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Ephrin-B2 Overexpression in the Vascular Endothelium

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March, 2006

Thesis presented in fulfilment of the degree of Doctor of Philosophy at the University of London

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To my family
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Abstract

Previous work has established that Eph family receptor tyrosine kinases and ephrin ligands control a wide range of morphogenetic processes in vertebrate embryos through cell-contact dependent signalling interactions. In the developing cardiovascular system, ephrin-B2, a transmembrane protein is expressed by arterial endothelial cells (ECs) whereas the cognate receptor EphB4 is predominantly found on the venous endothelium. Gene targeting studies in mice have demonstrated that both molecules are critically required for angiogenic remodelling of embryonic blood vessels and survival beyond midgestation.

To gain more insight into the role of ephrin-B2 in vascular development and its arterial expression, I have used the tetracycline-controlled expression systems to overexpress the ligand in the endothelium of all vascular beds (i.e. in arteries, veins and microvessels) of transgenic mice. In the course of this study, I have employed several different transgenic EC-specific driver lines in combination with tetracycline-controlled (tTA, Tet-OFF) and reverse tetracycline-controlled (rtTA, Tet-ON) transactivators. Ephrin-B2 overexpression triggers enhanced activation of EphB receptors particularly in the venous endothelium. This leads to severe vascular malformations such as oedema and haemorrhaging. Induction of ephrin-B2 expression at different stages of embryonic development controls not only vascular patterning and the recruitment of supporting pericytes and vascular smooth muscle cells but it can also trigger tissue-specific responses.

In summary, my work has established that ephrin-B2 is an important regulator of blood vessel morphogenesis throughout embryonic development. Some results suggest that the ligand may also be involved in pathological conditions such as fibrosis as ectopic expression of ephrin-B2 in the embryonic liver triggers the activation of hepatic stellate cells. The resulting increase in matrix deposition around hepatic blood vessels could represent early signs of a fibrotic phenotype.
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Chapter 1 | Introduction

During vertebrate embryogenesis, the cardiovascular system is the first functional organ system to develop and it is established through a series of complex processes that involve coordinated interactions between distinct cell lineages. Embryonic endothelial cells form the framework of arteries, veins and capillaries through which essential oxygen and nutrients are supplied to developing tissues and organs. Expansion of the embryonic vasculature through the regulated proliferation, migration and differentiation of endothelial cells can be divided into two discrete processes known as vasculogenesis and angiogenesis (Risau and Flamme, 1995). Commencing with the assembly of a primitive vessel labyrinth by endothelial precursor cells, the vascular system will be subsequently remodelled into a hierarchically organised network (Figure 1.1). This process continues during embryonic and postnatal growth without disruption of essential blood circulation within the organism.

1.1 Vasculogenesis

During vasculogenesis, mesoderm-derived endothelial cell precursors known as angioblasts differentiate and assemble into discrete blood vessels in situ to form a primitive tubular network. This primary capillary plexus consists of relatively uniformly sized endothelial channels. Mesoderm-inducing factors of the fibroblast growth factor family are crucial in inducing paraxial and lateral plate mesoderm to form angioblasts and haematopoietic cells. Vasculogenesis occurs at two distinct embryonic locations. In extra-embryonic tissues, angioblasts initially appear in blood islands in the splanchnic mesoderm adjoining the extra-embryonic endoderm in the posterior half of the embryo. In contrast to extra-embryonic angioblasts, which form structures that are closely associated with blood cells, intra-embryonic angioblasts are only rarely associated with blood cells (Ciau-Uitz et al., 2000; Cormier and Dieterlen-Lievre, 1988; Jaffredo et al., 1998; Olah et al., 1988). In avian embryos, the first intra-embryonic angioblasts, which form slightly later than the extra-embryonic angioblasts, are visible as discrete cells at bilateral sites near the head folds that correspond to the future endocardium, and slightly later at the lateral edges of the anterior intestinal portal (Coffin and Poole, 1988; Sugi and Markwald, 1996). Angioblasts subsequently become visible throughout broad
regions of the embryo proper. Although the extra-embryonic and intra-embryonic vessels will ultimately form a continuous vascular network, each area develops independently of the other. At present, it is not known if extra and intra-embryonic angioblasts are specified by the same mechanism, or whether different genetic pathways regulate their formation. Although the morphological events underlying vascular cord formation and endothelial tubulogenesis have been described in some detail, less is known about the signalling pathways involved in vascular development.

1.1.1 Mediators and signalling pathways involved in vasculogenesis

One of the most important signalling molecules involved in early blood vessel development is vascular endothelial growth factor A (VEGF-A), which acts through its high-affinity receptor VEGFR2 (Flk-1). VEGF activity is essential for the formation of blood vessels, and embryos lacking either VEGF or VEGFR2 develop few (or no) angioblasts and die early in development (Carmeliet et al., 1996; Ferrara et al., 1996; Shalaby et al., 1995). In various contexts, VEGF has been shown to act as a potent mitogen (Keyt et al., 1996; Park et al., 1993; Wilting et al., 1996), chemoattractant (Ash and Overbeek, 2000; Cleaver and Krieg, 1998; Waltenberger et al., 1994) and survival factor (Gerber et al., 1998). Additionally, the proper regulation of VEGF is crucial for the formation of normal endothelial channels (Drake et al., 2000; Fong et al., 1995; Fong et al., 1999). The early lethality of knockout embryos lacking VEGFR1 (Flt-1), another member of the VEGF receptor family, is attributed to the increase of haemangioblast commitment, resulting in the formation of disorganised vessels with supernumerary endothelial cells (Fong et al., 1999). VEGFR1 may act as a "decoy" receptor competing with VEGFR2. Several upstream factors have been shown to regulate VEGF and VEGFR2 expression. The transcription factor hypoxia-inducible factor 1 (HIF-1) and its family members play crucial roles in sensing changes in tissue oxygen tension and stimulating gene expression changes that enhance blood vessel growth into hypoxic tissues during post-gastrulation development (Ryan et al., 1998). Additional factors such as specificity protein 1 act in concert to regulate VEGF transcription (Pages and Pouyssegur, 2005). At the transcriptional level, there appear to be necessary roles for GATA family proteins as well as the homeodomain protein HoxB5 in upregulation of VEGFR2 during development (Wu et al., 2003). Another layer of regulation in endothelial development is ascribed to the ETS family of
transcriptional factors that direct downstream endothelial specific expression of VEGFR2, VEGFR1, the angiopoietin receptors, and MEF2C, a recently identified member of the MADS box superfamily of vascular developmental transcription factors (De Val et al., 2004).

Evidence supports BMP signalling as a proximal stimulus for VEGFR2 expression. Similar to VEGF, the transforming growth factor β (TGF-β) signalling pathway has been found to be required for mesoderm differentiation, haematopoiesis and blood vessel formation. Both TGF-β1 and TGF-β receptor 2 knockouts die at E10.5 due to defects in haematopoiesis and vasculogenesis (Dickson et al., 1995; Oshima et al., 1996), while ALK5 (a subtype of TGF-β receptor 1) null mice show normal haematopoiesis (Larsson et al., 2001). Nevertheless these embryos die at E10.5 and display defects in vessel formation in the yolk sac and placenta, which are caused by enhanced endothelial cell proliferation, impaired endothelial migration and suppressed fibronectin production. Fibroblast growth factors (FGFs) have also been implicated as proliferative agents during vascular development; however, the large number of FGF ligands, and the early lethality of knockout embryos have impeded research into the developmental roles of individual family members (Javerzat et al., 2002).

1.1.2 Endothelial tube formation

Although known signalling pathways are essentially linked to endothelial cell specification and proliferation, the specific signalling pathways required for vascular tubulogenesis have only been identified recently (Hogan and Kolodziej, 2002; Lubarsky and Krasnow, 2003). Within the embryo, the initial specification of angioblasts in the mesoderm is independent of tissue interactions with other germ layers (Vokes and Krieg, 2002). However, the first blood vessels within the embryo always form in mesoderm that is in close proximity to endoderm (Gonzalez-Crussi, 1971; Meier, 1980; Mobbs and McMillan, 1979; Pardanaud et al., 1989; Vokes and Krieg, 2002; Wilt, 1965). Furthermore, it was recently demonstrated that a signal originating from the endoderm is essential for the assembly of angioblasts into tubes (Vokes and Krieg, 2002).

Additional evidence has emerged that sonic hedgehog (SHH) signalling by the vasculogenic endoderm plays a central role in organizing specified angioblasts into vascular tubes. SHH is the first growth factor identified that specifically regulates
vascular tube formation (Vokes et al., 2004). An elegant study in zebrafish found that angioblasts migrate as individual cells to form a vascular cord at the midline. This transient structure is then stabilized by endothelial cell-cell junctions, and subsequently undergoes lumen formation to form a fully patent vessel. Downregulation of the VEGF signalling pathway does have an effect on the number of angioblasts but does not appear to influence their migratory behaviour. The study also indicated that the endoderm provides a substratum for endothelial cell migration and is involved in regulating the timing of this process, but that it is not essential for the direction of migration. In addition, the endothelial cells in endoderm-less embryos form properly lumenised vessels, contrary to what has been previously reported in Xenopus and avian embryos (Jin et al., 2005).

Endothelial cells have been shown to express vascular endothelial (VE)–cadherin (Lampugnani et al., 1992) and targeted deletion of the gene in mice leads to early embryonic death with associated severe vascular anomalies (Carmeliet et al., 1999; Gory-Faure et al., 1999; Radice et al., 1997). While Carmeliet et al. concluded that, due to the presence of lumenised vessels, the basis of the defects was a failure in the process of angiogenesis, Gory-Faure et al. proposed that extra-embryonic vasculogenesis was dependent on VE-cadherin activity, whereas intra-embryonic vasculogenesis was not. A recent study then established, that VE-cadherin function is not crucial to the process by which angioblasts make the transition to form a nascent vascular epithelium, but is critically required to prevent the disassembly of nascent embryonic blood vessels (Crosby et al., 2005).

1.2 Angiogenesis

Historically, the term angiogenesis was first used to describe the growth of endothelial sprouts from pre-existing postcapillary venules. More recently this definition has been extended to generally denote the growth and remodelling of the primitive vessel network which is formed by vasculogenesis. Angiogenic remodelling is achieved by both pruning and vessel enlargement, to form the interconnecting branching patterns characteristic for the mature vasculature. A different process, referred to as angiogenic sprouting, involves the sprouting from existing vessels into previously avascular tissue.
1.2.1 Vasodilation and permeability

Angiogenesis initiates with vasodilation, a process involving nitric oxide (NO). VEGF has been shown to induce NO synthase activity in endothelial cells (Carmeliet, 2000) (Figure 1.2). The increased levels of intracellular NO stimulate guanyl-cyclase to synthesise cyclic guanosine monophosphate (cGMP), which subsequently results in the relaxation of vascular smooth muscle cells (vSMCs). Vascular permeability increases in response to VEGF, thereby allowing extravasation of plasma proteins that lay down a provisional scaffold for migrating endothelial cells. This increase in permeability is mediated by the formation of fenestrations and vesiculo-vacuolar organelles. At the same time, in a process which involves Src kinases, platelet endothelial cell adhesion molecule (PECAM)-1 and VE-cadherin are redistributed (Eliceiri et al., 1999).

Although permeability is required for angiogenesis in order to allow the formation of new endothelial sprouts, excessive vascular leakage can lead to a variety of pathological scenarios. Therefore vascular permeability has to be tightly regulated through the orchestrated interaction of several molecules. The angiopoietins were discovered as ligands for the Ties, a family of receptor tyrosine kinases that are selectively expressed within the vascular endothelium. Angiopoietin-1 (Ang-1), a ligand of the endothelial Tie-2 receptor, is a natural inhibitor of vascular permeability, tightening pre-existing vessels. When acutely administered to adult vessels, Ang-1 protects against plasma leakage without profoundly affecting vascular morphology (Thurston et al., 2000). Unlike mouse embryos lacking VEGF or VEGFR2, embryos lacking Ang-1 or Tie-2 develop a rather normal primary vasculature. However this vasculature fails to undergo further remodelling.

1.2.2 Vessel destabilisation and matrix degradation

Before endothelial cells of pre-existing vessels are able to emigrate from their resident sites, inter-endothelial cell contacts must be loosened, perivascular support cells (mural cells) need to detach, the EC basement membrane must be degraded and the perivascular extracellular matrix needs to be remodelled.

Ang-2 was cloned based on its homology to Ang-1, and displayed similar high affinity for Tie-2, but – depending on the cell examined – Ang-2 could either activate or
antagonise Tie-2 signalling. Transgenic overexpression of Ang-2 in the embryonic endothelium resulted in embryonic death due to defects resembling those of Ang-1 or Tie-2 knockouts, demonstrating that Ang-2 could act as a Tie-2 antagonist in vivo, at least under some circumstances. This possibility became even more intriguing when Ang-2 expression profiles were examined. In adult animals, Ang-2 was induced in the endothelium of vessels undergoing active remoulding, such as sprouting or regressing vessels in the ovary (Goede et al., 1998), or in tumours (Hолash et al., 1999a; Holash et al., 1999b; Stratmann et al., 1998; Zagzag et al., 1999). These findings, together with the possibility that Ang-2 could act as a Tie-2 antagonist, led to the hypothesis that Ang-2 might provide a key de-stabilizing signal involved in initiating angiogenic remoulding (Hолash et al., 1999a; Holash et al., 1999b; Maisonpierre et al., 1997; Zagzag et al., 1999). This de-stabilisation may be required for subsequent endothelial cell migration.

Proteinases of the plasminogen activator, matrix metalloproteinase (MMP), chymase or heparanase families influence angiogenesis by degrading matrix molecules and by activating or liberating growth factors such as bFGF, VEGF and insulin growth factor-1 (IGF-1), sequestered within the extracellular matrix (Conway et al., 2001) (Figure 1.3). MMPs, in particular MMP-2, MMP-3 and MMP-9, play a critical role in degrading extracellular matrix and basement membrane structures, thereby permitting endothelial migration. Integrin receptors, particularly \( \alpha_5\beta_3 \), can recruit and activate MMPs, especially MMP-2, to localised microdomains on the cell membranes of blood vessels, where they conduct their proteolytic function (Brooks et al., 1996). The recruitment by integrins helps to regulate MMP activity, as excessive matrix degradation would impede endothelial invasion. The TGF-\( \beta \)1 signalling pathway is also implicated in this process. Targeted ablation of one of its type I receptors, the activin receptor-like kinase (ALK)-1, leads to lethality by E10.5 due to severe vascular abnormalities (Oh et al., 2000). The lumen of major blood vessels in ALK-1 mutants, including the dorsal aorta and branchial arches, are highly dilated and the capillary vessels are excessively fused. The fact that ALK-1 deficiency leads to increased levels of different plasminogen activators as well as Ang-2, suggests that the developing blood vessels in the knockouts may have undergone increased peri-vascular proteolysis and are more permeable, thus leading to excessive proliferation of ECs and capillary fusion.
1.2.3 Endothelial cell proliferation and migration

Once the path has been cleared, proliferating endothelial cells can migrate to distant sites. A delicate balance between angiogenic and angiostatic factors controls this process. Proliferating ECs are guided by gradients of chemotactic agents through the disintegrated basement membrane into the remodelled perivascular space. The signals that initiate and sustain angiogenesis are multiple and complex. Proangiogenic cytokines and growth factors include VEGFs, FGFs, angiopoietins, TGF-β, PDGFs, TNF-α, EGF, IL-8, and angiogenin, which are secreted by inflammatory cells (e.g., mastcells and macrophages), pericytes, keratinocytes (during epidermal wound healing), or tumour cells (Table 1.4). Some of these factors act directly by binding to their respective receptors on endothelial cells to induce proliferation and/or migration, while others act on local stromal or inflammatory cells to stimulate angiogenesis (Li et al., 2003; Weinstat-Saslow and Steeg, 1994).

Sprouting angiogenesis is critical to blood vessel formation, but the cellular and molecular controls of this process are poorly understood. In order to examine a possible involvement of VEGF receptor 1 (flt-1), Kearney et al. used time-lapse imaging of GFP-expressing vessels derived from stem cells to analyze dynamic aspects of vascular sprout formation (Kearney et al., 2004). Surprisingly, loss of flt-1 led to decreased sprout formation and migration, which resulted in reduced vascular branching. This phenotype was also seen in vivo, as flt-1−/− embryos had defective sprouting from the dorsal aorta. Rescue of the branching defect by a soluble flt-1 (sflt-1) transgene supports a model whereby flt-1 normally positively regulates sprout formation by production of sflt-1 that antagonizes VEGF signalling. Thus precise levels of bioactive VEGF-A and perhaps spatial localization of the VEGF signal are likely modulated by flt-1 to ensure proper sprout formation during blood vessel formation.

ECM and basement membrane components also transduce both pro- and anti-angiogenic signals by binding to integrins on endothelial cells. For example, intact fibrillar type IV collagen is bound by integrins α1β1 and α2β1 expressed on angiogenic endothelial cells and induces their proliferation and migration, while degraded type IV collagen does not bind these integrins and instead binds to αvβ3 integrin, inhibiting endothelial cell migration and proliferation (Kalluri, 2003). The ECM also acts as a sequestration/storage compartment for angiogenic growth factors such as VEGF, basic
FGF (bFGF), and TGF-β1, which can be released by proteolytic degradation (e.g. by MMPs) of the ECM (Kalluri, 2003). There are also multiple endogenous inhibitors of angiogenesis. Bioactive cleaved forms of collagens, which include endostatin, tumstatin, arrestin, and canstatin, bind to endothelial cell surface integrins to inhibit proliferation and migration (Kalluri, 2003; Kerbel and Folkman, 2002) (Table 1.4). Angiostatin, a proteolytic internal fragment of plasminogen, binds to ATP synthase and to angiomotin on the surface of endothelial cells to inhibit cell proliferation and migration (Moser et al., 1999; Troyanovsky et al., 2001). Other angiogenesis inhibitors are compounds that disrupt growth factor signalling, such as soluble forms of VEGF receptors, VEGFR-1 and neuropilin-1 (Veikkola et al., 2000). Proteins with thrombospondin-type motifs, such as thrombospondin-1 and -2 (TSP-1 and -2), platelet factor-4, and several members of the metalloprotease with ThromboSpondin-like repeats) family also show anti-angiogenic activity (Carpizo and Iruela-Arispe, 2000). Thrombospondin-1 binds to CD36, a class B scavenger receptor and collagen-binding protein, on endothelial cells, which results in inhibition of cell migration and induction of apoptosis (Carpizo and Iruela-Arispe, 2000).

Forkhead box O (Foxo) transcription factors are emerging as critical transcriptional integrators among pathways regulating differentiation, proliferation, and survival. Yet the role of the distinct Foxo family members in the angiogenic programme was only recently examined (Furuyama et al., 2004; Hosaka et al., 2004). Potente et al. found that Foxo1 and Foxo3a are the most abundant Foxo isoforms in mature endothelial cells and that overexpression of constitutively active Foxo1 or Foxo3a, but not Foxo4, significantly inhibits endothelial cell migration and tube formation in vitro (Potente et al., 2005). Silencing of either Foxo1 or Foxo3a gene expression leads to a profound increase in the migratory and sprout-forming capacity of endothelial cells. Gene expression profiling showed that Foxo1 and Foxo3a specifically regulate a non-redundant but overlapping set of angiogenesis - and vascular remodelling-related genes. Whereas Ang-2 was exclusively regulated by Foxo1, eNOS, which is essential for postnatal neovascularization, was regulated by Foxo1 and Foxo3a. In vivo, Foxo3a deficiency increased eNOS expression and enhanced postnatal vessel formation and maturation. The data suggest an important role for Foxo transcription factors in the regulation of vessel formation in the adult (Potente et al., 2005).
1.2.4 Arterio-venous specification

Arteries and veins can be anatomically distinguished within the adult circulatory system. Larger-diameter arterial vessels are exposed to the highest pressure and flow and are characteristically surrounded by a thick medial layer consisting mostly of vascular smooth muscle cells. In contrast, venous vessels carry efferent circulation of lower pressure, have less surrounding smooth muscle and possess specialized structures, such as valves, to ensure blood flow in a single direction. Although differences in fluid dynamics within the circulatory system play a role in determining the characteristic structure of an artery or vein, recent evidence suggests that the identity of endothelial cells lining these vessels is established before the onset of circulation by genetic mechanisms during embryonic development (Adams et al., 1999; Gerety et al., 1999; Lawson et al., 2001; Moyon et al., 2001; Wang et al., 1998). Molecular differences between arterial and venous endothelial cells have been demonstrated during embryonic development in chick, mouse, and zebrafish. In all of these vertebrate organisms, the transmembrane ligand ephrin-B2 is expressed in endothelial cells lining only arteries, while, in most cases, its receptor, EphB4, is expressed predominantly in venous endothelial cells (Lawson et al., 2001; Moyon et al., 2001; Wang et al., 1998). Artery-restricted expression of ephrin-B2 has also been demonstrated in adult mice and humans (Gale et al., 2001; Shin et al., 2001). Analysis of mice lacking ephrin-B2 (Adams et al., 1999; Wang et al., 1998) or EphB4 (Gerety et al., 1999), as well as Xenopus laevis injected with a dominant-negative form of EphB4 (Helbling et al., 2000), show that these factors are required for normal blood vessel development. However, they do not appear to be required for the determination of arterial or venous endothelial cell fate (Wang et al., 1998), suggesting the action of other upstream factors.

Little is known about the various pathways specifying the identity of arterial and venous SMCs, but recent genetic studies offer insight into the signals controlling arterial and venous identities of ECs. The Notch pathway, with its ligands (Delta-like-4, Jagged-1 and Jagged-2) and receptors (Notch-1, Notch-3 and Notch-4), promotes arterial fate of ECs by repressing venous differentiation (Lawson et al., 2001; Zhong et al., 2001). Sonic Hedgehog and VEGF act upstream, whereas Gridlock probably acts downstream of Notch to determine arterial fate, even before the onset of flow (Lawson et al., 2002; Zhong et al., 2001). ECs can differentiate into either arterial or venous ECs in embryonic development, in the neonatal retina and even in the adult heart, indicating
that ECs have a remarkable phenotypic plasticity (Stalmans et al., 2002; Visconti et al., 2002). Notch signalling, however, is also critical for proper maintenance of arteries. Mutations of the SMC-specific Notch-3 receptor, which disrupt SMC anchorage to the extracellular matrix (ECM) and impair SMC survival, cause degeneration of cerebral arterioles, leading to cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) (Kalimo et al., 2002). Recently Notch-3 was shown to be the first cell-autonomous regulator of arterial specification by regulating arterial differentiation and maturation of SMCs but not of endothelial cells (Domenga et al., 2004). Besides Notch, bone-marrow tyrosine kinase and neuropilin-1 (a VEGF_{164}-specific receptor) also influence arterial specification (Stalmans et al., 2002). By secreting VEGF, peripheral nerves promote arterial differentiation and provide a molecular explanation as to why arteries and nerves often run alongside each other in the body (Mukouyama et al., 2002).

The TGF-β signalling pathway has been also shown to be important in the regulation of arterial-venous identity. The human vascular disease called Hereditary Haemorrhagic Telangiectasia (HHT) is caused by the loss of normal arterial-venous distinction, leading to vascular malformations that subsequently result in haemorrhaging, shunting and emboli. Genetic studies have established that HHT is caused by mutations in both ALK1 and the TGF-β co-receptor endoglin (Azuma, 2000). Genetic targeting of these genes has shown that they are essential for the functional and molecular distinction between arteries and veins (Sorensen et al., 2003; Urness et al., 2000). Both knockouts die by E11.5 with severe arterio-venous malformations resulting from fusion of major arteries and veins as well as shunting between presumptive arterial and venous vascular beds. The arterial specific marker ephrin-B2 was down-regulated. Mutant embryos showed the loss of functional identity as intravascular haematopoietic clusters, which are normally restricted to arterial vessels, developed ectopically in veins.

In contrast to the concept of genetically controlled vessel fates, there are several emerging studies in the chick and quail that challenge this notion by showing that arterial-venous differentiation is controlled by haemodynamic forces. Two studies address whether endothelial cells are committed to an arterial or venous fate once expressing their specific markers (Moyon et al., 2001; Othman-Hassan et al., 2001). In quail embryos, ECs were isolated from either arteries or veins, which were
characterised by the expression of markers (ephrin-B2 and neuropilin-1 for the arteries, Tie-2 for the veins). Tissue pieces were then grafted into chick embryos and it was later analysed whether quail cells from veins had contributed to arteries or veins. The results revealed that endothelial cells of either arterial or venous origin are able to switch to the other arterial or venous identity and adopt their respective new marker expression. This was dependent on the embryonic stage of the quail cells as older ones were unable to switch but respected the arterial-venous identity of the host vessels. These findings indicate that there is some level of plasticity in the determination of vessel fate and the adoption of the new vessel identity could be mediated through local cues (such as vSMC interaction), blood flow, or blood pressure related signals in the endothelial cells.

Another study, using time-lapse video-microscopy to follow the formation of the vasculature of the chick yolk sac in real-time, provided evidence that haemodynamic flow mediates arterial-venous differentiation and patterning (Le Noble et al., 2004). It was shown that to form a mature vascular network consisting of paired and interlacing arterial-venous patterns, thin vessels from the arterial domain of the yolk sac must be disconnected from the arterial tree and reconnected to the venous system. This implies that endothelial plasticity is necessary to fashion normal growth of veins. To test the role of haemodynamic flow, the direction of blood flow in the developing vasculature was experimentally altered. This led to the reprogramming of the arterial-venous pattern suggesting that haemodynamic flow is a main regulator of yolk sac vascular development. However, in some areas of undifferentiated capillary beds of the yolk sac with disrupted blood flow, ephrin-B2 expression was detected, which is inconsistent with genetically predetermined expression in the absence of haemodynamic factors.

A recent study managed then to shed light into the mechanism involved in the establishment of the venous identity. Little was known so far about venous identity except that it involves EphB4 expression, because Notch signalling is not activated in veins. An unresolved question was how vein identity is regulated. In this study it was shown that COUP-TFII (also known as Nr2f2), a member of the orphan nuclear receptor superfamily, is specifically expressed in venous but not arterial endothelium (You et al., 2005). Ablation of COUP-TFII in a knock-in mouse model, enabled veins to acquire arterial characteristics (including the expression of the arterial markers NP-1 and Notch1) and recruit haematopoietic cell clusters. On the basis of their findings, the
group proposed a working model for the role of COUP-TFII in establishing arterial-venous identity. During normal development, COUP-TFII in venous endothelium suppresses NP-1, inhibits Notch signalling and the expression of arterial-specific genes. Without the effects of NP-1 and Notch signalling, EphB4, Flt4 and other factors important for the differentiation of the vein remain expressed and vein identity is maintained. Removal of COUP-TFII from the venous endothelium activates the expression of arterial markers NP-1, Jag1, Notch1 and ephrin-B2, leading to the acquisition of arterial functions and the induction of haematopoietic cell clusters. Notably, the acquisition of these arterial characteristics was not sufficient to convert mutant veins fully into arteries, because the expression of EphB4 is not totally lost, and arterial-venous fusion did not occur in the mutant mice. In contrast, ectopic expression of COUP-TFII in arterial ECs suppresses the expression of NP-1 and the downstream arterial markers. Arteries acquired more vein-like properties, resulting in the fusion of veins and arteries in transgenic embryos. Thus, the establishment and maintenance of arterial-venous identity is tightly regulated.

At this point, one can conclude that the development of arterial-venous identity involves an intricate interplay between genetic regulation and haemodynamic forces, each of which contributes a certain level of plasticity.

1.2.5 Vessel Maturation

Once new endothelial sprouts have reached their destination, a regulated process of blood vessels maturation is initiated. It requires recruitment of mural cells, generation of an extracellular matrix and specialisation of the vessel wall for structural support (Figure 1.4). Functional specialisation plays another vital role in order to meet local demands. All of these processes are orchestrated by physical forces as well as by a constellation of ligands and receptors whose spatio-temporal patterns of expression and concentration are tightly regulated. The origin, number, type and organisation of mural cells and the composition of the associated matrix depend on the location of the vessel and its function.

The mural cell population comprises of two main cell types, pericytes (PC) and vascular smooth muscle cells (vSMCs). Pericyte is the term for vascular mural cells embedded within the vascular basement membrane of blood microvessels, where they make
specific contacts with the endothelium. They are found around capillaries, precapillary arterioles, postcapillary venules and collecting venules. The association of pericytes to ECs does not only suppress the formation of new endothelial sprouts, but reduces as well the permeability of the endothelial monolayer thus preventing leakage through the vessel wall (Armulik et al., 2005; Betsholtz et al., 2005; Hirschi and D'Amore, 1996). Pericytes are related to vSMCs and are generally assumed to belong to the same cell lineage. They differ from vSMCs by their location relative to the endothelium, their morphology, and, to some extent, by their marker expression. However, the distinction between pericycle and vSMC morphology and location is not absolute.

Vascular SMCs are circumferentially arranged, closely packed and tightly associated with the abluminal side of the endothelial basal lamina of more mature and larger calibre blood vessels the endothelium. While capillaries, the most abundant vessels in our body consist of ECs, a surrounding basement membrane and a thin layer of pericytes, arterioles and venules have an increased coverage of mural cells. Precapillary arterioles are completely coated by vSMCs while the walls of larger vessels consist of three specialised layers. An intima composed of ECs, a media of vSMCs and an adventitia of fibroblasts, together with matrix and elastic laminae. The production of extracellular matrix and elastic fibers by vSMCs provides blood vessels with mechanical stability and elasticity, which is of particular importance for arteries that transport blood under high pressure from the heart to the periphery. SMCs and elastic laminae contribute to the vessel tone and mediate the control of vessel diameter and blood flow (Figure 1.4). Venous vessels that direct the blood stream under lower pressure from peripheral tissues back to the heart do not require a dense association of vSMCs (Jain, 2003).

1.2.5.1 PDGF-B/PDGFR-β signalling in mural cell recruitment

Previous work has established that the recruitment of mural cells and the generation of ECM is regulated by at least four molecular pathways: platelet-derived growth factor-B (PDGF-B) - PDGF-β receptor (PDGFR-β), sphingosine-1-phosphate-1 (S1P1) - endothelial differentiation shingolipid G-protein-coupled receptor-1 (EDG1), Angiopoietin-1 - Tie-2, and transforming growth factor-β (TGF-β) (Jain, 2003). Pericytes are attracted to proliferating endothelia by EC-derived PDGF and both PDGF
and its receptor are critical for the proper formation of stable blood vessels during development and tumorigenesis (Abramsson et al., 2003; Jain and Booth, 2003). Blood vessels in Pdgfb−/− animals are characterized by dilation, rupture, leakage, and hemorrhage and contribute to embryonic lethality (Bjarnegard et al., 2004; Lindblom et al., 2003). Importantly, PDGF and PDGF-receptor inhibitors disrupt mural cell association with ECs and block angiogenesis and tumour growth (Bergers et al., 2003). Not only the presence but also the level of PDGF-B is important for pericyte development as mice heterozygotes for a Pdgfb-null allele show reduced pericyte numbers compared with wild-type mice (Hammes et al., 2002).

Similarly, EDG1−/− mice exhibit a failure of mural cells to migrate to blood vessels (Kluck and Hla, 2002). The lack of EDG1 receptor, which is expressed on mural cells, may alter the EC matrix production or EC-mural interaction, and interfere with vessel maturation. Targeted deletion of Gαi13, a molecule downstream of EDG3, also yields an Edg1−/− knockout phenotype, and both PDGFB- and PDGFR-β-deficient mice exhibit markedly reduced expression of RGS5, a GTPase-activating protein for Ga, on their vascular plexi and small arteries (Cho et al., 2003).

### 1.2.5.2 Angiopoietin-Tie-2 signalling in the vascular wall

Genetic loss- and gain-of-function studies in mice show that the Ang-1-Tie-2 signalling loop is essential for vessel maturation and stabilisation. The Tie-2 receptor is generally held as being EC-specific (Davis et al., 1996; Sato et al., 1995), whereas its agonistic ligand, Ang-1, appears to be expressed mainly by peri-vascular and mural cells (Sundberg et al., 2002; Suri et al., 1996). Ang-1- or Tie-2-null mice die at midgestation from cardiovascular failure. Besides defective angiogenesis, blood vessels in these mutants show a poorly organised basement membrane and reduced coverage and detachment of pericytes (Dumont et al., 1994; Sato et al., 1995; Suri et al., 1996). The mechanism of vessel maturation by Ang-1 is far from clear. Notably in the absence of mural cells, Ang-1 restores a hierarchical order of the larger vessels, and rescued oedema and haemorrhage in the growing retinal vasculature of mouse neonates (Uemura et al., 2002). The role of Ang-2 seems to be contextual. In the absence of VEGF, Ang-2 acts as an antagonist of Ang-1 and destabilises vessels, ultimately leading to vessel regression. In the presence of VEGF, Ang-2 facilitates vascular sprouting.
1.2.5.3 TGF-β in endothelial/mural cell interactions

TGF-β1 promotes vessel maturation by stimulating the production of extracellular matrix (ECM) components and by inducing the differentiation of mesenchymal cells to mural cells (Chambers et al., 2003; Pepper, 1997). It is expressed in a number of cell types including ECs and mural cells. Depending on the context and concentration, it can act both, pro- or anti-angiogenic. The role of TGF-β in the control of mural cell-EC interactions has been demonstrated by genetic studies in mice. Targeted gene inactivation of the TGF-β receptor ALK-1 has led to embryonic lethality at E10.5 due to vascular defects caused by improper differentiation and recruitment of vSMCs to blood vessels (Oh et al., 2000). Knockout mice for TGF-β1, the type II ALK-5, the co-receptor endoglin, and downstream signalling molecules such as Smad5, have revealed the contribution of these molecules to the initial phases of angiogenesis as well as vessel maturation (Arthur et al., 2000; Bourdeau et al., 1999; Dickson et al., 1995; Larsson et al., 2001; Li et al., 1999; Yang et al., 1999). Carvalho et al. analysed TGF-β signalling in yolk sacs from endoglin knockouts and EC-specific knockouts of tfβrII and alk5 and found that the disrupted TGF-β signalling in ECs also impaired the TGF-β/ALK-5 signalling in adjacent mesenchymal cells, inhibiting their differentiation into vSMCs and association with the endothelial tubes (Carvalho et al., 2004). Therefore, TGF-β signalling in ECs promotes TGF-β expression, synthesis, and release by these cells, which, in turn, induces differentiation of vSMCs from surrounding mesenchymal cells but also reinforces TGF-β expression in the ECs themselves in an autoregulatory loop (Carvalho et al., 2004). In humans, mutations in ENDOGLIN and ALKI, which are both, expressed by endothelial cells, cause hereditary hemorrhagic telangiectasia (HHT) type 1 and 2, respectively. These diseases are characterised by bleeding caused by vascular malformations (Johnson et al., 1996; McAllister et al., 1994). Mural cell defects have been reported in several of the above mentioned mouse mutants and also in HHT (Braverman et al., 1990).

1.2.5.4 S1P/Edg signalling in endothelial/pericyte interactions

Sphingosine-1-phosphate (S1P) is a secreted sphingolipid engaged in cell communication through certain G-protein-coupled receptors denoted as S1P1 to S1P5. When added to cells, S1P triggers cytoskeletal, adhesive and junctional changes, affecting cell migration, proliferation and survival (Allende and Proia, 2002). Although
SIP₁ expression was originally described in endothelial cells, these receptors are expressed widely in cultured cells, including both endothelial and mesenchymal cells. Disruption of the slp1 (edgI) gene in mice causes mid/late-gestational lethality with vascular abnormalities involving defective vSMC/pericyte coverage of blood vessels (Liu et al., 2000). A similar defect was noticed in double knockouts for slp2 and slp3, whereas other double or triple knockouts slp1-3 showed more severe vascular defects and earlier lethality. SIP₁ signalling through Rac promotes trafficking of N-cadherin to polarised plasma membrane domains in ECs, thereby strengthening contacts with mural cells (Paik et al., 2004).

In that context, it was additionally shown that although N-cadherin is not required for the early events of angiogenesis, N-cadherin deficiency leads to an impairment of pericyte recruitment to nascent endothelium (Tillet et al., 2005). These data are consistent with a role of N-cadherin in promoting the stabilization of preformed vessels and not endothelial differentiation and sprouting per se. The finding that N-cadherin acts upstream of VE-cadherin (Luo and Radice, 2005) in vascular morphogenesis, together with earlier studies implicating SIP₁ as a major regulator of fundamental endothelial cell functions (Lee et al., 1999; Lee et al., 2001; Mehta et al., 2005) suggests that the mural cell deficiency in SIP₁ knockouts has a complex pathogenesis involving disrupted endothelium/pericyte interactions in combination with cell autonomous endothelial defects.

1.2.5.5 The role of Notch signalling in the vascular wall

Although Notch signalling in the vascular system has mainly been attributed to the establishment of arterio-venous identity (Shawber and Kitajewski, 2004) a recent study showed a role for Notch signalling in specification of the arterial characteristics of vSMCs. The arterial SMCs in Notch3⁻/⁻ mice appear to have lost some of their arterial characteristics and have instead acquired the characteristics of venous SMCs (Domenga et al., 2004). Importantly the endothelium in Notch3⁻/⁻ mice maintains the correct A-V identity. Notch3 is expressed by vSMCs of small and medium-sized brain arteries (Prakash et al., 2002) and NOTCH3 is mutated in CADASIL, a human stroke and dementia syndrome affecting the same type of vessel (Joutel et al., 1996). Although the pathogenesis of the vascular lesions in CADASIL is not fully understood, the studies of
Notch3\textsuperscript{-/-} mice suggest that it might involve problems with the specification of correct vSMC identity.

1.2.5.6 Involvement of Akt1 signalling in vessel maturation

Akt kinases control essential cellular functions, including proliferation, apoptosis, metabolism and transcription. Recent studies conducted using transgenic and knockout animals have indicated a role of this signalling pathway in pathological angiogenesis, vascular maturation and permeability in vivo. Many of the biological activities during embryonic angiogenesis ascribed to VEGF mediated signalling involve the activation of the phosphoinositide 3-kinase (PI3 kinase)-Akt signalling cascade. The interrelationship between VEGF and Akt pathway in vivo is complex, because PI3-kinase-Akt activation in turn mediates VEGF production in tissues (O'Neill and Abel, 2005). Although the phenotype of mice lacking single Akt genes is relatively mild (Chen et al., 2001), Akt1-Akt2 double-knockout mice die shortly after birth (Peng et al., 2003). A recent study helped to shed light on the vascular functions of Akt signalling. By using Akt1\textsuperscript{-/-} mice, Chen et al. showed that Akt1 is the predominant isoform of Akt in ECs and that besides enhanced pathological angiogenesis in Akt1\textsuperscript{-/-} mice, the vasculature of these mutants is immature and leaky (Chen et al., 2005). In a B16F10 tumour model in Akt1-deficient mice, tumour vessels showed a considerable reduction in SMC association and an about 50% reduction in basement membrane thickness compared to wild-type littermates. Thus Akt1\textsuperscript{-/-} blood vessels are characterised by a less developed basement membrane and deficient recruitment of mural cells. Further analysis revealed that the levels of laminin 5, the density of collagen fibrils and total amount of collagen in the skin of Akt1\textsuperscript{-/-} mice were significantly reduced. Additionally a decrease in the amount of Thrombospondin-1 and 2 (TSP-1, -2) in the tissues of Akt1\textsuperscript{-/-} mice was observed. At a mechanistic level, vascular leakage and enhanced angiogenesis in Akt1\textsuperscript{-/-} mice could be directly linked to the abnormalities in ECM deposition which are long-term consequences of deficient Akt activation. The impaired vascular maturation but not other observed aspects of vascular development seemed to be related to the endothelial nitric oxide synthase (eNOS) pathway in Akt1\textsuperscript{-/-} mice.
1.2.6 Angiogenesis in health and disease

When dysregulated, the formation of new blood vessels contributes to numerous malignant, ischemic, inflammatory, infectious and immune disorders. Historically the best known are cancer, psoriasis, arthritis, and blindness, but many additional common disorders such as obesity, asthma, atherosclerosis and infectious disease are included, and the list is still growing. Several congenital or inherited diseases are also caused by abnormal vascular remodelling. In addition, insufficient vessel growth and abnormal vessel regression not only cause heart and brain ischemia, but can also lead to neurodegeneration, hypertension, pre-eclampsia, respiratory distress, osteoporosis and other disorders. In the course of this introduction, the underlying mechanisms will only be discussed in more detail for cancer-related angiogenesis.

Without blood vessels, tumours can not grow beyond a critical size or metastasize. The observation that angiogenesis occurs around tumours was made nearly one hundred years ago. It is now widely accepted that the ‘angiogenic switch’ is ‘off’ when the effect of pro-angiogenic molecules is balanced by that of anti-angiogenic molecules, and is ‘on’ when the net balance is tipped in favour of angiogenesis. Various signals that trigger this switch have been discovered. These include metabolic stress, mechanical stress, immune/inflammatory response, and genetic mutations. Pro- and anti-angiogenic molecules can emanate from cancer cells, ECs, stromal cells, blood and the extracellular matrix. Their relative contribution is likely to change with tumour type and tumour site. It is also likely to change with tumour growth, regression and relapse.

Tumour vessels develop by sprouting or intussusception from pre-existing vessels. Circulating endothelial precursors, shed from the vessel wall or mobilised from the bone marrow, can also contribute to tumour angiogenesis (Asahara et al., 2000; Rafii, 2000). Tumour cells can also grow around an existing vessel to form a peri-vascular cuff. It is known that there are various molecular players involved in these different mechanisms of vascular growth (Carmeliet, 2000). Among these, members of the VEGF and Ang family have a predominant role. Several molecules, including a number of angiogenesis inhibitors, seem to be involved mainly in tumour angiogenesis. The temporal and spatial expression of these regulators is not well coordinated in tumours as in physiological angiogenesis and their mechanism of action is poorly understood. In addition, tumour vessels lack protective mechanisms that normal vessel acquire during growth. For example, they may lack functional peri-vascular cells, which are needed to protect
vessels against changes in oxygen or hormonal balance, provide them necessary vasoactive control to accommodate metabolic needs, and induce vascular quiescence (Benjamin et al., 1999). Finally, the vessel wall is not always formed by a homogenous layer of endothelial cells (Jain, 1988). Instead, it may be lined with only cancer cells or a mosaic of cancer and endothelial cells. The uncoordinated fashion in which tumour vessels form, generally results in chaotic vascular architecture and blood flow, high vascular permeability, non-uniform expression of surface markers and a lack of functional lymphatics. These characteristics represent a major challenge to cancer treatment since they complicate a targeted delivery of anti-cancer drugs to the tumour.

Since physiological angiogenesis during adulthood only occurs in processes like wound healing and the female reproductive cycle, angiogenesis-inhibitors have been recognised as promising therapeutics to fight cancer. Most anti-angiogenic agents in development target either VEGF-A and its receptors (Avastin, Vatalanib, VEGF-trap, IMC-1C11) or a combination of VEGF receptor with other growth factor receptors like epidermal growth factor receptor, PDGFR or Flt-3 (ZD6474, Sorafenib, Sunitinib). Yet others include MMPs (AE-941).

It remains a fact that angiogenesis is a critical factor in primary tumour growth and metastasis. Recent data supports the efficacy of inhibiting angiogenesis but monotherapy with these agents has not been promising particularly in patients with advanced disease. Currently combination therapy with chemotherapy has demonstrated a clinical benefit.
1.3 The Eph/ephrin gene family

In numerous processes that are vital for the development and maintenance of organism function, cells must communicate crucial information to respond appropriately to the changing environment. As such, receptor tyrosine kinases (RTKs) are transmembrane proteins, which, on receiving an external stimulus, respond by transmitting a signal to the inside of the cell. Of all the RTKs that are found in the human genome, Eph receptors, and their ephrin ligands, constitute the largest family.

Eph receptors have been intensely studied since their discovery nearly two decades ago. Nevertheless, new aspects of their distinctive mechanisms of action continue to emerge rapidly. At first, the main function attributed to this family of receptor tyrosine kinases was to guide growing neuronal processes during development towards their targets through repulsive effects. However, further studies have uncovered additional activities for Eph receptors in many other cell types. Eph/ephrin signalling is a key determinant not only of neural development, but also of cell morphogenesis, tissue patterning, angiogenesis and synaptic plasticity. Additional roles of Eph receptors in the biology of stem cells, immune function and blood clotting are also beginning to emerge.

In vertebrates 10 EphA (EphA1-EphA10) and 6 EphB receptors (EphB1-EphB6) (Murai and Pasquale, 2003; Pasquale, 2004) have been identified to date. The initial subdivision into A- and B-subclasses was based on similarities in the extracellular sequences, but also corresponds to the binding preference for either glycosylphosphatidylinositol anchor (GPI-anchor)-linked ephrin-A ligands or transmembrane ephrin-B ligands (Pasquale, 2004). Receptor-ligand interactions are promiscuous within each class, although binding affinities vary considerably (Himanen et al., 2004; Pasquale, 2004). Exceptions in the binding discrimination between classes are that ephrin-A5, at high concentration, can bind to EphB2, and that some ephrin-B ligands bind to EphA4 (Himanen et al., 2004; Pasquale, 2004).

1.3.1 Structure of Eph receptors

The extracellular part of Eph receptors includes the N-terminal ephrin-binding domain, a cysteine-rich region (containing an epidermal growth factor-like motif) and two
Fibronectin Type III repeats. It is separated by a membrane-spanning segment from the cytoplasmic part, which can be divided into four functional units. The juxtamembrane region that contains two conserved tyrosine residues, a classical protein tyrosine kinase domain, the sterile α-motif (SAM) and a PSD95/Dlg/ZO1 (PDZ)-domain binding motif (Figure 1.5). The structure of the SAM domain (~70 amino acids) indicates that it could form dimers and oligomers. The PDZ-binding motif – formed by the C-terminal four to five amino-acid residues - contains a consensus binding sequence that includes a hydrophobic residue (usually valine or isoleucine) at the very carboxyl terminus. The kinase domain of one receptor from each class (EphA10 and EphB6) lacks residues that are essential for catalytic activity, which indicates that these two receptors might not function by phosphorylating cytoplasmic target proteins (Murai and Pasquale, 2003). More Eph receptor and ephrin variants are generated by alternative splicing and their structures differ from the prototypical domain structure (Zisch and Pasquale, 1997).

1.3.2 Structure of ephrin ligands
Ephrin-B ligands are transmembrane proteins with an extracellular Eph-receptor binding domain that contains several receptor-binding interfaces. This receptor-binding domain is connected by a flexible linker to the transmembrane segment, which is followed by a short conserved cytoplasmic region (Figure 1.5). The cytoplasmic domain of ephrin-B ligands contains five conserved tyrosine residues, of which residues 312, 317 and 332, have subsequently been identified as the main in vivo tyrosine phosphorylation sites of activated avian ephrinB1 from neural tissue (Kalo et al., 2001). The striking conservation of the cytoplasmic domains, especially the last 33 residues, which also contain a PDZ-domain binding motif, strongly suggests a specific functional role for the C-termini of the ephrin-B molecules. Indeed, functional studies have identified the cytoplasmic domain of ephrin-Bs as involved in the bidirectional signalling (Adams et al., 2001; Bruckner et al., 1999; Cowan and Henkemeyer, 2001; Lu et al., 2001). The ephrin-A ligands have a similar extracellular organization but lack a cytoplasmic domain and are attached to the cell surface through a GPI-anchor (Figure 1.5).
1.3.3 Initiation of Eph/ephrin signalling

1.3.3.1 Receptor clustering

Interaction between Eph receptors and ephrins requires cell-cell contact because both families are anchored in the plasma membrane. The ensuing signals propagate bidirectionally into the Eph-receptor-expressing cells (in a process known as forward signalling) and the ephrin-expressing cells (reverse signalling) (Flanagan and Vanderhaeghen, 1998; Kullander and Klein, 2002; Murai and Pasquale, 2003; Wilkinson, 2001).

In contrast to most other RTKs, Eph receptors are activated when they are bound by clustered, membrane-attached ephrin ligands. Since the stoichiometry of Eph-ephrin binding is 1:1 (Lackmann et al., 1997), the minimal active complex should be comprised of two receptors and two ligands to allow clustering of two receptors. The mechanism of clustering has been revealed by structural studies of isolated Eph receptors and ephrins and their complexes (Himanen and Nikolov, 2003). The extracellular domain of ephrin-B2 forms dimers (Toth et al., 2001), which bind two Eph receptors via a high-affinity heterodimerization interface (Himanen et al., 2001). Heterodimers then interact with each other via low-affinity heterotetramerization interfaces and form ring-like structures in which one ephrin binds two receptors and vice versa (Himanen et al., 2001). A number of studies suggest that the degree of further Eph-ephrin clustering is important for the nature of the cellular response (Huynh-Do et al., 1999; Stein et al., 1998) (Figure 1.6). Experimental data from in vitro assays have shown that membrane clustering, which is required for receptor activation, can be mimicked by clustering tagged soluble ligands (Davis et al., 1994).

1.3.3.2 Activation

On ligand engagement, Eph receptors become extensively phosphorylated on tyrosine residues by transphosphorylation and also through Src family kinases that are often associated with the receptors (Kalo and Pasquale, 1999a; Knoll and Drescher, 2004). Phosphorylation promotes kinase activity by disrupting intramolecular inhibitory interactions that occur between the juxtamembrane segment and the kinase domain and by conferring a conformational order on the activation segment of the kinase domain.
that favours substrate binding (Wybenga-Groot et al., 2001; Zisch et al., 2000). Tyrosine phosphorylation by associated Src family kinases also changes the ephrin-B cytoplasmic domain into a conformation that may be more favourable for signalling (Palmer et al., 2002; Song, 2003). In vitro initiation of reverse signalling, monitored by tyrosine phosphorylation of ephrin-B cytoplasmic domain, was shown for primary neurons or ECs stimulated with the soluble ectodomain of Eph receptors (Palmer et al., 2002). Treatment of embryonic chicken retina with fibroblast growth factor (FGF) leads to phosphorylation of endogenous ephrin-B, presumably by the co-expressed FGF receptor (Chong et al., 2000). Likewise, stimulation of the endogenous PDGF receptor in NIH 3T3 fibroblasts that ectopically express ephrin-B1 induces rapid phosphorylation of ephrin-B1, which suggests that a direct interaction might occur between the PDGF receptor and ephrin-B1 (Bruckner et al., 1997).

1.3.3.3 Signal transmission

In addition to regulating kinase activity, tyrosine phosphorylation of Eph receptors and ephrins mediates interactions with signalling molecules that contain Src-homology-2 (SH2) domains (Brantley-Sieders et al., 2004a; Kalo and Pasquale, 1999a; Kullander and Klein, 2002; Murai and Pasquale, 2003; Prevost et al., 2003). Other signalling interactions - such as those with PDZ-domain-containing proteins and Rho-family guanine nucleotide exchange factors - are, in most cases, independent of tyrosine phosphorylation of the receptor (Noren and Pasquale, 2004). Phosphotyrosine phosphatases and the ubiquitin ligase Cbl can also be recruited to Eph/ephrin complexes and contribute to signal termination by dephosphorylation or internalization followed by degradation of the proteins, respectively (Freywald et al., 2002; Kikawa et al., 2002; Walker-Daniels et al., 2002; Wang et al., 2002). A series of elegant studies managed to establish that depending on the morphogenetic process, reverse signalling can be dependent (Cowan and Henkemeyer, 2001) or independent (Lu et al., 2001) of the phosphorylation of the ephrin-B cytoplasmic domain. There are as well examples in which the signalling event does not require the ephrin-B cytoplasmic domain as it has been shown for the migration of neural crest cells (Adams et al., 2001). In contrast, the hindbrain-segment-boundary formation and restriction of cell intermingling as well as the angiogenic remodelling of the embryonic vasculature critically rely on reverse signalling (Adams et al., 2001; Mellitzer et al., 1999; Xu et al., 1999). For the receptor
side, kinase domain-independent signalling events e.g. during the processes of synapse formation and synaptic plasticity in the adult nervous system were documented (Dalva et al., 2000).

1.3.4 Downstream signalling mediators

1.3.4.1 Cytoskeletal regulation

In most cases, Eph-ephrin interactions and signalling results in the rearrangement of the cytoskeleton. Accordingly, many of the downstream signalling cascades and mediators are involved in cytoskeletal dynamics. The Rho family of small GTPases has a central role in controlling the dynamic reorganization of the actin cytoskeleton required for cell migration and adhesion (Hall and Nobes, 2000). This family includes Cdc42, Rac, and Rho. The activity of Rho family GTPases is controlled by guanine nucleotide exchange factors (GEFs) that promote the exchange of GDP to GTP (Malliri and Collard, 2003; Schmidt and Hall, 2002).

The activation and/or direct binding of GEFs is one of the major mechanisms by which Eph receptors and ephrins regulate cell migration (Noren and Pasquale, 2004) and may underlie a key aspect of Eph-ephrin function: the localized regulation of the actin cytoskeleton at sites of cell-cell contact (Marston et al., 2003) that enables a directional attraction or repulsion response. The identification of Ephexin (Eph-interacting exchange protein), a novel GEF, provided a further mechanistic and functional link between EphA receptors and the cytoskeleton (Shamah et al., 2001). A number of GEFs binding to Eph receptors activate different Rho family members, including Rho (Ogita et al., 2003; Shamah et al., 2001), Rac1 (Penzes et al., 2003; Tanaka et al., 2004), and Cdc42 (Irie and Yamaguchi, 2002). In addition, the binding of Eph receptors to adaptor proteins, including Nck, Ras-GAP (Holland et al., 1997), Crk (Lawrenson et al., 2002; Nagashima et al., 2002), and Dishevelled (Tanaka et al., 2003), leads to modulation of Rho family GTPase activity.

Ephrin-B signalling involves some pathways implicated in regulation of the activity of Rho GTPases by Eph receptors, including GEFs (Tanaka et al., 2004), Dishevelled (Tanaka et al., 2003), and the Nck-related adaptor protein Grb4 (Cowan and Henkemeyer, 2001; Su et al., 2004). GEF-activity may also be modulated by Eph
receptors as well as by B class ephrins (Brantley-Sieders et al., 2004b; Maekawa et al., 2003; Schmidt and Hall, 2002; Steinle et al., 2003). In most cases, Eph-ephrin activation concurrently affects Rho, Rac, and Cdc42 and can shift the balance between their activated states. Eph-ephrin modulation of Rho family GTPases in neuronal cells regulates growth cone dynamics (Gallo and Letourneau, 2004), in which a shift of signalling towards RhoA results in growth cone retraction (Gallo et al., 2002; Shamah et al., 2001; Wahl et al., 2000), while prevalence of Rac1 and Cdc42 activity stimulates neurite extension (Tanaka et al., 2004). Similarly, in non-neuronal cells, increased Rac1 activation downstream of Eph receptors promotes spreading and migration (Brantley-Sieders et al., 2004b; Nagashima et al., 2002), and inhibition of Rac1 decreases cell spreading (Batlle et al., 2002; Deroanne et al., 2003). Whereas some GEFs and adaptor proteins that regulate Rho family GTPases, such as Dishevelled and Nck are widely expressed, others have restricted expression that could underlie cell type differences in responses (Ogita et al., 2003; Penzes et al., 2003; Shamah et al., 2001). Furthermore, the specificity of binding of some GEFs to EphA versus EphB receptors (Ogita et al., 2003; Shamah et al., 2001) could mediate the distinct responses to different Eph receptors that can occur within the same cells.

1.3.4.2 Effects on mitogenesis

The mitogen-activated protein kinase (MAPK) pathway is commonly activated by RTKs. This pathway plays a central role in regulation of key developmental processes such as proliferation, differentiation, and cell survival. Unlike many other RTKs, Eph receptors can engage both positive and negative regulators of the MAPK pathway. A positive effect of EphA and EphB receptors on MAPK activity has been found in many cell types. Depending on the cell type, Eph-mediated MAPK activation results in decrease of cell matrix adhesion (Pratt and Kinch, 2002; Zisch et al., 2000), activation of chemotaxis (Vindis et al., 2003), and stimulation of cell proliferation (Yu et al., 2003a) or differentiation (Aoki et al., 2004). On the other hand, EphA and EphB receptors can negatively regulate Ras/MAPK activity, resulting in suppression of cell proliferation (Miao et al., 2003), inhibition of cell matrix adhesion (Zou et al., 1999), and neurite retraction (Elowe et al., 2001).
1.3.5 Signalling read-out

1.3.5.1 Repulsive guidance

A repulsive response involving the retraction of cellular processes was the first discovered outcome of Eph forward signalling, and is a prominent effect in many cell types (Flanagan and Vanderhaeghen, 1998; Knoll and Drescher, 2002; Wilkinson, 2001) (Figure 1.7). Examples include the restriction of neural crest cells and motor axons from the caudal half of somites (Krull et al., 1997; Wang and Anderson, 1997), inhibition of axons from crossing the midline in the vertebrate CNS (Kullander et al., 2001; Williams et al., 2003; Yokoyama et al., 2001), guidance of motor axons in the limbs (Eberhart et al., 2002; Helmbacher et al., 2000) and preventing axons from exiting the CNS in Drosophila (Bossing and Brand, 2002).

1.3.5.2 Formation of tissue boundaries and inhibition of cell mixing

The inhibition of cell mixing between tissues or between distinct tissue domains is essential to maintain organised patterns. The roles of Eph receptors and ephrins in confining actively migrating cells to appropriate pathways, together with the complementary expression of these molecules within many tissues (Gale et al., 1996) may be relevant for many morphogenetic processes during vertebrate development. Using zebrafish animal caps as an experimental model, Mellitzer et al. were able to show that bidirectional activation of EphB2 and ephrin-B1 across an interface of adjacent cell populations inhibits cell intermingling (Mellitzer et al., 1999).

A further way in which Eph-ephrin interactions regulate cell behaviour has been found in studies of vertebrate mesoderm segmentation in which groups of mesenchymal cells aggregate and form epithelial somites. In the fused somites (fss/tbx24) zebrafish mutant, the lack of intersomitic boundaries and epithelial somites is accompanied by a lack of Eph/ephrin signalling interfaces, as this mutant expresses ephrin-B2 but not EphA4 in the presomitic mesoderm. By transplanting EphA4-positive wild-type donor cells into the prospective paraxial mesoderm, it could be demonstrated that boundaries can be induced in fss−/− mutants solely by the restoration of an Eph/Ephrin signalling interface (Barrios et al., 2003).
1.3.5.3 Attractive guidance

Adhesive and attractive responses have been also reported (Dravis et al., 2004; Holmberg et al., 2000), and these are sometimes mediated by the same Eph molecules and occur in the same types of cell. For example, ephrin-B1 can repel or attract different populations of EphB-expressing neural crest cells (Santiago and Erickson, 2002) and collateral branches from retinal axons (Hindges et al., 2002; McLaughlin et al., 2003). A number of studies have shown that Eph receptor activation can increase integrin-mediated adhesion of ECs in culture to ECM (Huynh-Do et al., 1999; Stein et al., 1998). For *in vivo* platelet aggregation, activation of EphA4 or ephrin-B1 synergises with thrombin receptor activation to promote integrin-mediated adhesion of platelets to fibrinogen (Prevost et al., 2002; Prevost et al., 2004).

Furthermore, it could be shown that EphB2 and ephrin-B2 are required for epithelial fusion that separates the urethra endoderm into the urinary and the alimentary tracts (Dravis et al., 2004). The incomplete septation of the urethra in EphB2 and ephrin-B2 mutants leads to a hypospadia phenotype similar to a common human birth defect. EphB2 and ephrin-B2 are coexpressed in cells that meet at the fusion site, suggesting that these regulate an adhesive response. Importantly, the analysis of gene knock-ins in which the cytoplasmic domain of EphB2 or ephrin-B2 is removed reveals that signalling through both components is required for the septation. This suggests a model in which the co-activation of EphB2 and ephrinB2 within the same cells underlies an adhesion response (Dravis et al., 2004).

1.3.5.4 Shaping and positioning of cellular processes

The most studied activity of Eph receptors and ephrins is in the establishment of topographically organized neuronal connections in the developing nervous system. Eph receptors and ephrins are also essential for the guidance of axonal projections that cross the midline of the nervous system. The elaboration of neuronal circuits involves the growth of axonal and dendritic processes towards predetermined targets and the development of specialized connections. The involvement of Eph/ephrin signalling will be discussed here for the example of dendritic spine formation.
During development, dendrites form extensive arborizations that are covered with motile filopodial protrusions. After these dendritic filopodia establish synaptic connections with axonal partners, they are replaced by structures called dendritic spines, which contain the postsynaptic compartment of the synapse (Murai et al., 2003). Mature spines acquire a distinctive mushroom-like shape, which is determined by their actin cytoskeleton and influences synaptic function (Yamaguchi and Pasquale, 2004). The morphological changes that occur during dendritic spine morphogenesis in hippocampal neurons require EphB forward signalling (Ethell et al., 2001; Henkemeyer et al., 2003; Penzes et al., 2003). Additionally, clustering of EphB receptors by ephrins drives the assembly of synaptic components such as neurotransmitter receptors and cytoplasmic PDZ-domain-containing proteins in cultured neurons (Dalva et al., 2000). Other evidence indicates that EphA receptors might regulate dendritic spine remodelling in the adult hippocampus in concert with ephrin-A ligands that are localized on glial cells (Murai et al., 2003). The repulsive surfaces of glial cell processes that surround synapses might therefore provide a framework that restricts spine movements through EphA/ephrin-A signalling. In a recent study, Moeller et al. showed that activation of the EphB receptor tyrosine kinases in cultured hippocampal neurons by their ephrin-B ligands induces morphogenesis of dendritic filopodia into dendritic spines (Moeller et al., 2005). This appears to occur through assembly of an EphB-associated protein complex that includes focal adhesion kinase (FAK), Src, Grb2, and paxillin and the subsequent activations of FAK, Src, paxillin and RhoA. These data suggest that EphB receptors are upstream regulators of FAK in dendritic filopodia, and that FAK-mediated RhoA activation contributes to assembly of actin filaments in dendritic spines.

Eph signals also regulate changes in cell shape outside the nervous system, such as those involved in epithelial branching morphogenesis, capillary sprouting and vascular remodelling.

**1.3.5.5 Eph/ephrin signalling in angiogenesis**

The formation of blood vessels is a striking example of tissue assembly that may involve both adhesive and repulsive responses to Eph/ephrin activation. A number of Eph receptors and ephrins are expressed by ECs (Adams et al., 1999; Daniel et al., 1996; Gerety et al., 1999), and a prominent role is played by EphB4 and ephrin-B2,
which are specifically expressed in veins and arteries, respectively. In addition, EphB and ephrin-B genes are expressed in mesenchymal tissues that ECs interact with (Adams et al., 1999; Gale et al., 1996; Gerety et al., 1999). As will be discussed in further detail in the Results chapter of this thesis, gene targeting experiments have revealed that EphB4 and ephrin-B2 are equally required for angiogenic remodelling (Adams et al., 1999; Gerety et al., 1999; Wang et al., 1998) and that endothelial expression of ephrin-B2 plays a critical role (Gerety and Anderson, 2002). These findings are suggestive of a requirement for activation of ephrin-B2 and/or EphB4 by interactions between endothelial cells to stimulate remodelling and assembly of new vessels (Adams et al., 2001; Cowan et al., 2004). Another role of EphB4/ephrin-B2 interactions may be to prevent fusion of arteries and veins, in particular when they are in close proximity.

Recent work has suggested that remodelling does not require signal transduction via ephrin-B2 (Cowan et al., 2004), and thus activation of EphB4 is sufficient to maintain segregation and enable angiogenesis both in veins and presumably indirectly in arteries. Ephrin-B expression in adjacent tissue can inhibit the formation of blood vessels and thereby confine sprouting to specific places such as between somites (Helbling et al., 2000). In vitro culture experiments indicate that EphB4/ephrin-B2 signalling between ECs and surrounding stromal cells can regulate the proliferation and mobility of ECs (Zhang et al., 2001). The results of these in vitro studies are consistent with diverse adhesive and de-adhesive responses of endothelial cells to Eph/ephrin activation. EphB4 can promote (Maekawa et al., 2003; Steinle et al., 2002) or inhibit (Fuller et al., 2003; Hamada et al., 2003) EC migration and adhesion. Similarly, ephrin-B2 activation has been reported to promote (Fuller et al., 2003; Hamada et al., 2003; Steinle et al., 2003) or inhibit (Zhang et al., 2001) assembly of endothelial cells. In contrast, EphB1 activation (Huynh-Do et al., 2002; Nagashima et al., 2002) and EphA2 activation (Brantley-Sieders et al., 2004b; Cheng et al., 2002) lead only to an increase in cell migration and angiogenic sprouting.

1.3.6 Disengagement of the Eph/ephrin signalling complex

In order to trigger repulsive cell separation, the Eph/ephrin signalling complex has to be disengaged. Several mechanisms allow the separation of two cell surfaces that adhere to
each other through Eph/ephrin contacts. Due to the high binding affinity and multivalency of the Eph/ephrin interactions, dissociation of the complexes does not appear to be feasible (Hattori et al., 2000; Zimmer et al., 2003). Instead, a form of endocytosis removes the adhesive Eph/ephrin complexes from the cell surfaces, allowing the cells to disengage. The internalized vesicles contain intact Eph/ephrin complexes and both of their surrounding plasma membranes (Mann et al., 2003; Marston et al., 2003; Zimmer et al., 2003). It is not known whether Eph/ephrin endocytosis also induces internalisation of other co-localised proteins, promotes the exchange of proteins between cells, or allows Eph/ephrin signalling from the cell interior. Another strategy to eliminate the signalling complexes and allow cell detachment involves proteolytic cleavage of ephrins. Interaction of ephrin-A2 with EphA3 activates the metalloproteinase ADAM10, which cleaves the ephrin extracellular domain (Hattori et al., 2000). Ephrin-B molecules can also be released from the cell surface following intramembrane cleavage by serine proteases of the rhomboid family, although it is not known how this activity is regulated (Pascall and Brown, 2004). It remains to be seen whether the released monomeric ephrins can inhibit further signalling and whether Eph receptor ectodomains might also be cleaved. Cell repulsion might also involve the inactivation of cell-cell and cell-ECM adhesion molecules by Eph receptors. Although there is evidence for crosstalk between Eph receptors and cell-cell adhesion molecules such as L1 and cadherins, the functional consequences remain unclear.

1.3.7 Ephs and ephrins in cancer

Due to their critical involvement in the control of cell migration, Ephs and ephrins have been implicated in cancer. Indeed, the first Eph receptor was identified in an erythropoietin-producing hepatoma cell line (Hirai et al., 1987). Since then numerous studies have reported high levels of Eph receptor expression in a wide variety of tumours (Berclez et al., 1996; Berclez et al., 2002; Easty et al., 1995; Hirai et al., 1987; Kiyokawa et al., 1994; Liu et al., 2002; Miyazaki et al., 2003; Saito et al., 2004; Wicks et al., 1992). In most of these cases there is a correlation between the degree of malignancy and the level of Eph expression. Moreover, high levels of Eph expression correlate with a poor degree of tumour differentiation and high degree of metastasis. A major question remains regarding the nature of the cellular responses caused by Eph
receptor overexpression. Eph receptor activation might enable invasion of ephrin-expressing adjacent tissues, or might lead to repulsion within the tumour tissue so that cells emigrate. Based upon their role in development, it can be predicted that loss of Eph or ephrin expression could similarly lead to invasion of adjacent tissue.

1.4 Conclusion and the aim of the thesis

In conclusion, the studies described here have shown that ephrin-B2 and its receptor, EphB4, are involved in early angiogenic remodelling of the embryo, but whether endothelial ephrin-B2 expression is essential in processes of blood vessel maturation and maintenance of the arterio-venous identity remains to be determined. The aim of the thesis was to use an inducible overexpression system to characterise of the role of ephrin-B2 in the vascular endothelium at progressed stages of embryonic development.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Model</th>
<th>Vascular Phenotype</th>
<th>Refs</th>
</tr>
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<td>Mouse</td>
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<td>(Monkley et al., 1996)</td>
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<td>Wnt3</td>
<td>Mouse</td>
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<td>Frizzled-5</td>
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<td>β-Catenin</td>
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<td>Indian hedgehog</td>
<td>ES/Mouse</td>
<td>Limited ability to remodel vasculature in yolk sac</td>
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<td>Sonic You</td>
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Table 1.1: Gene targeting experiments with vascular phenotypes (not exhaustive)
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<th>Signalling pathway</th>
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<td><strong>Cell–substrate adhesion</strong></td>
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<td>Shp2↑—FAK↓</td>
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<td>EphB2</td>
<td>R-Ras↓</td>
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<td>Cdc42↓, Rac1↓</td>
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<td>H-Ras↓—MAPK↓</td>
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<td>Vascular smooth muscle cell spreading↓</td>
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<td>Rac1↓—Pak1↓</td>
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<td>Grb4—FAK↑?</td>
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<tr>
<td><strong>Cell–substrate adhesion↑</strong></td>
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<td>Integrin-mediated adhesion↑</td>
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<td>TAK1/MKK4/MKK7↑—JNK↑</td>
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<td>Nck–NIK↑—JNK↑</td>
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<td>p110α/PI3K↑</td>
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<td>β1-integrin-mediated adhesion↑</td>
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<td>Src-family kinases↑</td>
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<td>Fyn↑—β-integrin↑—MAPK↑,↑—MAPK↑</td>
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<td>Focal complex stabilization↑</td>
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<td>Cas—Crk—Rap1↑</td>
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<td>Cell protrusions↓, Cell migration↓</td>
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<td>EphB</td>
<td>Cdc42↓, Rac1↓</td>
<td>(Miao et al., 2005)</td>
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<td>FGF-dependent endothelial cell migration and proliferation↑</td>
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<td>Syndecan-1 expression↑</td>
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<td>Chemotaxis through G-protein-coupled CXCR4 receptor↑</td>
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<td>PDZ-RGS↓—Gpsubunit↓</td>
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<td>p110α/PI3K↑</td>
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<td>Grb7</td>
<td>(Han et al., 2002)</td>
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<td>Cell migration↑</td>
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<td><strong>Cell invasion↑</strong></td>
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<td>Stat3↑</td>
<td>(Yuan et al., 2004b)</td>
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<td>Membrane ruffling↑</td>
<td>EphB1</td>
<td>Cas—Crk—Rac1↑</td>
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<td><strong>Endothelial cell migration↑</strong></td>
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<td>Endothelial cell migration↑</td>
<td>EphA2</td>
<td>PI3K↑—Rac1</td>
<td>(Brantley-Sieders et al., 2004b)</td>
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<tr>
<td>Endothelial cell migration↑</td>
<td>EphB</td>
<td>Src↑, PI3K↑—Akt</td>
<td>(Steinle et al., 2002)</td>
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<td>Endothelial cell migration↑, angiogenesis↑</td>
<td>EphB</td>
<td>PI3K↑</td>
<td>(Maekawa et al., 2003)</td>
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<td>Endothelial cell migration↑</td>
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<td>PI3K↑—Akt↑—MAPK↑</td>
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<td>PDZ-protein—JNK↑?</td>
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<td><strong>Axon growth and guidance</strong></td>
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<td>Retinal growth cone collapse↑</td>
<td>EphA4</td>
<td>Ephexin↑—RhoA↑</td>
<td>(Shamah et al., 2001) (Shah et al., 2005)</td>
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<td>Retinal growth cone collapse↑</td>
<td>EphA</td>
<td>Src↑, PI3K↑—nonclassical PKC↑</td>
<td>(Wong et al., 2004)</td>
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<td>Retinal and DRG growth cone collapse↑, endocytosis↑</td>
<td>EphA</td>
<td>Rac1↓ — Rac1↑</td>
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<td>Retinal growth cone collapse↑, endocytosis↑</td>
<td>EphA</td>
<td>Vav2↓—Rac1↑?</td>
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<td>Retinal growth cone repulsion↑</td>
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<td>Src-family kinases↑</td>
<td>(Knoll and Drescher, 2004)</td>
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<td>Retinal and DRG axon retraction↑</td>
<td>EphA</td>
<td>RhoA↑—Rho-kinase↑—F-actin turnover↑</td>
<td>(Gallo et al., 2002)</td>
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<tr>
<td>Neurite outgrowth↑</td>
<td>EphA2</td>
<td>Tiam1↑—Rac1↑</td>
<td>(Tanaka et al., 2004)</td>
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Table 1.2 (continued on next page)
<table>
<thead>
<tr>
<th>Outcome of the signal</th>
<th>Eph or ephrin</th>
<th>Signalling pathway</th>
<th>Refs</th>
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<tbody>
<tr>
<td>Neurite outgrowth</td>
<td>Ephrin-B1</td>
<td>Tiam1†  -Rac1†</td>
<td>(Tanaka et al., 2004)</td>
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<td>Neurite outgrowth</td>
<td>Ephrin-A5</td>
<td>Fyn†-β-integrin†-MAPK†, ?-MAPK†</td>
<td>(Davy et al., 2000; Davy and Robbins, 2000)</td>
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<tr>
<td>Microtubule redistribution in growth cones</td>
<td>Ephrin-B</td>
<td>Microtubule destabilizing protein SGC10†</td>
<td>(Suh et al., 2004)</td>
</tr>
</tbody>
</table>

**Dendritic spines**

| Dendritic spine morphogenesis                      | EphB          | N-WASP/Intersectin†-Cdc42†               | (Irie and Yamaguchi, 2002)                |
| Dendritic spine morphogenesis                      | EphB          | Kallrin†-Rac1†-Pak1†                   | (Penzes et al., 2003)                     |
| NMDA-receptor-dependent Ca²⁺ influx                | EphB          | Src†-NMDA-receptor†                    | (Takasu et al., 2002)                     |

**T-cells**

| T-cell chemotaxis                                | EphA          | Cdc42†, RhoA†                           | (Sharfe et al., 2002)                     |
| T-cell responses                                 | EphB6         | Rac1†-JNK†                              | (Freywald et al., 2003)                   |
| T-cell responses                                 | EphB6         | p38MAPK†                                | (Luo et al., 2002)                        |
| T-cell responses                                 | EphB6         | ZAP70†-LAT-MAPK†                       | (Luo et al., 2004)                        |
| T-cell responses                                 | EphB          | MAPK†, p38MAPK†                        | (Yu et al., 2003a; Yu et al., 2003b)      |

**Other**

| EphB endocytosis                                 | EphB6         | Rac1†-dynamin†                          | (Marston et al., 2003)                    |
| Ephrin-B endocytosis                             | Ephrin-B      | Dynamin†                                | (Parker et al., 2004b)                    |
| Endocytosis                                     | EphB          | Synaptopodin 1†                         | (Irie et al., 2005)                       |
| Endothelial cell proliferation                   | EphB          | PI3K†-Akt†                              | (Steinle et al., 2002)                    |
| Endothelial cell proliferation                   | Ephrin-B2     | Srl, PI3K†, MAPK†                      | (Steinle et al., 2003)                    |
| Sprouting angiogenesis                           | Ephrin-B      | Src-family kinases†                     | (Palmer et al., 2002)                     |
| Angiogenesis of lymphatic vessels                | Ephrin-B2     | PDZ-domain-containing protein           | (Makinke et al., 2005)                    |
| Vascular smooth muscle cell contractility        | EphA4         | Vsm-RhoGEF†-RhoA†                      | (Ogita et al., 2003)                      |
| Neurogenesis                                    | EphA          | Rap1†-MAPK†                             | (Aoki et al., 2004)                       |
| Dopaminergic neuronal fate                       | EphB1         | Transcription factor Nurr1†              | (Calo et al., 2005)                       |
| Astrocyte hypertrophy, proliferation, migration  | EphA4         | RhoA†                                    | (Goldshmit et al., 2004)                  |
| Glial scar formation                            | EphA4         | Jak2†-Stat3†                            | (Lai et al., 2004)                        |
| Cell sorting in frog animal caps                 | Ephrin-B1     | Dishevelled-RhoA†-Rho kinase†            | (Tanaka et al., 2003)                     |

Table 1.2: Eph receptor and ephrin signalling pathways that affect cell behaviour (Pasquale, 2005)

Only signalling pathways for which there is evidence that they affect cell behaviour are included. Most of the evidence for the signalling pathways is through in vitro cell culture experiments. Proteins shown to be binding partners or targets of Eph receptors are listed in several other reviews (Brantley-Sieders et al., 2004a; Kalo and Pasquale, 1999b; Kullander and Klein, 2002; Prevost et al., 2003). Cas, Crk-associated substrate; DRG, dorsal root ganglion; FAK, focal adhesion kinase; GEF, guanine nucleotide-exchange factor; Grb, growth-factor-receptor-bound; Jak, Janus kinase; JNK, Jun N-terminal kinase; LAT, linker for T-cell activation; MAPK, mitogen-activated protein kinases Erk1 and Erk2; MKK, MAPK kinase; NIK, Nck-interacting kinase; NMDA, N-methyl-D-aspartate; Pak, p21-activated protein kinase; PDZ-RGS, PDZ-domain-containing regulator of G-protein signalling; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; Shc, Src-homology-2 (SH2) domain-containing; Shp2, SH2-domain-containing protein tyrosine phosphatase; Stat, signal transducer and activator of transcription; TAK, HIV Tat-associated kinase; WASP, Wiskott–Aldrich syndrome protein; ZAP, zeta-chain-associated kinase, 70 kDa; †, increased activity or activity required; ‡, decreased activity; ↔ indicates a temporal sequence; ††, in vivo evidence.
<table>
<thead>
<tr>
<th>Eph or ephrin</th>
<th>Other signalling molecule</th>
<th>Outcome of the crosstalk</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eph</td>
<td>Integrins, receptor tyrosine kinases</td>
<td>MAPK activation</td>
<td>(Noren and Pasquale, 2004)</td>
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<tr>
<td>EphA</td>
<td>SDF-1-chemokine–CXCR4 receptor</td>
<td>T-cell chemotaxis</td>
<td>(Sharpe et al., 2002)</td>
</tr>
<tr>
<td>EphA</td>
<td>Fibronectin, laminin</td>
<td>Changes in growth cone turning</td>
<td>(Weinil et al., 2003)</td>
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<tr>
<td>EphA2</td>
<td>E-cadherin</td>
<td>EphA2 expression†, EphA2 activation, change in EphA2 subcellular localization</td>
<td>(Orsulic and Kemler, 2000; Zantek et al., 1999)</td>
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<td>EphA4</td>
<td>IFN-γ, LIF</td>
<td>Activation of astrocytes†</td>
<td>(Goldsmith et al., 2004)</td>
</tr>
<tr>
<td>EphB</td>
<td>Syndecan-2</td>
<td>Dendritic spine morphogenesis†</td>
<td>(Ethell et al., 2001)</td>
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<td>EphB</td>
<td>NMDA receptor</td>
<td>Clustering of NMDA receptors†, Ca2+ fluxes†</td>
<td>(Dalva et al., 2000; Takasu et al., 2002)</td>
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<td>EphB</td>
<td>TrkA neurotrophin receptor</td>
<td>Tyrosine phosphorylation of ARMS†</td>
<td>(Kong et al., 2001)</td>
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<td>EphB</td>
<td>Inflammatory cytokines, heparanase</td>
<td>Switch from anti-angiogenic to pro-angiogenic effects</td>
<td>(Yuan et al., 2004a)</td>
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<td>EphB2</td>
<td>L1</td>
<td>L1 tyrosine phosphorylation†</td>
<td>(Zisch et al., 1997)</td>
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<td>EphB2, EphB3</td>
<td>Ryk receptor</td>
<td>*Palate fusion?</td>
<td>(Trivier and Ganesan, 2002)</td>
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<td>EphB6</td>
<td>T-cell receptor</td>
<td>Co-clustering†, co-stimulation of T cells†</td>
<td>(Luo et al., 2002)</td>
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<td>EphB6</td>
<td>EphB1 receptor</td>
<td>EphB6 transphosphorylation†</td>
<td>(Freywald et al., 2002)</td>
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<td>Ephrin-A2</td>
<td>ADAM10 metalloprotease</td>
<td>Ephrin-A2 cleavage</td>
<td>(Hattori et al., 2000)</td>
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<td>Ephrin-B</td>
<td>Rhomboid family serine proteases</td>
<td>Ephrin-B cleavage</td>
<td>(Pascall and Brown, 2004)</td>
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<td>Ephrin-B</td>
<td>L1, Laminin</td>
<td>Changes in growth cone guidance</td>
<td>(Suh et al., 2004)</td>
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<td>Ephrin-B1</td>
<td>FGF receptor</td>
<td>*Cell–cell adhesion†</td>
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<td>FGF receptor</td>
<td>*Commitment to retinal cell fate†</td>
<td>(Moore et al., 2004)</td>
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<td>Ephrin-B1</td>
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<td>Chemoattraction in cerebellar neurons†</td>
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<td>Ephrin-B1</td>
<td>PDGF receptor</td>
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<td>(Bruckner et al., 1997)</td>
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<td>Tie2 receptor</td>
<td>Ephrin tyrosine phosphorylation†</td>
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<td>Ephrin-B2</td>
<td>Metabotropic glutamate 1 receptor</td>
<td>mGlur receptor-mediated stimulation of polyphosphoinositide hydrolysis†</td>
<td>(Calo et al., 2005)</td>
</tr>
</tbody>
</table>

**Table 1.3**: Crosstalk between Eph-ephrin pathways and other signalling pathways (Pasquale, 2005)

ADAM, a disintegrin and metalloprotease; ARMS, ankyrin repeat-rich membrane spanning; CXCR4, CXC chemokine receptor-4; FGF, fibroblast growth factor; IFN, interferon; LIF, leukemia inhibitory factor; mGlur receptor, metabotropic glutamate receptor; MAPK, mitogen-activated protein kinase; NMDA, N-methyl-D-aspartate; PDGF, platelet-derived growth factor; SDF-1, stromal-cell-derived factor-1. †, increased; †, decreased; *, in vivo evidence.
<table>
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<tr>
<th>Angiogenic Factors</th>
<th>Anti-angiogenic Factors</th>
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<tr>
<td>Acidic and basic fibroblast growth factor (aFGF, bFGF)</td>
<td>Interferons (IFN-α, IFN-β)</td>
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<td>Vascular endothelial growth factor family and their</td>
<td>Interleukins (IL-12, IL-18)</td>
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<tr>
<td>receptors (VEGF, VEGFR), Neuropilins, Semaphorins</td>
<td>Pigment epithelium-derived factor (PEDF)</td>
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<td>Platelet derived growth factor BB and receptors (PDGF-</td>
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<tr>
<td>BB, PDGFR)</td>
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<tr>
<td>Angiopoietins and their receptor (Ang1, Ang2 and Tie-2)</td>
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<tr>
<td>Hepatocyte growth factor (HGF)</td>
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<td>Eph receptors and their ephrin ligands</td>
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<td>Notch receptors and their ligands</td>
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<td>Insulin-like growth factor (IGF-1)</td>
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<td>Transforming growth factor-β1 (TGF-β1)</td>
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<td>Tumour necrosis factor-a (TNF-α)</td>
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<td>Interleukins (IL-1, IL-4, IL-6, IL-8)</td>
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<td>Sonic hedgehog (Shh)</td>
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<td>Endocrine gland-derived vascular endothelial growth</td>
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<td>factor (EG-VEGF)</td>
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<td>Prostaglandins (PGD-E1, PGD-E2)</td>
<td>Tissue inhibitor of metalloproteases (TIMP-1,</td>
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<td>Heparin</td>
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<td>Adhesion molecules (integrins: αvβ3, αvβ5, α5β1,</td>
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<td>Certain Matrix Metalloproteinases (MMPs)</td>
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<td>Plasminogen activators</td>
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<td>HIF-1α</td>
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<td>NOS, COX-2</td>
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<td>Bradykinin</td>
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<td>Histamine</td>
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<tr>
<td><strong>Table 1.4:</strong> Pro-and anti-angiogenic factors</td>
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</table>

The following table lists factors which assemble into a primitive vascular network (angiogenesis) and recruits smooth muscle cells (SMC) to cover the endothelial tube. This process results in a mature vascular network comprising of arterioles, capillaries and veins.
Figure 1.1: Formation of new blood vessels (Carmeliet and Collen, 2000)

Angioblasts differentiate into endothelial cells which assemble into a primitive vascular network (vasculogenesis). This first network matures via different modes of vascular growth (angiogenesis) and recruits smooth muscle cells (SMC) to cover the endothelial tubules. This process results in a mature vascular network comprising of established arteries, capillaries and veins.
Figure 1.2: Multiple steps and factors involved in angiogenesis (Distler et al., 2003)

First steps of angiogenesis involve vasodilation and increased vascular permeability with exudation of high molecular weight plasma proteins such as fibrinogen. After detachment of vascular smooth muscle cells (SMC), the vessel wall is destabilised and perivascular extracellular matrix (ECM) is degraded. This enables endothelial cells (EC) to proliferate and migrate along gradients of chemotactic agents in the hypovascularised areas. Once the target region has been reached, the ECs assemble and form tube-like structures. Finally, mesenchymal cells (MC) proliferate and differentiate into pericytes (PC) or vSMCs or that form the outer layers of the vessel wall.
Figure 1.3: Matrix transitions during angiogenesis (Kalluri, 2003)

Angiogenesis is associated with degradation and reformation of the vascular basement membrane (VBM). a) In response to growth factors and MMPs, the VBM undergoes degradative and structural changes. This transition from mature VBM to provisional matrix promotes the proliferation and migration of vascular endothelial cells. Growth factors, such as VEGF, bFGF and PDGF, are released from the BM, and are also produced by tumour cells, fibroblasts and immune cells. b) This induces formation of an intermediate, and then a new (mature) VBM. Together with the vascular endothelial cells and pericytes, the VBM mediates formation of a new blood vessel. The degraded VBM during this process has a crucial role in regulating angiogenesis.
Figure 1.4: Blood vessel wall composition of nascent versus mature vessels (Jain, 2003)

a) Nascent vessels consist of a tube of ECs. b) Capillaries, the most abundant vessels in our body. c) Arterioles and venules have an increased coverage of mural cells compared with capillaries. d) The walls of larger vessels consist of three specialized layers: an intima composed of endothelial cells, a media of SMCs and an adventitia of fibroblasts, together with matrix and elastic laminae.
Figure 1.5: General features of Eph receptors and ephrins (Kullander and Klein, 2002)

A schematic diagram showing an ephrin-expressing cell (top) interacting with an Eph-expressing cell (bottom). Structural domains and tyrosine phosphorylation sites (P) are indicated.
Figure 1.6: Activation of Eph/ephrin signalling by clustering (Pasquale, 2005)

a) Heterodimerisation of an Eph receptor with an ephrin is mediated by their high-affinity interface (indicated in red) and leads to intercellular adhesion. b) Tetramerisation occurs by the binding of two heterodimers through their low-affinity interfaces (in orange and dark blue). The proximity of two receptor kinase domains leads to transphosphorylation on tyrosine residues (pink circles), which causes conformational changes in the juxtamembrane segment that promote signalling. The ephrin membrane-proximal cytoplasmic segment also changes conformation after becoming phosphorylated by Src-family kinases.
Figure 1.7: Signalling components of activated Eph receptors and ephrins (adapted from Boyd and Lackmann, 2001)

An updated list of Eph/ephrin signalling pathways affecting cell behaviour and examples for crosstalks between Eph/ephrin and other signalling pathways can be found in tables 1.2 and 1.3.
# Chapter 2 | Materials and methods

## Materials

### 2.1 Chemicals and reagent

With the exception of those listed below, all chemicals/reagents were purchased from Sigma-Aldrich or BDH/Merck. Reagents were of analytical grade or the highest grade available.

<table>
<thead>
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<th>Chemicals &amp; Reagents</th>
<th>Company</th>
</tr>
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<td>Promega</td>
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<td>Mowiol®</td>
<td>Calbiochem</td>
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<td>Fluoromount®</td>
<td>Southern Biotechnology Associates, Inc.</td>
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<tr>
<td>Trypsin/EDTA</td>
<td>Invitrogen Life Technologies</td>
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<td>BCIP (X-phosphate), 4-toluidine salt, solution 50mg/ml</td>
<td>Boehringer Mannheim</td>
</tr>
<tr>
<td>NBT solution 100mg/ml</td>
<td>Boehringer Mannheim</td>
</tr>
<tr>
<td>Shandon Cryomatrix™</td>
<td>Thermo Electron Co.</td>
</tr>
<tr>
<td>X-Gal</td>
<td>Novabiochem</td>
</tr>
</tbody>
</table>

---

(Other buffer and solutions were prepared as described below.)
### 2.2 Buffers and solutions

All buffers and solutions were prepared as described below.

<table>
<thead>
<tr>
<th>Buffer &amp; Solutions</th>
<th>Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-agar</td>
<td>1% bacto-tryptone, 0.5% yeast extract, 170mM NaCl, 15% agar</td>
</tr>
<tr>
<td>L-broth</td>
<td>1% bacto-tryptone, 0.5% yeast extract, 170mM NaCl</td>
</tr>
<tr>
<td>Upper gel buffer (UGB) 4x</td>
<td>0.5M Tris-HCl, 0.4% SDS, pH 6.8</td>
</tr>
<tr>
<td>Lower gel buffer (LGB) 4x</td>
<td>1.5M Tris-HCl, 0.4% SDS, pH 8.8</td>
</tr>
<tr>
<td>SDS gel running buffer 10x</td>
<td>0.25M Tris, 1.92M Glycine, 1% SDS, pH 8.3</td>
</tr>
<tr>
<td>SDS gel transfer buffer 1x</td>
<td>0.025M Tris, 0.192M Glycine, 10% Methanol v/v</td>
</tr>
<tr>
<td>Sample buffer 2x</td>
<td>20% Glycerol v/v, 3% SDS w/v, 25mM TrisHCl, 0.05% Bromophenolblue w/v, 10mM DTT</td>
</tr>
<tr>
<td>PBS</td>
<td>140mM NaCl, 2.5M KCl, 10mM Na₂HPO₄ (pH7.2), 1.5mM KH₂PO₄</td>
</tr>
<tr>
<td>PBS-T</td>
<td>PBSA + 0.1% Tween-20</td>
</tr>
<tr>
<td>PCR Buffer 10x (pH 8.3)</td>
<td>100mM Tris-HCl, 15mM MgCl₂x6H₂O, 500mM KCl</td>
</tr>
<tr>
<td>PLC lysis buffer</td>
<td>50mM HEPES, 150mM NaCl, 10% Glycerol, 0.1% Triton X-100, 1.5mM MgCl₂, 1mM EGTA, 1% NP-40, 100mM NaF, 10mM Na₃P₂O₇, 1mM Na₃VO₄, Protease Inhibitor Cocktail (SIGMA P2714) 1:100</td>
</tr>
<tr>
<td>Triton Tris lysis buffer</td>
<td>10mM Tris pH 8.0, 1% Triton X-100 v/v</td>
</tr>
<tr>
<td>HBAH</td>
<td>0.5mg BSA, 0.1% NaN₃, 20mM HEPES pH 7.2, in Hank’s BBS (GIBCO)</td>
</tr>
<tr>
<td>Alkaline phosphatase substrate buffer (for AP-fusion protein binding assay)</td>
<td>2M Diethanolamine pH 9.8, 1mM MgCl₂, 200mg PNPP (P-nitrophenylphosphate disodium)</td>
</tr>
<tr>
<td>Alkaline phosphatase substrate solution (for embryo alkaline phosphatase whole-mount staining)</td>
<td>0.15mg/ml BCIP, 0.3mg/ml NBT, 2mM levamisole in alkaline phosphatase buffer</td>
</tr>
<tr>
<td>Alkaline phosphatase buffer (for embryo alkaline phosphatase whole-mount staining)</td>
<td>100mM Tris, 100mM NaCl, 50mM MgCl₂</td>
</tr>
<tr>
<td>Tail Buffer</td>
<td>50mM Tris-HCl (pH8.0), 100mM NaCl, 100mM EDTA, 1% SDS</td>
</tr>
<tr>
<td>TBE Running Buffer (1x)</td>
<td>89mM Tris base, 89mM boric acid, 2mM EDTA</td>
</tr>
<tr>
<td>TE</td>
<td>10mM Tris-HCl (pH 8.0), 1mM EDTA (pH 8.0)</td>
</tr>
</tbody>
</table>
2.3 Enzymes

All enzymes were obtained from New England Biolabs with the following exceptions.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calf intestinal alkaline phosphatase (CIP)</td>
<td>Roche</td>
</tr>
<tr>
<td>DNase I (RNase free)</td>
<td>Roche</td>
</tr>
<tr>
<td>DNA Polymerase I (Klenow)</td>
<td>Amersham Biosciencesc</td>
</tr>
<tr>
<td>Pfu Turbo® DNA Polymerase</td>
<td>Stratagene</td>
</tr>
<tr>
<td>Taq Polymerase</td>
<td>Qiagen</td>
</tr>
<tr>
<td>Proteinase K</td>
<td>Gibco BRL</td>
</tr>
<tr>
<td>RNA polymerases (T3, T7, Sp6)</td>
<td>Roche</td>
</tr>
<tr>
<td>RNase A</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

2.4 Immunoreagents

All antibodies were used at the concentrations indicated below. Applications: IHC immunohistochemistry; IP immunoprecipitation; IB immunoblot.
<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Isotype</th>
<th>Dilution</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Smooth Muscle Actin (SMA) non-conjugated Clone 1A4 (Cat.No. A2547)</td>
<td>Mouse (IgG)</td>
<td>1:400 IHC</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>α-SMA-Cy3 Clone 1A4 (Cat.No. C6198)</td>
<td>Mouse (IgG)</td>
<td>1:400 IHC</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>α-SMA-FITC Clone 1A4 (Cat.No. F3777)</td>
<td>Mouse (IgG)</td>
<td>1:400 IHC</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Collagen IV (Cat.No. AB756P)</td>
<td>Rabbit (IgG)</td>
<td>1:200 IHC</td>
<td>Chemicon</td>
</tr>
<tr>
<td>Desmin Clone D33 (Cat.No. M0760)</td>
<td>Mouse (IgG)</td>
<td>1:25 IHC</td>
<td>DAKO</td>
</tr>
<tr>
<td>Endomucin Clone V.7C7AK</td>
<td>Rabbit (IgG)</td>
<td>1:100 IHC</td>
<td>Gift from D. Vestweber</td>
</tr>
<tr>
<td>EphB2 (Cat.No. AF467)</td>
<td>Goat (IgG)</td>
<td>1:200 IHC</td>
<td>R&amp;D Systems</td>
</tr>
<tr>
<td>EphB4 (Cat.No. AF446)</td>
<td>Goat (IgG)</td>
<td>1:200 IHC</td>
<td>R&amp;D Systems</td>
</tr>
<tr>
<td>Ephrin-B2 (Cat.No. AF496)</td>
<td>Goat (IgG)</td>
<td>1:100 IHC</td>
<td>R&amp;D Systems</td>
</tr>
<tr>
<td>Fibronectin (Cat.No. F3648)</td>
<td>Rabbit (IgG)</td>
<td>1:200 IHC</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>FlagM2 (Cat.No. F3165)</td>
<td>Murine Cell Culture (IgG)</td>
<td>1:800 IP</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>GFP (Cat.No. A11122)</td>
<td>Rabbit (IgG)</td>
<td>1:500 IHC</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>Laminin α5 Clone 4G6</td>
<td>Rat (IgG)</td>
<td>1:500 IHC</td>
<td>Fiebinger Center</td>
</tr>
<tr>
<td>PECAM-1(CD31) Clone:MEC13.3</td>
<td>Rat (IgG)</td>
<td>1:100 IHC</td>
<td>PharMingen</td>
</tr>
<tr>
<td>Phospho-Histone H3 (Cat.No. 06-570)</td>
<td>Rabbit (IgG)</td>
<td>1:100 IHC</td>
<td>Upstate</td>
</tr>
<tr>
<td>Phospho-Tyrosine Clone 4G10</td>
<td>Mouse (IgG)</td>
<td>1:1000 IB</td>
<td>Upstate</td>
</tr>
<tr>
<td>Secondary Antibody</td>
<td>Isotype</td>
<td>Dilution</td>
<td>Company</td>
</tr>
<tr>
<td>-------------------------</td>
<td>---------</td>
<td>----------</td>
<td>------------------</td>
</tr>
<tr>
<td>Alexa Fluor® 488</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Anti-Rat IgG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-Mouse IgG</td>
<td>Goat</td>
<td>1:500</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>Anti-Rabbit IgG</td>
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<td></td>
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</tr>
<tr>
<td>Alexa Fluor® 546</td>
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<tr>
<td>Anti-Rat IgG</td>
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<td></td>
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<tr>
<td>Anti-Mouse IgG</td>
<td>Goat</td>
<td>1:500</td>
<td>Molecular Probes</td>
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<tr>
<td>Anti-Rabbit IgG</td>
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<td>Alexa Fluor® 350</td>
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<tr>
<td>Anti-Rat IgG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-Mouse IgG</td>
<td>Goat</td>
<td>1:500</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>Anti-Rabbit IgG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-Rat IgG conjugated</td>
<td>Goat</td>
<td>1:5000</td>
<td>Amersham</td>
</tr>
<tr>
<td>Anti-Mouse IgG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HRP conjugated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-Rabbit IgG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HRP conjugated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-Goat IgG conjugated</td>
<td>Donkey</td>
<td>1:5000</td>
<td>Amersham</td>
</tr>
<tr>
<td>Anti-Goat IgG conjugated</td>
<td>Rabbit</td>
<td>1:7000</td>
<td>Sigma</td>
</tr>
<tr>
<td>Anti-Goat IgG conjugated</td>
<td>Rabbit</td>
<td>1:300</td>
<td>Vector</td>
</tr>
</tbody>
</table>
## 2.5 Miscellaneous

The following table lists the remaining products that were used.

<table>
<thead>
<tr>
<th>Product</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Molecular Biology</strong></td>
<td></td>
</tr>
<tr>
<td>Oligonucleotides</td>
<td>Sigma Genosys</td>
</tr>
<tr>
<td>QIAGEN® Plasmid Maxi Kit</td>
<td>Qiagen</td>
</tr>
<tr>
<td>QIAprep® Spin Miniprep Kit</td>
<td>Qiagen</td>
</tr>
<tr>
<td>QIAquick® Gel Extraction Kit</td>
<td>Qiagen</td>
</tr>
<tr>
<td>QIAquick® PCR Purification Kit</td>
<td>Qiagen</td>
</tr>
<tr>
<td><strong>Cell Culture</strong></td>
<td></td>
</tr>
<tr>
<td>DMEM</td>
<td>Invitrogen Life Technologies</td>
</tr>
<tr>
<td>Foetal Calf Serum</td>
<td>Invitrogen Life Technologies</td>
</tr>
<tr>
<td>L-Glutamine 200 mM (100x)</td>
<td>Invitrogen Life Technologies</td>
</tr>
<tr>
<td>Penicillin-Streptomycin (100x)</td>
<td>Invitrogen Life Technologies</td>
</tr>
<tr>
<td>FuGENE 6™</td>
<td>Roche</td>
</tr>
<tr>
<td>PBS endotoxin-free</td>
<td>Cancer Research UK</td>
</tr>
<tr>
<td>Tissue culture plastic-ware</td>
<td>Corning, Falcon and NUNC</td>
</tr>
<tr>
<td><strong>Biochemistry</strong></td>
<td></td>
</tr>
<tr>
<td>30% Acrylamide/Bis Solution, 29:1</td>
<td>BioRad</td>
</tr>
<tr>
<td>Full-Range™ Rainbow Molecular Weight Markers</td>
<td>Amersham Biosciences</td>
</tr>
<tr>
<td>Phosphatase Inhibitor Cocktail I (P2850)</td>
<td>Sigma</td>
</tr>
<tr>
<td>Phosphatase Inhibitor Cocktail II (P5726)</td>
<td>Sigma</td>
</tr>
<tr>
<td>Protease Inhibitor Cocktail (P2714)</td>
<td>Sigma</td>
</tr>
<tr>
<td>DC Protein Assay (500-0116)</td>
<td>Biorad</td>
</tr>
<tr>
<td><strong>Immunohistochemistry</strong></td>
<td></td>
</tr>
<tr>
<td>ApopTag® Red In Situ Apoptosis Detection Kit (S7165)</td>
<td>Intergen</td>
</tr>
</tbody>
</table>
2.6 List of suppliers and distributors

<table>
<thead>
<tr>
<th>Company</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abcam Limited</td>
<td>Cambridge, UK</td>
</tr>
<tr>
<td>Applied Biosystems</td>
<td>Foster City, CA, USA</td>
</tr>
<tr>
<td>BD Biosciences</td>
<td>Oxford, UK</td>
</tr>
<tr>
<td>Boehringer Mannheim GmbH</td>
<td>Mannheim, Germany</td>
</tr>
<tr>
<td>Cambridge Biosciences</td>
<td>Cambridge, UK</td>
</tr>
<tr>
<td>Corning</td>
<td>Schiphol-Rijk, NL</td>
</tr>
<tr>
<td>DakoCytomation</td>
<td>Cambridgeshire, UK</td>
</tr>
<tr>
<td>Dynal Biotech UK</td>
<td>Wirral, UK</td>
</tr>
<tr>
<td>Invitrogen Life Technologies</td>
<td>Paisley, UK</td>
</tr>
<tr>
<td>Promega</td>
<td>Southampton, UK</td>
</tr>
<tr>
<td>Qiagen</td>
<td>West Sussex, UK</td>
</tr>
<tr>
<td>Novabiochem</td>
<td>Nottingham, UK</td>
</tr>
<tr>
<td>New England Biolabs</td>
<td>Hitchin, UK</td>
</tr>
<tr>
<td>Peprotech EC Ltd.</td>
<td>London, UK</td>
</tr>
<tr>
<td>Perkin-Elmer Co.</td>
<td>Foster City, CA, USA</td>
</tr>
<tr>
<td>R&amp;D Systems Inc.</td>
<td>Minneapolis, CA, USA</td>
</tr>
<tr>
<td>Sigma-Aldrich Company</td>
<td>Dorset, UK</td>
</tr>
<tr>
<td>Strathegic Scientific Limited</td>
<td>Cambridgeshire, UK</td>
</tr>
<tr>
<td>Stratagene</td>
<td>Amsterdam, NL</td>
</tr>
<tr>
<td>Thermo Electron Co.</td>
<td>Pittsburgh, PA, USA</td>
</tr>
<tr>
<td>Vector Laboratories Inc.</td>
<td>Burlingame, CA, USA</td>
</tr>
<tr>
<td>WWR International Ltd</td>
<td>Dorset, UK</td>
</tr>
</tbody>
</table>

4.7.3 Preparation of plasmid DNA

To screen colonies following ligation and transformation, single colonies were picked and inoculated in 5ml LB media containing the appropriate antibiotics, then grown overnight on a 37°C agitator. Half of the overnight culture was used for small-scale preparation of plasmid DNA, whilst the remainder was stored at 4°C. The QIAprep Spin Miniprep Kit was used for small-scale plasmid purification according to the manufacturer’s instructions. DNA was eluted in TE or sodium acetate buffer (Qiagen) and stored at -20°C. For large-scale plasmid preparations, 1/4 of the overnight culture was added to 2-4ml of glycerol plus antibiotics and incubated overnight at 37°C in an agitator. Extracts were prepared by centrifugation at 6000g for 15 min in a Beckman J211 centrifuge. The plasmid DNA was then purified using a QIAprep Spin Kit according to manufacturer’s instructions.
Methods

2.7 Preparation, manipulation and analysis of plasmid DNA

2.7.1 Enzymatic manipulation of DNA fragments
Restriction enzymes and T4 DNA ligase were purchased from NEB and used for restriction digestions and DNA ligations, respectively. DNA polymerase Klenow fragment (NEB) and dNTPs (Gibco BRL) were used to blunt end DNA fragments. Calf intestinal alkaline phosphatase or shrimp alkaline phosphatase (Boehringer Mannheim) were used to remove 5’ phosphate groups from DNA fragments.

2.7.2 Bacterial transformation
XL1-Blue MRF’ Supercompetent Cells (Stratagene) were used for cloning of DNA. 0.1μg DNA from ligations or 1ng of plasmid DNA was added to a 50μl aliquot of competent cells on ice and left for 30 minutes. The transformation was performed according to the manufacturer’s instructions. In brief, the bacteria were heat shocked at 42°C for 45 seconds before being placed back on ice for 1 minute. 500μl of L-broth was then added to the bacteria which were subsequently cultured for 60 minutes at 37°C with shaking at 150rpm. 100-300μl of bacterial culture was spread out using glass beads on to L-agar plates containing 100μg/ml of ampicillin or 50μg/ml of kanamycin.

2.7.3 Preparation of plasmid DNA
To screen colonies after ligation and transformation, single colonies were picked and inoculated in 5ml LB media containing the appropriate antibiotic, then grown overnight in a 37°C agitator. 2ml of the overnight culture was used for small scale preparation of plasmid DNA whilst the remainder was stored at 4°C. The QIAprep® Spin Miniprep Kit was used for small-scale plasmid purification according to the manufacturer’s instructions. DNA was eluted in TE or elution buffer (Qiagen) and stored at -20°C. For large-scale maxi preparations, 0.5ml of the overnight culture was added to 150ml LB plus antibiotic and incubated overnight at 37°C in an agitator. Bacteria were pelleted by centrifugation at 6000rpm for 15 min in a Beckman J2-21 centrifuge. The plasmid DNA was then purified using a QIAGEN® Maxi Kit according to manufacturer’s instructions.
DNA was dissolved in TE or elution buffer (Qiagen) to a concentration of 1mg/ml and stored at -20°C.

2.7.4 Quantification of nucleic acids

DNA was diluted in 10mM Tris-HCl pH 8.5 or water and placed in a quartz cuvette. Absorbance at 260nm and 280nm was read in a spectrometer with the correct respective DNA program set-up.

2.7.5 Purification of DNA from agarose gels

The percentage of agarose used in a gel was dependent on the size of DNA fragment that was to be purified: a 0.5kb fragment was typically run on a 2% gel whilst fragments of 3kb and larger were run on 1% or 0.8% gels. 1kb and 100bp DNA ladders (NEB) were run alongside to determine the fragment size. The gel slice containing the correct fragment was removed using a clean scalpel. DNA was then purified from the agarose using the QIAquick® Gel Extraction Kit according to the manufacturer’s instructions. DNA was resuspended in 10mM Tris-HCl pH 8.5 or TE.

2.7.6 Polymerase Chain Reaction (PCR)

Amplification of DNA products for cloning was performed using Stratagene’s Pfu Turbo PCR kit. Reactions were carried out according to the manufacturer’s instructions.

2.7.7 Sequencing of DNA

All DNA sequencing was performed by the DNA Sequencing Service (Cancer Research UK) using the ABI® BigDye™Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) according to the manufacturer’s instructions. Briefly, 0.5µg template DNA was added to 3.2pmol sequencing primer and 8µl BigDye™Terminator mix in a total volume of 20µl. PCR was carried out as recommended by the manufacturer and DNA products were ethanol precipitated at room temperature for 15 minutes by addition of 300µl 95% ethanol, 5µl 3M sodium acetate (pH4.5) and 80µl dH2O. After centrifugation at 13000rpm for 20 minutes, the pellet was washed with
500μl 70% ethanol, vortexed briefly, centrifuged for 5 minutes as described above, air dried, and then stored at -20°C until loading in the polyacrylamide sequencing gel.

2.8 Tissue culture and biochemical techniques

2.8.1 Transfection of tissue culture cells

HEK-293 (human embryonic kidney) cells were cultivated in DMEM medium supplemented with 10% FCS, L-Glutamine and Penicillin-Streptomycin in a 5% CO₂ tissue culture incubator (Heraeus HERAcell™) at 37°C. Depending on the experimental setting the transfection was either performed after the Calcium-Phosphate (CaPO₄) method or by using FuGENE 6 transfection reagent (Roche) according to the manufacturer’s instructions.

The CaPO₄ method was used for transfections in 10cm tissue culture dishes. Optimal transfection results were obtained with cell confluences between 30-50%. In brief, 10-15μg DNA were mixed with 500μl 0.25M CaCl₂ in a 1.5ml microtube. To this phosphate-DNA complex 500μl of 2xBBS (50mM BES, 280mM NaCl, 1.5mM Na₂HPO₄, pH 6.95) were added, gently vortexed and incubated at RT for 15 minutes. In the meantime the culture medium was removed from the cells and replaced by fresh complete medium. The calcium phosphate-DNA solution was then added drop-wise to the medium whilst swirling the plate. After 12-14 hours of incubation at 37°C and 3% CO₂, the transfection medium was removed, the cells once washed with endotoxin-free PBS and then incubated for further 24-36 hours with fresh complete medium at 37°C and 5% CO₂. The cells were used for immuno precipitation assays after having grown to 90% cell confluence.

MEF/3T3 Tet-OFF™ cells (Clontech Cat. No. C3018-1) were transfected in 6-well plates using FuGENE 6 transfection reagent (Roche) according to the manufacturer’s instructions.
2.8.2 Immunoprecipitation

90% confluent HEK-293 cells in 10cm tissue culture dishes were once washed with ice-cold endotoxin-free PBS, lysed on ice in 1ml PLC lysis buffer for 20-30 minutes and then harvested using a cell scraper. In order to precipitate the cell debris, the cell suspension was transferred to a cooled 1.5ml microtube, briefly vortexed and then centrifuged for 20 minutes at 13000rpm and 4°C. The supernatant was transferred to a new cooled 1.5ml microtube and the protein concentration was determined using the Lowry method according to the DC Protein Assay (Biorad 500-0116) in an Ultrospec 3100 pro spectrophotometer (Amersham Pharmacia BIOTECH AB). 2mg/ml total protein in 800µl lysis buffer were typically used for immunoprecipitation assays. To clear the cell lysate from proteins which non-specifically bind to Protein A or G sepharose, the samples were rotated for 1 hour at 4°C with 40µl of either sepharose beads type, depending on the antibody subtype used for the immunoprecipitation. After 5 minutes centrifugation at 5000rpm and 4°C, the cleared supernatant was transferred to a new cooled 1.5ml microtube. 35µl fresh sepharose beads and 1µg IP antibody were added to the sample and rotated overnight at 4°C. The precipitated proteins bound to the sepharose beads were washed 3 times in 1ml PLC lysis buffer and then denatured by boiling for 5 minutes in 40µl 2x sample buffer. The samples were then subjected to SDS-polyacrylamide gel electrophoresis.

For the immunoprecipitation from isolated murine organs, tissue lysates were prepared on ice in 0.5ml PLC lysis buffer in a 2ml round bottom microtube using a tissue homogeniser (Ultra-Turrax® T25 basic; IKA®-WERKE). After 30 minutes lysis on ice the cell debris was precipitated by centrifugation at 13000rpm and 4°C. All other steps were performed according to the protocol above.

2.8.3 SDS-polyacrylamide gel electrophoresis

Immunoprecipitated proteins were separated according to their size, shape and electrical charge using denaturing SDS-polyacrylamide gel electrophoresis. Typically 7 or 10 % polyacrylamide gels were used. To prepare a 10% resolving gel the following solutions were mixed together and immediately transferred into 1.5mm Invitrogen® Gel Cassette placed in a XCell SureLock™ Electrophoresis Cell (Novex®).
<table>
<thead>
<tr>
<th>10% Resolving Gel</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>2.90ml</td>
</tr>
<tr>
<td>4x Lower gel buffer LGB</td>
<td>1.75ml</td>
</tr>
<tr>
<td>30% Acrylamide/Bis Solution, 29:1</td>
<td>2.40ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>100μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>10μl</td>
</tr>
</tbody>
</table>

This gel mix was overlaid with 400μl isopropanol to generate an even gel front. After 30 minutes the gel was polymerised and the isopropanol was aspirated from the top. To prepare a 4% stacking gel, the following solutions were mixed together, subsequently pipetted onto the polymerised resolving gel and a 1.5mm 10 slot gel coomb was inserted.

<table>
<thead>
<tr>
<th>4% Stacking Gel</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>3.25ml</td>
</tr>
<tr>
<td>4x Upper gel buffer UGB</td>
<td>1.25ml</td>
</tr>
<tr>
<td>30% Acrylamide/Bis Solution, 29:1</td>
<td>0.55ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>40μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>20μl</td>
</tr>
</tbody>
</table>

After 30 minutes the stacking gel was polymerised, the coomb was removed and the resulting gel pockets briefly flushed with 1x SDS gel running buffer.

At this stage the upper and lower chamber of the electrophoresis cell were supplied with appropriate amounts of 1x SDS gel running buffer and the protein samples were loaded into the gel pockets using a Hamilton® Microliter™ Syringe. 10μl of Full-Range™ Rainbow Molecular Weight Markers (Amersham Biosciences RPN800) were pipetted into the front gel pocket in order to determine the correct molecular weight of the separated proteins in Western Blot analysis. To electrophoretically separate the protein samples, an initial current of 20mA (BIORAD® POWER PAC 3000) was applied, which was increased to 40mA after the samples had entered the resolving gel. The separated proteins were then transferred to a nitrocellulose membrane.
2.8.4 Western blotting

In preparation of the Semi-dry protein transfer the stacking gel was removed and the resolving gel soaked in 1x gel transfer buffer for 5 minutes. The transfer was performed in a Hoefer® Large Semiphor Transphor® Unit (Amersham Pharmacia, Biotech AB Serial No. 20067041) which was assembled as follows. 2 sheets of gel size Whatman® 3MM chromatography paper (Cat. No. 3030917), followed by 1 sheet Hybond™ ECL™ nitrocellulose membrane, both pre-soaked in 1x gel transfer buffer, were placed onto the bottom part (anode) of the electrophotography cassette and further wetted by adding 10ml 1x gel transfer buffer. The resolving gel was placed air bubble free on the nitrocellulose membrane and covered with 2 more sheets of pre-soaked Whatman® paper before the cassette lid (cathode) was lowered onto the assembled transfer layers. The protein transfer was performed for 2 hours at a current of 70mA per gel.

After the transfer, the electrophotography cassette was disassembled and the nitrocellulose membrane immersed in 5% dry milk powder in PBS-T for 1 hour at RT on an orbital shaker to block non-specific binding sites. This blocking step was followed by brief washing in PBS-T for 5 minutes. The membrane was then incubated with the primary antibody, diluted in blocking solution, for a minimum of 1 hour which was followed by a wash step in 3 changes of PBS-T for 10 minutes each. The incubation with the peroxidase labelled secondary antibody, diluted in blocking solution, was performed at RT for 30 minutes. The membrane was then washed again as before. For the detection equal volumes of ECL Detection Reagents 1 and 2 to give sufficient liquid to cover the membrane were mixed. Excess wash buffer was drained from the washed blots and the protein side uppermost placed on a sheet of SaranWrap. The prepared ECL detection reagent was then added directly to the side carrying the protein and incubated for 1 minute. Excess detection reagent was then drained off and the blot placed protein side down onto a fresh piece of SaranWrap. The SaranWrap was folded over the back of the blot to form an envelope. The blot was then placed, protein side up, in a film cassette. In the darkroom with switched-off lights a sheet of autoradiography film (Hyperfilm™ ECL™) was placed on top of the blot, the cassette closed and exposed for 1 minute. The film was then removed and developed in an IGP Compact2 developer. If required a second piece of film was exposed for an appropriate amount of time.
2.9 Generation of TetO-ephrin-B2 transgenic line

2.9.1 Cloning of ephrin-B2-ECFP cDNA
A DNA fragment encoding for the Enhanced Cyan Fluorescent Protein (ECFP) was inserted in frame into an ephrin-B2 cDNA using a unique endogenous ClaI restriction site located in the extracellular domain of the ligand. Additionally to the 5’ and 3’ ClaI sites, a full length FLAG sequence (GAC TAC AAG GAC GAC GAT GAC AAG) was included in the 3’ primer designed to PCR-amplify the ECFP-cDNA. The FLAG-tag was used in combination with an anti-FLAG M2 antibody to facilitate biochemical assays with the ephrin-B2 fusion protein. To verify the correct orientation of the ECFP-tag the resulting construct was sequenced. The correct localisation of fusion protein to the cell membrane was shown by exciting the internal ECFP tag with the appropriate ECFP filter using a Leica DM IRBE microscope 24 hours after transfection of the ephrin-B2-ECFP cDNA into HEK-293 cells. Furthermore the correct size of the fusion protein was verified by immunoprecipitation and Western blot analysis from transfected HEK-293 cells.

2.9.2 Protein binding assay with alkaline phosphatase fusion proteins
To determine and compare the binding capacities to EphB4 receptor, a protein binding assay with the generated ephrin-B2 fusion protein and the wild-type ephrin-B2 ligand was performed. For this purpose HEK-293 cells grown in 6-well tissue culture plates were transfected with either ephrin-B2-ECFP or wild type ephrin-B2 using the FuGENE 6 transfection reagent (Roche) according to the manufacturer’s instructions. 24 hours after the transfection the cell medium was aspirated and the cells twice rinsed with pre-warmed HBAH. The washed cells were then incubated with 1ml 0.5nM EphB4-AP (cell culture supernatant containing 0.1% NaN₃, buffered with 10mM HEPES) for 60 minutes at RT. EphB4-AP is a fusion protein consisting of the EphB4 receptor extracellular domain fused to the enzyme alkaline phosphatase. After a washing step with 2 changes of HBAH, the cells were lysed in 0.5ml Triton-Tris lysis buffer per well for 5 min. The cells were then collected and the well rinsed with further 0.4ml lysis buffer which was then added to the cell lysate. The samples were vigorously vortexed, incubated for 5 minutes at RT, again vortexed and then centrifuged at 13000rpm for 5 minutes. The
supernatant was transferred to a new 1.5ml microtube and incubated for 10 minutes at 65°C in a water bath in order to heat-inactivate endogenous alkaline phosphatase.

To assess the amount of EphB4-AP bound to the 2 different expressed ephrin-B2 ligands, an enzymatic reaction was used in which the alkaline phosphatase fused to EphB4 receptor catalyses a colorimetric reaction in AP-substrate buffer. To initiate this colour reaction 100μl AP-substrate buffer were added to 100μl of the heat inactivated lysate in a UV penetrable 96-well plate. The kinetics of the reaction were read in a SpectraMAX® Plus spectrophotometer (Molecular Devices; PO2243) at 405nm for 10-15min.

2.9.3 Whole-mount staining of embryos with alkaline phosphatase fusion proteins

Embryos at embryonic day 10.5 (E10.5) were dissected in ice-cold PBS and all extra-embryonic membranes removed. The PBS was then aspirated, the AP- fusion protein added (10nM AP fusion protein as cell culture supernatant, 10% goat serum, 0.02% NaN₃) and the embryos incubated at 4°C O/N gently shaking. The AP-solution was removed and the embryos washed in PBS for 2 hours. The embryos were briefly fixed (60% acetone, 3% formaldehyde, 20mM HEPES, pH7.0) followed by a PBS wash step. To inactivate endogenous alkaline phosphatase the embryos were incubated at 65°C for 20 minutes. The PBS was removed, replaced with alkaline phosphatase buffer and incubated for 5 minutes at RT. The staining reaction was performed in AP-substrate solution for a minimum of 4 hours at RT or O/N at 4°C gently shaking. To stop the colour reaction the AP-substrate solution was replaced with 1mM EDTA in PBS. The embryos were then post-fixed in 4% paraformaldehyde for 24 hours at 4°C.

2.9.4 Generation of TetO-ephrin-B2 expression construct

Ephrin-B2-ECFP cDNA was inserted into the tTA/rtTA responsive reporter plasmids pUHD10-3 (M. Gossen) using a unique BamHI restriction site. To verify the correct orientation of the ECFP tag the resulting construct was sequenced. The expression and correct localisation of the fusion protein was shown by transfection into MEF/3'T3 Tet-OFF™ cells (Clontech Cat. No. C3018-1), stably expressing the tetracycline-controlled
transactivator tTA. The correct localisation of fusion protein to the cell membrane was shown by exciting the internal ECFP tag with the appropriate ECFP filter using a Leica DM IRBE microscope 24 hours after transfection. The TetO-ephrin-B2 construct was then linearised with the restriction enzymes HindIII and XhoI and subsequently used for pronuclear injections into fertilised mice zygotes.

2.9.5 TetO-ephrin-B2 founder screening

The genomic insertion of TetO-ephrin-B2 was confirmed using an ECFP-specific genotyping PCR. Six identified founder were bred to the inducer lines Tie-2 tTA7770 (U. Deutsch, Berne, Germany) and VE-Cadherin tTA (L. Benjamin, Boston, USA). The transgene expression was confirmed by immunoprecipitation from isolated tissues. Based on TetO-ephrin-B2 protein expression levels and consistency of the exhibited phenotype founder 1 was chosen for further analysis.

2.9.6 Induction of TetO-ephrin-B2 expression in vivo using the Tet-ON expression system

Expression of TetO-ephrin-B2 using the Tet-ON system in older embryos was induced by addition of 2mg/ml doxycycline and 3% sucrose to the drinking water of pregnant females. The doxycycline water was kept protected from light and exchanged daily.

2.10 Analysis of genomic DNA

2.10.1 Isolation of genomic DNA from mouse tissue

Tail snips or yolk sacs were stored at -20°C until ready for processing. The mouse sample was incubated in 500µl Tail Buffer with freshly added Proteinase K at 100µg/ml. Digestion was carried out at 55°C overnight. 250µl of 6M NaCl was added and mixed for 5 minutes on an Eppendorff Mixer and the sample was centrifuged at 13000rpm for 7 minutes after which the supernatant was decanted into a fresh 1.5ml microtube containing 500µl isopropanol and mixed as before. After 7 minutes of centrifugation at 13,000rpm, the supernatant was decanted and the remaining pellet was
washed with 300 μl 70% ethanol and centrifuged as before. The pellet was then air dried and dissolved in 150μl of sterile water.

### 2.10.2 Genotyping Mouse Genomic DNA by PCR

Qiagen Taq polymerase was used for genotyping of mouse genomic DNA via PCR. A master mix was made up according to the number of samples and a typical cycling program is described in the table below.

<table>
<thead>
<tr>
<th></th>
<th>Volume (μl)</th>
<th>Temperature (°C)</th>
<th>Time (Minutes)</th>
<th>No. of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Template</td>
<td>1.0</td>
<td>94</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>PCR Buffer 10x</td>
<td>2.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dNTPs (2.5 mM of each)</td>
<td>2.5</td>
<td>94</td>
<td>1</td>
<td>30-35</td>
</tr>
<tr>
<td>Primer 1 (20μM)</td>
<td>1.25</td>
<td>Annealing Temp.</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Primer 2 (20μM)</td>
<td>1.25</td>
<td>72</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Pli Taq Polymerase</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distilled Water</td>
<td>16</td>
<td>72</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td>25</td>
<td>4</td>
<td>Forever</td>
<td></td>
</tr>
</tbody>
</table>

Embryos and mice were routinely genotyped by PCR using the primers listed below. All primers were manufactured by Sigma-Genosys.
<table>
<thead>
<tr>
<th>Mouse Line</th>
<th>Primer</th>
<th>Sequence 5' to 3'</th>
<th>Anneal. Temp.</th>
<th>Fragment Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tie-2-LacZ</td>
<td>Forward</td>
<td>HHFW3 GCC TAT GAG AGG ATA CCC CTA TTG</td>
<td>62°C</td>
<td>~720 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>LZREV1 GGA ACA AAC GGC GGA TTG ACC G</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tie-2 tTA 7770</td>
<td>Forward</td>
<td>HHFW1 GCA TAC CAT ACA TAG GTG GAG G</td>
<td>62°C</td>
<td>~608 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>rTAREV1 AAT GGC TAA GGC GTC GAG CAA A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TetO-ephrin-B2</td>
<td>Forward</td>
<td>CFP-5' ATG GTG AGC AAG GGC GAG GAG</td>
<td>63°C</td>
<td>~740 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CFP-3' CTT GTA CAG CTC GTC CAT GCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VE-Cadherin tTA</td>
<td>Forward</td>
<td>VETTA For GAC GCC TTA GCC ATT GAG AT</td>
<td>60°C</td>
<td>~333 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Tet7BHSDK LacZ</td>
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<td>LacZ FW4 CCG TCA CGA GCA TCA TCC TC</td>
<td>60°C</td>
<td>~608 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>LacZ REV4 GAC GAA ACG CCT GCC AGT ATT TAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>X-lacZ4</td>
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<td>PLaCZ-E CAAT ACT ATG CAG ACT CTG GAA GTG</td>
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<td>~600 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>PLaCZ-3 CAA ACT CAT CAA TGT ATC TTA TCA TGT C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immorto</td>
<td>Forward</td>
<td>Immos GCA TTG CCT GGA ACG CAG TGA GT</td>
<td>60°C</td>
<td>~380 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>Immoas GAA CAG ACT GTG AGG ACT GAG GG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tie-2 rtTA</td>
<td>Forward</td>
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<td>62°C</td>
<td>~566 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>M2 REV2 GCT TTT GAG CGA GTT TCC TTG TCG</td>
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<td></td>
</tr>
</tbody>
</table>
2.11 Histology, lacZ stainings, immunohistochemistry

2.11.1 Fixation and paraffin-embedding of tissues for sectioning
Mouse tissues were dissected and fixed overnight in 4% paraformaldehyde (PFA- made in PBS at pH 7.4) at 4°C. They were then washed sequentially in the following solutions: 30 minutes in PBS at 4°C, 30 minutes in 0.9% NaCl at 4°C, 2 x 15 minutes in 0.9% NaCl in 50% ethanol at RT, 2x 30 minutes in 70% ethanol in PBS at RT (tissues can be stored at this stage at 4°C), 60 minutes in 85% ethanol in PBS at RT, 60 minutes in 95% ethanol in PBS at RT, 3 x 60 minutes in 100% ethanol at RT, 2 x 30 minutes in Xylene at RT, overnight in Xylene at RT, 60 minutes in Paraffin/Xylene (1:1 ratio) at 56 °C, 4x 60 minutes in Paraffin at 56°C. The samples were finally transferred into embedding moulds filled with hot paraffin and allowed to solidify overnight at RT before cutting. The paraffin blocks were stored at RT. For sectioning, the moulds were mounted on the microtome chuck, sections were cut at a thickness of between 5-7μm, mounted onto coated glass slides, dried at 42°C and stored at RT.

2.11.2 Fixation and embedding of tissues for cryosectioning
Mouse tissues were freshly dissected, transferred to plastic tubes of appropriate size and immediately snap-frozen in liquid nitrogen. The tissues were then placed in embedding moulds filled with Shandon Cryomatrix™ and the moulds were gradually frozen on dry ice and stored at -80°C. For sectioning, the moulds were mounted on a cryostat chuck and frozen sections were cut at about -26°C at a thickness of between 7-10μm. The sections were thaw-mounted onto coated glass slides and stored at -80°C.

2.11.3 Immunohistochemical stainings on paraffin sections
General immunohistochemical stainings used on sections are described here. Additional steps were required for stainings using the CD31/PECAM-1 antibody are also included and are labelled as such. Slides were subjected to the initial treatments as follows: 2 x 10 minutes in Xylene, 2 minutes sequentially in 100% ethanol, 95%, 90%, 80%, 70%, 50%, 30% and finally 5 minutes in PBS. For PECAM-1 stainings, and these additional treatment steps were performed as follows: 60 minutes in PBS at 65°, 5 minutes in PBS at RT, 10 minutes in 0.3% H₂O₂ in methanol (all steps from here onwards are performed...
in a humid chamber), 2 minutes in PBS, 12 minutes in 0.1% Trypsin in PBS, 5 minutes in 0.1μg/ml Trypsin Inhibitor in PBS, 20 minutes in 2M HCl, and 2 minutes in PBS. All stainings proceeded as follows: 60 minutes in blocking solution (1% serum from the species in which the secondary antibody was made and 0.1% BSA in PBS), minimum 2 hours or overnight incubation in blocking solution containing primary antibodies at 4°C, 3 x 10 minutes in PBS, 30 minutes in blocking solution containing secondary antibodies, 3 x 10 minutes in PBS, 10 minutes in DAPI solution (nuclear marker, stock at 10mg/ml, 1:1000 dilution in PBS). Coverslips were mounted onto the tissue using Fluoromount® or Mowiol®.

2.11.4 Immunohistochemical stainings on paraffin sections using the Batlle Protocol
Detection of ephrin-B2 and EphB4 on paraffin sections was performed with goat anti-Ephrin-B2 (1:100), and anti-EphB4 (1:200) antibodies (R & D Systems), based on the protocol as described in Batlle et al., (2002), with modifications. In brief, following dewaxing and hydration, sections were pre-treated with peroxidase blocking buffer (120mM Na₂HPO₄, 43 mM citric acid, 30 mM NaN₃, 0.2% H₂O₂, pH 5.8) for 20 minutes at RT. Antigen retrieval was performed by boiling the samples in Na-citrate buffer (10 mM, pH 6.0) for 20 minutes. After boiling, the samples were allowed to cool down slowly to RT. The specimens were then blocked with 1% BSA in wash buffer (0.05M Tris pH7.5, 0.3M NaCl, 0.1% Tween-20) for 60 minutes. Incubation of antibodies was performed in 1% BSA in wash buffer overnight at 4°C. A bridging step was applied through the incubation of the samples with a rabbit anti-goat antibody for an hour at RT. This was followed by 30 minute incubation at RT, in the anti-rabbit HRP-conjugated polymer supplied in the Envision+ kit (Dako). Stainings were developed for 6 minutes at RT using the peroxidase substrates including tyramide-FITC.

2.11.5 Immunohistochemical stainings on cryosections
Slides were transferred from -80°C directly into 100% acetone at RT and fixed for 10 minutes. The slides were then air dried and washed in PBS (2 x 5 minutes). A SuperHT PAP-pen was used to create a liquid-repellent barrier surrounding the sections. The
tissues on the slides were blocked with 1% BSA and 1% serum from the species in which the secondary antibody was made in PBS for 30 minutes at RT. This was followed by the incubation of tissues with the primary antibodies (diluted in blocking solution) for 2 hours at RT. The slides were washed with PBS (3 x 10 minutes) and then incubated with secondary antibodies (diluted in blocking solution) for 30 minutes at RT, followed by another wash with PBS (3 x 10 minutes). The tissues were counterstained with the nuclear marker DAPI (10mg/ml, 1:1000 dilution in PBS) for 10 minutes at RT. Slides were then mounted onto the coverslips with Fluoromount® or Mowiol®.

2.11.6 Whole-mount immunohistochemistry with PECAM-1 antibody
This procedure was only required for whole-mount embryos using PECAM-1 antibody and was performed as follows. Embryos were dissected and fixed in 4% PFA overnight. The embryos were then subjected to the following treatments: 3 x 5 minutes in PBS, sequential dehydration for 15 minutes each in 25%, 50%, 75%, 100% methanol in PBS (embryos can be stored in 100% methanol at -20°C), 5 hours in 5% H₂O₂ in methanol (changing solution every hour), 2 x 15 minutes in 100% methanol, sequential rehydration for 15 minutes each in 75%, 50%, 25% methanol in PBS, 2 x 15 minutes in PBS, 2 hours in PBSMT (3% non-fat dry milk powder and 0.1% TritonX-100 in PBS), overnight incubation with PECAM-1 antibody diluted in PBSMT at 4°C, 5 x 1 hour washes in PBSMT at 4°C, overnight incubation with biotinylated secondary anti-rat antibody (diluted in PBSMT) at 4°C, 5 x 1 hour washes in PBSMT at 4°C. This was followed by overnight incubation in ABC-Reagent (Avidin-Biotin Complex 1:100 in PBSMT) at 4°C, 5 x 1 hour washes in PBSMT at 4°C, 1 hour in PBT (0.2% BSA and 0.1% TritonX-100 in PBS) at RT, incubation of embryos in DAB staining solution (DAB Staining Kit) in the dark at RT until sufficient staining has developed, 2 x 10 minutes in PBT, 2 x 10 minutes in PBS, overnight fixation in 2% PFA and 0.1% glutaraldehyde at 4°C, 3 x 5 minutes in PBS, 1 hour clearing in 50% glycerol in PBS, followed by transfer to 70% glycerol in PBS and storage at 4°C.
2.11.7 Apoptosis detection on paraffin and cryo sections
The detection of apoptotic cells on paraffin and cryo sections was achieved by using the ApopTag®Red In Situ Apoptosis Detection Kit (Intergen S7165) according to the manufacturer's instructions.

2.11.8 Whole-mount immunofluorescence staining of skin
Whole-mount skin samples were fixed in 4% PFA overnight, washed in PBS and subsequently blocked and permeabilised by incubation in 1% goat serum and 1% BSA in PBS-T for 2 hr at 4°C. Samples were incubated with primary antibodies in the same solution overnight at 4°C with gentle agitation. They were then washed four times for 1 hour each in PBS-T, followed by incubation with secondary antibodies for either 4 hours at RT or overnight at 4°C in 1% goat serum and 1% BSA in PBS-T. They were then washed four times for 1 hour as before, in PBS-T. The samples were mounted in-between glass slides and coverslips using Fluoromount® or Mowiol® mounting medium. The slides were flattened with a weight and finally analysed on a Leica DM IRBE microscope connected to a Hamamatsu Orca-ER® monochromatic camera or a Zeiss LSM510 Meta confocal microscope.

2.11.9 LacZ staining for the detection of β-galactosidase activity in transgenic tissues
Whole embryos (stages E9.5 to 13.5) or tissues and organs of both embryos and adults were dissected and fixed in LacZ fix solution (0.2% glutaraldehyde, 5mM EGTA, 2mM MgCl₂ in PBS) for 10-15 minutes, at room temperature on a rocker. The samples were washed several times (3 x 5 minutes) in wash buffer (2mM MgCl₂, 0.02% NP-40 in PBS). They were then incubated in LacZ staining buffer (containing 1mg/ml X-Gal, 2.12mg/ml potassium ferrocyanide, 1.64mg/ml potassium ferricyanide in wash buffer) at 37°C, until the desired level of staining had been obtained. The samples were then washed twice in PBS, post-fixed with 4% PFA overnight. After a brief wash in PBS the specimen were either directly photographed (whole embryos) using a Leica MZ FL III stereo microscope connected to a Photometrics Cool Snap® colour camera or embedded in Fluoromount® (skin samples) and then documented.
2.12 Hematoxilin-Eosin Staining

Slides were subjected to the initial treatments as follows: 2 x 10 minutes in Xylene, 2 minutes sequentially in 100% ethanol, 95%, 90%, 80%, 70%, 50% and 30%. This was followed by 30 seconds incubation in Hematoxilin and subsequent wash steps first in 2 x dH2O for 1 minute, 2 x tap water for 5 min and finally dH2O for 30 seconds, moving the slide-rack up and down. The Eosin staining was performed for 12 seconds and stopped by moving the slide-rack up and down for 5 times each in 95% EtOH and then 100% EtOH. After 2 x 1 minute incubations in Xylene the slides were air dried and then mounted in Entellan (CN Biosciences 107961).

2.13 Electron microscopy

Skin samples were fixed in 2.5% glutaraldehyde in Sorensens Phosphate buffer pH 7.4, post fixed in Osmium tetroxide in the same buffer and given to the Electon Microscopy Unit for processing. In brief, the samples were embedded in araldite, sections were cut on a Reichert Ultracut®, stained with lead citrate and uranyl acetate and analysed using a JEOL 1010 electron microscope.
Chapter 3 | Inducible overexpression of ephrin-B2 in the vascular endothelium

3.1 Introduction

The cardiovascular system is the first organ system that develops and reaches a functional state in the vertebrate embryo. The term “vasculogenesis” refers to the initial steps when the endothelial cell precursors, the angioblasts, differentiate from the mesenchyme to form a juvenile vascular system. This primary capillary plexus is subsequently remodelled into a mature vascular system by pruning and reorganisation processes, which are commonly summarised under the term angiogenesis. This complex remodelling process involves the recruitment of mural cells to stabilise the vessels and starts in the vasculature of many organs. Mural cell recruitment becomes critical during late embryonic development (Nehls and Drenckhahn, 1993) but the molecular mechanism is poorly understood.

Recent work in the mouse has demonstrated important roles for ephrin-B2 and its cognate EphB receptors in vascular development. Reciprocal expression of ephrin-B2 and EphB4 in arterial and venous endothelial cells, respectively, suggested ephrin-B2/EphB4 interactions at the arterial–venous interface (Wang et al., 1998; Adams et al., 1999; Gerety et al., 1999). Consistently, knockout mice lacking either ephrin-B2 or EphB4 die during embryogenesis and display similar cardiovascular defects (Wang et al., 1998; Gerety et al., 1999). Angiogenic remodelling in mutant yolk sac and embryos was defective, resulting in arrest at the primitive capillary plexus and embryonic lethality around embryonic day E10.5. Due to the early lethality of the ephrin-B2 knockout and the phenotypic similarity to EphB4 null-mice it was so far not possible to understand the role of ephrin-B2 in most processes of vascular development.

In the course of this study a gain-of-function approach was used to gain more insight into the function of ephrin-B2 during vascular morphogenesis. For this purpose, I generated an in vivo model in which ephrin-B2 was overexpressed on arterial and ectopically expressed on venous endothelial cells. This model allowed me to study the role of ephrin-B2 in establishing and maintaining the arterio-venous identity. Results
also indicate an involvement of the ligand in mural cell association to maturing blood vessels.

3.1.1 The Tetracycline-controlled mammalian gene expression system

Vascular defects and arrested morphogenesis in knockout mice has led to the identification of several important signalling molecules and pathways that regulate blood vessel formation processes (Table 1.1). A frequent limitation in such studies is that null mutants are embryonic lethal at E9.5-E11.0 so that the analysis of gene function is confined to these early stages of vascular development. These limitations can be overcome by inducible and cell-type specific gene targeting systems such as the Cre/loxP method in mouse. Another way to study the function of a gene critical for embryonic development is a gain-of-function approach in which a gene under study can be specifically overexpressed in a tissue of interest.

The Tet Technology comprises two complementary control circuits, initially described as the tTA dependent and rTA dependent (Gossen and Bujard, 1992; Gossen et al., 1995) expression systems. They are now commonly referred to as the Tet-OFF System (i.e. tTA based) and the Tet-ON System (i.e. rTA based).

In each system, a synthetic tetracycline controlled transcription factor (tTA or rTA) interacts with a responsive promoter, TetO, to induce expression of a gene under study (Figure 3.1). Expression is regulated through the administration of the effector substance tetracycline or one of its derivatives (e.g. Doxycycline). These substances act at the level of DNA binding of tTA and rTA transcription factors. rTA requires a tetracycline ligand for DNA binding, whereas the interaction between tTA and DNA is prevented by tetracycline. Thus, the two versions of the Tet System respond to tetracyclines in opposite ways, making them complementary as each system has its unique characteristics and strengths.

Several reports about the “leakiness” can be found. The creators of this system define leakiness of a minimal promoter-tet operator construct such as P\textsubscript{hCMV}\textsuperscript{-1}, as the
intrinsic activity of such a sequence upon transfer into cells. The intrinsic activity of the minimal promoter may vary in different cell lines. It may also change when additional sequence elements, which could function as enhancers, are introduced into the vector. When a transcription unit, controlled by a proper minimal promoter-tet operator sequence, is integrated into the chromosome, the situation changes profoundly. After packaging into chromatin suppression, the residual activity of such promoters is drastically reduced. On the other hand, minimal promoters also can function as enhancer traps, which become activated by nearby enhancers. There may also be transcriptional read-through from outside promoters. Thus, in stable cell lines or transgenic mice the so-called leakiness is primarily a function of a particular integration site.

Both the tTA and rtTA system are complementary and the decision which one to use depends on the particular experimental strategy. To keep a gene switched off and to induce it rapidly at a given time, the rtTA system may be preferable. On the other hand, to keep a gene active and to turn it off occasionally, the tTA system may be more suitable.

3.2 Results

3.2.1 Validation of ephrin-B2-ECFP binding capacity to EphB4 receptor

In order to achieve inducible overexpression of ephrin-B2, a tetracycline responsive ephrin-B2 transgenic line (TetO-ephrin-B2) was generated. The ephrin-B2 ligand used for this study was genetically modified to facilitate the analysis of the gain-of-function model. To allow localisation of the protein on the cellular level, the ligand was fused to Enhanced Cyan Fluorescent Protein (ECFP), using an internal restriction site in the ephrin-B2 cDNA. The site chosen for the insertion is located in the N-terminal part of the extracellular domain of the mature protein. The ECFP tag in the TetO-ephrin-B2 fusion protein can be immunolabelled with an anti-GFP antibody. Originated from the Green Fluorescent Protein (GFP), the Cyan and Yellow Fluorescent Proteins (CFP, YFP) were generated by introducing point mutations into the original GFP sequence
changing the emission spectra of the resulting protein. The antibody recognition site remained thereby unaltered. Additionally to the ECFP insertion, a FLAG sequence encoding an epitope that is specifically recognised by an anti-FLAG antibody was added by PCR to the 3'-end of the ECFP sequence. The purpose of this modification was the facilitation of biochemical experiments.

To verify that binding capacity of the fusion protein to the EphB4 receptor is comparable with endogenous ephrin-B2 ligand, an in vitro EphB4-AP binding assay was performed (Figure 3.2). By expressing either wild-type ephrin-B2 or ephrin-B2-ECFP in HEK-293 cells and incubation with the EphB4 receptor fusion protein, it could be shown that there was no significant difference in the amount of EphB4-AP bound to ephrin-B2-ECFP in comparison to the unmodified ligand. Thus the genetic modifications in the ephrin-B2 ligand did not alter the binding characteristics to its receptor. The tested ephrin-B2-ECFP cDNA was subsequently inserted into the Tet-response vector pUHD 10-3 and then used for pronuclear injections into fertilised mice zygotes to generate the TetO-ephrin-B2 transgenic line.

3.2.2 Choice and characterisation of inducer lines

In order to direct TetO-ephrin-B2 expression in vivo to endothelial cells (ECs), inducer lines were chosen in which tTA/rtTA expression is driven by EC-specific promoters. These lines were Tie-2 tTA7770, Tie-2 rtTA-M2 14974 (both obtained from U. Deutsch, Berne, Switzerland) and VE-Cadherin tTA (obtained from L. Benjamin, Boston, USA). To verify endothelial cell specific expression, the inducer lines were bred into a background of NZL-2 reporter mice (Baron et al., 1995) expressing lacZ protein under the control of the tTA/rtTA responsive promoter. Embryos resulting from these breedings were isolated between embryonic days E11.5 and 15.5 and subjected to β-galactosidase staining. It could be shown that the three different inducer lines specifically and sufficiently induce gene expression in a wide range of vascular beds, including arteries, veins and microvessels (Figure 3.3 and (Sun et al., 2005)) and thus were suitable to induce TetO-ephrin-B2 expression in the Tet-ON/Tet-OFF overexpression model.
3.2.3 Induction of TetO-ephrin-B2 overexpression in the vascular endothelium

Six TetO-ephrin-B2 founder lines, confirmed by ECFP-specific genotyping PCR, were obtained by pronuclear injection of the linearised TetO-ephrin-B2 DNA construct into fertilised mice zygotes. The F1 generation from these founder lines was subsequently bred to Tie-2 tTA7770 and VE-Cadherin tTA inducer mice (Tet-OFF) and embryos were dissected at embryonic day E10.5. With both inducer lines, double transgenic embryos dissected from founder line 1 consistently showed severe developmental defects such as poorly vascularised yolk sacs, growth retardation, haemorrhages, oedema, inflated pericardium and arrested heart development (Figure 3.4). No living double transgenic embryos of this founder line were found after embryonic day E12.0. Under the same conditions the five remaining founder lines showed either no or inconsistent developmental anomalies. Anti-FLAG immunoprecipitation from embryo lysates of the different lines revealed that ephrin-B2-ECFP expression was undetectable in founder lines 2, 3 and 4 and low in founder line 5 (data not shown). Only founder line 1 showed significant transgene expression and was therefore used for further analysis.

3.2.3.1 Induction schemes and gross-morphological defects

The overexpression of ephrin-B2 at later stages of embryonic development was of particular interest since it allowed the examination of the maturation and maintenance of blood vessels, processes in which the role of the ligand is still poorly understood. For this purpose, different induction schemes using the Tet-ON system were tested. In a first set of experiments, pregnant TetO-ephrin-B2 female mice were mated with Tie-2 rtTA-M2 14974 males, and subsequently induced with doxycycline from embryonic day E9.5 to 14.5, at which point they were sacrificed (Figure 3.5). Freshly isolated double transgenic embryos from these breedings showed haemorrhages and severe oedema, suggesting that the overexpression of ephrin-B2 causes vascular defects. With this induction regime mutant embryos did not survive beyond day E15.5.
In a second approach, the induction was performed from embryonic day E12.5 to 18.5. Double transgenic embryos from these litters displayed significant oedema, haemorrhages and blood-filled lymphatic vessels in the skin (Figure 3.5). Furthermore lymphatic vessels in the mesentery and the intestine contained blood (Figure 3.5). These defects were incompatible with survival beyond birth.

Both chosen induction schemes led to severe developmental abnormalities caused by vascular defects. To conclusively show the correlation between mutant phenotypes and transgene expression, a series of biochemical experiments was carried out.

3.2.4 Biochemical validation of transgenic ephrin-B2 expression and signalling capacity in vivo

To confirm that the observed developmental defects were caused by the expression of transgenic ephrin-B2 and the activation of endogenous EphB receptors, immunoprecipitation experiments were performed. In order to obtain sufficient amounts of total protein and compare protein expression in different organs, E18.5 embryos from the late stage induction scheme (E12.5-18.5) were used for the biochemical analysis described below.

Immunoprecipitation with anti-ephrin-B2 antibody yielded the endogenous ligand as well as the ephrin-B2-ECFP fusion protein from whole organ lysates of double transgenic embryos (Figure 3.6). Western blot analysis verified not only the expression of the fusion protein which was visible as a 75kDa band in transgenic animals but also that the expression level of the endogenous ligand remained unchanged under these conditions. Although I had shown previously in vitro that the ephrin-B2-ECFP fusion protein can bind to the EphB4 receptor (Figure 3.2), the in vivo functionality of this interaction had yet to be determined.

Eph receptor tyrosine phosphorylation commonly serves as direct indicator for ephrin ligand induced receptor activation and forward signalling. Therefore tyrosine phosphorylation of EphB4 receptor was evaluated by immunoblotting with an anti-phosphotyrosine specific antibody after pull-down with anti-EphB4 antibody from
pooled E18.5 heart/lung embryo lysates. A significant increase in EphB4 tyrosine phosphorylation and thus receptor activation was detected in lysates prepared from double transgenic embryos compared to wild type litter mates (Figure 3.7). This result demonstrated that the transgenic approach did not only lead to the expression of the ephrin-B2-ECFP but also that the fusion protein was presented in the correct way to engage cognate Eph receptors.

To determine whether ephrin-B2 overexpression has an effect on the total level of EphB4 receptor protein, pooled lysates prepared from the major inner organs of E18.5 embryos (heart, lung, liver, kidneys and intestine) were analysed by immunoprecipitation and EphB4 immunoblot. This showed that EphB4 levels in mutant embryos were significantly reduced in comparison to control littermates (Figure 3.8). Recent studies have shown that trans-endocytosis and degradation of the ligand-receptor complex represents one of several mechanisms to terminate Eph/ephrin signalling between neighbouring cells (Mann et al., 2003; Marston et al., 2003; Zimmer et al., 2003). The decreased level of EphB4 receptor protein can therefore be interpreted as a further indication for up-regulated Eph/ephrin signalling in ephrin-B2 overexpressing mice. It also turned out that the Eph/ephrin expression patterns and levels depend on the specific organ analysed. Only a slight decrease in the level of EphB4 expression resulted in lysates from mutant heart/lung whereas a more significant reduction was seen in lysates from pooled heart, lung, liver, kidneys and intestine.

3.3 Discussion

As ablation of ephrin-B2 expression in the entire mouse as well as selectively in ECs leads to embryonic lethality around midgestation, it has been so far impossible to study the role of the ligand in the second half of embryonic development. Here, I have generated an inducible gain-of-function model to approach this problem and gain insight into the role of the transmembrane ligand in the regulation of vascular morphogenesis.

By utilising the Tetracycline-controlled mammalian expression system in combination with endothelial cell specific promoters, it was possible to successfully overexpress
ephrin-B2 in mouse embryos. I was able to show that an increase in ephrin-B2 expression at different stages of embryonic development leads to severe phenotypes which are most likely directly caused by vascular defects. The overexpression from earliest stages of embryonic development using the Tet-OFF inducer lines resulted in early embryonic lethality around E11.0. The affected embryos displayed a phenotype reminiscent of ephrin-B2 global knockout embryos thus underlining the importance of this ligand during development. These phenotypic similarities suggest that ephrin-B2 levels need to be carefully controlled in the endothelial lining of blood vessels. Loss or overexpression of the ligand is equally incompatible with the normal regulation of blood vessel morphogenesis.

A major advantage of the Tet-ON system is that transgene expression can be quickly triggered through the administration of Tetracycline/Doxycycline at any time point during development. This approach revealed that an increase in ephrin-B2 expression in the vascular system leads to severe developmental defects at a range of embryonic stages. Biochemical analysis of mutant embryos confirmed not only the expression of the ephrin-B2 transgene but also showed activation of its interaction partner EphB4, as monitored by an increase in tyrosine phosphorylation of the receptor. An additional indication for increased forward signalling was the decreased level of total EphB4 receptor protein, which is consistent with previous publications reporting internalisation and degradation of the receptor-ligand signalling complex.

These results did not only verify the functionality of the ephrin-B2 gain-of-function model but also supplied first evidence that overexpression of ephrin-B2 ligand leads to severe developmental defects throughout embryonic development. The obtained data justified the utilisation of this model in further studies aimed at understanding the cellular and molecular mechanisms leading to these abnormal developmental processes.
Figure 3.1: The tetracycline/doxycycline controlled expression systems.

The Tet Technology comprises two complementary control circuits: The Tet-Off (i.e. tTA dependent) and the Tet-On expression systems (i.e. rTA dependent). In each system, a synthetic tetracycline/ doxycycline controlled transcription factor (tTA or rTA) interacts with the responsive promoter, teto, to induce expression of the gene under study (ephrin-B2-ECFP). Expression is regulated through the administration of the doxycycline. Doxycycline acts at the level of DNA binding of tTA and rTA transcription factors. rTA requires a doxycycline ligand for DNA binding, whereas the interaction between tTA and DNA is prevented by doxycycline. To achieve tissue specific induction, tTA/rTA expression is controlled by Tie-2 or VE-Cadherin-tTA promoters which are largely endothelial specific.
Figure 3.2: Ephrin-B2 fusion to ECFP does not affect its binding to EphB4 receptor

EphB4-AP binding assay on HEK-293 cells transiently transfected with either wild-type ephrin-B2 or ephrin-B2-ECFP. The graph represents the average of 4 independent binding experiments. The genetic modification does not significantly affect the binding capability of ephrin-B2-ECFP to EphB4 receptor.
Figure 3.3: Blood vessel specific induction of TetO-controlled lacZ expression by different tTA/rtTA inducer lines

(A-F) β-galactosidase staining of whole embryos and different embryonic tissues in the NZL-2 reporter line background. (A-C) β-galactosidase activity was evident in vascular structures throughout E15.5 Tie-2 tTA 7770 embryos. (A) Whole embryo. (B) Detail of lacZ-positive blood vessels in the brain and (C) mesenteries. (D-F) Induction of lacZ expression in Tie-2 rtTA embryos. (D) β-galactosidase activity was shown in blood vessels throughout E11.5 embryos (induction by IP injection of the pregnant female with 2mg/ml doxycycline 20 hours pre-dissection), (E) lacZ-positive blood vessel in E18.5 skin and (F) intestine (induction by 2mg/ml doxycycline + 3% sucrose in the drinking water of the pregnant female from E12.5-18.5). [A-D with permission from U. Deutsch, Berne, Switzerland].
Figure 3.4: ephrin-B2 overexpression in the early embryo leads to severe developmental defects

(A+B) Freshly dissected embryos at E10.5. (B) Double transgenic embryos present severe growth retardation, haemorrhages, an inflated myocardium and arrest in heart development compared to the wild-type littermates (A).
Figure 3.5: rtTA-controlled ephrin-B2 overexpression leads to severe developmental defects at various stages during embryonic development

Freshly dissected embryos at (A) E14.5 and (B, C) E18.5. Doxycycline induction in (A) from E9.5-14.5. (A) Double transgenic embryo exhibits severe oedema and haemorrhages compared to control littermate. (B) Induction after midgestation (E12.5-18.5) leads to extensive oedema and blood filled lymphatic vessels in the skin and mesenteries (C) of mutant embryos. Induction was performed with 2mg/ml doxycycline + 3% Sucrose in the drinking water of pregnant females.
Figure 3.6: Induction of TetO-ephrinB2 expression *in vivo*

Ephrin-B2 was immunoprecipitated from pooled E18.5 embryo organ lysates (heart, lung, liver, intestine, kidneys). The immuno blot was performed with the same anti-ephrin-B2 antibody used for the IP. Wild-type ephrin-B2 is detected as double band representing different glycosylation states of the protein. In mutant embryos additionally to the wild type bands a higher molecular weight band representing the ECFP tagged TetO-ephrinB2 fusion protein can be detected. Induction of transgene expression from E12.5-18.5 with 2mg/ml doxycycline + 3% sucrose in the drinking water of the pregnant female.
Figure 3.7: Increased EphB4 receptor tyrosine phosphorylation in TetO-ephrin-B2 embryos

EphB4 was immunoprecipitated from pooled E18.5 embryo heart/lung lysates with an anti-EphB4 antibody. (A) The immuno blot with an anti-phospho-tyrosine antibody reveals a significant increase in EphB4 tyrosine phosphorylation in heart/lung lysates from mutant embryos. (B) EphB4-reprobe of the same blot after stripping. Induction of transgene expression as in Figure 3.6.
Figure 3.8: Decreased levels of EphB4 receptor total protein in TetO-ephrin-B2 embryos

EphB4 was immunoprecipitated from pooled E18.5 embryo organ lysates (heart, lung, liver, intestine, kidneys) with an anti-EphB4 antibody. The immunoblot with the IP antibody shows a significant decrease in the total level of EphB4 receptor expression in TetO-ephrin-B2 embryos. Induction of transgene expression as in Figure 3.6.
Chapter 4: Analysis of the early phenotype: 
ephrin-B2 overexpression from the onset of 
vascular development

4.1 Introduction

The discovery that the largest known family of receptor tyrosine kinases, the Eph receptors, and their ephrin ligands are expressed throughout the vascular network sparked considerable interest in the field of vascular biologists. Their functions had previously mainly been studied in the central nervous system in the formation of tissue boundaries and in other morphogenetic processes in the developing embryo. It had been shown that Ephs and ephrins regulate the topographic map formation in the retinotectal/retinocollicular system (Cheng et al., 1995; Drescher et al., 1995; Frisen et al., 1998; Nakamoto et al., 1996) and play essential roles in the formation and fasciculation of brain commissures (Henkemeyer et al., 1996; Orioli et al., 1996; Park et al., 1997). They have additional important functions in patterning of embryonic structures of the brain (Xu et al., 1995; Xu et al., 1996) and somites (Durbin et al., 1998). Furthermore, ephrins control migration of neural crest cells into branchial arches and somites (Krull et al., 1997; Smith et al., 1997; Wang and Anderson, 1997). In these systems, ephrin–Eph interactions are thought to be mainly repulsive, that is, navigating growth cones or migrating cells expressing Eph receptors would turn away from cells expressing the corresponding ephrin ligand (Drescher et al., 1995; Nakamoto et al., 1996; Brennan et al., 1997; Krull et al., 1997; Wang and Anderson, 1997). Repulsive interactions and complementary expression patterns suggested that ephrins and Eph receptors define spatial boundaries in the developing embryo (Gale et al., 1996).

Recently, it has been discovered that Eph/ephrin molecules are also present on endothelial cells as well as mural cells in the vascular system. In particular the distinct expression patterns of the transmembrane ligand ephrin-B2, which was found to be expressed on arteries while its EphB4 receptor is predominantly localised on veins (Wang et al., 1998) led to speculations about a possible involvement of this ligand/receptor pair in the establishment and maintenance of the arterio-venous
boundary. To get an insight into a possible function of ephrin-B2 in vascular development, conventional knockout approaches were used to inactivate the gene in mice (Wang et al., 1998; Adams et al., 1999). The analysis of the resulting null mice revealed a complete arrest of angiogenesis leading to early embryonic lethality around embryonic day E10.5, confirming the substantial role of the molecule during vascular development. However, ephrin-B2 expression is not restricted to vascular endothelial cells, and it has been proposed that its essential function may be exerted in adjacent mesenchymal cells. A study by Adams et al. (1999) subsequently depicted a much more complex system of Eph/ephrin interaction in the vascular system by showing the co-expression of multiple ephrins and Eph receptors in the yolk sac and embryonic vasculature. Subsequently, Gerety et al. generated mice in which ephrin-B2 is specifically deleted in the endothelium of the developing vasculature and the endocardial lining of the heart (Gerety and Anderson, 2002). It turned out that this cell-type specific deletion of ephrin-B2 results in angiogenic remodelling defects very similar to those seen in global ephrin-B2 mutants.

For example, mutant embryos died at midgestation and were dramatically underdeveloped compared to wild-type littermates at E9.5. The mutant vasculature was disorganised and less intricately developed than that of wild-type embryos. Furthermore, the angiogenic remodelling of the yolk sac was arrested at the primary plexus stage. Another striking feature of the EC-specific deletion was the ablated remodelling of the anterior cardinal vein (ACV), which arises initially from the fusion of small diameter vessels present in the lateral mesenchyme of the hindbrain and head (Coffin and Poole, 1988). The vascular-specific knockout embryos furthermore exhibited angiogenic remodelling defects of the head vasculature and intersomitic vessels as well as an arrest in heart development. The study concluded that ephrin-B2 is required specifically in endothelial and endocardial cells for angiogenesis, and that ephrin-B2 expression in perivascular mesenchyme is not sufficient to compensate for the loss of ephrin-B2 in these vascular cells. But further interpretation of these results was complicated by the early embryonic lethality of these knockout embryos and the fact that a targeted inactivation of EphB4 (Gerety et al., 1999) essentially phenocopied the severe defects of ephrin-B2 null mice. To study the role of the ephrin-B2 ligand throughout embryonic development, I established an inducible gain-of-function approach to overexpress ephrin-B2 on the arterial endothelium and ectopically express it on venous ECs.
4.2 Results

By cross-breeding endothelial cell-specific Tet-OFF driver lines to TetO-ephrin-B2 mice, ephrin-B2 was successfully overexpressed from the onset of development. Interestingly, double transgenic embryos at E10.5 from these breedings showed similar vascular malformations as ephrin-B2 null embryos.

4.2.1 Validation of ephrin-B2 overexpression at embryonic day E9.5

Ephrin-B2 expression can be detected in the embryonic vasculature from embryonic day E8.25 by using an ephrin-B2-TaulacZ knock-in line (Wang et al., 1998). Conventional detection methods, like immunohistochemical techniques proved to be less reliable in visualising the ligand at these early stages in development in my hands. In an attempt to circumvent this limitation, ephrin-B2 expression was studied by an EphB4-AP whole-mount binding assay. For this purpose, the TetO-ephrin-B2 line was bred to the Tet-OFF inducer line Tie-2 tTA7770 and embryos dissected at E10.5. The fusion protein between the EphB4 receptor and alkaline phosphatase only bound in detectable amounts to the very large vessels in the brain of wild-type embryos whereas double transgenic embryos displayed staining in many vascular beds (Figure 4.1). Stained blood vessel-like structures could be identified in the head and trunk as well as intersomitic vessels. These results suggested successful overexpression of ephrin-B2 in the vasculature of early embryos.

4.2.2 Arrested angiogenic remodelling and perturbed blood vessel development in TetO-ephrin-B2 embryos

In order to examine the overall vascular morphology in the ephrin-B2 overexpressing embryos, whole-mount antibody staining for PECAM-1 was performed on E9.5 embryos (Figure 4.2). Already at this early stage in development, ephrin-B2-overexpressing embryos were slightly growth retarded compared to control littermates. Mutants exhibited defects in the general angiogenic remodelling, which was most apparent in the vasculature of the head and trunk. While wild-type embryos showed progressed angiogenic remodelling of the head vasculature into smaller and larger
diameter vessels, this process appeared to be arrested at the primary capillary plexus stage in ephrin-B2-overexpressing embryos. Additionally the anterior cardinal vein (ACV) failed to form properly similar to what has been reported for ephrin-B2 and EphB4 knockout mice.

4.2.3 Decreased EphB4 expression on venous endothelial cells and reduction in smooth muscle actin positive cells

Immunohistochemical stainings on E9.5 embryo sections were used to examine the cause for the observed defect in angiogenic remodelling further. Immunolabelling for ephrin-B2 and its receptor EphB4 in combination with an anti α-smooth muscle actin counterstaining, revealed complete loss of EphB4 expression in endothelial cells around the anterior cardinal vein at embryonic day E9.5 (Figure 4.3). At the same time there was no ectopic ephrin-B2 expression detectable on the ACV. This might be an indication of ephrin-B2 induced, increased inter-endothelial signalling leading to internalisation and degradation of the ligand/receptor complex. The dorsal aorta in the mutant embryo showed prominent TetO-ephrin-B2 expression whereas endogenous expression of the ligand in control embryos was below detection limits. Additionally, there seemed to be a significant reduction in α-smooth muscle actin positive cells around the dorsal aorta of double transgenic embryos.

4.3 Discussion

As initial experiments have indicated (chapter 3), overexpression of ephrin-B2 at different stages during embryonic development leads to severe developmental defects. The observed phenotype in early embryos was almost identical to ephrin-B2 null mice. Compared to wild-type littersmates, mutant yolk sacs were poorly vascularised (not shown) and growth retardation from embryonic day E9.5 was observed. Haemorrhaging and oedema could be seen consistently. Furthermore, the development of the heart was arrested and mutant embryos exhibited a strongly inflated pericardium. Further analysis revealed severely affected angiogenic remodelling of the developing vasculature. The mutant head vasculature failed to develop properly from the honeycomb-like capillary plexus containing vessels of equal diameter into a hierarchical network of smaller and
larger diameter vessels. Formation of the anterior cardinal vein was compromised. The observed vascular defects can not solely be explained by the delay in development because angiogenic remodelling in mutant embryos is already defective at E8.5, that is, before the onset of growth retardation (not shown).

Taken together, these data underline the important role of the ephrin-B2 ligand during vascular morphogenesis. Immunohistochemical stainings revealed a loss of EphB4 receptor expression in the endothelium of the anterior cardinal vein of mutant embryos. At the same time, it was not possible to detect ectopic expression of ephrin-B2 on the venous endothelium. A possible explanation for this finding could be inter-endothelial signalling triggered by cis-interaction between ectopically expressed ephrin-B2 and its receptor EphB4 on the same vessel. As mentioned in chapter 3, recent work has shown that trans-endocytosis and degradation of the ligand-receptor complex represents one of several mechanisms to terminate Eph/ephrin signalling between neighbouring cells.

The fact that ephrin-B2 overexpressing arterial vessels displayed disrupted vSMC coating gave a first indication of effects on blood vessel supporting cells. vSMCs have been shown to express EphB2, EphB3 and EphB4 receptor (Foo et al., in press) which can bind and signal via ephrin-B2. The observed effect of increased EC-specific ephrin-B2 expression on vSMCs might point towards an involvement of the ligand in the regulation of mural cell association to maturing blood vessels, a process which is still poorly understood. Since ephrin-B2 overexpression in the developing vasculature leads to early lethality similar to the ephrin-B2 null mice, the inducible Tet-ON system was considered to be better suited to examine the role of ephrin-B2 at later stages of embryonic development.
Figure 4.1: tTA-induced ephrin-B2 overexpression in the early embryo

(A+B) EphB4-AP whole-mount staining on E10.5 embryos. The EphB4 fusion protein binds to available ephrin-B2 ligand. (A) Using this technique only the major vessels in the head (black arrowheads) of the wild-type embryo could be detected. (B) In comparison, vessel-like structures in the head (black arrows), intersomitic vessels (white arrows), the dorsal aorta and anterior cardinal vein (red arrow) can be seen in the double transgenic embryo, indicating a significant ephrinB2 overexpression.
Figure 4.2: Ephrin-B2 overexpression in the early embryo leads to arrested angiogenic remodelling

(A-F) PECAM-1 whole-mount staining on E9.5 embryos. (B) Mutant embryos display a delay in development compared to control embryos (A) already at E9.5. (D) The angiogenic remodelling of the head vasculature into smaller and larger diameter vessels is perturbed in double transgenic embryos. (F) In addition the formation of the anterior cardinal vein from smaller vessels is ablated (black arrow). (E) Anterior cardinal vein of a wild-type embryo (white arrow).
Figure 4.3: Ephrin-B2 overexpression in the early embryo leads to perturbed vSMC association and loss of venous EphB4

(A+C) Immunodouble staining for ephrin-B2 and α-SMA on E9.5 embryo transversal sections. (A) Prominent ephrin-B2 expression can be detected on mutant DA exhibiting altered morphology. The dorsal aorta shows a poor association with vSMCs. (C) In contrast, the wild-type DA shows a continuous layer of vSMCs but ephrin-B2 expression is below detection level. (B+D) Immunodetection of EphB4 and α-SMA. (B) Loss of EphB4 receptor expression in mutant ACV (dashed lines) can be observed. (D) EphB4 expression on control ACV. DA: dorsal aorta; ACV: anterior cardinal vein.
Chapter 5 | Overexpression of ephrin-B2 at later stages of development

5.1 Introduction

The maturation of vascular beds, involves the recruitment of mural cells, the deposition of extracellular matrix molecules and the specialisation of vessels wall for structural support and specific functional roles. In its simplest embodiment, vascular development can be thought of as a series of processes involving blood vessel formation, stabilisation, branching, remodelling and pruning as well as specialisation. The timing of most of these events overlaps, allowing the vasculature to evolve seamlessly to maturation. Furthermore, numerous examples show that individual molecules tend to have multiple functions during the development of the vascular network (Jain, 2003).

Previous work has established that the recruitment of mural cells and the generation of ECM is regulated by at least four molecular pathways: PDGF-B - PDGFR-β, S1P1 - EDG1, Angiopoietin-1 - Tie-2, and TGF-β (Jain, 2003). The potential involvement of the Eph/ephrin signalling in these processes could so far not be studied due to the early embryonic lethality of the ephrin-B2 and EphB4 null mice.

The results presented in the previous chapter have shown that the combination of ephrin-B2 overexpression in arterial and ectopic expression in venous endothelial cells causes severe cardiovascular defects in the developing early embryo. Intriguingly, this gain-of-function approach produced a phenotype almost identical to ephrin-B2 full knockouts resulting in early embryonic lethality. Thus overexpression of ephrin-B2 from very early stages of embryonic development proves to be similar developmentally restrictive as the deletion of the ligand. To circumvent this limitation, the Tet-ON system was used to trigger TetO-ephrin-B2 expression at later stages in embryonic development. Thus it was possible to examine potential roles of ephrin-B2 in morphogenetic processes at progressed stages of vascular development such as mural cell recruitment or the maintenance of the arterio-venous identity.
5.2 Results

5.2.1 Validation of TetO-ephrin-B2 inducibility at later stages of development

It was important to determine whether the Tie-2 rtTA-M2 14974 driver line could induce TetO-ephrin-B2 expression throughout the entire embryonic vasculature. For this purpose, immunohistochemical stainings for ephrin-B2 were performed to assess expression levels in different organs of induced and non-induced E18.5 embryos. Significant ephrin-B2 overexpression on arteries as well as ectopic expression on veins could be confirmed in all tissues analysed, i.e. heart, lung, liver, intestine, skin (Figure 5.1). In all these tissues, transgene expression appeared to be restricted to the endothelial lining of blood vessels. This initial analysis of the transgenic expression pattern revealed also that two of the examined tissues, embryonic skin and liver, display distinct morphological differences in ephrin-B2-overexpressing embryos compared to wild-type littermates. Both tissues were subsequently studied in more detail.

5.2.2 Ephrin-B2 overexpression leads to pronounced vascular defects in embryonic skin

Embryonic skin morphogenesis is a complex and highly dynamic process of continuous cell-cell interactions. Vascularisation of the skin has to be flexible and efficient due to the rapidly increasing requirement for oxygen and nutrient supply in the growing organism. The embryonic skin therefore provides a valuable and readily accessible model to study blood vessel development and maturation.

5.2.2.1 TetO-ephrin-B2 expression in embryonic skin

Upon dissection, mutant E16.5-18.5 skin appeared to be thinner and less rigid to tension. Mutant head skin as well as back skin frequently tore in the process of detaching from the embryo while this was not the case for control animals. Judged by Hematoxilin/Eosin staining, the general skin morphology in mutants looked less dense
compared to wild-type skin (Figure 5.2). Immunolabelling for ephrin-B2 on skin sections showed an altered morphology of ephrin-B2 overexpressing blood vessels. Vascular integrity appeared compromised as indicated by oedema and hemorrhaging. Immunolabelling of the overexpressed ephrin-B2 with an anti-GFP antibody showed that transgene expression in the skin is largely restricted to medium and larger diameter blood vessels in subdermal layers (Figure 5.3). No TetO-ephrin-B2 expression could be detected in capillaries just below the epidermis. In order to specifically examine the effect of ectopic ephrin-B2 expression on venous vessels, the venous endothelial-specific marker endomucin (Brachtendorf et al., 2001) was used for immunolabelling. α-SMA counterstaining, routinely used to visualise blood vessel supporting vascular smooth muscle cells (vSMCs), showed very often a patchy and discontinuous pattern compared to wild type skin. This effect seemed to be more pronounced on venous vessels.

### 5.2.2.2 Perturbed mural cell association to ephrin-B2 overexpressing blood vessels

The recruitment of mural cells, i.e. vascular smooth muscle cells (vSMCs) and pericytes becomes increasingly critical for the development of a mature and functional vascular network (Carmeliet, 2003; Jain, 2003). Pericytes (PC) extend numerous cellular processes and make direct contact with the endothelial cells from newly formed vascular beds, capillaries and postcapillary venules. This interaction suppresses the formation of new endothelial sprouts and prevents leakage through the vessel wall by reducing the permeability of the endothelial monolayer (Armulik et al., 2005; Betsholtz et al., 2005; Hirschi and D'Amore, 1996). In contrast, vSMCs attach in one or multiple layers to the abluminal side of the endothelial basal lamina of more mature and larger calibre blood vessels. It is still not clear whether pericytes differentiate into smooth muscle cells or are gradually lost and replaced by vascular smooth muscle cells. The production of extracellular matrix and elastic fibers by vSMCs provides blood vessels with mechanical stability and elasticity, which is of particular importance for arteries that transport blood under high pressure from the heart to the periphery. Accordingly, fewer vSMCs are found on venous (low-pressure) vessels that direct the blood stream from peripheral tissues back to the heart (Jain, 2003).
Results from the initial ephrin-B2 overexpression experiments indicated a potential effect on the proper association of vSMC to blood vessels in the embryonic skin. To examine the mural cell association to ephrin-B2 overexpressing vessels, immunohistochemical stainings for α-SMA and Desmin were analysed. α-SMA selectively marks vSMCs whereas Desmin labels both vSMCs and PCs. The performed co-staining did not only confirm a loss of vSMCs but also highlighted that pericytes detach from the affected vessels (Figure 5.4). In fact, by analysing Desmin staining on its own it was not possible to identify the walls of mutant blood vessels. While in wild-type skin the regular layer of pericytes outlines the round lumen of blood vessels, the pericytes coverage in mutants was too irregular to depict vessel-like structures. A significant number of Desmin-positive cells could be detected in the surrounding tissue. As mentioned in Chapter 3, freshly isolated double transgenic embryos from late stage induction experiments could be identified by prominent haemorrhages, oedema and blood filled lymphatic vessels in the skin and intestine. Previous work has established that such phenotypes can be caused by mural cell defects (Bjarnegard et al., 2004; Lindblom et al., 2003). It appears therefore likely that endothelial ephrin-B2 expression can control mural cell association which also provides a potential explanation for the vascular defects observed in transgenic embryos.

A useful tool to examine association of vSMCs and pericytes to maturing blood vessels is the X-LacZ4 reporter line (Tidhar et al., 2001) in which at late developmental stages and in the adult, lacZ staining marks vascular smooth muscle cells and pericytes in most vascular beds. By introducing this allele into the EC-specific Tet-ON overexpression system, it was possible to analyse the mural cell coverage to maturing blood vessels. Back skin samples of triple transgenic embryos were analysed by whole-mount β-galactosidase staining and compared to skin specimen from control littermates carrying only the lacZ reporter allele but neither of the induction elements. At first sight, mural cell staining seemed significantly less pronounced in mutant embryos (Figure 5.5). It was possible to follow lacZ-positive small diameter blood vessel to the centre of the back skin in controls whereas lacZ-positive vessels in the mutant terminated in considerable distance from the midline. Upon dissection, blood often remains in venous vessels of the skin, which can help to distinguish arteries and veins if these vessels happen to run side by side. It was apparent that the association of LacZ-positive cells to veins, identified by this criterium was significantly reduced in mutants. In fact, smaller
diameter vessels in mutant embryos appeared to be entirely without mural cell attachment whereas the vasculature of similar calibre was never completely devoid of LacZ-positive cells in wild-types. It is noteworthy at this point that the study which initially characterised the X-LacZ4 line showed down-regulation of the lacZ transgene associated with SMC-activation upon injury. On the other hand, Foo et al. (in press) showed recently that mural cells in which ephrin-B2 function had been deleted - leading to perturbed mural cell association to microvessels - continue to express the reporter. Taken together, the results from the lacZ reporter analysis and the data obtained from immunohistochemical stainings for mural cells indicate that ephrin-B2 overexpression in the endothelium causes the loss of blood vessel supporting cells.

5.2.2.3 Analysis of matrix deposition around ephrin-B2 overexpressing blood vessels

While pericytes make direct contact to endothelial cells, vSMCs are separated from the endothelium by a layer of extracellular matrix called the basement membrane. Despite its small size and amorphous appearance, the basement membrane has multiple important functions. It separates the endothelium and the stroma, and serves to regulate the passage of macromolecules and cells. Furthermore, growth factors can be bound by the basement membrane and released in a controlled fashion to influence cell proliferation, migration, differentiation as well as synthesis and remodelling of the basement membrane. The most abundant and ubiquitous components of the basement membrane include the glycoproteins laminin and entactin, collagen IV, fibronectin and perlecan.

Since mural cell coverage of ephrin-B2 overexpressing cells was shown to be perturbed (Chapter 5.2.2.2), it was interesting to determine whether the loss of vSMCs was a secondary effect caused by the loss of pericytes or the result of direct interactions with ephrin-B2 overexpressing ECs. For endothelial cells to make direct contact with vSMCs, the basal lamina would have to be partially degraded. To address this question, the expression patterns of different extracellular matrix components were analysed by immunohistochemical staining. Immunolabelling of fibronectin and α-SMA showed reduced deposition of fibronectin (Figure 5.6) around mutant blood vessels. Since
several studies have shown that vSMCs themselves synthesise fibronectin, the loss of this cell population would naturally lead to decreased fibronectin deposition. The expression patterns of Collagen IV or Laminin 5 did not reveal any differences between ephrin-B2 overexpressing and wild type blood vessels. It thus appears unlikely that the loss of vSMCs from mutant blood vessels is caused by degradation of the basement membrane. Nevertheless, it cannot be excluded that changes in basement membrane composition might affect vSMC association.

5.2.2.4 Cell apoptosis around TetO-ephrin-B2 expressing vessel in the subdermal layer

The loss of a cell population can be the result of insufficient proliferation, cell death, detachment and migration or simply the loss of marker molecule expression. To determine the cause for the decrease in α-SMA and Desmin positive cells around ephrin-B2 overexpressing blood vessels, a cell death detection assay was used to evaluate changes in cellular apoptosis in embryonic skin (Figure 5.7). Wild-type skin showed very low levels of apoptosis and positive staining was only found in shedding cornified keratinocytes of the epidermis and in hair follicles. In contrast, mutant skin samples exhibited a significant increase in apoptotic cells within the dermis. To identify this apoptotic cell population, immunolabelling for different endothelial and mural cell markers was performed. It turned out that neither vSMCs (detected by immunolabelling for α-SMA) nor ECs (detected by anti-PECAM-1 immunostaining), comprise the apoptotic cell population (data not shown). The harsh tissue treatment for the apoptosis detection affected the epitope accessibility for the anti-Desmin antibody. It was therefore not possible to determine whether the increase of cell death is a sign of general necrosis caused by insufficient blood/oxygen supply or an indicator for pericyte apoptosis.

5.2.2.5 Enrichment of TetO-ephrin-B2 in EC protrusions

The analysis of the skin phenotype so far had been mainly based on immunohistochemical stainings on skin sections. Skin whole-mount stainings are more
suitable to study the overall vascular architecture, mural cells association and branching pattern. The partial loss of vSMCs shown by immunostainings on mutant skin sections was confirmed by skin whole-mount immunolabelling with anti-PECAM-1 and anti-α-SMA antibodies (Figure 5.8). Smooth muscle cell coverage was found to be disrupted and patchy, a defect which was mainly seen in blood vessels which morphologically appeared to be veins. Furthermore vSMCs were sometimes detected on vessels which were, judged by their morphological appearance, of lymphatic origin. Apart from the collecting ducts lymphatic vessels do not possess smooth muscle cell coverage in the wild-type situation.

In order to determine the overall expression and localisation of the ephrin-B2 transgene, anti-GFP skin whole-mount staining was performed. This revealed that a significant number of TetO-ephrin-B2 expressing blood vessels displayed endothelial protrusions showing a concentration of the ephrin-B2-ECFP fusion protein (Figure 5.9). Very fine, almost spine-like EC extensions were seen on larger calibre vessels, reaching through the layer of vSMCs. With decreasing vessel diameter and hence less mural cell association, more foot or knob like protrusions were observed. Immunodetection of the ephrin-B2 fusion protein with an anti-GFP antibody in skin transversal sections had implicated a restriction of transgene expression to medium and larger calibre vessels in the dermis (Figure 5.3). Nevertheless anti-GFP whole-mount staining of skin revealed expression of the ephrin-B2 fusion protein in small diameter blood vessels and even in the finest EC protrusions. Unfortunately, it was not possible to confirm these results by staining with anti-ephrin-B2 antibodies. Detection of endogenous ephrin-B2 is only possible on paraffin sections and requires rather harsh antigen retrieval as well as signal amplification. Hence it was not possible to examine ephrin-B2 protein localisation in whole-mounts of wild-type and transgenic skin. The use of other endothelial markers like PECAM-1 or endomucin, which would have allowed a direct comparison of endothelial protrusions in mutant and wild-type skin, did not conclusively show differences in the number or length of endothelial processes. This may be due to the localisation of these marker proteins, which may be different from ephrin-B2.
5.2.2.6 Ultrastructural analysis of the endothelial monolayer

Electron microscopic analysis was used to examine in more detail the endothelial morphology and association of blood vessel supporting cells to the endothelial monolayer. In preparation of the electron microscopy, low magnification pictures of the specimen were taken (Figure 5.10). Judged by the stained nuclei, mutant skin samples exhibit a significant decrease in tissue density, which is probably a result of oedema and increased liquid pressure in the mutant tissue. Furthermore vastly dilated and blood filled vessel, probably lymphatics, were visible. This dilation may be a secondary effect caused by the accumulation of blood and fluid in the lymphatic network.

High magnification electrographs show distinct morphological differences of the endothelial monolayer in mutant skin compared to wild-type specimen (Figure 5.10). Numerous endothelial cell extensions were visible and in many cases the endothelial lining was very thin. Examples in which the endothelial thinning led to rupture of the monolayer were frequently detected. In these affected blood vessels, pericytes appeared to have detached from the endothelial monolayer. Thus, the electron microscopic analysis supported the data obtained by immunohistochemistry, showing a loss of mural cells from ephrin-B2 overexpressing blood vessels and the formation of EC protrusions.

5.2.3 Ectopic ephrin-B2 expression in embryonic liver leads to early signs of fibrosis

Liver development comprises multiple stages and is influenced by hormonal factors as well as intercellular and matrix–cellular interactions. The fetal liver represent the major site of haematopoiesis during embryonic development, but acquires additional various metabolic functions near birth. For the liver to serve as a site for haematopoiesis by midgestation, proper vascular development is essential. A series of studies managed to establish that endothelial cells provide a crucial growth stimulus to the hepatic bud even before the blood vessels are formed. During embryonic development, blood supply of the liver is of venous origin and arterial marker expression such as ephrin-B2 only commences after birth (Figure 5.11). The embryonic liver therefore represents a unique
setting to study ectopic expression of arterial markers in an exclusively venous environment.

5.2.3.1 Activation of Hepatic Stellate cells

Similar to the already documented vascular beds of other organs, hepatic endothelial cells express significant amounts of ephrin-B2 in mutant embryos. The important difference to other organs analysed is that there are no arterial vessels present in the developing liver at this stage of development. Analysis of the hepatic vasculature revealed a significant increase in α-SMA/Desmin positive cells around ephrin-B2 expressing blood vessels (Figure 5.11). It is very likely that this cell population represents hepatic stellate cells (HSCs). HSCs, a mesenchymal cell type in hepatic parenchyma, have unique features with respect to their cellular origin, morphology, and function. Normal, quiescent HSCs function as major vitamin A-storing cells containing over 80% of total vitamin A in the body. They are located between parenchymal cell plates and sinusoidal endothelial cells, and extend well-developed, long processes surrounding sinusoids in vivo as pericytes. However, HSCs are known to be 'activated' or 'transdifferentiated' to a myofibroblast-like phenotype lacking cytoplasmic lipid droplets and long processes in pathological conditions such as liver fibrosis and cirrhosis. HSCs are the predominant cell type producing extracellular matrix (ECM) components as well as ECM degrading metalloproteases in hepatic parenchyma, indicating that they play a pivotal role in ECM remodeling in both normal and pathological conditions. Recent findings have suggested that HSCs have a neural crest origin because their gene expression profile is similar to neural cell types as well as smooth muscle cells and myofibroblasts. The morphology and function of HSCs are regulated by ECM components as well as by cytokines and growth factors in vivo and in vitro. HSC activation by ectopic ephrin-B2 expression might provide clues for pathological settings such as liver fibrosis.
5.2.3.2 Increased matrix deposition around TetO-ephrin-B2 positive hepatic blood vessels

Since HSCs are the predominant cell type producing extracellular matrix in the liver, the expression patterns of different matrix components were examined. Immunohistochemical stainings of E18.5 liver sections for fibronectin showed significantly increased expression around ephrin-B2 expressing hepatic vessels. The dot-like fibronectin expression pattern seen in controls changed to a continuous staining in mutants (Figure 5.12). In a similar fashion, Laminin 5 deposition increased, whereas Collagen IV seemed only slightly upregulated by the expression of ephrin-B2. By α-SMA counterstaining, the increased Laminin 5 deposition could be directly linked to activated hepatic stellate cells and there was an almost complete overlay of the two markers. These changes described above were not linked to altered proliferation or apoptosis in the embryonic liver (data not shown).

5.3 Discussion

Due to the early lethality of ephrin-B2 null mice, it has so far not been possible to analyse the function of the transmembrane ligand during later stages of vascular development. By employing the rtTA-controlled mammalian expression system (Tet-ON), I was able to successfully induce ephrin-B2 overexpression on arterial and ectopically express it on venous endothelial cells at various stages of embryonic development. Embryos in which ephrin-B2 overexpression was induced from embryonic day 12.5 (E12.5) displayed severe vascular defects around E17.5, detectable upon dissection as extensive oedema and haemorrhages. Extravasated blood was found in lymphatic vessels of the skin and the mesenteries. These defects caused embryonic lethality around E18.5. Detailed analysis of these mutant embryos revealed that mural cells failed to attach properly to maturing blood vessels in the embryonic skin. The association of vSMCs and pericytes to blood vessels was severely compromised. Depending on the severity of the exhibited phenotype, pericytes were partially or completely detached from blood vessels and even dispersed in the surrounding tissue. vSMCs showed in several cases ectopic association to what appeared to be lymphatic capillaries, which do not have a mural cell coating in the wild-type controls.
Immunodetection of the ephrin-B2-ECFP fusion protein showed not only prominent expression in the transgene but also its concentration in endothelial cell protrusions. Due to technical limitations, it was not possible to compare ephrin-B2 localisation between mutant and wild-type skin as commercially available anti-ephrin-B2 antibodies can not be used successfully in skin whole-mount immunostainings. Although the analysis of immunostainings with endothelial-specific markers like PECAM-1 or endomucin confirmed the presence of EC-protrusions in both wild-type and mutant endothelial cells, it proved difficult to assess whether the number of these processes is increased by ephrin-B2 expression.

A series of recent studies linked ephrin-B2 expression to the formation of cellular protrusions in other cell types. While Henkemeyer et al. reported that ephrin-B2 mediated activation of EphB receptors accelerates dendritic spine formation in cultured hippocampal neurons (Henkemeyer et al., 2003), Moeller et al. showed that EphB receptors regulate dendritic spine morphogenesis through recruitment/phosphorylation of FAK and Rho activation (Moeller et al., 2005). A recent study managed to show that the overexpression of ephrin-B2 leads to the formation of multiple lamellipodia, enhanced polymerisation of actin fibers, and induction of focal adhesion complexes and constitutive activation of focal adhesion kinase in B16 mouse malignant melanoma cells (Meyer et al., 2005).

It has been shown that growing blood vessels navigate along stereotyped paths towards their target similar to axonal growth cones (Carmeliet and Tessier-Lavigne, 2005). Specialised endothelial tip cells at the leading front of such vessels extend numerous filopodia and react to chemoattractant and repellent guidance cues that act over long or short distances (Carmeliet and Tessier-Lavigne, 2005). As shown by Lu et al., (2004) disrupted expression of the Unc5b gene in mice, or of Unc5b or netrin-1a in zebrafish, leads to aberrant extension of endothelial tip cell filopodia, excessive vessel branching and abnormal navigation. So far it could not be shown if endothelial tip cells express ephrin-B2. To conclusively link ephrin-B2 overexpression with EC-protrusion formation in vivo, the difficulties in endogenous ephrin-B2 detection have to be overcome. In situ hybridisation might pose an alternative to approach this question. Additionally, the isolation of primary endothelial cells or aortic ring explants in which
TetO-ephrin-B2 expression can be induced might provide a valuable tool to gain deeper insight into these processes.

Despite the technical limitations, I was able to show for the first time that endothelial ephrin-B2 controls the association of mural cells to maturing blood vessels. Published data for A-class Ephs and ephrins indicates that the amount of receptor/ligand interactions determines the functional read-out of the signal (Hansen et al., 2004). Increased expression of ephrin-B2 on endothelial cells might affect the association of pericytes by increased forward signalling via Eph receptors expressed on PCs. It was not possible to determine whether the observed loss of vSMCs is a result of direct interaction with ephrin-B2 overexpressing ECs. Nevertheless, the loss of PCs might directly affect the association of vSMC to the maturing blood vessel. The observed vascular defects appeared to be much more pronounced on veins. A possible explanation for this observation could be that ectopic expression of ephrin-B2 ligand on EphB4-positive venous endothelial cells will lead to inter-endothelial interactions and signalling events. Furthermore, in contrast to arteries, venous vessels maintain some pericycle attachment throughout development. This might allow prolonged interaction between ephrin-B2 overexpressing ECs and Eph receptor expressing PCs.

During embryonic development, the blood supply to the liver is of venous origin and there is no detectable expression of arterial markers such as ephrin-B2. The expression of arterial markers commences only after birth with a switch in the hepatic blood supply. The ectopic expression of ephrin-B2 in the embryonic liver led to the activation of hepatic stellate cells. The activation or transdifferentiation of this cell population has been linked to pathologies such as liver fibrosis and cirrhosis as HSCs represent the predominant cell type producing extracellular matrix (ECM) components in the liver. As a consequence of ephrin-B2 expression in this venous environment, HSC activation resulted in increased deposition of different ECM components, which might possibly present an early stage of a fibrotic phenotype. Ongoing long term ephrin-B2 overexpression experiments in adult mice will help to gain deeper insight into the molecular pathways involved. It will be interesting to determine whether ephrin-B2 overexpression in adulthood will also result in increased matrix deposition and HSC activation. It will as well be important to address whether ephrin-B2 is up-regulated in human liver fibrosis.
Figure 5.1: Induction of TetO-ephrin-B2 expression throughout the embryo at later stages of development

(A-R) Double immuno labeling of ephrin-B2 and α-SMA on sections of different organs from E18.5 embryos. Overexpression of ephrin-B2 on arterial ECs and ectopic expression on veins can be detected. (A-F) sections through blood vessels in the heart wall, (G-L) transversal sections through mesenteric vessels, (M-R) sections trough vessel in the lung. Induction of transgene expression from E12.5-18.5 with 2mg/ml doxycycline + 3% sucrose.
Figure 5.2: Mutant skin appears thinner and less rigid to tension

(A-D) H+E staining of E18.5 skin sections (A+C) and transversal sections through the thorax of wild-type and mutant embryos as indicated. Mutant back skin is thinner and exhibits decreased tissue density as a result of oedema caused tissue pressure. Transversal sections through the mutant embryo reveal extensive oedema (arrow in B) compared to the control (D). (A+C 200x magnification; B+D 50x magnification)
Figure 5.3: ephrin-B2 overexpressing blood vessels exhibit change in morphology and perturbed vSMC association

(A-F) Double immunostaining for ephrinB2 and α-SMA on skin sections. (A) ephrin-B2 overexpression leads to changes in blood vessels morphology compared to control skin (D). (B) Poor vSMC association coincides with ephrin-B2 gain-of-function on mutant blood vessels. (G-L) Double immunodetection of GFP and α-SMA in mutant skin. (G) TetO-ephrin-B2 expression appears to be restricted to medium and larger diameter blood vessels in subdermal layers of the skin. (J-L) Microvessels underneath the epidermis test negative for GFP expression. (M-R) Double immunostainings for endomucin and α-SMA. (N and arrow in O) The observed decrease in vSMC association in mutant skin is more pronounced on the venous endothelium. A: artery; V: vein (630x magnification)
Figure 5.4: Poor vascular smooth muscle cell and pericyte association to morphologically altered mutant blood vessels in E18.5 skin

(A-F) Double immuno labelling for α-SMA and desmin on E18.5 skin sections. (A-C) Mutant blood vessels exhibit altered morphology and discontinuous α-SMA staining. desmin-positive pericytes appear to detach from these vessels (arrows in A). (D-F) Wild-type blood vessel show prominent coating by α-SMA and desmin-expressing cells. (400x magnification)
Figure 5.5: Decreased mural cell association in mutant back skin

(A-D) Freshly dissected skin from E18.5 embryos in the X-LacZ4 background were assayed for β-gal activity labelling mural cells. (B) Smaller diameter venous vessels show decreased mural cell association in mutant skin. (A) Veins of similar diameter show mural cell coverage in control skin (arrows in A). (D) The overall coverage of blood vessels by stained cells in mutant back skin appears significantly reduced compared to control (C). (A+B 400x magnification; C+D 50x magnification)
Figure 5.6: Decreased fibronectin deposition around mutant blood vessels with altered vSMC association

(A-F) Immuno labelling of fibronectin and α-SMA on E18.5 skin sections. Mutant blood vessels with discontinuous vSMCs association show a decrease in fibronectin deposition compared to blood vessels in the control embryo (D-F). No difference was detected for either Collagen IV (G-L) or Laminin 5 (M-R) deposition. (630x magnification)
Figure 5.7: Increased apoptosis in the skin of ephrinB2 overexpressing embryos

(A-F) Detection of apoptotic cells in sections of E18.5 back skin. (A) Endothelial cell-specific ephrin-B2 overexpression leads to cell apoptosis in sub-epidermal layers of the skin, which (arrows in C) is free of apoptosis in wild-type (D). (200x magnification)
Figure 5.8: Perturbed vSMC association to ephrin-B2 overexpressing blood vessels

(A-I) Whole-mount immunofluorescence staining on E18.5 skin for α-SMA and PECAM-1. (B) The disrupted and patchy α-SMA staining depicts failure of vSMCs to attach properly to ephrin-B2 overexpressing blood vessels. (G-I) Ectopic localisation of α-SMA positive cells to lymphatic vessel. (G) PECAM-1 positive vessels, judged by their bag-like morphology, of lymphatic origin (dashed lines in I). (400x magnification)
Figure 5.9: Ephrin-B2-ECFP is enriched endothelial cell protrusions

(A-F) Whole-mount immunofluorescence staining of E18.5 mutant skin for GFP and α-SMA. (A+C) The anti-GFP staining reveals significant ephrin-B2 transgene expression in the skin vasculature. The protein appears to be concentrated in EC protrusions which are filopodia or spine-like on larger diameter blood vessels and knob-like on smaller diameter vessels (200x magnification). (D-F) Higher magnification of the EC protrusions. (D) 400x magnification of GFP-positive knob-like structures on blood vessels without mural cell coverage (white arrows). (E, F) 630x magnification of filopodia-like EC extensions (red arrow heads in F). The fine protrusions can end in lamellipodia-like end structures (white arrowheads in E).
Figure 5.10: Ultrastructural analysis of blood vessels in embryonic skin confirms EC-protrusions and defective pericyte association

Skin sections of E18.5 wild type and mutant embryos show dilated (*red arrows in B*) and blood-filled lymphatic vessels (*black arrows in B*) in the skin of double transgenic embryos. *(B)* Dermal tissue architecture is affected by oedema as judged by the decreased nuclei density in the mutant skin specimen. *(C-E)* Electron micrographs reveal impaired interaction between pericytes (PC) and endothelial cells (EC) in mutant blood vessels *(D)*. The endothelial monolayer presents numerous luminal and abluminal protrusions (*arrowheads in D*). *(E)* Rupture of the endothelial monolayer and haemorrhaging in mutant skin.
Figure 5.11: Venous expression of ephrin-B2 leads to activation of hepatic stellate cells.

(A-C) Double immuno labelling for EphB4 receptor and α-SMA on E18.5 wild-type liver sections. (A) EphB4 expression can be detected on hepatic sinusoidal ECs. (D-J) Immunohistochemical staining for ephrin-B2 and α-SMA. (D) Strong ectopic ephrin-B2 expression can be observed on mutant hepatic ECs compared to unstained blood vessels in the control liver (G). (E) Activated hepatic stellate cells (HSCs) upregulate α-SMA expression (arrows). (J-O) Double immunodetection of Desmin and α-SMA. (J) Activated HSCs show an upregulation of Desmin expression (arrowheads).
Figure 5.12: Activation of hepatic stellate cells leads to increased extracellular matrix deposition around hepatic blood vessels

(A-F) Double immuno labelling for fibronectin and α-SMA on E18.5 liver sections. (A) A significant increase in fibronectin deposition can be detected around mutant blood vessels compared to wild type (D). (G-L) Double immuno detection of Laminin 5 and α-SMA. (H) A strong increase in Laminin 5 deposition around ephrin-B2 expressing hepatic blood vessels can be observed which almost completely overlaps with the α-SMA counterstaining (arrowheads in I). (M-R) Immuno labelling for Collagen IV and α-SMA. A moderate increase of Collagen type IV deposition is detectable around mutant hepatic vessels.
Chapter 6 | Discussion & concluding remarks

Over the past few years, a series of studies have demonstrated the essential role of ephrin-B2 in the developing vascular system, where the molecule is predominantly expressed by arterial endothelial cells (Wang et al., 1998). Ephrin-B2 null mutant mice are lethal at midgestation due to severe malformation of the vasculature including the lack of angiogenic remodelling (Adams et al., 1999; Gerety et al., 1999). Similar defects occur in EphB4 deficient mice and in a subset of mice lacking the two receptors EphB2 and EphB3 (Adams et al., 1999). These receptors are binding partners for ephrin-B2 suggesting that reciprocal receptor-ligand interactions and the downstream signal transduction cascades are essential for normal blood vessel formation. The early lethality of global as well as EC-specific ephrin-B2 knockout mice prevented so far the analysis of the role of this transmembrane ligand at later stages of embryonic development. In the work presented here, I have generated an inducible EC-specific ephrin-B2 overexpression system to circumvent this limitation and to examine the role of ephrin-B2 in processes such as mural cell association and maintenance of arteriovenous integrity during blood vessel maturation. The analysis of this system outlined the importance of Eph/ephrin-B2 interaction in the vascular system throughout embryonic development.

It has been previously reported that EC-specific overexpression of ephrin-B2 does not lead to pronounced developmental defects (Oike et al., 2002). In this study, transgenic embryos overexpressing ephrin-B2 on endothelial cells developed only minor vascular malformations such as haemorrhaging and decreased capillary network formation in the head region. Likewise, capillary formation in the postnatal brain was only modestly reduced. These findings stand in stark contrast to my own work in which, irrespective of the analysed developmental stage, the upregulation of ephrin-B2 expression leads to severe vascular defects and embryonic lethality. A possible explanation for this discrepancy could lie in the verification of transgene expression. In the bicistronic expression construct used by Oike et al., an ephrin-B2 cDNA and a lacZ reporter gene, separated by an internal ribosome recognition site (IRES), were placed under control of a single promoter. The use of such bicistronic expression constructs is not without problems and a variety of technical limitations such as a lack of robust expression of
either protein or generally lower expression levels compared to monocistronic expression vectors have been reported (Jankowsky et al., 2001). The spatial expression pattern of the ephrin-B2 transgene was never directly assessed in the transgenic animals generated by Oike et al., and critical conclusions were based on correlative results obtained with the lacZ reporter. In this context it is also noteworthy that Oike et al. did not report whether Eph receptor phosphorylation (i.e. activation) and therefore Eph/ephrin signalling were increased in their transgenic mice. In the work presented here, I used a series of biochemical and immunohistochemical experiments to conclusively show proper expression, localisation and signalling capacity of the ephrin-B2 transgene. Consequently, discrepancies between the two studies may be attributable to differences in the levels and patterns of transgene expression and the successful triggering of signalling processes.

6.1 The role of ephrin-B2 expression in mural cell association

There is accumulating evidence that ephrin-B2 and EphB4 play important roles in the different cell populations forming blood vessels throughout development and, probably, in the adult organism. But the underlying regulatory mechanisms are still poorly understood. Recent work from our laboratory (Foo et al., in press) established that mural cells require ephrin-B2 for normal association with small diameter vessels. To study the role of the transmembrane ligand in pericytes and vSMCs, ephrin-B2 was specifically deleted in the mural cell population by breeding transgenic Pdgfrb-Cre mice (S.S.F. and R.H.A., unpublished) into a background of mice carrying a loxP-flanked version of the Efnb2 gene (Grunwald et al., 2004). The resulting mutants developed to term but died shortly after birth due to respiratory failure. Efnb2ΔPC/vSMC embryos present a variety of vascular defects. Freshly isolated E18.5 mutant embryos display oedema and extensive haemorrhaging in the skin, the mesenteric lymphatics and the lung, which is very similar to the phenotype observed at the same embryonic stage in mutants in which endothelial ephrin-B2 overexpression was induced after midgestation.
This shows the importance of ephrin-B2 expression in endothelial and mural cells for blood vessel maturation.
Detailed analysis of ephrin-B2 loss-of-function mutants (Foo et al., in press) revealed that the microvessel architecture was disrupted. Examining of the spatial organisation of pericytes and vSMCs associated with microvessels showed that although there was no significant difference in the numbers of mural cells covering the mutant vasculature compared to control tissues, smooth muscle cells were scattered in Efnb2ΔPC/vSMC embryos, resulting in an incomplete coverage of microvessels. The presence of rounded vSMCs suggested a requirement of ephrin-B2 expression in these cells for proper spreading and attachment. Similarly, the association of pericytes, which normally wrap tightly around capillaries, was clearly perturbed in mutant microvessels (Foo et al., in press). The situation is very similar to ephrin-B2 overexpressing vasculature, in which small diameter vessels exhibit insufficient vSMCs/PCs coverage. But compared to the situation on Efnb2ΔPC/vSMC blood vessels, the reduced perivascular cell coverage can be attributed to a loss of associated mural cells. In fact smooth muscle actin and desmin-positive cells were abundantly scattered around ephrin-B2 overexpressing blood vessels.

On the ultrastructural level, Efnb2ΔPC/vSMC pericytes associate poorly with capillaries and abnormal deposits of extracellular matrix can be seen in gaps separating PCs from the endothelium (Foo et al., in press). Similarly, ultrastructural analysis confirmed detachment of PCs from the endothelial monolayer in the skin of ephrin-B2 overexpressing transgenic mice, although there was no apparent increase in the deposition of ECM around blood vessels. While Foo et al. had not addressed the role of Eph/ephrin molecules in EC-mural cell interaction, the results presented in this thesis show that ephrin-B2 plays a critical role in the control of this process. Neither ephrin-B2 overexpression in ECs nor inactivation of the gene in mural cells had any appreciable effect on arterio-venous identity as judged by expression of specific molecular markers. This finding is in line with previous studies (Gerety et al., 1999; Wang et al., 1998) suggesting that the Eph/ephrin system regulates features that are characteristic for arteries and veins (such as differentiating mural cell recruitment) but not AV cell fate decisions.
6.2 Expression of Ephs and ephrins during adulthood and in pathological settings

Postnatal neovascularisation is an important mechanism involved in numerous pathological processes such as ischemic cardiovascular diseases (myocardial and limb ischemia), tumourigenesis and wound healing, but also in physiological processes such as in the female reproductive system. The mis-regulation of blood vessel development contributes to a growing list of pathological conditions and, in particular, cancer (see Chapter 1.2.6 and 1.3.7). The analysis of expression profiles of different cancer types revealed that ephrins and Ephs are upregulated in variety of tumours, especially in the more aggressive stages of tumour progression (Dodelet and Pasquale, 2000). For example, EphA2 is up-regulated in breast, liver, and prostate cancer, glioblastoma, esophageal squamous cell carcinoma, ovarian cancer, and melanoma (Surawska et al., 2004). Ephrin-B2 in particular was shown to be up-regulated in melanomas, ovarian cancer, liver cancer and kidney cancer (Bohme et al., 1993; Fox and Kandpal, 2004; Vogt et al., 1998; Walker-Daniels et al., 1999), where the transmembrane protein may affect tumour cell behaviour but also interactions with surrounding tissue and blood vessels.

It has been previously shown that ephrin-B2 continues to specifically mark arteries in the adult, with its expression progressively extending to the vascular smooth muscle cell layer towards to end of embryonic development (Gale et al., 2001; Shin et al., 2001). Furthermore, ephrin-B2 can be detected at sites of neoangiogenesis in adults and it has been shown to be up-regulated on tumour vasculature in different mouse tumour models (Gale et al., 2001; Shin et al., 2001). The morphology of tumour vessel has been studied extensively in recent years as these vessels have been recognised as a clinically important therapeutic target. The abnormalities of tumour vessels provide the potential for targeting these vessels without destroying the normal vasculature. On the other hand structural and functional abnormalities, which can lead to leakiness and ischemic areas within the tumours, affect the accessibility of drugs to cancer cells. This poses a substantial hindrance to effective and targeted cancer treatment. As reported by Morikawa et al., pericytes found on blood vessels of different human and mouse tumours in mouse xenograft models present abnormally loose association to endothelial
cells and extend numerous cytoplasmic processes deep into the tumour tissue (Morikawa et al., 2002). In fact, the disrupted mural cell coverage around the examined tumour vessels very much resembles the observed phenotype in ephrin-B2 overexpressing embryos. This raises the question if ephrin-B2 expression levels on tumour ECs could be responsible for the insufficient association of mural cells to sprouting tumour vessels. The normalisation of intra-tumour vessels could potentially facilitate the delivery of specific anti-cancer drugs into the tumour. To understand the precise mechanisms by which ephrin-B2 mediates mural cell association in physiological and pathological conditions may well provide important clues for such therapies.

Other studies have also indicated that ephrin-B2 may regulate blood vessel formation in the adult, for example, by promoting interactions between arterial and venous vessels (Hayashi et al., 2005). In this study a hind limb ischemia model was used to show that ephrin-B2 mRNA was significantly up-regulated in the ischemic limb at 24 hours, after initial upregulation of VEGF mRNA after 6 hours. In order to confirm EC-specific upregulation of ephrin-B2, an in vitro EC tube formation assay with human microvascular ECs (HMVECs from adults) was performed. Quantitative RT-PCR analysis demonstrated that ephrin-B2 transcription was significantly upregulated in tube-forming ECs from 4 to 15 hours during the process of vascular tube formation. The group went on to examine how ephrin-B2 could affect postnatal arterial and venous neovascularisation in vivo. By using a corneal micropocket assay in ephrin-B2/LacZ mice, the induction of postnatal neovascularisation by ephrin-B2 or VEGF was assessed. Although both, ephrin-B2 as well as VEGF could induce significant neovascularisation in corneal avascular areas, the area around the ephrin-B2 pellet exhibited weaker circumferential neovascularity compared to the VEGF induction. Furthermore the arterial neovascularure induced by ephrin-B2 was limited, with shorter length and fewer branches at the capillary level than that induced by VEGF. In turn the effect of ephrin-B2 ligand induced neovascularisation seemed to have a more pronounced effect on venous vessels, judged by the capillary ratio between arterial and venous vessels. At this point it remains unclear whether this effect involves altered endothelial cell-pericyte interaction as they were observed in my own study. It appears possible that ephrin-B2 stimulates neoangiogenesis by loosening pericyte contacts. In conclusion, the emerging picture is that ephrin-B2 may play a critical role in adult
neovascularisation. There are also strong indications that ephrin-B2 is involved in wound healing processes (Hafner et al., 2005a; Hafner et al., 2005b).

6.3 Therapeutic potential of Eph/ephrin signalling inhibition

Looking at the potential contribution of EphB4/ephrin-B2 signalling in pathological scenarios, it is not surprising that increasing attention is being paid to the evaluation of the therapeutic potential of soluble forms of EphB4 and ephrin-B2 in treating vascular proliferative disorders and cancers (Kertesz et al., 2005; Martiny-Baron et al., 2004; Zamora et al., 2005). There are first indications that soluble forms of ephrin-B2 and EphB4 can reduce neovascularisation in a model of proliferative retinopathy (Zamora et al., 2005). Kertesz et al. showed that a soluble version of the EphB4 receptors (sEphB4) can block the activation of EphB4 and ephrin-B2, suppress EC migration, adhesion and tube formation in vitro, and inhibit the angiogenic effects of VEGF and bFGF in vivo. More importantly, the localised (in the implanted tumour) as well as systemic administration of sEphB4 led to an inhibition of tumour growth in a murine xenograft model. As recently shown by Noren et al., EphB4 receptor promotes tumour growth by stimulation angiogenesis through ephrin-B2 by attracting ephrin-B2-positive ECs to the tumour (Noren et al., 2004). The data suggest that endothelial ephrin-B2 promotes the formation of new blood vessels in EphB4-positive tumour tissue, in addition to promoting vascular remodelling. Using a different approach, a recent study found that overexpression of ephrin-B2 by tumour cells markedly decreased tumour growth in a mouse xenograft model (Liu et al., 2004). It was also shown that KM12L4 human colon cancer cells stably transfected with ephrin-B2 produced higher tumour microvessel density and lower tumour cell proliferation than did parental or vector-transfected control cells (Liu et al., 2004). Tumours from ephrin-B2-transfected cells had significantly decreased blood volume. Liu et al. proposed that the decrease in blood perfusion might result from dysfunctional vessels, although ephrin-B2 transfection increased tumour vessel density.

The data presented in this thesis outlines the importance of endothelial ephrin-B2 expression for mural cell attachment to the maturing vasculature. With these new insights, the targeting of Eph/ephrin signalling for therapeutic reasons has to consider
the potential effects on blood vessel integrity. This might be used as an advantage for certain pathological conditions but poses as well a potential complication for the treatment of tumours. It is also noteworthy that most anti-angiogenic drugs target VEGF, which has shown to act as an upstream regulator of ephrin-B2 (Lawson et al., 2002). The ablation of VEGF function might affect mural cells association by interfering with ephrin-B2 signalling.

6.4 Conclusions

In the context of this thesis it might initially surprise that the deletion of ephrin-B2 in mural cells leads to similar defects as the overexpression of the ligand on endothelial cells. Both studies consistantly indicate that ephrin-B2 plays a crucial role in the association of pericytes and vascular smooth muscle cells to the maturing vasculature. Thus, the regulation of this process appears to require precisely balanced expression of ephrin-B2 on ECs, PCs and vSMCs to control the attachment of mural cells to the endothelial monolayer. There are examples in which the same receptor/ligand pair can trigger repulsive or adhesive cellular responses, depending on the density of presented ligands/receptors and the cellular context (Eberhart et al., 2002; Helmbacher et al., 2000; Huynh-Do et al., 1999). Overexpression of ephrin-B2 on endothelial cells might tip this balance to a repulsive signalling read-out. PCs and vSMCs have been shown to express several Eph receptors, which can bind and thereby signal through ephrin-B2. As shown by biochemical experiments, elevated ephrin-B2 levels in the endothelium triggered increased EphB4 activation and led ultimately to a decrease in total levels of EphB4 receptor protein, possibly caused by internalisation and degradation of the ligand/receptor signalling complex (Chapter 3.2.4).

Despite phenotypical similarities between the loss of ephrin-B2 function in mural cells and the ephrin-B2 overexpression on ECs in vivo, there are obvious differences in characteristics of how these three cells types can interact with each other. While the loss of ephrin-B2 function has a direct effect on the interaction between Eph receptor expressing vSMCs and PCs and between PCs and ECs, the presence of a vascular basement membrane separating ECs from the smooth muscle layer suggests that the loss of vSMCs may be a secondary effect caused by the effects of endothelial ephrin-B2. On
the other hand, endothelial processes presenting transgenic ephrin-B2-ECFP fusion protein were frequently seen to protrude the subendothelial basement membrane and the surrounding smooth muscle layer so that direct EC-vSMC interactions appear possible (Chapter 5.2.2.5).

As discussed in Chapter 5, the fact that ectopic expression of ephrin-B2 ligand on venous ECs leads to a more pronounced loss of mural cells might be explained by interendothelial interactions and signalling events of EphB4 positive venous ECs encountering ectopically expressed ephrin-B2. Furthermore, in contrast to arteries, venous vessels maintain some pericyte attachment throughout development. This might allow prolonged interaction between ephrin-B2 overexpressing ECs and Eph receptor expressing PCs. The presence of an arterial marker on veins did not affect the venous identity but rather lead to the destabilisation of venous vessels due to the loss of blood vessel supporting cells.

The ectopic expression of ephrin-B2 in the embryonic liver produced early signs of a fibrosis. Although these results are preliminary, the postnatal effects of ephrin-B2 overexpression on hepatic sinusoidal ECs should be investigated. Overall, the work presented in the context of this thesis underlines the importance of ephrin-B2 expression during later stages of development. The coordinated expression of this ligand is crucially involved in the proper association of vascular smooth muscle cells and pericytes to the maturing vasculature.

6.5 Future Perspectives

Although it was possible to reveal the importance of ephrin-B2 signalling in mural cell association to the endothelial monolayer, the underlying inter- and intracellular signalling events need to be elucidated in more detail. The isolation of primary cells (ECs, vSMCs and PCs) from TetO-ephrin-B2 mice will help to study cellular processes and interaction of these cell populations in vitro. The inducibility of ephrin-B2 expression under different culture conditions will facilitate the establishment of a variety of in vitro assays, ranging from co-culturing, scratch to cell migration assays.
Finally, it should be well worth to combine the ephrin-B2 overexpression system with animal models of human pathologies to investigate the role of the ligand in disease processes.
References


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