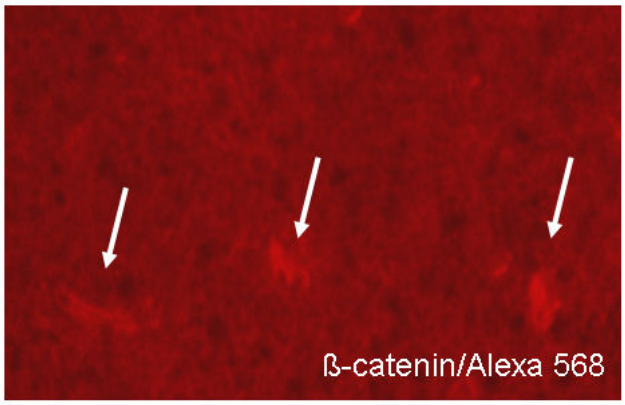


Figure 18 ß-catenin is expressed in vessels of the brain.
Immunofluorescence staining against ß-catenin in sections of wild-type mouse brain. Arrow heads indicate ß-catenin staining of medium-size brain vessels.

In order to evaluate the influence of ischemia/hypoxia on Dkk-1 and ß-catenin expression, we used brains from mice that had been subjected to ischemic stroke by middle cerebral artery occlusion (MCAO) for 60 minutes. Thereafter, occlusion was released and the animals were killed 24 or 48 hrs later. The brains of the mice were isolated and frontal sections of the brain were stained with antibodies directed against Dkk-1. Fig. 19A shows the border of the infarcted area 24 hrs after stroke and it can be seen that Dkk-1 expression is down-regulated in the neurons of the infarct area. As expected, the demarcation area of the ischemic stroke is characterized by the astrocytic wall depicted in Fig 19B by the green GFAP staining. As in

normal brains, the Dkk-1 antibody specifically stained neurons and there was no indication of blood vessel staining. The same pattern of neuronal Dkk-1 expression as described above was also observed 48 hrs after stroke (not shown).

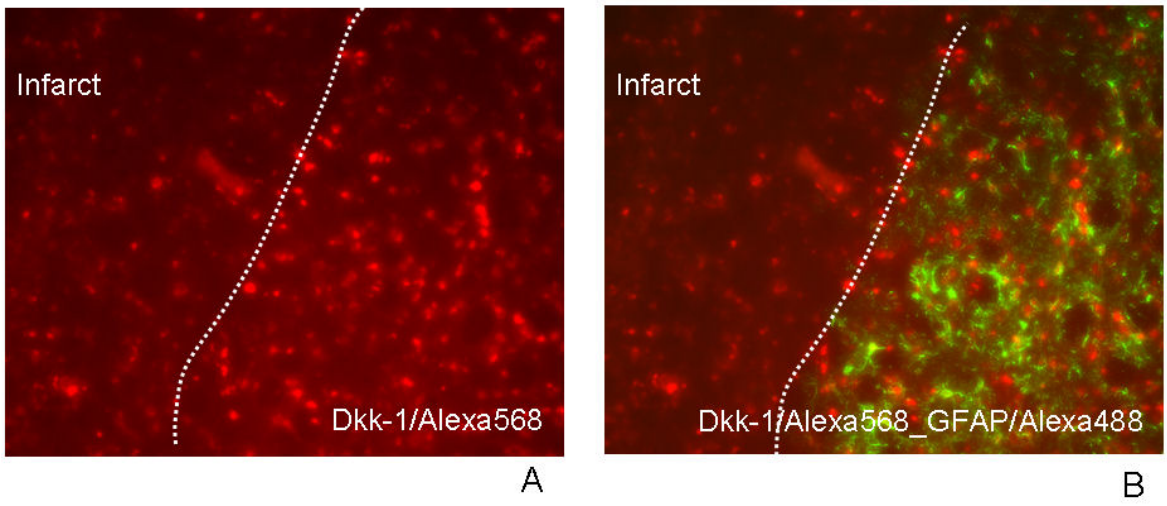
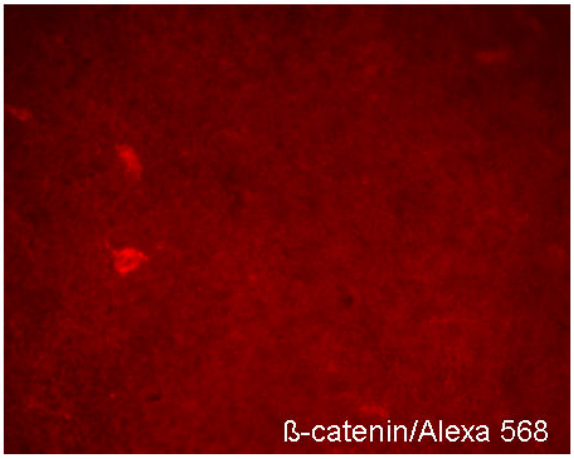


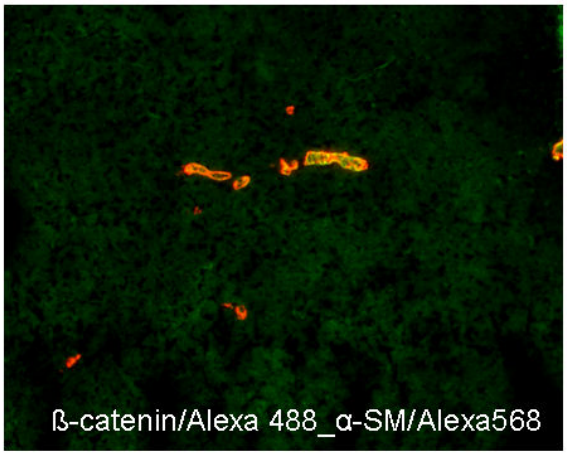
Figure 19 Dkk-1 expression in neurons decreases in the area of infarction.
Immunoflorescence staining against Dkk-1 in sections of mouse brain 24 hrs after MCAO (magnification 20x). The parts shown on the pictures are taken from the border zone of the infarct. (A) On the side of the infarct, Dkk-1 staining is diminished. (B) Dkk-1 expression (red color) stays restricted to neurons, revealed by the co-staining with the astrocyte marker GFAP.

In a further set of experiments, the expression of ß-catenin as a central component of the Wnt signaling pathway was analyzed in the mouse brain 48 hrs after ischemic stroke. Again, frontal sections of the mouse brain were examined. The expression of ß-catenin in the non-infarcted contralateral hemisphere was restricted to blood vessels (Fig. 20A). Double staining with the smooth muscle marker α-smooth muscle actin (α -SM) revealed that ß-catenin was located in the smooth muscle layer (media) of these vessels (Fig. 20B). Double staining with ß-catenin and the endothelial marker PECAM-1 showed no co-localization, indicating that ß-catenin expression in the contralateral hemisphere is restricted to the smooth muscle layer (Fig. 20C).

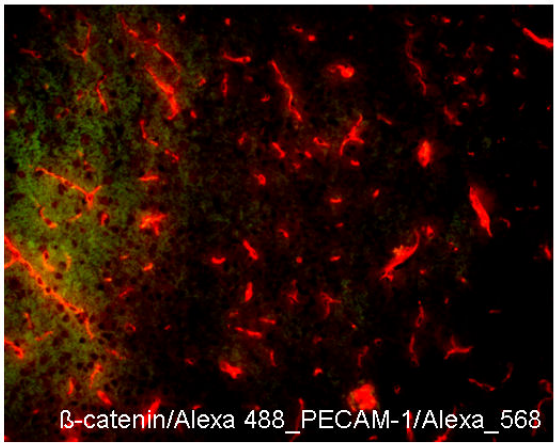
In contrast to the unaffected hemisphere, there was a shift in the expression pattern of ß-catenin in the infarct area 48 hrs after induction of stroke. Fig. 20D shows blood vessels in the infarcted area which are positive for ß-catenin. Arrows indicate examples of these vessels. To elucidate the localization of ß-catenin staining, we performed co-staining with the endothelial marker PECAM-1. Fig. 20E demonstrates the co-localization of ß-catenin and PECAM-1 indicating an endothelial localization of ß-catenin in brain blood vessels after infarction.



A



B



C

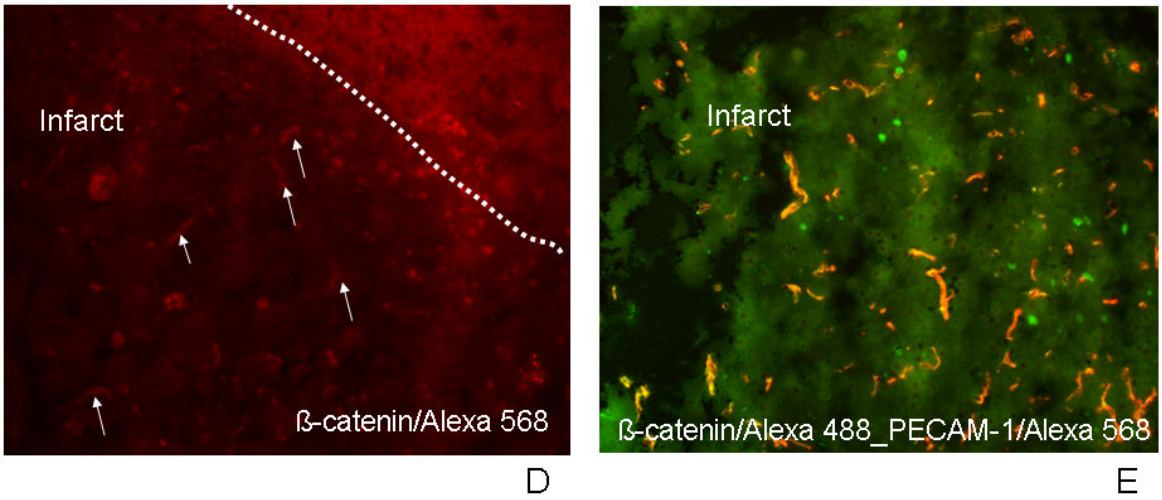


Figure 20 ß-catenin expression shifts from a predominant smooth muscle expression in the contralateral hemisphere to an endothelial expression in the border zone of the infarction 48 hrs after MCAO. Immunofluorescence staining against ß-catenin in sections of mouse brain 48 hrs after MCAO (magnification 20x). (A) The contralateral hemisphere is not affected by the infarct. ß-catenin is expressed in medium-sized vessels. (B) Co-stainings with the smooth muscle marker α-actin (red color) and (C) the endothelial marker PECAM-1 (CD 31) (red color) show that ß-catenin expression locates to the smooth muscle layer of these vessels (B) and not to the endothelium (C). (D) In the border zone of the infarction, ß-catenin is found to be expressed in small vessels (magnification 40x). (E) Co-staining of ß-catenin (green color) and the endothelial marker PECAM-1 (CD 31 red). Orange color due to staining overlap reveals that ß-catenin expression has changed to an endothelial expression pattern.

Taken together, the in-vivo experiments demonstrate that 1) Dkk-1 is expressed in the great peripheral arteries but not in brain vessels. 2) Dkk-1 expression in brain is restricted to neurons and decreases in the infarcted area. 3) ß-catenin expression shifts from a predominant localization in the media of brain vessels to endothelial cell expression in the infarcted area.

5. Discussion

5.1. Expression of Dkk-1 and ß-catenin in endothelial cells in-vitro

During the last years, reports from various investigators have shown that several components of the Wnt signaling pathway are expressed in vascular cells. Functional analysis of some of these factors suggests that active Wnt signaling via ß-catenin plays an important role in vessel formation and remodeling. Wnt antagonism, on the other hand, exerted by a large variety of secreted extracellular antagonists such as WIF, sFRPs and Dkks, is thought to be associated with vessel stability (Goodwin & D´Amore, 2002).

In HUVECs, expression of components of the Wnt signaling pathway such as members of the Wnt, Fz, Tcf and Lef gene families have been detected (Wright et al., 1999; Masckauchan et al., 2005). There is an increasing body of evidence that the canonical Wnt signaling pathway through ß-catenin is involved in angiogenesis by promoting endothelial proliferation, migration and survival (for HUVECs see: Masckauchan et al., 2005; Skurk et al., 2005; Kim et al., 2006). Promotion of angiogenesis by ß-catenin in HUVECs, as assessed by transduction of HUVECs with ß-catenin, might be mediated in part through the transcriptional activation of VEGF (Skurk et al., 2005).

In contrast, only little is known about the expression and function of secreted Wnt antagonists in endothelial cells. In HUVECs, FrzA/sFRP1, a member of the sFRP family, was shown to exert an anti-proliferative effect (Ezan et al., 2004). The same observation could be made for Dkk-1 in the tumor cell line NIH3T3 (Fedi et al., 1999). Our finding that *Dkk-1* is strongly expressed in HUVECs shows that another potent secreted Wnt antagonist is present in mature endothelial cells pointing to more complex regulation of the Wnt signaling pathway in endothelial cells.

Wnt signaling is commonly observed during embryogenesis. In vascular development, a role for Wnt/ß-catenin signaling has also been implicated (Goodwin & D´Amore, 2002). For example, in mouse development, Wnt5a, Wnt10b and Fz5 are expressed in vascular endothelial cells of blood vessels in the embryonic yolk sac. It has been assumed that Wnt5a and Wnt10b are ligands in Fz5-dependent angiogenesis in the yolk sac (Ishikawa et al., 2001). Wnt2 deficient mice show an abnormal placental vasculature (Monkley et al., 1996) and ß-catenin expression is regulated during vascular development and degeneration in avian mesonephros, a model for vascular development (Nacher et al., 2005). Another study shows that conditional inactivation of ß-catenin in endothelial cells of mouse embryos results in alterations in vascular patterning and increased vessel fragility (Cattelino et al., 2003). In the present study we show that *ß-catenin*, the central component of the canonical Wnt signaling pathway, is expressed in embryonic endothelial progenitors participating in early vasculogenesis. Interestingly, Wnt antagonists such as Dkk-1 and FrzA/sFRP1 also seem to be involved in vascular development. It was shown that Dkk-1 and FrzA/sFRP1 were temporarily expressed in the aorta during mouse embryogenesis (Monaghan et al., 1999) and during embryonic vascular development in the inner lining of the endothelial cell layer of the visceral yolk sac, respectively (Jaspard et al., 2000).

Our results demonstrate that the Wnt antagonist *Dkk-1* is expressed in eEPCs, which represent immature endothelial progenitors from mice that are involved in early vascular development. These cells show the special characteristics of immature endothelial precursors and express endothelial cell specific markers such as *Thrombomodulin* and *Tie-2 (*Hatzopoulos et al., 1998). Wnt/ß-catenin signaling in vascular development is of special interest because many of the signals relevant to blood vessel development are reactivated during adult blood vessel formation (Ferguson et al., 2005).

5.2. Expression of Dkk-1 in activated endothelial cells

In vascular development, angiogenesis and vasculogenesis contribute to the formation of the vasculature. Differentiation and maturation of endothelial precursors is one of the crucial steps in the formation of a functional endothelium (Ferguson et al., 2005; Jain, 2003; Carmeliet, 2003). In vitro, eEPCs have the ability to differentiate when the cells are treated with RA/cAMP. Stimulation of eEPCs with RA/cAMP leads to the induction of a set of genes specific to the endothelial lineage (e.g. *Flk-1, vWF, P-selectin, M-CSF*) proving a new model to study the transition of immature endothelial cells to a mature endothelium. This model is highly valuable to identify genes regulated in endothelial differentiation and helps to understand the molecular mechanisms underlying endothelial development (Hatzopoulos et al., 1998). In this study, we show that upon differentiation of eEPCs with RA/cAMP *Dkk-1* is down-regulated indicating a function in endothelial differentiation. It is interesting to note that Wnt signaling has been implicated in the differentiation of the vasculature. ß-catenin has been shown to increase endothelial cell differentiation (Skurk et al., 2005), which is consistent with our findings that *Dkk-1* expression decreased upon differentiation possibly to allow active Wnt signaling via ß-catenin. A recent report shows that during in-vitro differentiation of embryonic stem cells (ES) various members of the Wnt signaling pathway, among them *Dkk-1,* are differentially regulated. It is demonstrated that Wnt signaling activity is likely to promote endothelial cell differentiation (Wang et al., 2006).

Our findings that *Dkk-1* is down-regulated upon endothelial differentiation with RA/cAMP has also interesting implications for a role of Dkk-1 in angiogenesis since as mentioned above signaling during embryonic vascular development is recapitulated in adult vessel formation (Ferguson et al., 2005). Moreover, treatment of eEPCs with cAMP represents an activation process which is displayed in activation and inhibition of a variety of genes (Vajkoczy et al.,

2003). This activation of endothelial cells is also observed during the early stages of angiogenesis when quiescent endothelial cells are stimulated by pro-angiogenic factors.

In another model of endothelial activation, we investigated the effect of hypoxia on *Dkk-1* expression. Hypoxia is an important stimulus for angiogenesis and EPCs that contribute to postnatal vasculogenesis (Carmeliet, 2003; Asahara et al., 1999; Takahashi et al., 1999). It is known from previous studies that eEPCs when used in animal models in vivo respond to hypoxia since they could be shown to home preferentially to hypoxic lung metastases that have a low vessel density. In vitro, eEPCs are activated by hypoxia and respond with an increase in VEGF expression (Wei et al., 2004). In another model of hypoxia eEPCs were shown to enhance vascularization and tissue recovery in acute and chronic ischemia (Kupatt et al., 2005). Similar to eEPCs, HUVECs also respond to hypoxia with an increased *VEGF* expression in vitro (Namiki et al., 1995). Our results confirm these data, eEPCs as well as HUVECs increased their *VEGF* expression when challenged with hypoxia. At the same time a down-regulation of *Dkk-1* expression was observed in both cell types.

ß-catenin is known to promote proliferation and angiogenesis in HUVECs. This pro-angiogenic effect is thought to be meditated in part through the induction of VEGF by ß-catenin (Skurk et al., 2005; Kim et al., 2006). Our experiments with HUVECs showed, however, that *ß-catenin* does not respond to hypoxia, at least at the RNA level. Although transcriptional regulation of ß-catenin, measured in our experiments by RT-PCR, was not observed during hypoxia, this does not exclude that hypoxia leads to stabilization accumulation of cytosolic ß-catenin protein, which subsequently can interact with Lef/Tcf transcription factors contributing to specific gene expression such as *VEGF*.

Wnt antagonists like Dkk-1 and FrzA/sFRP1 are secreted proteins which are thought to promote vessel stability (quiescence) and expression of these antagonists may decrease in angiogenic conditions in order to allow active Wnt signaling. FrzA/sFRP1 is expressed in

quiescent bovine aortic endothelial cells (BAECs) in vitro and in vivo and inhibits proliferation of BAECs when these cells were transiently transfected with a plasmid coding FrzA/sFRP1 or when the purified FrzA/sFRP1 protein was added to the cells (Dupláa et al., 1999). This antiproliferative effect is probably due to an inhibition of the Wnt/ß-catenin pathway (Ezan et al., 2004). In addition, Dupláa and coworkers (1999) showed that BAECs expressed barely detectable levels of FrzA/sFRP1 when the cells were sparse and proliferating, but high expression levels when the cells were grown to confluency.

In line with these observations, we find that eEPCs 6 hrs after plating express low levels of *Dkk-1,* which strongly increase after 24 hrs of culturing. This finding can be explained by assuming that passaging and exchange of medium represents an activation mechanism, which causes the cells to proliferate and therefore to inhibit the transcription of *Dkk-1*. When eEPCs are confluent 24 hrs later and the proliferation stimulus has decreased, *Dkk-1* expression is enhanced.

5.3. Influence of Dkk-1 overexpression in HUVECs on VEGF and components of Wnt signaling

Our experiments with HUVECs challenged with hypoxia showed that *Dkk-1* and *VEGF* expression was regulated in opposite directions, whereas *ß-catenin* expression remained unchanged. Since *VEGF* and *ß-catenin* are important pro-angiogenic factors we investigated whether *DKK-1* had a direct influence on the expression of *VEGF* and *ß-catenin*.

To accomplish this goal, we prepared a plasmid coding *DKK-1* for gain-of-function experiments. A pBK-CMV phagemid vector (Stratagene) carrying the powerful CMV enhancer/promoter was chosen to allow sufficient overexpression of *DKK-1,* which was cloned into the multiple cloning site of the vector (MCS). As expected, transfection of HUVECs with

the pBK-CMV-DKK-1construct resulted in an increased *DKK-1* expression at the RNA level. Unfortunately, our attempts to detect DKK-1 in transfected HUVECs on the protein level by Western Blot analysis failed. However, when we repeated the transfection experiment in eEPCs, Western Blot analysis was successful and revealed an enhanced DKK-1 expression on the protein level (data not shown).

In HUVECs, transduction of ß-catenin was shown to induce VEGF expression (Skurk et al., 2005). In line with that observation, we found that HUVECs overexpressing the Wnt antagonist *DKK-1* expressed lower level of *VEGF*. In BAECs, however, Dufourcq and co-workers (2002) could not find a difference in VEGF expression when the cells were treated with the Wnt antagonist FrzA/sFRP1. In contrast to the down-regulation of *VEGF*, *DKK-1* overexpression in HUVECs had neither an influence on the expression of *ß-catenin* nor on other components of the Wnt signaling pathway such as *LRP6* or *DVL*. This finding points to a specific regulation of *VEGF* by *DKK-1* in HUVECs.

To further validate our results more experiments are needed, in particular it should investigated whether the changes in *VEGF* expression can be confirmed on the protein level and *VEGF* expression can be increased by interfering with DKK-1 through RNAi experiments.

5.4. Expression of Dkk-1 and ß-catenin in vessels and adult mouse tissues in-vivo

In a study identifying genes involved in the induction and maintenance of the quiescent, differentiated phenotype of vascular endothelium, the Wnt antagonist FrzA/sFRP1 was found to be highly expressed in the endothelium of adult bovine aorta (Duplàa et al., 1999). Similar results are presented in the present study by demonstrating a high expression level of *Dkk-1* in large peripheral arteries and in the endothelium of mouse aorta. It should be emphasized that

the endothelium of the aorta is quiescent, highly polarized and nonproliferating. As has been shown by our in vitro experiments, this is a condition associated with a high expression of *Dkk-1*.

In contrast, ß-catenin has been shown to be involved in endothelial cell proliferation. Accumulation of cytosolic and nuclear ß-catenin was observed in proliferating vessels of different tumors of the central nervous system (Yano et al., 2000a; Yano et al., 2000b; Eberhart et al., 2000). ß-catenin has also been described in neovascularization processes after myocardial infarction and overexpression of ß-catenin enhanced angiogenesis in a model of hind limb ischemia in mice (Blankesteijn et al., 2000; Kim et al., 2006). It has been observed that in the adult vasculature ß-catenin is rarely accumulated in the cytoplasm or the nucleus (Goodwin & D´Amore, 2002). In line with these findings, we observed no particular endothelial expression of ß-catenin in larger arteries (and veins) of the myocardium, but a predominant smooth muscle cell expression.

Wnt signaling is one of the main regulatory signaling pathways that plays a role in the development and maintenance of organ systems. Our findings that *Dkk-1* and *ß-catenin* are expressed in various mouse organs underscore this importance of Wnt signaling. Although *Dkk-1* is only weakly expressed in most tissues, this finding presents new results concerning the tissue distribution of *Dkk-1* in adult mice since in previous studies Dkk-1 was only detected in placenta tissue and the eye (Krupnik et al., 1999; Monaghan et al., 1999).

5.5. Expression of Dkk-1 and ß-catenin in normal and ischemic mouse brain

In the present study, we found that *Dkk-1* was down-regulated upon hypoxia in endothelial cells in vitro. In vivo, *Dkk-1* was expressed in large arteries of the mouse. These observations

prompted us to investigate whether in an animal model of tissue hypoxia Dkk-1 was also down-regulated in vessels.

To our surprise, we could not detect Dkk-1 in vessels of the mouse brain as in the aorta. Instead, we found a highly specific neuronal expression of Dkk-1 as validated by co-staining brain sections with the neuronal marker NeuN or the astrocyte marker GFAP. Our observations are in line with the findings of Krupnik et al. (1999) for Dkk-3, another member of the Dkk family. Dkk-3 was found to be expressed in neurons of the cortex and the hippocampus by in situ hybridization in adult mice. Using Dkk-1 knockout mice it could be demonstrated that Dkk-1 is necessary for the patterning of the forebrain and for the induction of head formation in Xenopus laevis (Mukhopadhyay et al., 2001; Glinka et al., 1998). In contrast to our observations, a recent report shows that in brains of patients suffering from Alzheimer´s disease Dkk-1 immunostaining is observed in the temporal cortex and white matter whereas age-matched controls show no Dkk-1 expression (Caricasole et al., 2004). A more recent study from the same group demonstrates that Dkk-1 was induced in the hippocampus of gerbils and rats subjected to a transient global ischemia as well as in cultured cortical neurons challenged with the excitatory amino acid N-methyl-D-aspartate (NMDA) as an in vitro model of excitotoxic neuronal death (Cappucio et al., 2005). In gerbils, no immunostaining for Dkk-1 could be observed in the hippocampus of controls whereas in control rats a low expression of Dkk-1 could be observed. It should be noted, however, that Cappucio at al. (2005) show an increased Dkk-1 expression in ischemic gerbil brains in non-neuronal cells and blood vessels in defined areas (CA1 and CA3 regions) of the hippocampus. In contrast to these observations, we found no indication of Dkk-1 expression in mouse brain vessels neither in wild-type animals nor in ischemic animals subjected to focal ischemia by MCAO. Our findings, however, indicate that Dkk-1 is not only expressed in neurons of the hippocampus but also in neurons of the cortex and subcortical areas of wild-type mice independent of hypoxia. Upon hypoxia, we observed an

increase in GFAP staining around the infarct which is in accordance with previous reports showing an astrocytic reaction to brain ischemia (Chen et al., 1993; Clark et al., 1993). Astrocyte functions are known to influence neuronal survival including glutamate uptake, glutamate release, free radical scavenging, water transport and the production of cytokines and nitric oxide (Chen & Swanson, 2003). In contrast to the observations of Cappucio et al. (2005), we found a decrease of Dkk-1 expression in neurons in the infarcted area rather than an increase. Diminished Dkk-1 expression in the border zone of the infarction co-localized with a decreased NeuN staining. This may not necessarily be due to neuronal loss, since NeuN immunoreactivity readily decreases after metabolic perturbations although neurons still preserve their integrity (Ünal-Çevik et al., 2004). In the core of the infarction, however, no staining for either Dkk-1or NeuN was observed (data not shown). The different results of our study compared with those of Cappucio and co-workers (2005) may be due to different experimental procedures such as global versus focal ischemia. In the case of global ischemia vessels were occluded for only a few minutes whereas in our model of focal ischemia MCA occlusion lasted for 60 minutes. In addition, tissue specificity may have played a role, since in our model of MCAO the hippocampus was not affected and therefore Dkk-1 regulation was not expected.

Our in vitro results have shown that ß-catenin mRNA levels were not regulated upon hypoxia in HUVECs. In vivo, however, there is a report demonstrating that ß-catenin is induced by hypoxia in a mouse model of hindlimb ischemia (Kim et al., 2006). In another study, ß-catenin was found in endothelial cells of newly formed and pre-existing vessels within the infarcted area of the rat heart. Outside the infarction area or in sham operated animals there was no indication of endothelial staining of ß-catenin, suggesting a role for this Wnt signaling component during neovascularization after myocardial infarction (Blankesteijn et al., 2000).

In the present study, we used a mouse model of brain ischemia to evaluate the expression and regulation of ß-catenin in blood vessels. Similar to the findings of Blankesteijn and co-workers (2000), we observed that ß-catenin was expressed by endothelial cells of the border zone of the brain infarct 48 hrs after MCAO. This finding represents a shift of ß-catenin expression from a predominant vascular smooth muscle cell expression in the unaffected contralateral hemisphere to the endothelium in the penumbra.

It is known from previous reports that 24 hrs after the induction of brain ischemia by MCAO, neovascularization starts to appear in rodent stroke models (Marti at al., 2000; Conway et al., 2003). After 48 hrs, a strong increase of PECAM-1 positive endothelial cells was observed at the border zone around the infarcted area. PECAM-1 staining of endothelial cells co-localizes with markers of cell proliferation suggesting an angiogenic reaction characterized by endothelial cell proliferation and vessel growth (Marti et al., 2000). Our observation that ß-catenin expression is shifted from smooth muscle expression in the unaffected part of the brain to an endothelial expression in the border zone of the infarction may indicate that ß-catenin plays an important role in the angiogenic response after ischemic stroke.

6. Summary

Angiogenesis and neovascularization depend on endothelial cell proliferation and migration. Active Wnt-signalling through its central component, ß-catenin, is thought to play a role in angiogenesis. In recent work, it has been emphasized that ß-catenin plays an important role in neovascularization after myocardial infarction, in cell proliferation and cell migration and angiogenesis. Another key factor in promoting endothelial cell proliferation, migration and angiogenesis is *vascular endothelial growth factor* (*VEGF),* which could be shown to be a target gene of ß-catenin. In contrast, inhibition of Wnt signalling is thought to be associated with vessel stability and vessel regression. Dickkopf-1 (Dkk-1) is a well characterized and potent inhibitor of Wnt signaling which is thought to specifically antagonize canonical Wnt signaling.

The aim of the present study was to investigate the expression of *Dkk-1* in endothelial cells in vivo and in vitro and elucidate its regulation in conditions of endothelial activation. A further purpose of the study was to compare expression of *Dkk-1* under hypoxic conditions in vitro and in vivo with the expression of *ß-catenin* and investigate a possible influence of *Dkk-1* on the pro-angiogenic factors *ß-catenin* and *VEGF*.

In the present work, we demonstrated that Dkk-1 is expressed in mouse embryonic endothelial progenitor cells (eEPCs) and human umbilical vein endothelial cells (HUVECs) in vitro, which represent immature endothelial precursors and fully mature endothelial cells, respectively. We found that *Dkk-1*expression is down-regulated in eEPCs activated by a differentiation stimulus (RA/cAMP), by an angiogenic stimulus (hypoxia) or by an endothelial growth stimulus (fresh medium). Stimulation of HUVECs with hypoxia confirmed the down-regulation of *DKK-1*

expression under hypoxic conditions. Overexpression of *DKK-1* in HUVECs revealed that *DKK-1* suppressed *VEGF* expression, but had no influence on *ß-catenin* expression.

In vivo, Dkk-1 expression was found in large peripheral arteries and in the endothelium of mouse aorta, which represents a quiescent, highly polarized and non-proliferating endothelium, but not in vessels of the brain. Instead, our in vivo experiments in mouse brains showed that Dkk-1 expression in the brain is restricted to neurons. In an animal model of brain ischemia neuronal expression of Dkk-1 decreased in the infarcted area.

In the present study, we further demonstrated that *ß-catenin* was also expressed in eEPCs and HUVECs. In vitro, stimulation of HUVECs with hypoxia had no effect on *ß-catenin* expression. In vivo, ß-catenin expression could be found in larger arteries of the heart and brain with a predominant expression in the smooth muscle layer. In an animal model of brain ischemia, ß-catenin shifted its expression from a predominant localization in the media of brain vessels in the unaffected contralateral hemisphere, to an endothelial cell expression in the infarcted area 48 hrs after MCAO.

We conclude that *Dkk-1* expression might be associated with the quiescent state of endothelial cells compared to the activated state in which *Dkk-1* expression is down-regulated. In maintaining the quiescent state *Dkk-1* might interfere with *VEGF* expression. An animal model of brain ischemia showed that Dkk-1 is probably not involved in neovascularization after brain infarction since it is not expressed in small vessels. In contrast, ß-catenin could be found in endothelial cells in the border zone of infarction, pointing to a role of ß-catenin in neovascularization after brain ischemia.

7. Zusammenfassung

Endothelzellproliferation und -migration sind Komponenten der Angiogenese und Neovaskularisation. Aktive Signaltransduktion über den Wnt Signalweg und dessen zentrale Komponente ß-catenin wird mit Angiogenese in Verbindung gebracht. In neueren Arbeiten konnte gezeigt werden, dass ß-catenin eine wichtige Rolle in der Neovaskularisation nach einem Myokardinfarkt, in der Zellproliferation, der Zellmigration und der Angiogenese spielt. Eine weitere wichtige Schlüsselkomponente bei der Vermittlung von endothelialer Zellproliferation und -migration ist der Vascular endothelial growth factor (VEGF), von dem gezeigt werden konnte, daß er ein Target Gen von ß-catenin ist. Im Gegensatz dazu ist eine Inhibition des Wnt Signalweges mit dem Erhalt von Gefäßen und Gefäßregression assoziiert. Dickkopf-1 (Dkk-1) ist ein gut charakterisierter und potenter Inhibitor der Wnt Signaltransduktion, von dem angenommen wird, das er ein spezifischer Inhibitor des kanonischen Wnt Signalweges ist.

Das Ziel der vorliegenden Arbeit war einerseits die Expression von Dkk-1 in Endothelzellen in vivo und in vitro zu untersuchen und andererseits die Regulation von Dkk-1 in der Endothelzellaktivierung zu betrachten. Ein weiteres Ziel war es die Expression von Dkk-1 unter hypoxischen Bedingungen mit der Expression von ß-catenin zu vergleichen, und zu untersuchen, ob Dkk-1 einen Einfluß auf proangiogenetische Faktoren wie ß-catenin und VEGF haben könnte.

Die vorliegende Arbeit zeigt, dass Dkk-1 in vitro sowohl in embryonic endothelial progenitor cells (eEPCs) als auch in human umbilical vein endothelial cells (HUVECs) exprimiert wird. Diese Zellen sind Beispiele für unreife endotheliale Vorläuferzellen und für reife Endothelzellen. Es zeigte sich, dass die Dkk-1-Expression sowohl in eEPCs, die mit einem Differenzierungsstimulus (RA/cAMP) aktiviert wurden, als auch durch angiogenetische Stimuli

(Hypoxie) oder einen endothelialen Wachstumsstimulus (frisches Medium) supprimiert wird. Stimulation von HUVECs mit Hypoxie bestätigte die in den eEPCs beobachtete Supression der Dkk-1 Expression. Überexpression von Dkk-1 in HUVECs zeigte, dass Dkk-1 die VEGF-Expression supprimierte während die Expression von ß-catenin nicht beeinflusst wurde.

In vivo konnte eine Dkk-1 Expression in den großen peripheren Arterien gefunden werden. Untersucht wurde die Mausaorta als Beispiel für ein ruhendes, polarisiertes und nicht-proliferierendes Endothel, in dem sich die Dkk-1 Expression vornehmlich im Endothel lokalisieren ließ. In kleineren Gefäßen wie den Hirngefäßen zeigte sich keine Dkk-1 Expression. Stattdessen war im Mausgehirn eine Expression von Dkk-1 in Neuronen zu beobachten. In einem Tiermodell zur Hirnischämie zeigte sich eine verminderte Expression von Dkk-1 in Neuronen des Infarktgebietes.

Die vorliegende Arbeit demonstriert weiter, dass ß-catenin sowohl in eEPCs und HUVECs exprimiert wird. In vitro hatte eine Stimulation von HUVECs durch Hypoxie keinen Effekt auf die ß-catenin Expression. In vivo konnte eine ß-catenin Expression in den größeren Arterien des Herzens (Koronargefäße) und des Gehirns mit einer vornehmlichen Lokalisation in der glatten Muskelschicht der Arterien beobachtet werden. Ein Tiermodell zur Hirnischämie zeigte, dass sich die ß-catenin Expression von einer dominanten Expression in der Media der Hirngefäße in der vom Infarkt nicht betroffenen Hemisphäre zu einer endothelialen Expression in Gefäßen des Infarktgrenzgebietes 48 hrs nach MCAO veränderte.

Wir schließen daraus, dass eine starke Dkk-1 Expression mit dem ruhenden Phänotyp des Endothels im Gegensatz zu einer Supprimierung der Dkk-1 Expression im aktivierten Endothel assoziiert sein könnte. Beim Erhalt des ruhenden Endothels könnte Dkk-1 mit der Expression von VEGF interagieren. Im Tiermodell zur Hirnischämie zeigte sich kein Hinweis auf eine Rolle von Dkk-1 in der Neovaskularisation nach Infarkt, da keine Expression von Dkk-1 in Gehirngefäßen gefunden werden konnte. Im Gegensatz dazu zeigte ß-catenin eine Expression in

Endothelzellen in der Infarktgrenzzone, was möglicherweise eine Rolle von ß- catenin in der Neovaskularisation nach einem Hirninfarkt anzeigt.

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9. Acknowledgements

I would like to thank PD Dr. med. Christian Kupatt, I. Medizinische Klinik, Universitätsklinikum Grosshadern, for receiving me into his research group and his helpful advice accompanying the progress of my thesis. Dr. Hatzopoulos, chief of the vascular genetics group at GSF, for his enthusiastic energy, his never tiering interest in scientific research and his expert adivice that made it a pleasure to work with him. Nicola, Heike, Mathias, Matthias, Iris and Albert supported my work with their help, technical adivice and many exciting discussions about my thesis for which I am truly thankful. Finally, I would like to thank GSF research Centre in Grosshadern for proving support for my thesis work.

10. Abbreviations

µg	microgram
µl	microlitres
° C	celcius
%	percent
A	adenine
A	absorption
APC	adenomatous polyposis coli
BAECs	bovine aorta endothelial cells
BBE	bovine brain extract
BLAST	Basic Local Alignment Search Tool
BMP	bone morphogenetic protein
bp	base pair
BSA	bovine serum albumin
C	cytosine
C3H	mouse strain
Ca^{2+}	$Calcium^{2+}$
cAMP	cyclic Adenosine-Monophosphate
CD	cord blood-derived
cDNA	complementary desoxyribonucleic acid
CKI	casein kinase I
CMV	cytomegalie virus
Cox	cyclooxigenase
CO_2	carbon dioxide

Cy	fluorescence dye, conjugated to secondary antibodies
DH5α	E. coli bacterial strain
ddH_2O	doubly destilled water
Dkk/DKK	Dickkopf
DMEM	Dulbecco´s modified eagle medium
DMSO	dimethylsufoxide
DNA	desoxyribonucleic acid
DNAse	desoxyribonuclease
dNTP	Deoxyribonucleosidtriphosphate
Dvl/DVL	Dishevelled
E. coli	Escherichia coli
ECs	Endothelial Cells
EDTA	Ethylendiamintetraacetate
eEPCs	Embryonic Endothelial Progenitor Cells
EPCs	endothelial progenitor cell
et al.	et alii
ES	embryonic stem cells
FBS	fetal bovine serum
FGF	fibroblast growth factor
Fig.	figure
FITC	Fluorescein-isothiocyanate (dye conjugated to secondary antibodies)
Flk-1	fetal liver kinase 1 or VEGFR-2
FrzA (sFRP1)	frizzeled related protein A
FrzB	frizzled related protein B

Fz	frizzled
G	guanine
g	gramm
GA	Gentamicin/AmphotericinB
GAPDH	glycerinealdehyde-3-phosphate dehydrogenase
GFAP	glial fibrillary acidic protein
GSK	glycogen snthetase kinase
hEGF	human epidermal growth factor
HEPES	N-(2-Hydroxyethyl)piperazin-N`-2-ethansufloacid
hFGF	human fibroblast growth factor
HIF	hypoxia inducible factor
h/hrs	hour/hours
HUVECs	human umbilical vein endothelial cells
IGF	Insulin like growth factor
IgG	Immunoglobuline G
IP	isoelectric point
kb	kilobases
Krm	Kremen
l	liter
LB medium	Luria-Bertani medium
LDL	low density lipoprotein
Lef	lymphoid enhancer binding factor
LRP	lipoprotein-related protein
MCA	middle cerebral artery
MCAO	middle cerebral artery occlusion

MCS	multiple cloning site
M-CSF	macrophage colony stimulating factor
min	minutes
ml	millilitres
mM	millimol
MMP	matrix metalloproteinase
mw	molecular weight
mRNA	messenger ribonucleic acid
NCBI	National Center for Biotechnological Information
NeuN	neuron-specific nuclear protein
ng	nanogram
NMDA	N-methyl-D-aspartate
HIH3T3	tumor cell line
nm	nanometers
O_2	oxygen
OD	optical density
O/N	overnight
PBS	Phosphate buffered saline
PCP	planar cell polarity
PCP/CE	Planar cell polarity/Convergent extension
PCR	Polymerase Chain Reaction
PECAM	Platelet Endothelial Cell Adhesion Molecule
PLGF	placental growth factor
pmol	picomol
RA	retinoic acid

RNA	ribonucleic acid
RNAi	ribonucleic acid interference
RNase	Ribonuclease
rpm	rounds per minute
RT	reverse transcriptase
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
sec	seconds
sFRP	secreted frizzled related protein
T	thymin
T17b	murine eEPC line
Taq Polymerase	polymerase from thermophilus aquaticus
TBE	Tris-borate EDTA
Tcf	T-cell-specific transcription factor
Tris	Tris(hydroxymethyl)-aminomethane
U	Unit
UV	ultra violet
V	Volt
VE-cadherin	vascular endothelial cadherin
VEGF	Vascular Endothelial Growth Factor
VEGFR	vascular endothelial growth factor-receptor
vWF	von Willebrand factor
WIF	Wnt inhibitory factor
Wnt	named after the Drosophila *wingless (wg)* and the mouse *Int-1* genes
w/v	weight/volume

11. Curriculum vitae

Name:	Julia Johanna Laura Maria
Date of birth:	04 November 1979
Place of birth:	Munich, Germany
Marital status:	unmarried

Education:

09/1986-07/1990	Grundschule Tutzing
09/1990-07/1996	Gymnasium Tutzing
08/1996-06/1999	Gymnasium Christianeum, Hamburg
11/1999- 04/2006	Medical faculty, Ludwig-Maximilians-Universität., Munich

Degrees:

25 Juni 1999	Abitur, Gymnasium Christaneum Hamburg
21 August 2001	Ärztliche Vorprüfung
29 August 2002	Erster Abschnitt der ärztlichen Prüfung
15 March 2005	Zweiter Abschnitt der ärztlichen Prüfung
20 April 2006	Dritter Abschnitt der ärztlichen Prüfung
11 May 2006	Approbation /licence to parctice as a doctor

Positions held:

01/2007	Assistant doctor, I. Medizinische Klinik, Zentralklinikum Augsburg