The Role of EphrinB2 in Blood Vessel Development

Shane Siang Chin Foo

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Vascular Development Laboratory, Cancer Research UK, London
Department of Anatomy, University College London

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

To my family

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Abstract

The development of the vascular network is a complex process that is controlled by precisely balanced angiogenic and anti-angiogenic factors. Several studies have established that ephrinB2 and its receptor EphB4 are important regulators of angiogenic remodelling in the embryonic vasculature. The ablation of either gene in mice leads to fatal cardiovascular defects and early lethality. As ephrinB2 is expressed in different cell types within blood vessels, specifically in arterial endothelial cells, pericytes and vascular smooth muscle cells, the function of the molecule in each cell population has remained unclear.

To determine the role of ephrinB2 expression in the luminal endothelial lining of blood vessels, tissue-specific knockout mice were generated with the Cre-loxP method. The resulting mutants displayed fatal defects in the development of blood vessels and the heart that resembled the phenotype of the global ephrinB2 null mice. This demonstrated that ephrinB2 is essential in the endothelial cells and that its expression in other cell types within the cardiovascular system is not sufficient to compensate for this loss.

Pericytes and vascular smooth muscle cells, so-called mural cells, are associated with the endothelium and are essential for the formation of a stable and mature vascular network. To study the role of ephrinB2 in mural cells that express progressively increasing levels of the ligand during the second half of embryonic development, a transgenic mouse line expressing Cre recombinase under the control of a fragment from the PDGFRβ gene was established. This permitted the generation of mural cell-specific ephrinB2 knockouts in which the investment of pericytes and smooth muscle cells in the microvasculature was impaired so the vessel wall assembly was defective. Consequently, these mutants displayed oedema, haemorrhaging and presumably died of respiratory arrest. These findings demonstrate that ephrinB2 expression in mural cells is essential for the maturation of the microvessels but apparently not for the remodelling of the endothelium.

As insufficient support by mural cells and the resulting increase in blood vessel permeability is a relevant factor in human disease, such as diabetic retinopathy and tumours, the findings presented here argue that the ligand may be a potential target for therapeutic intervention.

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In the earliest stages of development, the embryo survives in the absence of the vasculature as it initially receives the oxygen and nutrients through diffusion from the yolk sac and placenta. However, this is quickly changed due to the increasing demand for oxygen and nutrients required for its growth. The embryo then rapidly forms a highly vascularised system through two processes known as vasculogenesis and angiogenesis (Figure1.1). The resulting mature vascular system ensures that an adequate blood flow is maintained to provide the cells of the organism with sufficient supply of nutrients and oxygen, as well as the removal of waste and by-products.

1.1 Vasculogenesis

The initial formation of a primitive vascular network in the embryo is established at the time when somites begin to form, by a process known as vasculogenesis. The first phase of the construction of the embryonic vasculature, begins with the differentiation of mesenchymal cells from the lateral plate mesoderm of the yolk sac into haemangioblast, which are the precursors of both blood cells and endothelial cells (Shalaby et al., 1997). These cells condense into aggregates known as blood islands, of which the inner cells become haematopoietic stem cells (blood cells precursors), while the outer cells develop into angioblasts (blood vessels precursors). The angioblasts then differentiate *in situ* and assemble into endothelial cell cords, which then extend and fuse together to form connecting tubes. These later develop into a capillary network known as the primary capillary plexus. While the initial vascular plexa are still emerging, first steps of remodelling occur, thus transforming the uniform primary plexa into more complex secondary structures.

In the embryo proper, the primitive vascular network includes the major vessels such as the aortic arches, the dorsal aorta and the cardinal veins, as well as honeycomb-like plexa connecting these principle vessels. While in the yolk sac, the assembly of endothelial cells gives rise to the vitelline vein, which allows the delivery of nutrients,

waste removal and promotes gaseous exchange between the maternal and embryonic circulation. Molecules from different growth factor families act in concert to promote vasculogenesis. Both vascular endothelial growth factor (VEGF) and transforming growth factor β (TGF-β) signalling pathway are found to be required for mesoderm differentiation, haematopoiesis and blood vessel formation. VEGF-A and VEGFR2 (flk-1) knockouts in mice are lethal at E8.5-11 due to defects in angioblast differentiation, haematopoiesis and blood vessel formation (Carmeliet et al., 1996; Ferrara et al., 1996; Shalaby et al., 1997). In contrast, the lethality in VEGFR1 (flt-1) embryos is attributed to the increase of haemangioblast commitment, thereby resulting in the formation of disorganized vessels due to overcrowding of the endothelial cells (Fong et al., 1999). 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) knockouts show normal haematopoiesis (Larsson et al., 2001). However, they still 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, *in vitro*.

1.2 Angiogenesis

The second process, known as angiogenesis, refers to the formation of new blood vessels through the modification of pre-existing vascular structures. In this process the primary vascular network is remodelled, pruned and enlarged into distinct arteries, veins and interconnecting capillary beds that are characteristic of the mature vasculature. Angiogenesis is a complex biological process involving the delicate co-ordination of multiple steps that are controlled by an exquisite balance between activating and inhibitory effectors (Figure 1.2).

1.2.1 Intussusception

Angiogenesis is normally thought of as the formation of new capillaries and vessels mediated through the sprouting, migration and proliferation of endothelial cells that are

resident in the capillaries, a process coined as sprouting angiogenesis. Nonsprouting angiogenesis by means of intussusception is another important process involved in the expansion and modification of vessels and capillary formation. It involves the partitioning of existing vessel lumens into smaller ones by formation and insertion of interstitial tissue structures or pillar tissues into the vessel lumen. This process was first observed in the rapidly expanding postnatal lung capillary bed, but has since been detected in a variety of tissues and organs in embryonic as well as adult angiogenesis (Patan, 2004).

1.2.2 Vasodilation and permeability

The vasodilation of pre-existing vessel is the initial process in angiogenesis and involves nitric oxide (NO) (Carmeliet, 2000). Remarkably, vascular endothelial growth factor (VEGF) induces NO synthase activity in endothelial cells. The increased levels of intracellular NO stimulate guanyl-cyclase to synthesise cyclic guanoside monophosphate (cGMP), which subsequently results in the relaxation of vascular smooth muscle cells (vSMCs). VEGF is also involved in increasing vascular permeability of endothelial cells through the formation of fenestrae, a process that is restricted to the endothelium of glomeruli, gastrointestinal tract, endocrine organs and different areas of the brain (Zachary and Gliki, 2001). More generally, vascular permeability can be increased by the disruption of cell-to-cell adhesion, caused by the phosphorylation of components of intercellular endothelial adherens and tight junctions (Zachary and Gliki, 2001). VEGF stimulation leads to the phosphorylation of adhesion molecules including vascular endothelial (VE)-cadherin and β-catenin, as well as the tyrosine phosphorylation of tight junction molecules, occludin and zona occluden (ZO)-1. To a certain extent, this allows plasma proteins from the blood stream, such as fibrinogen and plasminogen, to extravasate through inter-endothelial cell junctions into the surrounding tissue (Carmeliet, 2000). These plasma proteins then provide temporary structural support for the migration of activated endothelial cells.

Control of vascular permeability is not only critical during developmental angiogenesis, but also for the normal physiology of the adult vasculature. Excessive vascular leakage is a factor in pathological conditions such as intracranial hypertension or circulation

collapse, and therefore blood vessel permeability must be tightly regulated. However, the signalling mechanism regulating this effect remains largely obscure.

Angiopoietin1 (Ang1), is an inhibitor of vascular permeability and is expressed in mural cells (consisting of vSMCs and pericytes). Targeted gene inactivation studies have revealed its important role in remodelling and promoting stabilisation of the vessels, through mediating tight association between endothelial and mural cells, and interactions with extracellular matrix molecules. Ang1 knockout mice die at midgestation (E12.5) and display cardial defects as well as generalised vascular remodelling and branching defects (Suri et al., 1996). Mutant analysis showed a failure of myocardial and mural cell recruitment to the vessel wall and aberrant organisation of extracellular matrix elements such as collagen fibres. Although not as severe, these phenocopy defects seen in knockout mice of the cognate receptor, Tie2, a tyrosine kinase expressed in the endothelium (Sato et al., 1995). Mutants lacking Tie2 die at E10.5, displaying growth retardation of the heart and defective vascular remodelling. Further evidence of Ang-1 association with the regulation of vascular permeability, are provided by its overexpression in the skin of transgenic mice under the control of the keratin (K)-14 promoter (Suri et al., 1998). These mice display larger, more numerous and more highly branched vessels. More interestingly, subsequent studies of these mice show that overexpression of Ang-1 makes dermal vessels resistant to the increase in permeability (i.e. leakiness) caused by inflammatory agents or by overexpression of VEGF (Thurston et al., 1999). Further corroborating data is provided by another study using the mouse neonatal retina where the vascular network is formed in the absence of mural cells (Uemura et al., 2002). In this setting, the vasculature is poorly remodelled and leaky, but the application of Ang-1 is remarkably able to restore a hierarchical order of the larger vessels and rescue oedema and haemorrhage. As there are many pathologies associated with microvascular leakage, such as inflammatory diseases and diabetic retinopathy, Ang-1 has the therapeutic potential to reduce plasma leakage in these conditions.

1.2.3 Vessel destabilisation and matrix degradation

Before the ECs of pre-existing vessels are able to invade out of their resident site, inter-endothelial cell contacts must be loosened, mural cells need to detach, the EC basement membrane must be degraded and the perivascular extracellular matrix needs to be remodelled. A known trigger of such processes is Ang-2, an antagonist of Ang-1, that inhibits Tie2 signalling and causes destabilisation of the vessels leading to subsequent endothelial cell migration. Proteases, such as the plasminogen activators, chymases, matrix metalloproteases (MMP), and Heparanase families, are important effectors in these processes as they degrade the matrix molecules and liberate sequestered growth factors such as bFGF, VEGF and insulin-like growth factor (IGF)-1 from the matrix (Conway et al., 2001). 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 $\alpha_v\beta_3$, can recruit and possible 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-β1 signalling pathway is also implicated in this process. Targeted ablation of one of its type I receptors for TGFβ, 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 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 perivascular proteolysis and are more permeable, thus leading to excessive proliferation of ECs and capillary fusion.

1.2.4 Endothelial cell proliferation and migration

Once the physical barriers provided by the basement membrane are dissolved, proliferating endothelial cells are free to form sprouts and to migrate to distant sites. A

delicate balance between angiogenic and angiostatic factors controls these processes. Proliferating ECs are guided by gradients of chemotactic agents through the disintegrated basement membrane into the remodelled perivascular space. These processes involved in active angiogenesis are tightly regulated during the development of the embryo and adult organism, and are mediated through the interplay between various isoforms of VEGF, angiopoietins, fibroblast growth factors (FGFs), ephrins, PDGF-B and their respective receptors, as well as many other molecular factors (Table 1.1). VEGF and the angiopoietins appear to be specific mitogens for endothelial cells, whereas FGFs induce proliferation in a wide variety of cell types. Several of these angiogenic molecules have had their distinct function and contribution revealed through the generation of knockout mice, despite considerable molecular redundancies (Table 1.2). Inhibitors of angiogenesis include endostatin, angiostatin and Thrombospondins (Table 1.1).

1.2.5 Lumen formation and vessel stabilisation

Once migrating endothelial cells have reached the target area, they assemble into a monolayer and form tube-like structures enclosing a lumen. The intercalation of endothelial cells and vessel fusion of pre-existing cells enables vessels to increase their lumen size and length to meet changing demands. Interestingly, the different isoforms of VEGF-A have been shown *in vivo* to be involved in determining vessel size and branching behaviour of the growing microvessel network. Mutant mice that only express the solubleVEGF120 isoform, without heparin-binding capabilities, developed capillary networks with fewer branch points and larger luminal diameter. In contrast, mice solely expressing VEGF188, the heparin-binding isoform, exhibited the formation of ectopic and abnormally thin vessel branches, while mutants lacking either VEGF164 or one of its co-receptors neuropilin (Nrp)-1 showed no vascular branching defects. The mytocyte enhancer binding factor 2C (MEF2C), as well as the integrins $\alpha_v\beta_3$ and α_5 are also involved in lumen formation, whereas Thrombospondin-1 appears to act as an inhibitor (Carmeliet, 2000).

Once nascent endothelial tubes are formed, they must be stabilised by mural cells. To achieve this, the endothelium secretes factors that cause the mesenchymal cells in the

surrounding tissue to proliferate and migrate to the abluminal surface of the premature vessels, in a process known as vascular myogenesis. Then, these primordial mural cells differentiate either into pericytes, which are located within the basement membrane, or into vSMCs, which are found wrapped around the outer face of the basement membrane (Figure 1.3). VSMCs cover the majority of the vascular network but are absent from capillaries, while pericytes cover even the smallest capillary branches. There is a gradual transition from vSMCs to pericytes on the arterial side of capillaries and then back to vSMCs on the venular side.

1.3 Arterial or venous fate, determined by genetic predisposition or by haemodynamic force?

It was previously thought that the structural difference between arteries and veins is functionally determined by blood flow. Arterial vessels, which receive blood at high pressure from the heart, are characteristically surrounded by a thick layer of vSMCs (tunica media)(Figure 1.4). Venous vessels, on the other hand, carry efferent circulation of lower pressure, thus have less ensheathing smooth muscle, and contain valves to prevent blood back-flow (Figure 1.4). There is now a growing body of evidence indicating that molecular differences do exist between arterial and venous endothelial cells and are predetermined before the onset of blood circulation through genetic regulation.

A first key finding, indicative of arterial-venous specific gene expression profiles, was that ephrinB2 is specifically expressed in arteries while one of its cognate tyrosine kinase receptors, EphB4 is specifically expressed in veins (Wang et al., 1998; Adams et al., 1999; Gerety et al., 1999). Ablation of the genes for ephrinB2 or EphB4 in mouse, results in lethality, defective remodelling of the yolk sac vascular plexus, decreased branching of cranial capillaries, abnormal sprouting of intersomitic vessels and decreased vascularisation of the neural tube. In both knockouts, angiogenesis is arrested, affecting both arteries and veins. The expression pattern of ephrinB2 and EphB4 was determined by the knock-in of the lacZ reporter cassettes into the endogenous gene loci. Homozygous embryos (knockouts) still expressed lacZ in

specific vessels, showing that the respective promoter/enhancer elements are still active and that ephrinB2 and EphB4 are not required for the initial specification of arterial and venous fate. It is believed that interactions between ephrinB2 and its receptor EphB4 modulate angiogenesis in two ways. At the border between arterial and venous capillaries the "attractive" interactions might help to ensure that arterial and venous capillaries are connected. In non-border regions, they may ensure that the fusion of capillaries to make larger vessels respects arterial-venous identity possibly through repulsive signals (Yancopoulos et al., 1998).

Gridlock, a basic-helix-loop-helix (bHLH) transcription factor, is an early determinant for arterial specification. Angioblasts expressing gridlock in the lateral posterior mesoderm, favour differentiation into an arterial cell fate (Zhong et al., 2000). Interestingly, the removal of gridlock expression in zebrafish, by genetic mutations or morpholino-mediated gene knockdown, ablates some arteries and leads to an expansion of the venous domain, as determined by diminution of ephrinB2 expression and increase in EphB4-positive structures (Zhong et al., 2001). Furthermore, gridlock has been shown to be downstream of Notch, as interference with Notch signaling using blocking agents reduces arterial and increases venous domains, similar to what has been observed for gridlock mutants. Notch signalling is associated with cell fate determination in various cell types and for the definition of tissue boundaries (Zhong et al., 2001). Inhibition of Notch in zebrafish leads to the loss or arterial markers including ephrinB2 and ectopic expression of venous markers. Mice lacking Notch1, one of four Notch receptors known in mammals, display vascular defects with disruption at the anterior bifurcation of the aorta (Krebs et al., 2000; Lawson et al., 2001). More recently, it has been reported that VEGF acts downstream of sonic hedgehog (Shh) and upstream of the Notch pathway to control the determination of arterial cell fate in zebrafish (Lawson et al., 2002). The loss of either VEGF or Shh leads to the loss of arterial identity, i.e. ephrin-B2a expression, while their overexpression resulted in ectopic expression of arterial markers. Furthermore, arterial differentiation was rescued in embryos lacking Shh activity by injection of VEGF mRNA. Likewise, defective VEGF signalling was rescued by activation of the Notch pathway. Genetic studies in mice lacking the expression of the Notch ligands Delta-like (Dll)-4 (arterial endothelium specific) or Jagged1 (endothelium specific), revealed critical roles in angiogenic remodelling in the yolk sac and in the embryo but their involvement in vessel identity is

unclear ((Xue et al., 1999) and unpubished data from Mailhos et al.). However, mutations in the Notch signalling pathways has been linked to several human syndromes that display vascular defects. Mutations in the Notch3 gene causes cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), a hereditary adult-onset condition causing stroke and vascular dementia (Joutel et al., 1996). Mutations in the Jagged (Jag)-1 gene causes Alagille syndrome, which is a developmental disorder characterised by abnormalities in multiple organs including the liver, heart, eye, and kidneys (Li et al., 1997).

The TGFβ 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 or 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 TGFβ 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 (Urness et al., 2000; Sorensen et al., 2003). Both knockouts die by E11.5 with severe arteriovenous malformations resulting from fusion of major arteries and veins as well as shunting between presumptive arterial and venous vascular beds. The arterial specific marker, ephrinB2 was downregulated. 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 (ephrinB2 and neuropilin-1 for the arteries, Tie2 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 reveal 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 chick 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, ephrinB2 expression was detected, which is inconsistent with genetically predetermined expression in the absence of haemodynamic factors.

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. The precise mechanism of how this arterial-venous fate is established and whether one of the two contributing factors is prevalent remains to be uncovered.

1.4 Interactions between mural cells and endothelial cells

Besides stabilising the vessels by inhibiting endothelial proliferation and migration, and by stimulating the production of extracellular matrix, mural cells protect the newly formed vessel from rupture and regression and play an important role in haemostatic control. Larger vessels become covered by additional muscular coatings that provide additional elastic and vascomotor properties necessary to accommodate increased blood

flow (Figure 1.4). This process is also influenced by mural cell-mediated deposition of elastin fibres and extracellular components. Once the final shape of the vessel is established, mural cells mediate many specialised functions in vascular beds. They control haemostasis, provide a blood barrier and scavenge plasma molecules that pass through the endothelium, regulate vascular permeability in the glomerulus, and control vessel remodelling and plasticity (Carmeliet and Collen, 2000). An example of the ability of vSMCs to adapt to specialised functions, are the mesangial cells in kidney glomeruli. Mesangial cells control the intussusception of capillaries in the glomerulus and thereby increase the surface area for filtration of the blood.

1.5 Molecules involved in mural cell differentiation, recruitment, growth, and remodelling

The origin of smooth muscle cells is very complex and depends on their location. Smooth muscle cell differentiation can occur by the following: 1) differentiation of mesodermal cells through differentiation signals secreted by the endothelium; 2) transdifferentiation of ECs into vSMCs or of bone marrow precursors and macrophages triggered by the autocrine release of angiopoietin-1; 3) transformation of epicardial cells to coronary smooth muscle; and 4) differentiation of the mesectoderm of the neural crest into vSMC (Berk, 2001). The complexity of the origin of vSMC suggests that growth factors and their receptors may control vSMC differentiation and growth in different vascular beds. Smooth muscle differentiation involves transcriptional events mediated by the serum response factor, *Prx-1* and *Prx-2*, *CRP2/SmLIM*, capsulin, and members of the *Hox*, *MEF2* and *GATA* family, which are not reviewed here (see (Gittenberger-de Groot et al., 1999)).

In terms of molecular mechanisms that regulate interactions between mural cells and ECs, at least four pathways are found to be involved. Key molecular components of these include (a) Ang-1 and Tie2; (b) platelet-derived growth factor B (PDGF-B) and PDGF receptor β (PDGFRβ); (c) sphingosine-1-phosphate-1 (S1P1) and endothelial differentiation sphingolipid G-protein-coupled receptor 1(EDG1); and (d) transforming

growth factor (TGF)-β (Jain, 2003). Other molecules contributing to mural cell differentiation and recruitment include heparin binding EGF-like factor (HBEGF), Endothelin-1 and the Eph/ephrin system (Berk, 2001).

Of the factors mentioned above, PDGF-B and TGF-β have been studied in most detail. Besides stimulating differentiation of SMC from mesoderm, both are also involved in vSMC recruitment to the vessel wall. PDGF-B, which is produced by immature endothelial cells, can induce mesenchymal cell proliferation and migration towards the endothelial cells *in vitro* (Hirschi et al., 1999). Its essential role in mural cell recruitment has been demonstrated *in vivo* through the generation of knockout mice lacking PDGF-B or its cognate receptor, PDGFR-β. Both mutants display similar vascular defects and pericyte loss (Soriano, 1994; Lindahl et al., 1997; Hellstrom et al., 1999). The cytokine TGF-β1 can inhibit the activities of other angiogenic factors in EC proliferation and migration, but can also induce vessel maturation by stimulating extracellular matrix production and mural cell differentiation. Co-culture systems of mesenchymal and endothelial cells led to TGF-β1 activation and the induction of pericyte/vSMC differentiation (Hirschi et al., 1998; Darland and D'Amore, 2001). At the same time, endothelial cell proliferation and migration becomes inhibited (Sato et al., 1990). TGF-β1 is expressed in multiple cell types including mural cells and ECs, and its pro- or anti-angiogenic role is determined by the context and its concentration. Low concentrations were found to synergistically enhance angiogenesis, induced by factors such as VEGF and bFGF, while high concentrations were inhibitory (Jain, 2003). The role of TGF-β in the control of mural cell-EC interactions, has been demonstrated by genetic studies in mice. Target 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 vSMC 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 (Carmeliet, 2000).

Besides molecular regulators, mentioned above, local physical factors such as hypoxia, mechanical forces, injury and flow dynamics control the recruitment of mural cells.

Despite our knowledge of many relevant signalling receptors and interacting molecules, the overall mechanism by which mural cells exert their multiple functions with endothelial cells *in vivo* is not well defined. Open questions include the in-depth knowledge of the mechanism of mural cell recruitment, how endothelial tubes are ensheathed, how they differentiate to serve local functions and the role of local extracellular matrix and endothelium. Acquiring thorough knowledge of such processes may provide the basis for therapeutic approaches.

1.6 The super family of receptor tyrosine kinases

Receptor tyrosine kinases (RTKs) have been recognised as critical mediators of numerous morphogenic processes during embryonic development. They also play essential roles during blood vessel development and its maturation. Examples are the vascular endothelial growth factor receptor (VEGFR), Tie, platelet derived growth factor receptor (PDGFR), fibroblast growth factor receptor (FGFR) and Eph receptors (Risau and Flamme, 1995; Gale and Yancopoulos, 1999). Some of these molecules and their ligands regulate vasculogenesis and different aspects of angiogenesis including the recruitment of mural cells and the associated remodelling of extracellular matrix. The roles of PDGF-B/PDGFRβ and Eph/ephrin are reviewed here while the well characterised VEGF/VEGFR and angiopoeitin/Tie-2 receptor families are reviewed elsewhere (see (Daniel and Abrahamson, 2000; Yancopoulos et al., 2000)).

1.7 Eph/ephrin gene family

The function of Ephs and ephrins have predominately been studied in the nervous system, where they are involved in patterning of the rhombomeres in the hindbrain, in axon pathfinding, and the guidance of migrating neural crest cells (Flanagan and Vanderhaeghen, 1998). More recently, Eph receptor and their ligands have emerged as important mediators of vascular growth and development.

The Eph receptors, with 14 members, constitute the largest class of RTKs identified. The first receptor was named for its expression in an erythropoietin-producing

hepatocellular (Eph) carcinoma cell line from which it was first isolated. Their respective family of binding ligands was termed "ephrin" (Eph receptor interacting molecules)(EN Committee, 1997) and consists of 8 members. Both Eph receptors and ephrins are divided into two subclasses, class A and B, on the basis of binding specificity and structure. In general, EphA receptors (EphA1-8) promiscuously bind to ephrinA ligands (ephrinA1-5), which are attached to the cell by a glycosylphosphotidylinositol (GPI) anchor. EphB receptors (EphB1-6) interact with ephrinB ligands (ephrinB1-3) contain a hydrophobic transmembrane region and a highly conserved short cytoplasmic domain. There are some members that have binding affinities for both classes of molecules such as EphA4, capable of ephrinB2 and ephrinB3 binding, and ephrinB1, capable of binding to EphA2. The interaction of Eph receptors with cell surface-bound ephrins makes them unique because other families of RTKs bind to soluble ligands. Furthermore, Eph-ephrin interactions mediate bi-directional signalling, whereby signal transduction pathways are activated downstream of ligands as well as receptors.

1.7.1 Structure of Eph receptors

Similar to other RTKs, Eph receptors contain a single transmembrane domain. The extracellular region consists of a highly conserved globular ligand binding domain, made up of immunoglobulin-like motifs, followed by a cysteine-rich domain and two fibronectin type III repeats (Figure1.5). The structural difference in the ligand-binding domain, dictates the specific binding affinities to either ephrin A or B class ligands. Fibronectin type III repeats may be involved in receptor dimerisation and/or interactions with other proteins such as NMDA receptors (Dalva et al., 2000). The intracellular region consists of a juxtamembrane region, a conserved tyrosine kinase domain, a sterile-α-motif (SAM) domain, and a PSD95/Dlg/ZO1 (PDZ)-binding motif. The function of the conserved Eph SAM domain remains elusive, as its deletion does not impair EphA4 function *in vivo* (Kullander et al., 2001). However, structural studies have revealed its potential involvement in modulating receptor dimerisation and clustering (Stapleton et al., 1999; Thanos et al., 1999), or even bind adapter proteins (Boyd and Lackmann, 2001).

1.7.2 Signalling Mechanism Through Eph receptors

Based on structural studies, it is thought that unbound ephrins cluster together to form homodimers, which subsequently bind to Eph receptors with a 1:1 stoichiometry. Upon ligand and receptor binding, they must reorient themselves and adopt the required active conformation to initiate signalling into both interacting cells. Crystallographic studies of the EphB2-ephrinB2 complex have revealed a unique mode of ligand-receptor recognition where two receptors bind two ligand molecules to form a ring heterotetramer complex, which fix the orientation of participating ligands and receptors ((Himanen et al., 2001), Figure 1.6). A further step in the activation and signalling of Eph receptors is the transphosphorylation of two tyrosine residues in the juxtamembrane region, as determined by structural studies on EphB2 (Wybenga-Groot et al., 2001). This phosphorylation results in a conformational change that allows the tyrosine kinase domain to be activated. The kinase activity is then stimulated by the phosphorylation of the activation loop within the kinase domain, which subsequently exposes newly autophosphorylated tyrosines for interaction with SH2/SH3 domains of target scaffolding/adaptor proteins. The phosphorylated tyrosine residue in both the juxtamembrane and kinase domain, serves as docking sites for a series of signalling proteins such as Ras-GTPase activating protein (RasGAP), Src and Abl family of non-receptor tyrosine kinases, low molecular weight phosphotyrosine phosphatase (LMW-PTP), phospholipase Cγ, phosphatidylinositol 3-kinase, and adaptor proteins SLAP, Grb2, Grb10, and Nck ((Brückner and Klein, 1998; Flanagan and Vanderhaeghen, 1998; Kalo and Pasquale, 1999); Figure 1.7). The C-terminal PDZ-binding domain of some Eph receptors are involved in interaction with PDZ-domain-containing molecules such as such as AF6, Pick1, Syntenin, and Grip1 and Grip2 (Cheng et al., 2002). Many of these proteins are believed to function as scaffolds for the assembly of multiple signalling protein complexes at the membrane.

1.7.3 Structure and signalling through A-class ephrins

Both A- and B-class ephrins share a unique conserved extracellular receptor-binding domain. But unlike the B-class, ephrinA ligands do not have a cytoplasmic domain and are tethered to the cell membrane by the hydrophobic GPI anchor. Nevertheless, there is evidence suggesting that ephrinAs have intrinsic signalling capabilities. Genetic studies in *C. elegans* in which the GPI-anchored ephrin homologues were mutated, suggest that they are capable of reverse signal transduction in response to engagement with the single Eph receptor VAB-1 (Chin-Sang et al., 1999; Wang et al., 1999). Furthermore, studies in cell culture have shown that the GPI-anchor protein CD59 can transmit an intracellular signal (Murray and Robbins, 1998) and that ephrinA5 is able to modulate cellular adhesion via Fyn in an integrin-dependent manner, when bound to its cognate Eph receptor (Davy et al., 1999; Davy and Robbins, 2000). The precise mechanisms governing ephrinA signalling remain to be uncovered.

1.7.4 Structure and signalling through B class ephrins

EphrinB ligands possess a single transmembrane domain and a short cytoplasmic region, consisting of five conserved tyrosine residues and a C-terminal PDZ-binding motif (Figure 1.5). The tyrosine residues can be phosphorylated and serve as docking sites for proteins containing SH2/SH3 domains (Figure 1.6), while the PDZ-binding motif attracts PDZ-domain containing molecules. All these structural motifs control the binding and subsequent activation of specific Eph receptors, often referred to as "forward" signalling. This simultaneously mediates membrane localisation and may subsequently lead to activation events through the B-class ligands itself that may eventually eliciting distinct biological responses, in a process termed "reverse" downstream signalling.

Much attention has been given to the study of signalling events occurring in B class ligands upon receptor engagement. It has been reported that the cytoplasmic tyrosine residues of ephrinBs become phosphorylated (Figure 1.6), which may lead to a conformational changes that in turn results in the recruitment of interacting cytoplasmic proteins and the activation of intracellular signalling cascades (Song, 2003). An

assortment of adaptor proteins has been found to interact with the PDZ-domain of ephrinBs. These include syntenin, Grip1/2, Pick1, the phospho tyrosine phosphatase FAP-1, PHIP that is related to Par-3, and more recently, PDZ-RGS3 (Lin et al., 1999; Lu et al., 2001). Association with these PDZ-domain containing proteins may facilitate the localisation of ephrinB1 into sphingo-lipid/cholesterol-enriched raft membranes, which could contribute to clustering and downstream signalling (Bruckner et al., 1999). Interestingly, it has been demonstrated that EphB receptor engagement of ephrinB1 causes rapid recruitment of src-family protein tyrosine kinase (SFKs) to raft membranes, leading to transient Src family kinase (SFK) activation (Palmer et al., 2002). The PDZ domain containing protein tyrosine phosphatase PTP-BL is subsequently recruited to the ligands cytoplasmic domain and can mediate its dephosphorylation.

The first evidence of reverse signalling by ephrins comes from genetic studies of EphB2 and its role in axon guidance in the central nervous system. Mice lacking EphB2 show defects in axon guidance in the formation of the anterior commisure while this develops normally in mutants expressing a catalytically inactive form of EphB2 (Henkemeyer et al., 1996). This suggests that the ephrinB ligands are able to actively guide axons in the absence of functional Eph receptor signalling. This is further supported by studies of retinal axon pathfinding in the optic disc. Axon guidance was found to be independent of the kinase domain of both EphB2 and EphB3, thus reliant on ephrinB2 reverse signalling (Birgbauer et al., 2000). In addition, it has been shown that the cytoplasmic domain of ephrinB2 acquires an intrinsic reverse signalling role that is essential for proper vascular morphogenesis (Adams et al., 2001). Several additional ephrinB2 knock-in mice have been generated in which the cytoplasmic domain was either truncated, replaced or modified ((Cowan et al., 2004) and unpublished data). Although the outcome of these studies is controversial, the cytoplasmic domain of ephrinB2 may regulate blood vessel and/or heart development. The use of an animal cap assay generated from a combination of lineage tracer dye and RNA injections into zebrafish embryos, has demonstrated that bidirectional signalling between ephrinB-EphB leads to restricted cell intermingling between adjacent cell populations (Mellitzer et al., 1999). It was shown that unidirectional activation through either Ephs or ephrinB2 was sufficient to restrict cell-cell communications between different cell populations, with the decrease in gap junction formation, despite intermingling (Mellitzer et al., 1999).

In vitro studies have uncovered that ephrinB1 reverse signalling activates the JNK pathway leading to changes in cell morphology that is independent of tyrosine phosphorylation (Xu et al., 2003). It was shown that the adaptor protein Grb4 is recruited to tyrosine-phosphorylated ephrinB1 via its SH2 domain, which resulted in the reorganisation of the actin cytoskeleton and led to a rounded cell morphology (Cowan and Henkemeyer, 2001). Furthermore, the association of ephrinB1 with Dishevelled (Dsh) was found to be induced by stimulation with the clustered EphB2 ectodomain (Tanaka et al., 2003). This led to the activation of RhoA GTPase and Rho kinase, and subsequently, cell repulsion.

1.7.5 From attraction to repulsion

As mentioned above, binding and activation of ephrins and Ephs require direct cell-to-cell interaction. The biological outcome of such interactions depend on cell type and context, but a general motif feature is the guidance of cell movements and the extension of cellular processes, which alters cell shape. Signals often involve the repulsion of Eph and ephrin expressing cells either in a unilateral or a mutual manner. A well studied example is the formation of hindbrain rhombomeres where Ephs and ephrins help to create boundaries between the cells of adjacent structures (Xu et al., 1999). Eph-ephrin signalling is also required for the fasciculation of axon tracts, guidance of axonal growth cones, guided migration of neural crest cells, and for the formation of topological maps in the brain (Holland et al., 1998; Holder and Klein, 1999).

The confusion still remains over how the initial adhesive interaction between Eph and ephrin proteins is converted into a repulsive signal. One possible solution to this problem may be ligand cleavage by the metalloprotease Kuzbanian/ADAM10 in response to binding between EphA3 and ephrinA2 (Hattori et al., 2000). The shedding of the ligand led to the disengagement of the cells involved and permitted efficient axon retraction in an *in vitro* assay. However, the relevance of this process *in vivo* and for the B-class ephrins remains unknown.

At sites of cell-cell contacts, endocytosis may be an alternative mechanism for the termination of adhesion between EphB-ephrinB complexes, which then permits

detachment and repulsion (Marston et al., 2003; Zimmer et al., 2003). These endocytosis events require full-length receptor or ligand and activation of the small GTPase Rac, which is in turn required for internalisation of the Eph/ephrin complex. This process leads to the retraction of lamellipodia and causes the cells to disengage.

1.7.6 Eph/ephrin function in embryonic blood vessel development

Recent studies including gene inactivation experiments in mice, cell culture systems, and angiogenic assays have demonstrated essential roles of ephrin ligands and their cognate receptors in the morphogenesis of the embryonic vasculature.

1.7.6.1 Expression pattern in the vasculature

Expression patterns for ephrins and Eph receptors are highly dynamic during embryogenesis so that the sites of expression and receptor-ligand interaction change rapidly during development (Holder and Klein, 1999). Several members of Eph/ephrin molecules are expressed in the developing cardiovascular system.

The A-class ligand, ephrinA1 has been detected by *in situ* hybridisation at multiple sites of vascular development in the mouse embryo. These include the endocardial cells lining the heart, the dorsal aorta, the primary head veins, intersomitic vessels and the limb bud vasculature (McBride and Ruiz, 1998). EphrinA1 is a chemoattractant for endothelial cells and has angiogenic activity in vitro as well as in cornea pocket assays in vivo (Pandey et al., 1995; Daniel et al., 1996). EphA2, which is also expressed in the vasculature, is an important interactor of ephrinA1. A dominant negative form of EphA2 has been shown to inhibit capillary tube-like formation in human venous endothelial cells (Ogawa et al., 2000). Furthermore, EphA2 has been found to be co-expressed with ephrinA1 in various implanted tumours lines in mice as well as in the cells of surgically removed human tumours, thus implicating possible involvement in tumour neovascularisation (Ogawa et al., 2000). However, neither the ephrinA1 nor the EphA2 null mice exhibit any vascular phenotype ((Chen et al., 1996) and unpublished data).

Important insights into the expression of B-class Eph RTKs and ephrins came from the insertion of β-galactosidase (β-gal) reporter cassettes into the mouse ephrinB2 and EphB4 genes (Wang et al., 1998). As this leads to β-gal expression under the control of the endogenous promoter and enhancer elements, the expression of ephrinB2 in arterial endothelial cells and of its cognate receptor, EphB4 in venous endothelial cells, was revealed. However, later studies reported low levels of ephrinB2 expression in venous endothelial cells as well as weak and patchy expression of EphB4 in the arteries (Shin et al., 2001). Nevertheless, ephrinB2 and EphB4 remain good markers for arterial and venous endothelial cells from the earliest stages of development in many species and are therefore widely used to define arterial-venous identity. It has also been shown that ephrinB2 expression expands to pericytes and vSMCs in the second half of embryonic development that persists into adulthood (Gale et al., 2001; Shin et al., 2001).

Other Eph/ephrin molecules are also expressed in the vascular system. EphrinB1 transcripts have been found in the endothelium but are not restricted to arterial or venous domains (Adams et al., 1999). In the endothelium of the developing kidney, ephrinB1 is co-expressed with EphB1 (Daniel et al., 1996). Clustered ephrinB1 fusion protein promotes capillary-like assembly and cell attachment in endothelial cells (Stein et al., 1998). Both ephrinB1 and ephrinB2 induce capillary sprout formation in cultured microvascular endothelial cells *in vitro*, similar to responses triggered by VEGF or Angiopoietins (Adams et al., 1999). EphB3 predominantly localises on veins but is also seen in some arteries in the yolk sac and in aortic arches (Adams et al., 1999). EphB2 and ephrinB2 are expressed by mesenchymal cells surrounding certain blood vessels, suggesting a putative role in vessel wall development through mesenchymal-endothelial interactions (Adams et al., 1999).

1.7.6.2 Insight into B-class Eph/ephrin function during blood vessel development from gene targeting studies

Many members of B-class Eph/ephrin molecules are expressed in the developing cardiovascular system, including ephrinB1, ephrinB2, EphB2, EphB3 and EphB4. For some of these genes, their involvement in vascular morphologies has been firmly established through targeted inactivation studies in mice.

The knockout of ephrinB2 resulted in severe cardiovascular defects, growth retardation, and embryonic lethality at E10.5 with 100% penetrance (Wang et al., 1998; Adams et al., 1999). Mutant hearts were inflated, incompletely looped and displayed reduced myocardial trabeculation. Vascular morphogenesis was arrested at the primary capillary plexus stage and angiogenic remodelling, such as branching and sprouting of vessels in the head and yolk sac, was impaired. Moreover, the dorsal aorta, with a low penetrance, and the anterior cardinal vein, with complete penetrance, were disrupted suggesting that ephrinB2 is required for some aspects of vasculogenesis. Overall, the vascular defects were observed in both arterial and venous domains of the vascular network. However, as ephrinB2 is only expressed on arterial endothelium, this may not be cell autonomous. The generation of knock-in mice (ephrinB2$^{\Delta C/\Delta C}$) expressing truncated ephrinB2 without the cytoplasmic domain, corroborates with this view (Adams et al., 2001). These mice displayed defects in angiogenic remodelling that where reminiscent of those observed in ephrinB2 knockout mice, confirming that the ligand reverse signalling through the cytoplasmic tail is critical during embryonic vascular development. However, replacement of the cytoplasmic domain by β-gal permitted vascular morphogenesis and full embryonic development (Cowan et al., 2004).

The ablation of EphB4 expression in mice, also resulted in mid gestation lethality and mimicked the phenotype of the ephrinB2 knockout (Gerety et al., 1999). This signified the essential role of reciprocal ephrinB2/EphB4 interactions and the mediated bidirectional signalling for fundamental steps during vascular morphogenesis.

Targeted disruption of either EphB2 or EphB3 showed no overt defects in blood vessel development. However, 30% of mice lacking both receptors displayed similar vascular

defects (Adams et al., 1999). The partial penetrance phenotype suggests that other EphB receptors may compensate for EphB2 and EphB3 function in the vasculature.

1.7.6.3 EphrinB2-mediated interactions between endothelial cells and perivascular cells

In recent years, there has been a steady increase in evidence for the involvement of ephrinB2 in the communication between the endothelium and perivascular cells of mesenchymal origin. In ephrinB2 knockout embryos, mesenchymal or pericyte cells surrounding the blood vessel are abnormally rounded compared to the normally elongated morphology (Wang et al., 1998). Angl expression, normally produced by cells supporting the endothelium was reduced in ephrinB2 knockout mice, as well as the expression of the cognate receptor Tie2 in endothelial cells (Adams et al., 1999). As Tie2 expression was also reduced in ephrin$^{\Delta C/\Delta C}$ mice, ephrinB2 reverse signalling may be required for endothelial-mesenchymal cell interactions. However, it is still unclear whether ephrinB2 directly regulates Tie2. As embryonic development proceeds, ephrinB2 expression in the arterial endothelial cells progressively extends to the surrounding vascular smooth muscle cells and pericytes (Gale et al., 2001; Shin et al., 2001). The involvement of ephrinB2 in promoting EC-mural cell proliferation and interaction has been highlighted by *in vitro* experiments involving OP9 stromal cells that over-express ephrinB2 (Zhang et al., 2001). This promoted growth and sprouting of ephrinB2- expressing endothelial cells as well as the recruitment and proliferation of smooth muscle cells. In addition, it has been reported that the activation of EphB2 and its ligands can promote smooth muscle cell proliferation in cultured bovine vascular smooth muscle cells (Woods et al., 2002).

In summary, data available to date indicates that the activation of Eph-ephrins signalling can induce vSMC proliferation *in vitro* but the actual roles *in vivo* require further investigation.

1.7.6.4 Roles of ephrinB2 in tumorigenesis?

Ephrin ligands and Eph receptors are frequently over-expressed in a wide variety of cancers affecting breast, lung and the gastrointestinal system as well as in melanomas and neuroblastomas. Several reports have also indicated their roles in tumour angiogenesis (for comprehensive reviews, see (Dodelet and Pasquale, 2000; Nakamoto and Bergemann, 2002)). Mice harbouring a LacZ cassette in the ephrinB2 gene demonstrated expression of ephrinB2 in blood vessels at sites of neovascularisation and in tumours formed by Lewis lung carcinoma and B16 melanoma cells (Gale et al., 2001; Shin et al., 2001). It is therefore conceivable that ephrinB2 may have an intrinsic role in the formation of the tumour vasculature and may be a potential therapeutic target along with EphB4. It has been shown that melanoma cells expressing soluble EphB4 (sEphB4) proliferate normally as a monolayer, but at a reduced rate when grown as three-dimenstional cultures *in vitro* (spheroids) or in subcutaneous tumours *in vivo* (Martiny-Baron et al., 2004). Analysis of the tumour revealed a reduction in microvessel density.

1.8 PDGF/PDGFR Gene Family

Platelet-derived growth factor (PDGF) was originally purified from platelets isolated from blood serum plasma. Since then, it has been characterised as a potent mitogen for many cell types, particularly those of mesenchymal or neuro-ectodermal origin, including fibroblasts, smooth muscles, epithelial and glia cells (Heldin and Westermark, 1999; Betsholtz et al., 2001). In cell culture assays, PDGF is involved in many aspects of cellular signalling events controlling proliferation, migration, differentiation survival as well as the deposition of extracellular matrix (ECM) and the release of tissue remodelling factors (Hoch and Soriano, 2003). The generation of knockout mice has revealed that many of the cellular responses to PDGF are crucial for mouse embryonic and postnatal development of several tissues and organs. PDGF has been also associated with pathological diseases such as atherosclerosis, fibrotic diseases, tumour malignancies, diabetic retinopathy and glomerulosclerosis (Heldin and Westermark, 1999) (Lindblom et al., 2003).

1.8.1 The PDGF signalling pathway

The PDGF family is comprised of four ligands, PDGF-A, -B, and the recently discovered PDGF-C and –D. The ligands exist as secreted disulphide-bonded homodimers, but only PDGF-A and –B are able to form heterodimers. PDGFs are recognised by two tyrosine kinase receptors, PDGFRα and PDGFRβ, which can form homodimers and heterodimers with different affinities with the ligands (Figure 1.8).

Mature PDGF-A and –B chains consist of around 100 amino acid residues and display 60% homology. From a structural aspect, PDGF belongs to the PDGF/VEGF super-family of growth factors as it consists of eight cysteine residues that are also evident in the VEGF isoforms and placenta growth factor (Heldin and Westermark, 1999). Two of these residues are used to form interchain disulphide bridges that stabilise the dimer, while the remaining six residues are involved in forming interchain disulphide bonds.

Mature PDGFα and β receptors have the molecular size of ~170 kDa and ~180 kDa respectively. Structurally, they consist of an extracellular ligand-binding domain with five immunoglobulin-like domains, a transmembrane domain, and an intracellular region that harbours a juxtamembrane domain and a split tyrosine kinase domain (Ronnstrand and Heldin, 2001). Upon PDGFR engagement with the ligand, this leads to a sequence of events beginning with the dimerisation of the receptor. This allows subsequent transphosphorylation of the tyrosine residues between the two receptors that results in the activation of the kinase domain. This leads to the autophosphorylation of several key tyrosine kinase residues that are important for further phosphorylation of subsequent downstream substrates (Ronnstrand and Heldin, 2001). Tyr849 and Tyr857 are the key tyrosine residues located in the tyrosine kinase domain of PDGFRα and PDGFRβ respectively, which regulate the kinase activity. In addition, the phosphorylation of Tyr579 and Tyr581 found in the juxtamembrane of PDGFRβ was found to be required for complete kinase activation and the association of the effector molecule Src (Baxter et al., 1998). The phosphorylation of these tyrosine residues leads to the recruitment of different signalling molecules containing Src homology 2 (SH2) domains, which are capable of binding to the other phosphorylated tyrosine residues. Molecules containing SH2 domains that bind to PDGFRs include the Src family kinases

(SFKs), the regulatory p85 subunit of phosphoinositol 3′-kinase (PI3-kinase), the GTPase-activating protein RasGAP, the adaptor molecule Grb2 and Shc, and the tyrosine phosphotase SHP-2. These molecules bind to specific residues and mediate diverse roles in signalling events that affect cell mitogenesis, chemotaxis and survival (Heldin and Westermark, 1999; Pawson et al., 2001). As 13 tyrosine residues have been identified in PDGFRβ so far, of which many binding partners have been identified, each one could regulate the initiation of the different intracellular signalling pathways (Ronnstrand and Heldin, 2001). Following PDGFR activation, the receptor is subsequently internalised and rapidly degraded.

Besides the investigation of signalling transduction pathways downstream of PDGFRs in tissue culture, there is some evidence suggestive of crosstalk between other receptor pathways and PDGFRs. For example, Brückner and co-workers reported that PDGF could induce ephrinB1 phosphorylation (Bruckner et al., 1999).

1.8.2 In vivo studies

The involvement and importance of PDGF-A, -B and PDGFRα and β in developmental processes, has been elucidated by the generation of knockout mice (Leveen et al., 1994; Soriano, 1994; Bostrom et al., 1996; Soriano, 1997). The PDGF-B, PDGFRα and β knockouts are lethal at prenatal stages while PDGF-A mutants die during early postnatal life. These studies uncovered the role of PDGFs in the control of proliferation, migration and survival of certain populations of glial progenitor cells and mesenchymal cells. These cells are subsequently involved in development of the cardiovascular system, the epithelial organs, the central nervous system and the skeleton (Betsholtz, 2004). An extensive series of point mutations have also been generated in the mouse to investigate the requirement of specific signal transduction pathways downstream of the PDGFRβ (Tallquist and Kazlauskas, 2004). Remarkably, mutant mice with the loss of six effector binding sites (F7) do not phenocopy null mutants and are viable. This suggests that PDGFRβ must retain some signalling capacity even though known signal transducers are unable to directly bind to the receptor. However F7 mice displayed a reduction in vSMCs in various tissues including the eye, heart and kidney.

1.8.3 Involvement of PDGF-B and PDGFRβ in blood vessel development

PDGF-B and PDGFRβ knockouts die at late gestation from extensive microvascular haemorrhaging and show defective formation of kidney glomeruli and oedema. These defects suggest that PDGF-B/PDGFRβ signalling promotes the proliferation of vSMC/pericyte progenitors and their recruitment to new blood vessels during embryogenesis (Figure 1.9).

Sprouting ECs express high levels of PDGF-B at their tips. This then acts as a short-range paracrine factor to attract surrounding PDGFRβ expressing pericytes and subsequently vSMCs, which then cover the nascent blood vessels. The perturbation of the PDGF-B/PDGFRβ signalling interactions by gene inactivation in mice, led to severe shortage of mural cell coverage of blood vessels, dilation of microvessel, microaneurysms and widespread bleeding. The endothelium is the critical source of PDGF-B for vSMC/pericyte recruitment, as demonstrated by the generation of endothelial-specific PDGF knockout mice (Enge et al., 2002). Furthermore, the loss of a single allele of either PDGF-B or PDGFRβ reduced the pericyte number and suggested that gene dosage is important.

An additional control mechanism that may further regulate the short-range interaction of PDGF-B is the binding of PDGF-BB molecules to the surrounding extracellular matrix, through a heparan sulphate proteoglycans (HSPG) binding sequence, termed the "retention motif". Mice lacking this motif exhibit reduced pericyte density and impaired pericyte investment to the microvessel wall (Lindblom et al., 2003). The reported loose attachment of the pericytes to the abluminal surface of the endothelium and protruded cellular processes of these cells away from the vessels suggested that PDGF-B retention and restricted diffusion (which may create a concentration gradient), is critical for proper association of pericytes to the microvessel wall.

Mesangial cells are a specialised form of mural cell that are restricted to the kidney. They normally constitute ~30% of the glomerular cells and appear to promote intussusception of invading vascular loops into the capillary network in the glomeruli.

The presence of mesangial cells is essential for the generation of large-surface areas needed for efficient blood filtration (Betsholtz, 2004). The defective formation of the glomeruli in PDGF-B and PDGFRβ mutants has been attributed to the almost complete lack of mesangial cells. Furthermore, both PDGF-B retention mutants and endothelial-specific knockouts exhibit inflated glomerular capillary loops due to delayed mesangial cell recruitment. However, this defect was corrected one month after birth and the required physiological level of mesangial cells had been recruited. It is possible that other molecules, such as PDGF-D, could be involved and are able to compensate for the loss of PDGF-B in mutants. Nevertheless, PDGF-B retention mutants develop glomerulosclerosis and proteinuria as adults.

1.9 Conclusion and the aim of the thesis

In conclusion, the studies described here have shown that ephrinB2 and its receptor, EphB4, are involved in early angiogenic remodelling of the embryo, but whether endothelial ephrinB2 expression is essential for blood vessel development remains to be determined. The work described above has also established that PDGFRβ signalling is an important regulator of mural cell proliferation and recruitment. Expression studies indicate that Eph/ephrin molecules may be also involved in these processes, especially ephrinB2. The aim of the thesis was to characterise of the role of ephrinB2 in endothelial and mural cell populations.

Angiogenic factors
Acidic and basic fibroblast growth factor (aFGF, bFGF)
Vascular endothelial growth factor family and their receptors (VEGF, VEGFR)
Platelet derived growth factor BB and receptors (PDGF-BB, PDGFR)
Angiopoeitins and their receptor (Ang1, Ang2 and tie2)
Hepatocyte growth factor (HGF)
Ephrins and their Eph receptors
Pleiotropin
Insulin-like growth factor (IGF-1)
Transforming growth factor-β1 (TGF-β1)
Tumour necrosis factor-a (TNF-α)
Interleukins (IL–1, IL-4, IL-6, IL-8)
Histamine
Bradykinin
Prostagladins (PGD-E1, PGD-E2)
Heparin
Adhesion molecules (integrins: $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_5\beta_1$, VE-cadherin)
Metalloproteases (collagenase, stromelysin, MMP-2)
Angiogenin

Anti-angiogenic factors
Interferons (IFN–α, IFN-β)
Thrombospondins (TSP-1, TSP-2)
Tissue inhibitor of metalloproteases (TIMP-1, TIMP-2, TIMP-3)
Endostatin (collagen XVIII fragment)
Angiostatin
Prolactin
Platelet factor 4 (PF-4)
Interleukins (IL–12,IL-18)
Heparinase
Prolactin
Vasostatin, calreticulin

Table 1.1: Angiogenic and anti-angiogenic factors

Molecules	Embryonic stage of lethality	Vascular Phenotype
HIF1$\alpha^{-/-}$ or HIF1$\beta^{-/-}$	10.5	Defective yolk sac vasculogenesis with intact endothelial cell differentiation
VEGF-A$^{-/-}$	10.5	No dorsal aorta, defective haematopoiesis
VEGFR1$^{-/-}$ (flt1)	8.5-9.5	Endothelial cell differentiation intact. Vascular disorganisation and angioblast overpopulation
VEGFR2$^{-/-}$ (flk1)	E8.5-9.5	Endothelial and haemangioblast migration and differentiation failure
VEGFR3$^{-/-}$	9.5	Vasculogenesis and angiogenesis intact, large vessels disorganised with lumen defects
Ang1$^{-/-}$	E10.5	Defective organisation , remodelling
Ang2$^{-/-}$	E12.5-P	Defect in vessel integrity, haemorrhaging
Tie1$^{-/-}$	E13.5—14.5	Defective vascular integrity/endothelial survival in angiogenesis, oedema and haemorrhaging
Tie2$^{-/-}$	E10.5	Defective organisation and remodelling, sprouting and heart trabeculation
ephrinB2$^{-/-}$	E10.5	Defective organisation and remodeling, sprouting, heart trabeculation, and extra-embryonic vessel fussion
EphB2$^{-/-}$/EphB3$^{-/-}$	E10.5 (only 30% penetrance)	Defects in sprouting, remodelling and organisation
EphB4$^{-/-}$	E10.5	Defective organization and remodeling, sprouting, heart trabeculation, and extra-embryonic vessel fussion
PDGF BB$^{-/-}$	E16-19	Dilated heart and aorta, microvessel leakage and microaneurysm
PDGFR$\beta^{-/-}$	E16-19	Dilated heart and aorta, microvessel leakage and microaneurysm
VE-cadherin$^{-/-}$	E10.5	Defective anterior large vessels and failure to establish yolk sac vascular plexus
TGFβ1$^{-/-}$	E10.5	Failure of endothelial differential, haematopoiesis and vessel formation

Table 1.2: Molecules required for proper embryonic vascular development

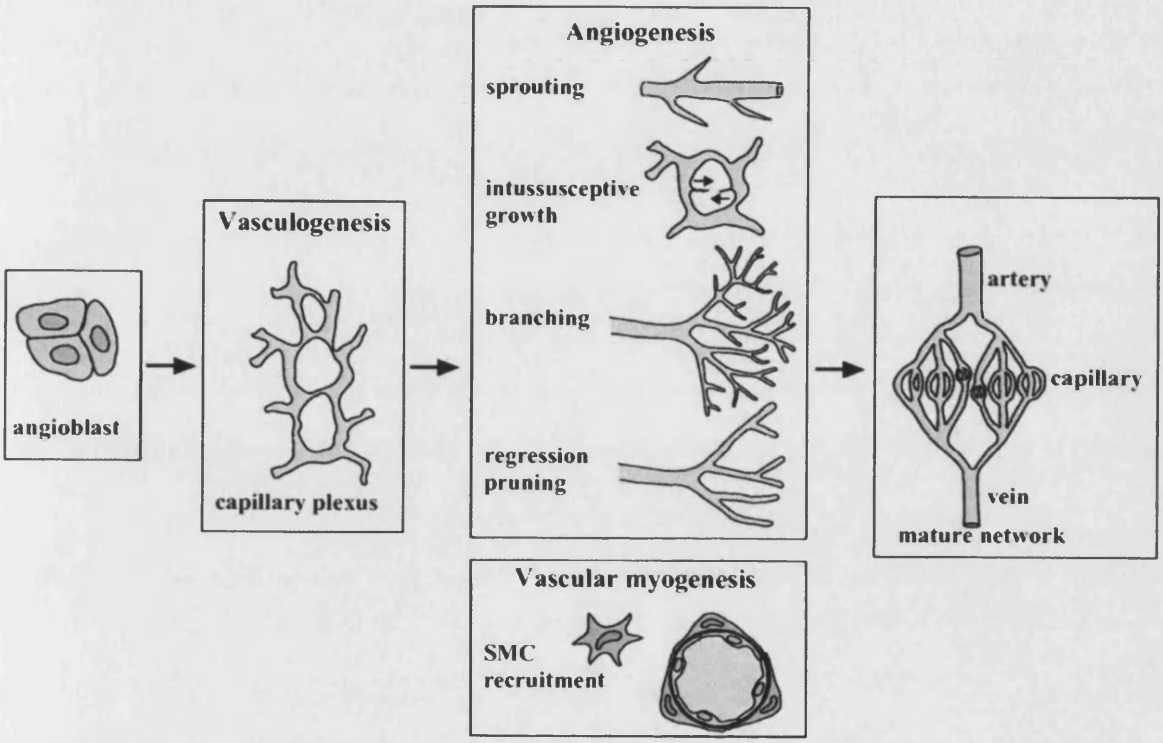


Figure 1.1: Formation of new blood vessels

(Carmeliet P. and Collen D., (2000) Journal of Pathology 190:387-405)

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.

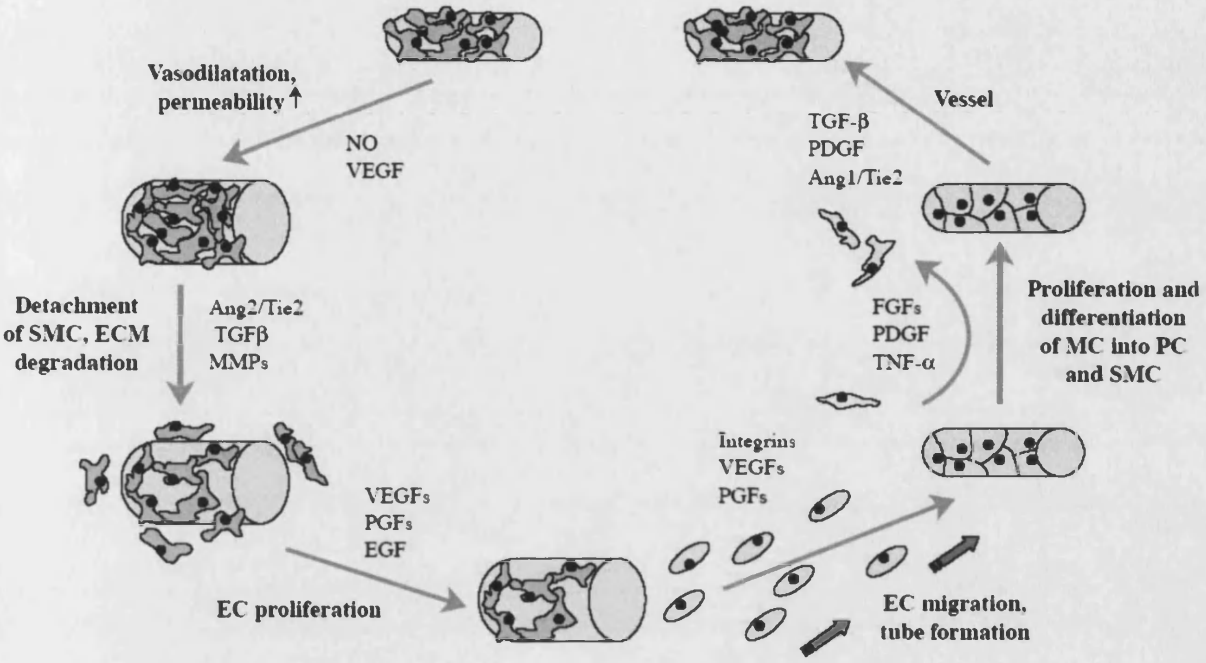


Figure 1.2: Multiple steps and factors involved in angiogenesis
(Distler J. W. et al. (2003) Q J Nucl. Med 47(3):140-161)
First steps of angiogenesis involve vasodilation and increased vascular permeability with exudation of high molecular weight plasma proteins such as fibrinogen. After detachment of 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.

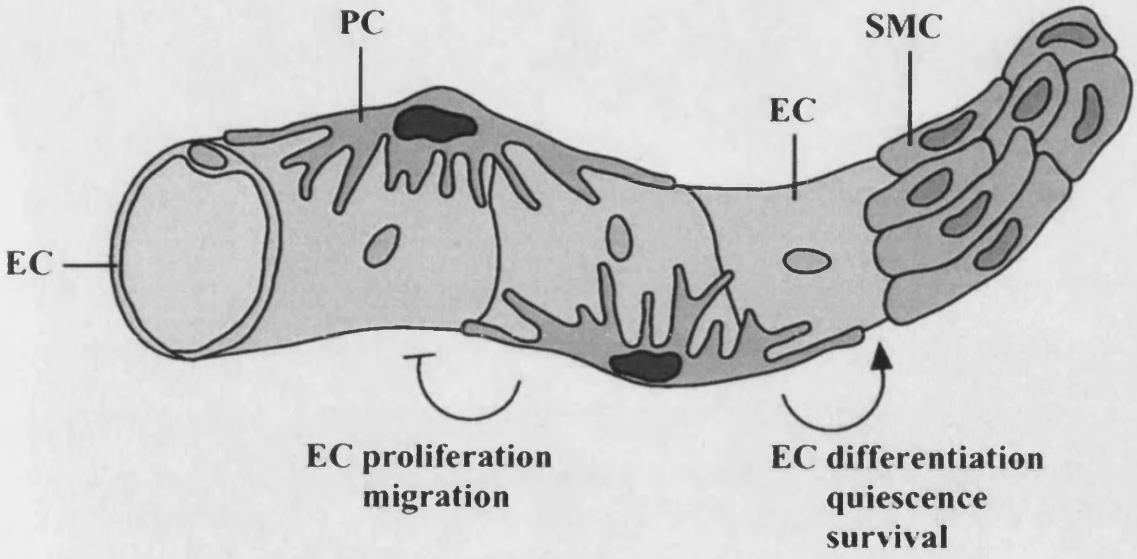


Figure 1.3: Mural cell interactions with endothelial cells

(Carmeliet P. and Collen D., (2000) Journal of Pathology 190:387-405) Mural cells, comprising of smooth muscle cells and pericytes, significantly determine vascular development. They inhibit proliferation and migration, and induce differentiation, quiescence and survival of endothelial cells.

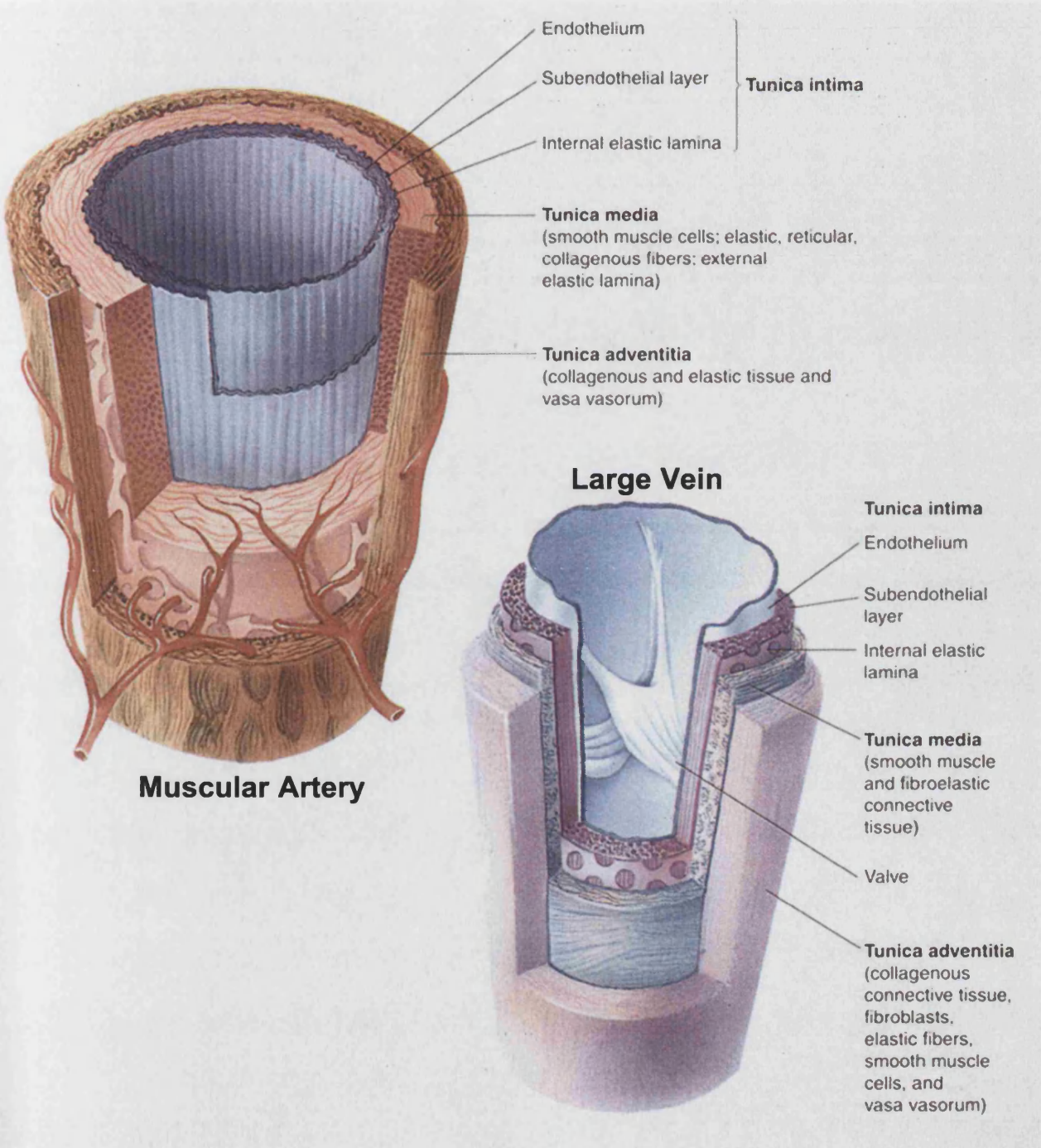


Figure 1.4: Structural differences between a muscular artery and a large vein with their multiple layers

(Gartner L. and Haitt. J., (2000) Color Atlas of Histology Page:154)

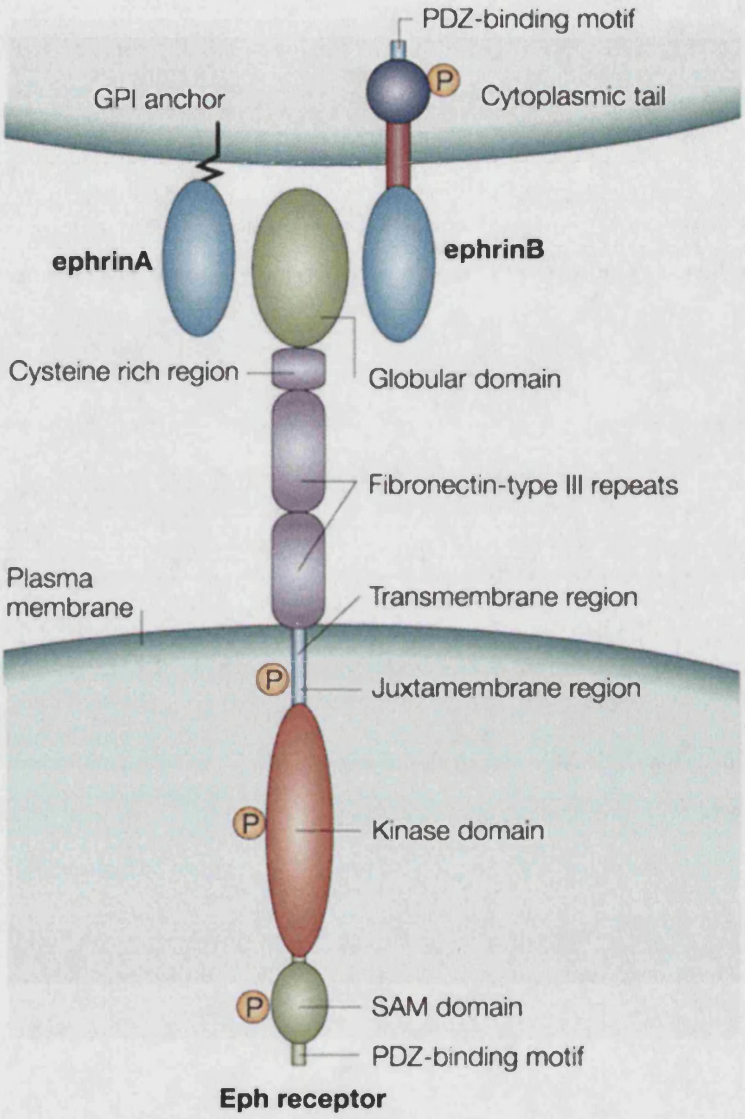


Figure 1.5: General features of Eph receptors and ephrins

(Kullander K. and Klein R., (2002) Nat. Rev. Mol. Cell. Biol. 7:475-486) 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.

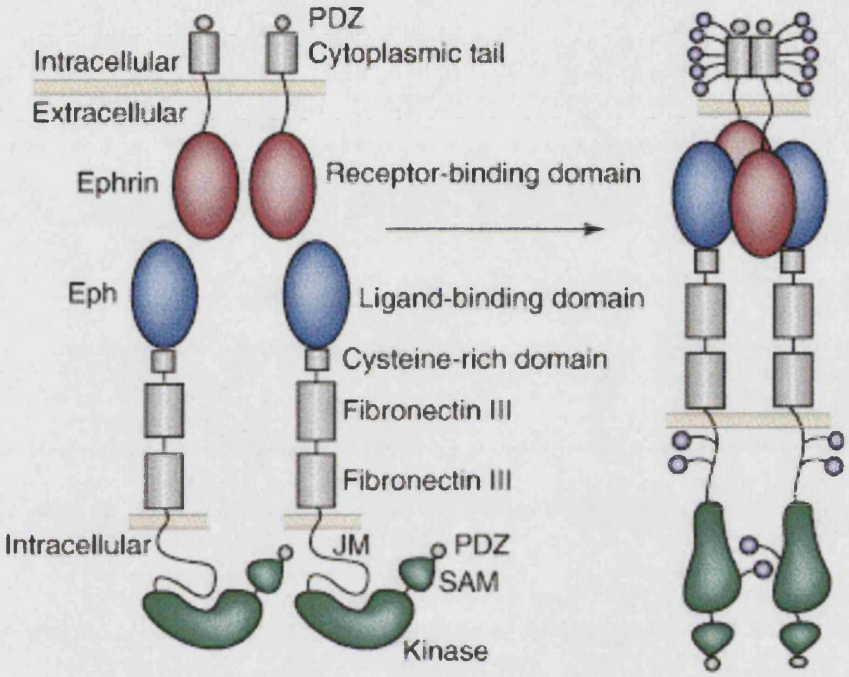


Figure 1.6: Activation of bidirectional Eph-ephrinB signalling pathway

(Himanen J. and Nikolov D., (2003) Trends Neurosci. 26(1):46-51
Diagram showing that Eph and ephrin molecules form a 2:2 circular heterotetramer, which leads to tyrosine phosphorylation in their cytoplasmic domains. The receptor binding domain of the B-class ephrins is shown in red, the ligand binding domain of Ephs in blue, and kinase domain and sterile-α-motif (SAM) in green. All the other structural elements are indicated in grey and the phosphate groups are purple.

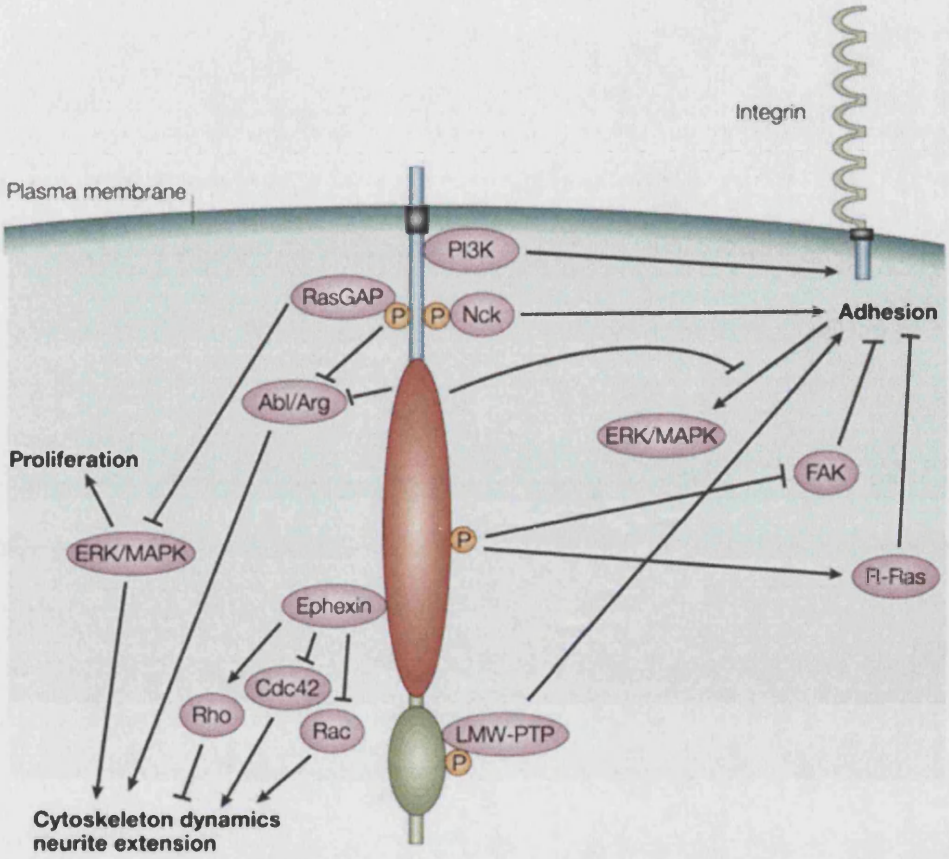


Figure 1.7: Adaptor interactions with Eph receptors

(Kullander K. and Klein R., (2002) Nat. Rev. Mol. Cell. Biol. 7:475-486) Diagram showing the adaptor molecules that interact with Eph receptors. Selected molecular cascades and biological effects are indicated.

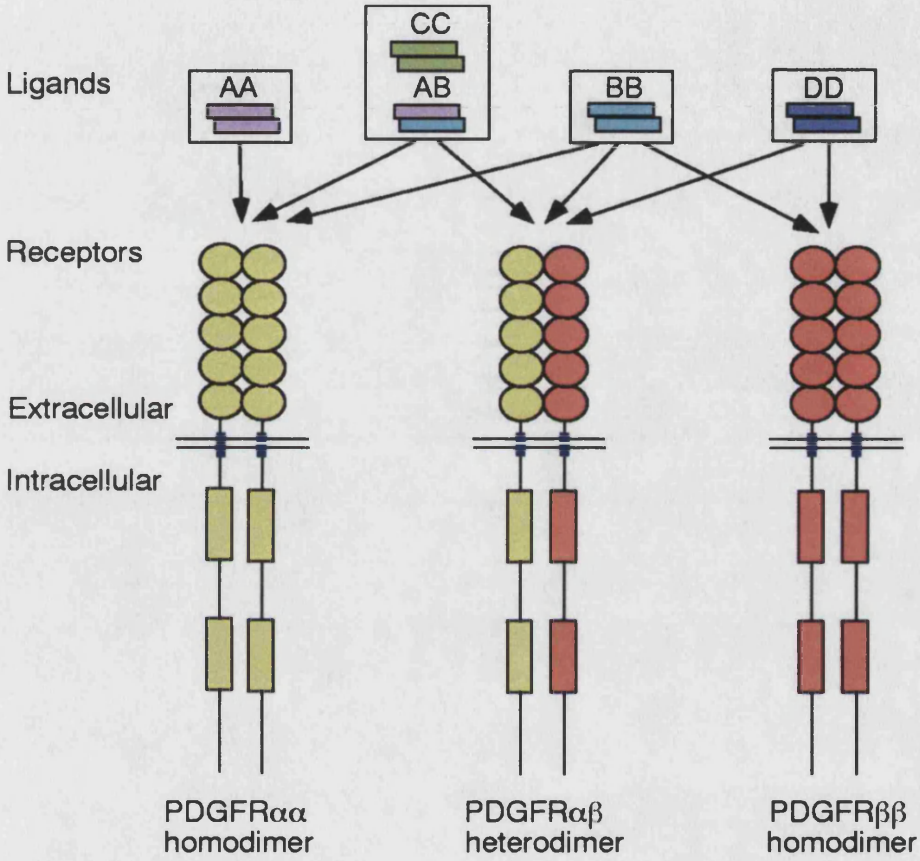


Figure 1.8: PDGF ligands and PDGFR receptors

(Hoch R. and Philippe S., (2003) Development 130(20):4769-4784)
A schematic diagram showing the homo- and heterodimeric complexes of the four known PDGF family ligands and their interactions with the cognate receptors PDGFRαα, αβ and ββ.

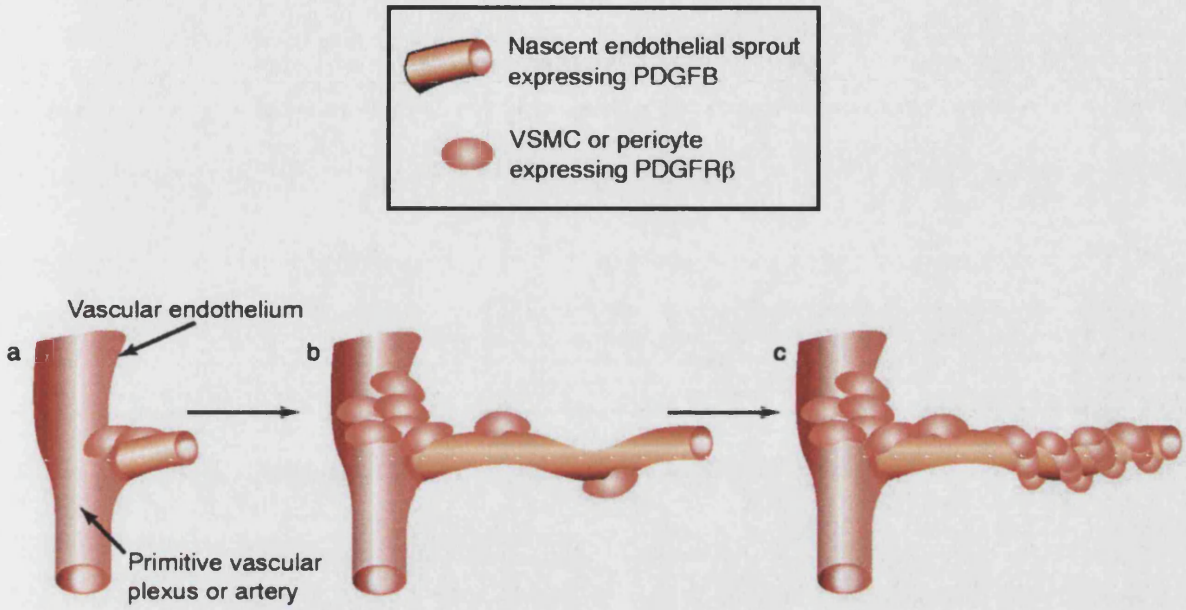


Figure 1.9: Vascular mural proliferation, survival and migration

(Hoch R. and Philippe S., (2003) Development 130(20):4769-4784)
During angiogenic remodelling, PDGFB is expressed in nascent vascular endothelial sprouts (a) leading to the proliferation of PDGFRβ-expressing pericytes and vascular smooth muscle cells (VSMC) near arterial walls and primitive vascular plexa (b). PDGF also directs the migration of mural cells along the endothelial sprouts (b,c). Upon reaching their destination, vSMC and pericytes associate tightly with the endothelium (c) and form the outer walls of the vessel wall. Survival and anti-proliferative factors produced by the mural cells stabilise nascent vessels.

Chapter2 | Materials and methods

Materials

2.1 Chemicals and reagents

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.

Chemicals & Reagents	Company
Agarose	Invitogen Life Technologies
dNTPs	Promega
Mowiol®	Calbiochem
TRIzol™ Reagent	Gibco BRL
Trypsin/EDTA	Invitogen Life Technologies
Vectashield®	Vector Laboratories Inc
Shandon Cryomatrix™	Thermo Electron Co.
X-Gal	Novabiochem

2.2 Buffers and solutions

All buffers and solutions were made as described below. "RT" indicates room temperate, "Filter" indicates sterilisation using a 0.2μm filter.

Buffer & Solutions	Contents
L-agar	1% bacto-tryptone, 0.5% yeast extract, 170mM NaCl, 15% agar
L-broth	1% bacto-tryptone, 0.5% yeast extract, 170mM NaCl
PBS	140mM NaCl, 2.5mM KCL, 10mM Na_2HPO_4 (pH7.2), 1.5mM KH_2PO_4
PBS-T	PBSA + 0.1% Tween-20
PCR Buffer 10x (pH 8.3)	100mM Tris-HCL, 15mM $MgCl_2 \cdot 6H_20$, 500mM KCL
SSC 20x	3M NaCl, 0.3 Sodium Citrate
Tail Buffer	50mM Tris-HCL (pH8.0), 100mM NaCl, 100mM EDTA, 1% SDS
TBE Running Buffer (1x)	89mM Tris base, 89mM boric acid, 2mM EDTA
TE	10mM Tris-HCL (pH 8.0), 1mM EDTA (pH 8.0)

2.3 Enzymes

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

Enzyme	Company
AMV Reverse Transcriptase	Roche
Calf intestinal alkaline phosphatase (CIP)	Roche
DNase I (RNase free)	Roche
DNA Polymerase I (Klenow)	Amersham Biosciencecs
Pfu Turbo® DNA Polymerase	Stratagene
Proteinase K	Gibco BRL
RNA polymerases (T3, T7, Sp6)	Roche
RNase A	Sigma

2.4 Immunoreagents

All antibodies were used at the concentrations indicated below. They were all used for immunoflourescence unless stated otherwise.

Primary Antibody	Isotype	Dilution	Company
α-Smooth Muscle Actin (SMA) Unconjagated (Clone 1A4)	Mouse (IgG)	1:300	Sigma Aldrich
α-SMA-Cy3(Clone 1A4)	Mouse (IgG)	1:300	Sigma Aldrich
β-Galactosidase (ab616)	Rabbit (IgG)	1:1,000	Abcam
Anti-digoxigenin AP-conjugate	Sheep (Fab fragments)	1:2,000	Roche
Desmin (Clone D33)	Mouse (IgG)	1:200	Dako
EphB2 (Catalogue No. AF467)	Goat (IgG)	1:200	R&D Systems
EphB3 (Catalogue No. AF432)	Goat (IgG)	1:200	R&D Systems
EphB4 (Catalogue No. AF446)	Goat (IgG)	1:200	R&D Systems
Ephrin-B2 (Catalogue No. AF496)	Goat (IgG)	1:200	R&D Systems
LYVE-1	Rabbit (IgG)	1:1,000	Kari Alitalo (gift)
PECAM-1(CD31-Clone:MEC13.3)	Rat (IgG)	1:300	PharMingen

Secondary Antibody	Isotype	Dilution	Company
Alexa Fluor® 488 Anti-Goat IgG	Donkey	1:500	Molecular Probes
Alexa Fluor® 488 Anti-Mouse IgG	Goat	1:500	Molecular Probes
Alexa Fluor® 488 Anti-Rabbit IgG	Goat	1:500	Molecular Probes
Alexa Fluor® 350 Anti-Rabbit IgG	Goat	1:500	Molecular Probes
Cy™3-conjugated Anti-Rat IgG	Goat	1:500	Jackson

2.5 Miscellaneous

The following table lists the remaining products that were used.

Product	Company
Molecular Biology	
Oligonucleotides	Sigma Genosys
QIAGEN® Plasmid Maxi Kit	Qiagen
QIAprep® Spin Miniprep Kit	Qiagen
QIAquick® Gel Extraction Kit	Qiagen
QIAquick® PCR Purification Kit	Qiagen
Cell Culture	
DMEM	Invitrogen Life Technologies
E4 Medium 10x	Cancer Research UK
Foetal Calf Serum	Invitrogen Life Technologies
Interferon γ	Gibco BRL
L-Glutamine 200 mM (100x)	Invitrogen Life Technologies
LipofectAMINE™	Invitrogen Life Technologies
PBS	Cancer Research UK
Penicillin-Streptomycin (100x)	Invitrogen Life Technologies
Tissue Culture Plastic-ware	Corning, Falcon and NUNC

2.6 List of suppliers and distributors

Company	Location
Abcam Limited	Cambridge, UK
Applied Biosystems	Foster City, California, USA
BD Biosciences	Oxford, UK
Boehringer Mannheim Gmbh	Mannheim, Germany
Cambridge Bioscience	Cambridge, UK
Corning	Schiphol-Rijk, Holland
DakoCytomation	Cambridgeshire, UK
Dynal Biotech UK	Wirral, UK
Invitrogen Life Technologies	Paisley, UK
Promega	Southampton, UK
Qiagen	West Sussex, UK
Novabiochem	Nottingham, UK
New England Biolabs	Hitchin, UK
Peprotech EC Ltd.	London, UK
Perkin-Elmer Co.	Foster City, California, USA
R&D Systems Inc.	Minneapolis, USA
Sigma-Aldrich Company	Dorset, UK
Stratech Scientific Limited	Cambridgeshire, UK
Stratagene	Amsterdam, Holland
Thermo Electron Co.	Pittsburgh, USA
Vector Laboratories Inc	Burlingame, California, USA
VWR International Ltd	Dorset, UK

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. 0.1mg 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 2 minutes. 500μl of L-broth was then added to the bacteria which were subsequently cultured for 45 minutes at 37°C with shaking at 150 RPM. 150-400μl 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 5 ml LB media containing the appropriate antibiotic, then grown overnight in a 37^{0}C agitator. 2 ml of the overnight culture was used for small scale preparation of plasmid DNA whilst the remainder was stored at 4^{0}C. The QIAprep® Spin Miniprep Kit was used for small-scale plasmid purification according to the manufactures instructions. DNA was eluted in TE or elution buffer (Qiagen) and stored at -20°C.

For large-scale maxi preparations, 0.5 ml of the overnight culture was added to 150 ml LB plus antibiotic and incubated overnight at 37^{0}C in an agitator. Bacteria were pelleted by centrifugation at 6000 RPM for 15 min in a Beckman J2-21 centrifuge. The plasmid DNA was then purified using a QIAGEN® Maxi Kit according to manufacturers

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 or RNA were diluted in 10 mM 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 or RNA 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 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 manufacturers instructions. DNA was resuspended in 10 mM Tris-HCl pH 8.5 or TE.

2.7.6 Polymerase Chain Reaction (PCR)

Amplification of DNA products for cloning were performed using Stratagene's *Pfu* Turbo PCR kit. Reactions were carried out according to the manufacturers instructions.

2.7.7 Sequencing of DNA

All DNA sequencing were performed by the DNA Sequencing Service (Cancer Research UK) using the ABI® BigDye™Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) according to the manufacturers 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 were carried out as recommended by the manufacturer and DNA products were ethanol precipitated at room temperature for 15 minutes by the addition of 300μl 95% ethanol, 5μl 3M sodium acetate (pH4.5) and 80μl dH_2O. After centrifugation at 14,000 RPM for 20 minutes, the pellet was washed with

500μl 70% ethanol, vortexed briefly, centrifuged as described above for 5 minutes, air dried, and then stored at -20°C until loading in the polyacrylamide sequencing gel.

2.8 Generation of transgenic lines using the PDGFRβ genomic fragment

2.8.1 Generation of the PDGFRβ-LacZ transgenic line

The PDGFRβ genomic fragment was obtained as a gift from Dr. Christer Betsholtz. A reporter construct, containing the internal ribosomal entry site fused to the LacZ sequence (IRES-LacZ), was inserted into exon-4 of the 5.1 PDGFRβ genomic fragment, via the EcoRV site (Figure 4.1). This PDGFRβ-LacZ construct was linearised with the restriction enzyme HpaI and subsequently used for pronuclear injections into fertilised mice zygotes. A total of 12 transgenic founder lines were generated.

2.8.2 Generation of the PDGFRβ-Cre transgenic line

A construct containing the internal ribosomal entry site fused to the Cre sequence (IRES-Cre), was inserted into exon-4 of the 5.1 PDGFRβ genomic fragment as before (Figure 4.4). This PDGFRβ-LacZ construct was linearised with the HpaI and subsequently used for pronuclear injections into fertilised mice zygotes. A total of 13 transgenic founder lines were generated.

2.9 Analysis of genomic DNA

2.9.1 Isolation of genomic DNA from mouse tissue

Tail snips or yolk sacs were stored at -20°C until ready for processing. 500μl Tail Buffer (50mM Tris-HCL (pH8.0), 100mM NaCl, 100mM EDTA, 1% SDS and freshly added Proteinase K at 100μg/ml) was added to the mouse sample. Digestion was carried out at 55°C overnight or for a minimum of 6 hours. 250μl of 6M NaCl was added and mixed for 5 minutes on an Eppendorf Mixer and the sample was centrifuged at 13,000 RPM for 10 minutes after which the supernatant was decanted into a fresh 1.5ml microtube containing 500μl isopropanol and mixed as before. After 10 minutes of centrifugation at 13,000 RPM, the supernatant was decanted and the remaining pellet

was washed with 70% ethanol and centrifuged as before. The pellet was then air dried and dissolved in 150μl of TE.

2.9.2 Genotyping Mouse Genomic DNA by PCR

Pic *Taq* polymerase (Cancer Research UK) 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.

	Volume (µl)	Temperature (°C)	Time (Minutes)	No. of Cycles
DNA Template	1.0	94	1	1
PCR Buffer 10x	2.5			
dNTPs (2.5 mM of each)	2.5	94	1	
Primer 1 (20µM)	1.25	Annealing Temp.	1	30-35
Primer 2 (20µM)	1.25	72	1	
Pic *Taq* Polymerase	0.5			
Distilled Water	16	72	10	1
Total Volume	**25**	4	Forever	

Embryos and mice were routinely genotyped by PCR using the primers listed below. All primers were manufactured by Sigma-Genosys.

Mouse Line	Primer		Sequence 5' to 3'	Anneal. Temp.	Fragment Size
ephrinB2 Conditional Non-Recombinant	Forward	B2Cs1	CTT CAG CAA TAT ACA CAG GAT G	62°C	~370 bp lox
	Reverse	B2Cas1	TGC TTG ATT GAA ACG AAG CCC GA		~270 bp WT
ephrinB2 Conditional Recombinant	Forward	B2Cs1	CTT CAG CAA TAT ACA CAG GAT G	62°C	~370 bp KO (conditional)
	Reverse	B2Cas3	AAT ACT GTT ACT ACA GGG TCC		
ephrinB2 KO	Forward	pFloxs3	CTG GGG ATG CGG TGG GCT CTA	62°C	~1300 bp KO
	Reverse	Elf2as6	GGG CCG CAG TAA GTT TAA AAC		
Tie1-Cre	Forward	Cre1	GCC TGC ATT ACC GGT CGA TGC AAC GA	70°C	~720 bp
	Reverse	Cre2	GTG GCA GAT GGC GCG GCA ACA CCA TT		
Tie2-LacZ	Forward	HHFW3	GCC TAT GAG AGG ATA CCC CTA TTG	62°C	~720 bp
	Reverse	LZREV1	GGA ACA AAC GGC GGA TTG ACC G		
Tie2CreER^{T}	Forward	Cre1	GCC TGC ATT ACC GGT CGA TGC AAC GA	70°C	~720 bp
	Reverse	Cre2	GTG GCA GAT GGC GCG GCA ACA CCA TT		
SMMHC-Cre	Forward	Cre1	GCC TGC ATT ACC GGT CGA TGC AAC GA	70°C	~720 bp
	Reverse	Cre2	GTG GCA GAT GGC GCG GCA ACA CCA TT		
PDGFRβ-LacZ	Forward	βR-LacZs	ACA TGA CAT CAA CCA TAT CAG C	62°C	~240 bp
	Reverse	βR-LacZas	GTG AAA CCT CGC TGG TGG		

Mouse Line	Primer		Sequence 5' to 3'	Anneal. Temp.	Fragment Size
PDGFRβ-Cre	Forward	Cre1	GCC TGC ATT ACC GGT CGA TGC AAC GA	70°C	~720 bp
	Reverse	Cre2	GTG GCA GAT GGC GCG GCA ACA CCA TT		
PDGFRβ-KO	Forward	βR1	ACA ATT CCG TGC CGA GTG ACA G	65°C	~320 bp KO
	Forward	βR3	ATC AGC CTC GAC TGT GCC TTC TAG		~114 bp WT
	Reverse	βR2	AAA AGT ACC AGT GAA ACC TCG CTG		
XlacZ4 (Pericyte-LacZ)	Forward	PLacZ-E	CAAT ACT ATG CAG ACT CTG GAA GTG	55°C	~600 bp
	Reverse	PLacZ-3	CAA ACT CAT CAA TGT ATC TTA TCA TGT C		
Immorto	Forward	Immos	GCA TTG CCT GGA ACG CAG TGA GT	60°C	~380 bp
	Reverse	Immoas	GAA CAG ACT GTG AGG ACT GAG GG		
Rosa26Reporter	Forward	Rosa1	AAA GTC GCT CTG AGT TGT TAT	55°C	~370 bp transgenic
	Reverse	Rosa2	GCG AAG AGT TTG TCC TCA ACC		~600 bp WT
	Reverse	Rosa3	GGA GCG GGA GAA ATG GAT ATG		

2.10 Analysis of products following total RNA isolation

2.10.1 Isolation of total RNA from cells in culture

TRIzol® Reagent was used to isolate total RNA according to the manufacturers instructions. Total RNA pellets were air dried, resuspended in RNase-free water and stored at -80°C.

2.10.2 Isolation of total RNA from mouse tissue

Freshly dissected tissue samples were homogenised using a pestle and mortar or immediately snap-frozen in liquid nitrogen for later use. Total RNA was isolated using the TRIzol® Reagent following the manufacturers instructions.

2.10.3 Synthesis of first strand cDNA

Using 1μg of total RNA as a template, reverse transcription was carried out resulting in first strand cDNA synthesis, using the AMV Reverse Transcriptase (Roche) according to the manufacturers instruction. The resulting product, in a volume of 20μl, was stored at -20°C.

2.10.4 Synthesis of second strand cDNA by PCR and mRNA profile

Using the first strand cDNA as template, second strand synthesis and amplification was performed by PCR. By designing different specific primer pairs that lie in exons flanking the intronic regions, an expression profile was generated of endogenous mRNAs from the isolated cells in which the total RNA was extracted from. The typical cycling program for the PCR is shown in Section 2.7.2 and the primers used are listed below.

Gene of Interest	Primer		Sequence 5' to 3'	Anneal. Temp.	Fragment Size
Desmin	Forward	Desmin-s	CGA GCT GCT GGA CTT CTC CCT	64°C	~220 bp
	Reverse	Desmin-as	GCT GCA TCC ACA TCC GCT C		
PDGFRβ	Forward	PDGFRβ-s	AAT TCC GTG CCG AGT GAC AG	62°C	~510 bp
	Reverse	PDGFRβ-as	ACG TAG CCA TTC TCG ATC ACA G		
PECAM-1	Forward	PECAM-1s	CAC CTC GAA AAG CAG GTC TC	64°C	~680 bp
	Reverse	PECAM-1as	CAG CTT CAC TGC TTT GCT TG		
EphB2	Forward	Nuks1	GCA CTT CGC TGG TGGCAG CTC	70°C	~970 bp
	Reverse	Nukas1	CTT GAT GCT GGT CTG GTA CTC		
EphB3	Forward	Sek4s1	GCT GGT GAG TTT GGG GAA GTG	68°C	~990 bp
	Reverse	Sek4 as1	GTG ACC CCA ATC CTT AGC AG		
EphB4	Forward	Myk1s1	CAG GTG GTC AGC GCT CTG GAC	64°C	~380 bp
	Reverse	Myk1as1	ATC TGC CAC GGT GGT GAG TCC		

2.11 Histology, lacZ stainings, immunohistochemistry and *in situ* hybridisation

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 in 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 transfered 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 slides, dried at 42°C and stored at RT.

2.11.2 Fixation and embedding of tissues for cryosectioning

Mouse tissues were dissected and either frozen immediately or fixed before being frozen (aorta samples were frozen immediately). In the latter case, tissues were fixed in 4% PFA overnight at 4°C, followed by equilibration of the tissues in cryoprotectant (20-30% phosphate buffered sucrose solution) overnight at 4°C. 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 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%, 25% 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% H_2O_2 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: 30 minutes in blocking solution (1% serum from the species in which the secondary antibody was made and 0.1% BSA in PBS), overnight incubation in blocking solution containing primary antibodies at 4°C, 3 x 5 minutes in

PBS, 60 minutes in blocking solution containing secondary antibodies, 3 x 5 minutes in PBS, 5 minutes in DAPI solution (nuclear marker, stock at 10mg/ml, 1:1000 dilution in PBS), 2 x 5 minutes in PBS. Coverslips were mounted onto the tissue using Vectashield® or Mowiol®.

2.11.4 Immunohistochemical stainings on paraffin sections using the Batlle Protocol

Detection of ephrinB2, EphB2, EphB3 and EphB4 on paraffin sections was performed with goat anti-EphrinB2 (1:200), goat anti-EphB2 (1:200), goat anti-EphB3 (1:200), and anti-EphB4 (1:400) antibodies (all from R & D Systems), based on the protocol as described in Batlle et al., 2002, with modifications. In brief, following dewaxing and hydration, sections were pretreated with peroxide blocking buffer (120 Na_2HPO_4, 43 mM citric acid, 30 mM NaN_3, 0.2% H_2O_2, 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. Incubation of antibodies was performed in 1% BSA in PBS overnight at 4°C. A bridging step was applied through the incubation of the samples in the rabbit anti-goat antibody for an hour at RT. This was followed by a 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 peroxide substrates including tyramide-FITC and tyramide-Cy3.

2.11.5 Immunohistochemical stainings on cryosections

Slides were transferred from -80°C directly into 100% methanol at RT and fixed for 10 minutes. The slides were then air dried and washed in PBS (2 x 5 minutes). Applying the PAP-pen created a barrier surrounding the sections. The tissues on the slides were blocked with 2% BSA 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 5 minutes) and then incubated with secondary antibodies (diluted in blocking solution) for 1 hour at RT, followed by another wash with PBS (3 x 5 minutes). The tissues were counterstained with the nuclear marker DAPI (10mg/ml, 1:1000 dilution in PBS) for 5 minutes at RT. Slides

were finally washed in PBS (2 x 5 minutes) and the tissues were mounted onto the coverslips with Vectashield® 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% H_2O_2 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 x1 hour washes in PBSMT at 4°C, 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 stainings have occurred, 2 x 10 minutes in PBT, 2 x 10 minutes in PBS, overnight fixation in 4% PFA 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 Whole-mount immunofluorescent staining of skin

Whole-mount skin samples were fixed in 4% PFA overnight, washed in PBS and gradually dehydrated to 100% methanol. This was followed by gradual rehydration in PBS and then incubation in 10% goat serum in PBS containing 0.1% TritonX-100 (PBX) 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 five times for 1 hour each in PBX, followed by overnight incubation with secondary antibodies, at 4°C in 10% goat serum in PBX. They were then washed five times for 1 hour as before, in PBX. The samples were mounted in-between glass slides and coverslips using

Vectashield® or Mowiol® mounting medium. The slides were flattened with a weight and finally analysed on a Leica microscope or a Zeiss LSM510 Meta confocal microscope.

2.11.8 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 $MgCl_2$ 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 and stored in PBS at 4°C.

2.11.9 Embedding of lacZ-stained tissue

As LacZ stainings tends to fade away during dehydration and Xylene treatments, the incubation times in the different solutions were reduced as follow: 15 minutes in 0.9% NaCl in 50% ethanol at RT, 2 x 15 minutes 70% ethanol in PBS at RT, 30 minutes in 85% ethanol in PBS at RT, 30 minutes in 95% ethanol in PBS at RT, 2 x 45 minutes in 100% ethanol at RT, 2 x 30 minutes in Xylene at RT, 30 minutes in Paraffin/Xylene (1:1 ratio) at 56°C, 4 x 60 minutes in Paraffin at 56°C. The samples were transferred into embedding moulds filled with hot paraffin and allowed to solidify overnight at RT.

2.11.10 Preparation of embryo powder

Embryo powder was needed to preabsorb the Anti-Digoxigenin-AP antibody used for in situ hybridisations and was prepared as follows. A litter of E12.5 embryos were dissected and homogenised in a minimum amount of cold PBS, using a pestle and mortar. Four times the volume of ice cold acetone was added, vortexed and placed on ice for a further 30 minutes. The sample was centrifuged at 10,000 RPM at 4°C for 10 minutes and the floating remains were removed with the supernatant. After rinsing the

pellet with ice-cold acetone, it was placed on filter paper to air dry completely. This was then ground to powder using a pestle and mortar and stored at -20°C.

2.11.11 Whole-mount *in situ* hybridisation with ephrinB2 riboprobes

For the generation of digoxigenin-(DIG)-labelled RNA probes for in situ hybridisation, a construct containing a 890 bp fragment of ephrinB2, extending from nucleotide 4 to 890 (extracellular domain), was linearised to prepare antisense and sense RNA probes. The probes were synthesised with a DIG RNA Labelling Kit (Roche) according to the manufacturers instructions. Dissected E9.5 embryos were fixed overnight in 4% PFA and gradually dehydrated in 100% methanol and stored at -20°C. For whole-mount in situ hybridisation of ephrinB2 Riboprobes on E9.5 embryos, the procedure is briefly described here. Embryos were gradually rehydrated in 75%, 50%, 25% methanol in PBS (15 minutes each) and then subjected to the following treatments: 2 x 5 minutes washed in PBT (0.1% Tween-20 in PBS) at RT, 1 hour bleaching in 6% H_2O_2 in PBT at RT with rocking, 3x 5 minutes washes in PBT at RT, a 15 minute treatment with 10μg/ml Proteinase K in PBT at RT with rocking, a 10 minute wash with 2mg/ml glycine in PBT (freshly made) at RT with rocking, and 2 x 5 minute washes in PBT at RT. Embryos were transferred to 2 ml tubes and 1ml of Prehybridisation Solution (Prehyb.- 50% Formamide, 5x SSC (pH 4.5), 50μg/ml yeast RNA, 1% SDS, 50μg/ml Heparin) was added and incubated at 70°C for 1 hour. Hybridisation Solution (Prehyb. with ~1μg/ml DIG Riboprobe) was made up and kept at 37°C. Prehyb. was then aspirated and replaced with 1ml Hybridisation solution and allowed to hybridise overnight at 70°C. The following wash steps were done as follows: 3 x 30 minute washes in pre-warmed Solution 1 (50% Formamide, 5x SSC (pH 4.5), 1% SDS) at 70°C with rocking, 3 x 30 minute washes in Solution 3 (50% Formamide, 2x SSC (pH 4.5)), 3 x 5 minutes washes in fresh TBST (1% Tween-20 and 2mM Levimasole) at RT with rocking, 2.5 hours in blocking solution (10% sheep serum in TBST) at RT with rocking. In the meantime the antibody solution was prepared as follows: In a 2ml tube, 0.5ml of TBST and 3mg of embryo powder was added and rocked for 30 minutes at 70°C. The sample was vortexed for 10 minutes, then cooled on ice. After adding 5μl sheep serum and 1μl of Anti-Digoxigenin-AP antibody, it was rocked for 1 hour at 4°C, followed by a centrifugation at 13,000 RPM at 4°C for 10 minutes. The supernatant was collected into a 15ml tube and diluted with 1% sheep serum in TBST to bring the volume up to 2ml, followed by a 10 minutes centrifugation at 4,000 RPM at 4°C. The blocking serum

was removed from the embryos and 1ml of antibody mix was added and mixed gently. This was then removed and an additional 1ml of antibody mix was added and incubated overnight at 4°C with rocking. The post antibody hybridisation washes were carried out as follows: 3 x 5 minute washes in TBST at RT with rocking, 5 x 1 hour washes in TBST at RT with rocking, overnight wash in TBST at 4°C with rocking. For the detection steps, the following procedures were followed: 3 x 10 minute washes in NTMT (1% Tween-20 and 2mM Levimasole) at RT with rocking, followed by incubation of embryos in staining solution (3.5μl BCIP (Roche) and 4.5μl NBT (Roche) in 1ml NTMT) in the dark with rocking until sufficient staining levels was achieved. 2 x 10 minute washes in PBT were performed at RT with rocking, followed by overnight fixation in 4% PFA at 4°C, then 2 x 5 minute washes in PBT and stained embryos were finally stored at 4°C.

2.11.12 Selection for YFP positives cells from embryonic skin using FACS

Timed matings were set up between PDGFRβ-Cre and Rosa26R lines. Yellow Fluorescent Protein (YFP) expressing embryos at E18.5, due to Cre mediated recombination, were easily identified by using a UV lamp under the microscope, using a GFP filter. The skin of these embryos were dissected out and incubated for 2 hours at 37°C, in a 5ml solution containing 2.5ml Collagenase (0.2μM) and 2.5ml of 2x Trypsin solution, supplemented with 3μM EDTA in PBS. The solution was then neutralised by adding 10ml of 10% Foetal Calf Serum (FCS) in DMEM (complete media) and incubating for 2 minutes. After a 1,000 RPM spin in a centrifuge for 5 minutes, the eluate was passed through a sieve with a pore size of 100μm (removing cell clumps and debris) and collected. An additional 5ml of complete media was passed through the sieve and the total collected sample was centrifuged at 1,000 RPM for 5 minutes, after which the supernatant was decanted and the remaining cells were resuspended in 3 ml of DMEM containing 2% FCS. This sample was then given to the Fluorescence Activated Cell Sorter Department where they sorted and collected YFP expressing cells.

2.12 In vitro analysis of isolated mouse cells

2.12.1 Aortic ring assay

The aorta was isolated from E18.5 ephrinB2$^{lox/+}$ mice embryos and placed in 1x E4 medium for dissection. The fibroadipose tissue was removed followed by the removal of the tunica adventitia layer. The aorta was then cut at 1-2mm intervals to obtain aortic rings and these were used for cultures according to the methodology of Bonanno et al. (2000) with modifications (unpublished). Briefly, the matrix used consisted of 30μg/ml Collagen Type IV, 1mg/ml Collagen Type I, 15μM NaOH, 10x E4 media, with appropriate amount of water. 250μl of this collagen mix was added to each well of a 4-well dish and left at 37°C for ~15 minutes to set. Each ring was stuck to the surface of the wells using 2μl of the liquid collagen mix, placed in a 37°C incubator for 5 minutes for the ring to adhere to the matrix and finally, 250μl of 10% FCS DMEM, supplemented with L-glutamine and penicillin/streptomycin, were added. The medium was replaced every two days.

2.12.2 Selection of endothelial cells using Dynabeads CD31

Cells that sprouted from the aortic ring assay were selected for endothelial cell populations using the Dynabeads® CD31 (Dynal Biotech) according to the manufacture's instructions.

2.12.3 Culturing smooth muscle cells isolated from the aorta

The "immorto" mice, where cells could be immortalised using the temperature sensitive SV40 antigen, were used for deriving SMCs. The aorta was isolated from the adult progeny, placed in DMEM and the adventitial layer removed. The aorta was cut longitudinally into ~ 5mm x 5mm pieces and placed into a 6-well dish, with the lumen touching the plastic. 10% FCS DMEM supplemented with 50u/ml IFNγ, was then added and cultured at 33°C. Once a significant number of cells had sprouted out (potententially SMCs), the aorta pieces were then removed, and the remaining cells were propagated in the same culturing conditions. These resulting cells were checked for their smooth muscle cell identity by staining with the αSMA antibody.

2.12.4 Aortic piece assay for assessing SMC sprouting and migration in response to PDGF-B

Mutant and control embryos were harvested at E18.5 and their aortas were dissected out in DMEM. Once the adventitial layers were removed, the aortas were cut longitudinally into ~ 5mm x 5mm pieces and placed into 4-well dishes coated with 250μg/ml of Collagen I, with the lumen side in contact with the matrix. The well was then filled with 200μl of DMEM supplemented with 0.5% foetal calf serum and 20ng/ml of soluble PDGF. The aortic pieces were then examined and followed for levels of sprouting and migration differences between mutants and controls.

2.13 Preparation of Tamoxifen and 4-Hydroxytamoxifen

In vials containing Tamoxifen and 4-Hydroxytamoxifen powder, peanut oil was added to obtain a concentration of 1mg/ml. The vials were placed in a beaker with ice, and then subjected to sonication (Sanyo Soniprep 150) at a frequency of 10 kHz for a total of 5 minutes at one minute intervals to allow the solution to cool. This was then stored at 4°C.

2.14 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 viewed in a JEOL 1010.

Chapter3 | Investigating the role of ephrinB2 in endothelial cells

3.1 Introduction

The cardiovascular system is the first organ system to develop and reach a functional state during embryonic development. "Vasculogenesis" refers to the initial steps when the endothelial cell precursors, the angioblasts, are differentiated from the mesenchyme to form a juvenile vascular system (Risau and Flamme, 1995). This primary capillary plexus is subsequently remodelled into a mature vascular system by pruning and reorganisation of endothelial cells in a process called "angiogenesis". This complex remodelling process involves the recruitment of mural cells to stabilise the vessels and starts in the vasculature of many organs during late embryonic development (Nehls and Drenckhahn, 1993), but the molecular mechanism is poorly understood.

3.1.1 The Cre-loxP 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. A frequent limitation in such studies is that null mutants are embryonic lethal at E9.5-E11 or exhibit complex phenotypes that do not allow the identification of the function of the investigated gene during vascular development. These limitations can be overcome by cell-type specific and inducible gene targeting systems such as the Cre-loxP approach, which exploits the site-specific recombinase activity of a gene product from bacteriophage P1 (Vasioukhin et al., 1999; Branda and Dymecki, 2004). The 38 kDa "Cre" recombinase specifically recognises the 34 bp sequences termed "loxP" sites. The system requires the crossing of two mouse lines, one that carries the target with an insertion of two loxP sites (known as "floxed"), while the other line expresses Cre recombinase under the control of a cell/tissue specific promoter and may be inducible. Consequently, Cre activity and gene activation will be restricted to specific cell-types or tissues (Figure 3.1). The first step of this approach involves the insertion of the loxP flanked targeting construct into a specific gene locus

by homologous recombination in embryonic stem (ES) cells. The construct contains a selection marker, usually the neomycin phosphotransferase (*Neo*) gene, to facilitate the identification of ES cells where recombination has occurred. However, selection markers may interfere with the normal transcription and splicing of the targeted and neighbouring genes, and may affect or compromise protein expression (Ohno et al., 1994). A widely used method to avoid such effects is to flank selection markers with "Frt" recognition sites and to remove them using "Flp", a recombinase from the yeast *Saccharomyces cerevisiae* (Dymecki, 1996).

Even though the Cre/loxP system is a powerful tool for manipulating the mouse, it has its own intrinsic limitations, predominantly due to the fact that it relies on a partial promoter sequence (possibly enhancers as well) to drive *Cre* expression. Inappropriate or unexpected *Cre* expression may lead to recombination in cell-types other than the desired ones. The intrinsic nature of a promoter and its regulation will affect the temporal and spatial pattern of recombination. Complications may also arise in complex breeding schemes where multiple conditional alleles need to be combined with specific transgenes. Cre expression and activity can be monitored using Rosa26R reporter mouse line where a triple polyadenylation sequence is flanked by loxP sites that is fused to the *lacZ* sequence and integrated in the Rosa26 locus (Soriano, 1999), a site in which promoter elements lead to ubiquitous expression. Breeding of these mice with specific Cre lines enables the monitoring of the activity and timing of Cre mediated recombination *in vivo* because the deletion of the loxP-flanked triple polyadenylation sequence results in constitutive β-galactosidase expression.

A limitation of the Cre-loxP system is the lack of control over the onset of Cre expression. This is particularly important if gene inactivation occurs early in development and leads to defects that will compromise other developmental processes without a direct involvement of the targeted gene. Such problems can be addressed by temporal regulation of Cre recombinase *in vivo*. This can be accomplished with tetracycline controlled gene expression systems, interferon-inducible expression or drug inducible forms of Cre (Garcia and Mills, 2002). In the latter case, the ligand binding domain of a mutated human oestrogen receptor is fused to Cre ($CreER^T$), so that the fusion protein requires the administration of the synthetic oestrogen antagonist

tamoxifen (or its derivative 4-hydroxytamoxifen) for its translocation into the nucleus and consequently, elimination of the loxP-flanked sequence (Figure 3.1)(Zhang et al., 1996).

3.1.2 Previous studies on ephrinB2

EphrinB2 marks arterial endothelial cells from the onset of angiogenesis during mouse embryonic development and is an important regulator of blood vessel development. Mice lacking ephrinB2 (ephrinB2$^{KO/KO}$) are lethal by midgestation (E11) and display angiogenic remodelling arrest in the head and yolk sac and defective myocardial trabeculation in the heart (Wang et al., 1998; Adams et al., 1999). As development proceeds, its expression progressively extends from the arterial endothelium to surrounding vascular smooth muscle cells and pericytes (Gale et al., 2001; Shin et al., 2001). Expression of ephrin-B2 has been shown to be downregulated in many adult tissues but is strongly expressed during neovascularisation associated with corpus luteum formation, wound healing and tumour angiogenesis (Gale et al., 2001; Shin et al., 2001).

Global ablation of ephrinB2 leads to severe cardiovascular defects in embryos, growth retardation and midgestation lethality. As multiple cell types in the cardiovascular system express ephrinB2, the specific requirement of the gene is difficult to address by global gene targeting.

3.2 Results

3.2.1 Generation ephrinB2 Conditional Line

The early lethality displayed by ephrinB2 knockout embryos has limited insight into its specific role in vascular morphogenesis. Mutants displayed defective remodeling of the yolk sac vascular plexus and decreased branching of cranial capillaries, as well as abnormal sprouting of intersomitic vessels and decreased vascularization of the neural tube. However, it is possible that some of these defects could be caused by malfunctions

of the heart and defects in extra-embryonic tissues. It is noteworthy that gene targeting of many other genes, including those involved in the Notch, Tie and VEGFR pathways, have resulted in very similar phenotypes.

To determine the specific role of ephrinB2 in endothelial cells (ECs), as well as in other cell types, a conditional ephrinB2 mouse line has been generated (Figure 3.2)(Grunwald et al., 2004) and has been used in the work presented here. Two different ephrinB2 conditional lines have been used, one contained the *Neo* cassette ($ephrinB2^{loxNeo/+}$), whereas *Neo* has been excised ($ephrinB2^{lox/+}$) by breeding to the Flp deleter mice (Dymecki, 1996) in the second line.

As interbreeding of $ephrinB2^{loxNeo/+}$ heterozygotes did not produce any homozygous offspring at weaning age, different stages of embryonic development was investigated by timed matings of animals. Freshly dissected $ephrinB2^{loxNeo/loxNeo}$ embryos at E9.5 displayed growth retardation as well as morphological abnormalities in the head, heart and back regions. Whole-mount stainings for PECAM-1, an endothelial cell marker, revealed severe vascular malformations such as arrest of vascular remodelling in the head, disrupted formation of the posterior cardinal vein and second branchial arch as well as inflation of the pericardium (Figure 3.3) These defects are similar to those observed in ephrinB2 knockouts. Homozygous mutants for both strains displayed similar lethality by E11 with full penetrance. In summary, the phenotypic similarities seen in $ephrinB2^{loxNeo/loxNeo}$ and ephrinB2 null mutants suggest that the presence of the *Neo* cassette in the conditional allele impairs expression of ephrinB2. Accordingly, Flp recombinase-mediated removal of *Neo* permitted the generation of $ephrinB2^{lox/lox}$ homozygotes at the expected ratio so this line was used for subsequent studies.

3.2.2 Validation of the conditional targeting using PGK-Cre

The conditional ephrinB2 line ($ephrinB2^{lox}$) had to be validated to ensure that the floxed-targeted locus was accessible to Cre as a prerequisite for recombinase-mediated deletion. For this purpose, the ephrinB2 gene was inactivated globally ($ephrinB2^{\Delta global}$ mutants), with the help of a transgenic line expressing Cre in a ubiquitous manner, to investigate whether resulting embryos would recapitulate the ephrinB2 knockout

phenotype. In PGK-Cre mice, Cre expression is driven by the promoter of endogenous 3-phosphoglycerate kinase (Lallemand et al., 1998). As this enzyme is involved in glycolysis necessary for every cell, the PGK promoter is active ubiquitously. Cross-breeding of the PGK-Cre and ephrinB2$^{lox/lox}$ mice allowed the generation of ephrinB2$^{\Delta global}$ mutants (Figure 3.4) which were then analysed. Whole-mount stainings of E9.5 embryos with anti-PECAM-1 antibody revealed an array of anomalies in cardiovascular development and angiogenic remodelling defects in mutants that were indistinguishable from ephrinB2$^{KO/KO}$ embryos (Figure 3.5). ephrinB2$^{\Delta global}$ animals were dead by E11 with full penetrance. Furthermore, whole-mount *in situ* hybridisation for *ephrinB2* (efnb2) mRNA in ephrinB2$^{\Delta global}$ mutant embryos showed no detectable transcripts (Figure 3.6) and proved that ubiquitous ablation was achieved.

3.2.3 Investigating the role of ephrinB2 in endothelial cells using the Tie1-Cre line

To determine the role of ephrinB2 in endothelial cells, ephrinB2$^{lox/lox}$ mice were combined with the Tie1-Cre transgenic line. This line restricts Cre expression and activity to the vascular endothelium of vascular structures from stage E8.5 onwards. Structures with Cre expression include the dorsal aorta, the yolk sac vasculature, and the developing heart tube (Gustafsson et al., 2001). Expression in many different vascular beds is also evident at later stages of embryonic development, and persists into adulthood. Additionally, sites of expression at later stages of development include the glomeruli of kidneys, brain, alveolar capillaries in the lung and endocardial cells lining the heart.

Endothelial cell-specific ephrinB2 mutants (ephrinB2$^{\Delta EC}$) were growth retarded, their yolk sacs were pale and they displayed very few blood vessels due to remodelling defects (Figure 3.7). Similar defects were seen in ephrinB2$^{\Delta global}$ mutants. PECAM-1 staining of whole ephrinB2$^{\Delta EC}$ embryos revealed severe vascular anomalies including arrested angiogenesis in the head vasculature, defects in the posterior cardinal vein and the dorsal aorta, as well as inflated pericardium (Figure 3.5). Nevertheless, mutants were alive and displayed a beating heart. Despite these similarities, the phenotype of ephrinB2$^{\Delta EC}$ mutants seemed less severe than what has been seen in full knockouts.

Furthermore, *in situ* hybridisation for the presence of ephrinB2 mRNA showed an overall reduction in transcript levels throughout the ephrinB2$^{\Delta EC}$ embryos in comparison to controls, which is presumably caused by the growth retardation evident at E9.5 (Figure 3.6). EphrinB2 mRNA was absent in the larger vessels, intersomitic vessels and the dorsal aorta. EphrinB2 transcripts in the mutant head region and in the branchial arches appear to correspond to expression in neural crest and mesenchymal cell populations.

3.2.4 Inducible ephrinB2 ablation in endothelial cells with Tie2-CreER^T transgenic

As the lethality of ephrinB2$^{\Delta EC}$ mutants prevents studies in late embryos and adults, it was impossible to study the role of the ligand in most tissues and organs. To circumvent this problem, a previously uncharacterised inducible endothelial-specific Tie2-CreER^T line (E. Wagner, unpublished) was acquired. This line hosts a transgene in which Cre was fused to the hormone-binding domain of oestrogen receptor (ER^T) that has been modified so that it can only be activated by binding of tamoxifen or 4-hydroxytamoxifen. Since the inducible Tie2-CreER^T mice had not been sufficiently characterised, the line was bred into a Rosa26R Cre reporter background to study its suitability for gene inactivation experiments.

3.2.4.1 Characterisation of Tie2-CreER^T activity in embryos

To study Tie2-CreER^T activity in mouse embryos, single intraparitoneal injections of either tamoxifen or its more potent metabolite 4-hydroxytamoxifen (4OH-TM) were administered to females at different stages of pregnancy (E9.5 to E17.5). The injected concentration was 5mg/40g body weight, which is and significantly below the reported lethal dose (Hayashi and McMahon, 2002). Embryos were harvested 24 hours post injection and assayed for β-galactosidase expression as a measure of Cre activity. This regime did not lead to detectable lacZ staining and no obvious adverse effects due to toxicity were noticed (data not shown).

In a second approach, 4OH-TM, at a dose of 3mg/40g body weight, was administered by intraperitoneally injection for three days in succession. This regime was applied to female mice at different stages of gestation starting from E9.5. Embryos were collected 24 hours after the final injection and subjected to β-galactosidase stainings. Even though the analysis revealed barely detectable levels of β-galactosidase activity for the vast majority of the embryos (data not shown), mosaic vascular staining was obtained for a fraction of double transgenic embryos. Of these samples, one embryo at E11 in particular, displayed widespread staining in blood vessel-like structures (Figure 3.8). As some embryos showed signs of growth retardation or oedema and because of an increased incidence of reabsorption (data not shown), it appears that toxic effects caused by the triple injection regime are more significant than for single injection.

3.2.4.2 Induction of Tie2-CreER^T activity in adult mice and in tumours

The Tie2-CreER^T line was also assessed for its capability of inducing Cre expression in adults. For this purpose, regimes of three or five injections of 5mg/40g body weight of 4OH-TM, spaced by 24-hour intervals, were administered intraperitoneally in 12 week old mice. The mice were sacrificed 24 hours after the final injection and isolated organs and tissues were assayed for lacZ expression, but revealed no specific staining in lung, kidney, heart, aorta, skin or intestine (data not shown). Several of the sacrificed animals had discoloured livers indicative of side effects. It has been reported that tamoxifen is converted into α-hydroxytamoxifen in the rodent liver where it can cause damage and induce hepatocarinomas if administered chronically (Wogan, 1997), which may explain the observation above.

Alternatively, "timed release" pellets from Innovative Research of America were implanted subcutaneously to induce Cre expression. This application route presents several advantages: a) animals are only exposed to the stress of a single implantation procedure, b) "peak and valley" effects are avoided, and c) rapid removal of tamoxifen through metabolic processes is prevented. Animals that had received 100mg of tamoxifen over a period of 22 days were sacrificed and various organs were analysed for β-galactosidase activity. Blue staining indicative of EC-specific Cre activity was observed in various tissues and organs (Figure 3.9), including heart, aorta, lung and tail

skin. Thus, timed release pellets are, in principle, suitable for the induction of Cre activity in adult tissues and organs. Furthermore, no toxic effects were observed.

To investigate whether the Tie2-CreER^T line could be used for studies in the tumour vasculature, Tie2-CreER^T/Rosa26R double transgenic mice received timed release pellets and, with a delay of three days, a single subcutaneous injection of two million Lewis Lung Carcinoma cells. These mice had to be sacrificed 11 days after the injection of the cells because tumours had grown to a size of 150-200 mm^3, close to the legal limit of 250 mm^3. The analysis of dissected tumours revealed lacZ expression that corresponds to a population of small vessels and capillaries (Figure 3.9). However, Cre induction appeared incomplete and this approach was not pursued any further.

3.3 Discussion

The early lethality displayed by ephrinB2 null embryos has limited the study of its role in blood vessel morphogenesis and development. From the analysis of the knockouts, it is difficult to determine whether the vascular defects are independent from the aberrations in the heart and extra-embryonic tissues. The Cre/loxP system may permit cell type-specific deletion of the ephrinB2 gene to study specific roles of the ligand. To establish the suitability of an ephrinB2^{lox} conditional line for experimental studies, these mice were bred to PGK-Cre transgenics expressing Cre ubiquitously. The resulting ephrinB2$^{\Delta global}$ mutants were lethal by E11 and displayed vascular malformations that were indistinguishable from the ephrinB2 knockout phenotype. In addition, *in situ* hybridisation of whole ephrinB2$^{\Delta global}$ embryos confirmed the absence of ephrinB2 mRNA suggesting that its ubiquitous ablation was achieved. The data validated the suitability for tissue-specific gene inactivation experiments. Furthermore, Grunwald and colleagues have also successfully used this line to remove ephrinB2 in the developing nervous system and in postnatal forebrain with Nestin-Cre and CamKIIα-Cre transgenic lines, respectively (Grunwald et al., 2004).

To investigate the role of ephrinB2 in the endothelial lining of blood vessels, the conditional line was bred with EC-specific Tie1-Cre transgenics. EphrinB2$^{\Delta EC}$ mutant embryos were lethal by E11 and displayed cardiovascular defects similar to null

mutants. Some ephrinB2$^{\Delta EC}$ mutants displayed a less severe phenotype, which could be due to incomplete deletion of the loxP-flanked ephrinB2 gene fragment in the EC population. Similar work with a different EC-specific Cre, published by Gerety and co-workers, showed that mutants were phenotypically indistinguishable from global ephrinB2 knockouts (Gerety and Anderson, 2002). However, the mutants generated by Gerety and colleagues harbour only one conditional allele in a null background. Therefore Cre-mediated recombination had to occur at only one allele leading to increased chances for a complete deletion in the endothelium. On the other hand, some abnormalities in these mutants could be caused or enhanced due to the haploinsufficiency of ephrinB2 expression in cell populations outside the endothelium, independent of Cre mediated recombination. Defects seen in ephrinB2^{loxNeo} homozygotes support that hypomorphic phenotypes can be caused by reduced ephrinB2 expression. The results presented here showed that ephrinB2 expression in endothelial cells is essential for normal development. The role of cardiac defects or changed haemodynamic parameters remains to be determined.

To avoid the early lethality of ephrinB2$^{\Delta EC}$ mutants and address the role of ephrinB2 in the late embryonic and adult vasculature, the previously uncharacterised inducible Tie2-CreER^{T} line was acquired, and bred into Rosa26R Cre reporter background. Single or multiple intraperitoneal injections of pregnant females from timed matings with either tamoxifen or 4OH-TM resulted in poor Cre activity that was considered insufficient for functional studies. Likewise, CreER^{T} activation in adult tissues and organs could not be achieved by 4OH-TM injection.

The application of tamoxifen through timed release pellets led to lacZ expression in various vascular beds, especially in the coronary vessels of the heart. This suggests that the continuous release of tamoxifen over a longer period of time produces better results than injection schemes. Nevertheless, reporter activation in the endothelium of adult tissues and in xenograft tumours was incomplete and, presumably, too low for functional studies with the ephrinB2^{lox} line.

These results might be caused by a limited capability for Cre induction of the Tie2-CreER^{T} line. Successful inducible gene inactivation in the adult endothelium has not been reported so far and is a major technical challenge in the field. The application

regime and biological availability of tamoxifen or 4OH-TM are further factors that could influence the activation of $CreER^T$ in transgenic mice. In fact, recent work from the laboratory shows that a modified protocol of tamoxifen preparation and injection leads to significantly improved reporter activation in Tie2-$CreER^T$/Rosa26R double transgenics.

The studies described here have also highlighted the toxicity of high doses of tamoxifen and 4OH-TM in embryonic and adult mice, a side effect that can have significant impact on functional studies. Peanut oil, in which tamoxifen and 4OH-TM was suspended, may also contribute to the toxicity and little is known about its effects in systemic administration. No systemic toxicity was noticed in adult mice with pellet implantations indicating that the absence of oil or other carrier substances, along with the longer lasting application of lower tamoxifen doses, might be beneficial. Further work is needed to address this question.

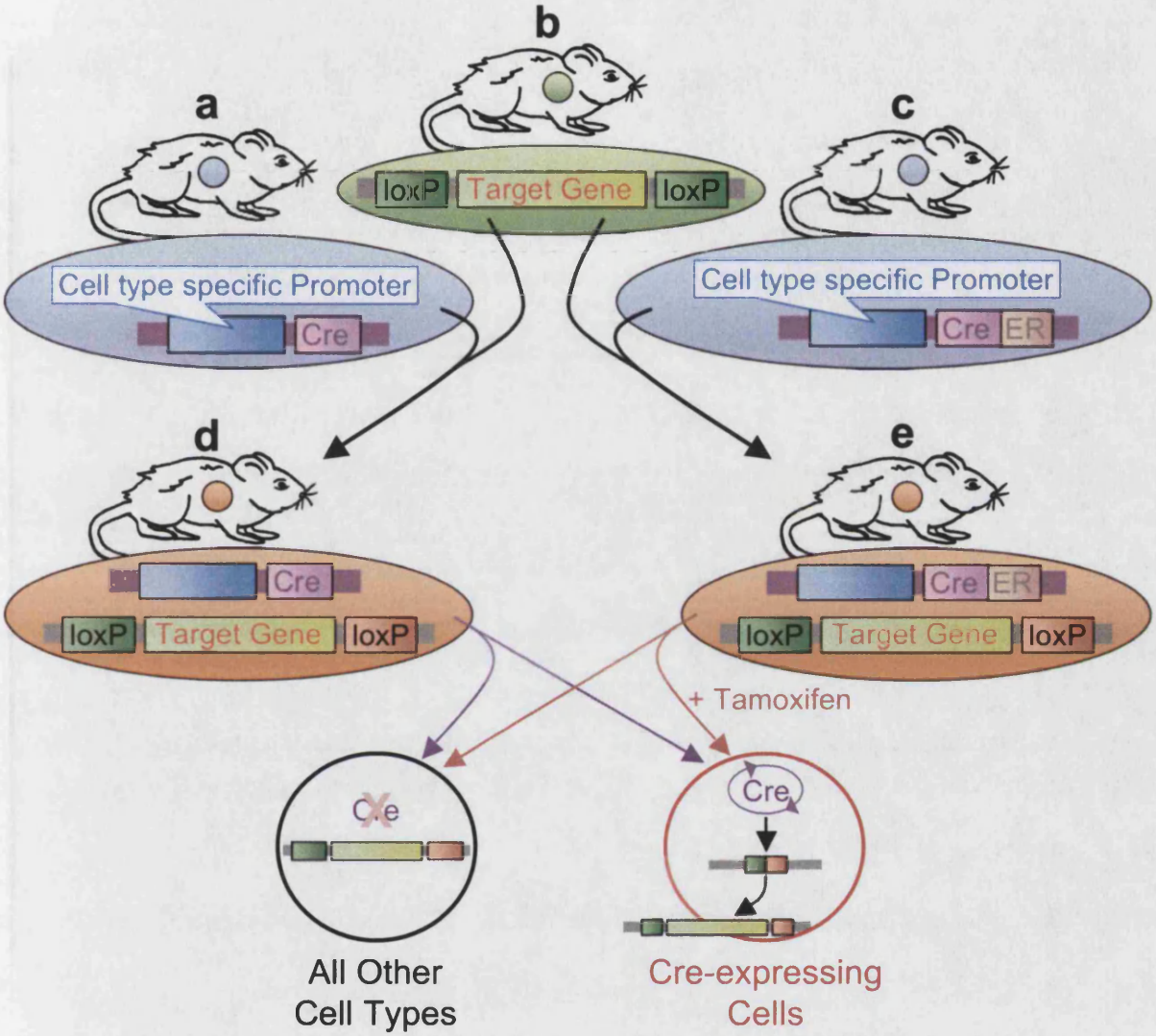


Figure 3.1: Generation of cell type specific knockouts using the Cre/loxP conditional system

Mice expressing Cre (a) or CreER (c) in specific cell types are bred with mice containing a target gene fragment flanked by loxP sites (b). In the Cre containing progenies (d), Cre expressing cells will mediate gene fragment deletion through recombination. While in the CreER containing progenies (e), Cre expression should only occur upon tamoxifen administration, thereby mediating cell type specific deletion.

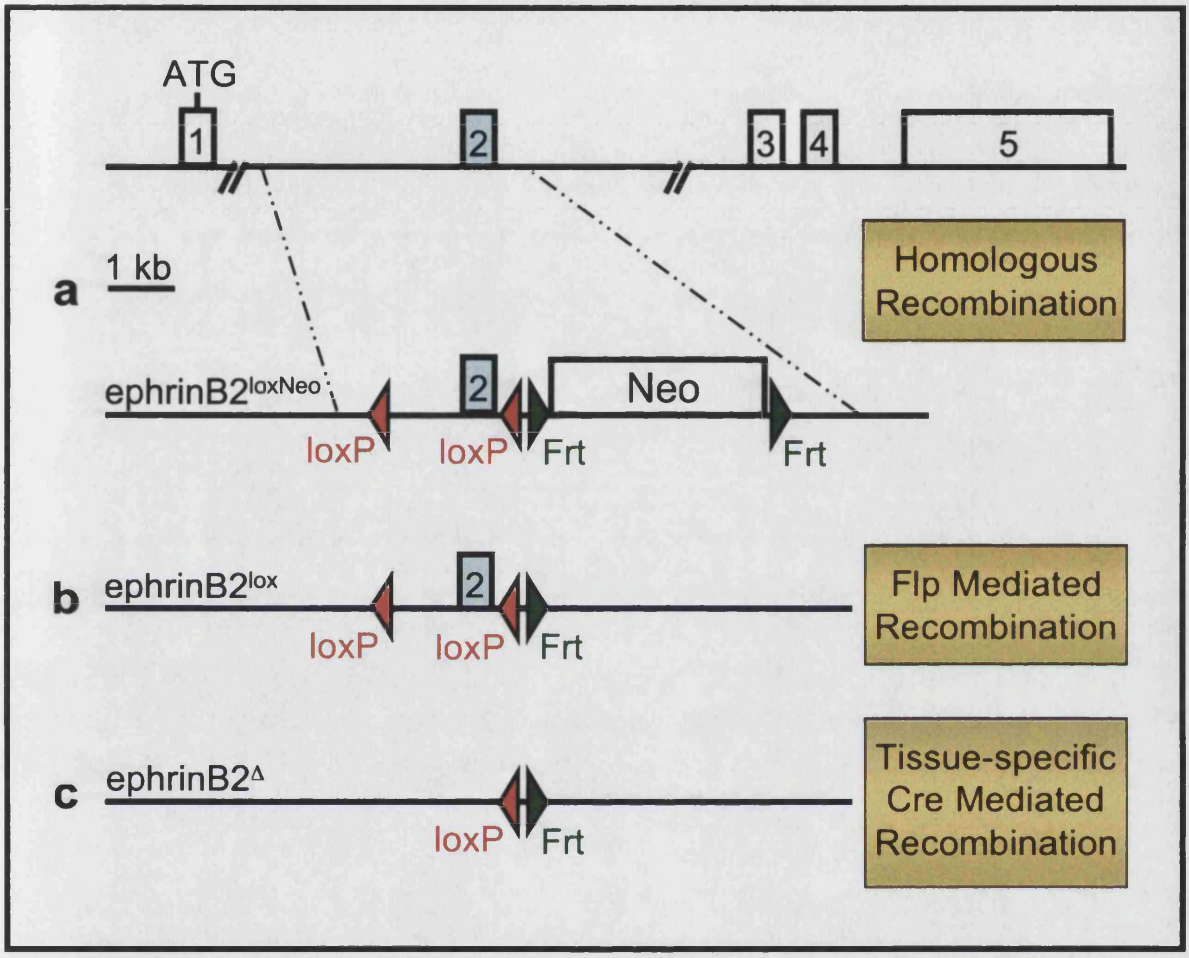


Figure 3.2: EphrinB2 conditional targeting vector and different recombination events

Schematic representation of the ephrinB2 conditional targeting vector. The loxP and Frt sites flank exon-2 and the neomycin (Neo) resistance selection marker respectively (a). Crossing with the Flp recombinase mice removed the Neo cassette (b). Cell type-specific deletion of the ephrinB2 fragment mediated by breedings with various Cre lines (c).

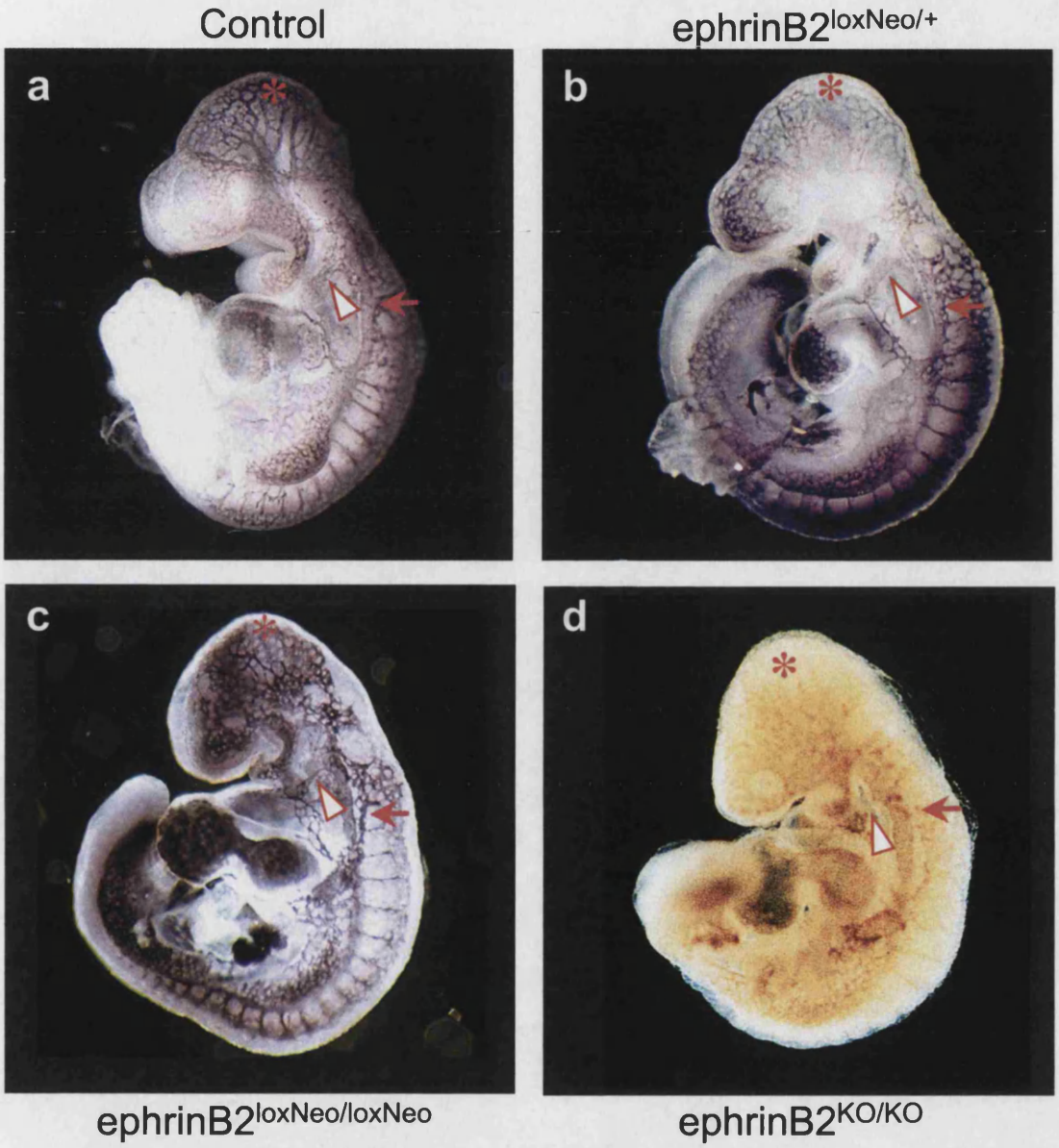


Figure 3.3: Homozygous ephrinB2^{loxNeo} display vascular defects that are similar to the ephrinB2 knockouts

PECAM-1 staining on whole-mount embryos at E9.5 embryos labelling the vascular network. The vascular morphology of the ephrinB2$^{loxNeo/+}$ mutants (b) is indistinguishable from wildtype controls (a). EphrinB2$^{loxNeo/loxNeo}$ mice (c) displayed severe defects in blood vessel development that were similar to those seen in the ephrinB2 knockout (d). Malformations disrupted assembly of the posterior cardinal vein (arrow) and second branchial arch (arrowhead) as well as causing defective angiogenic remodelling in the head region (asterisk).

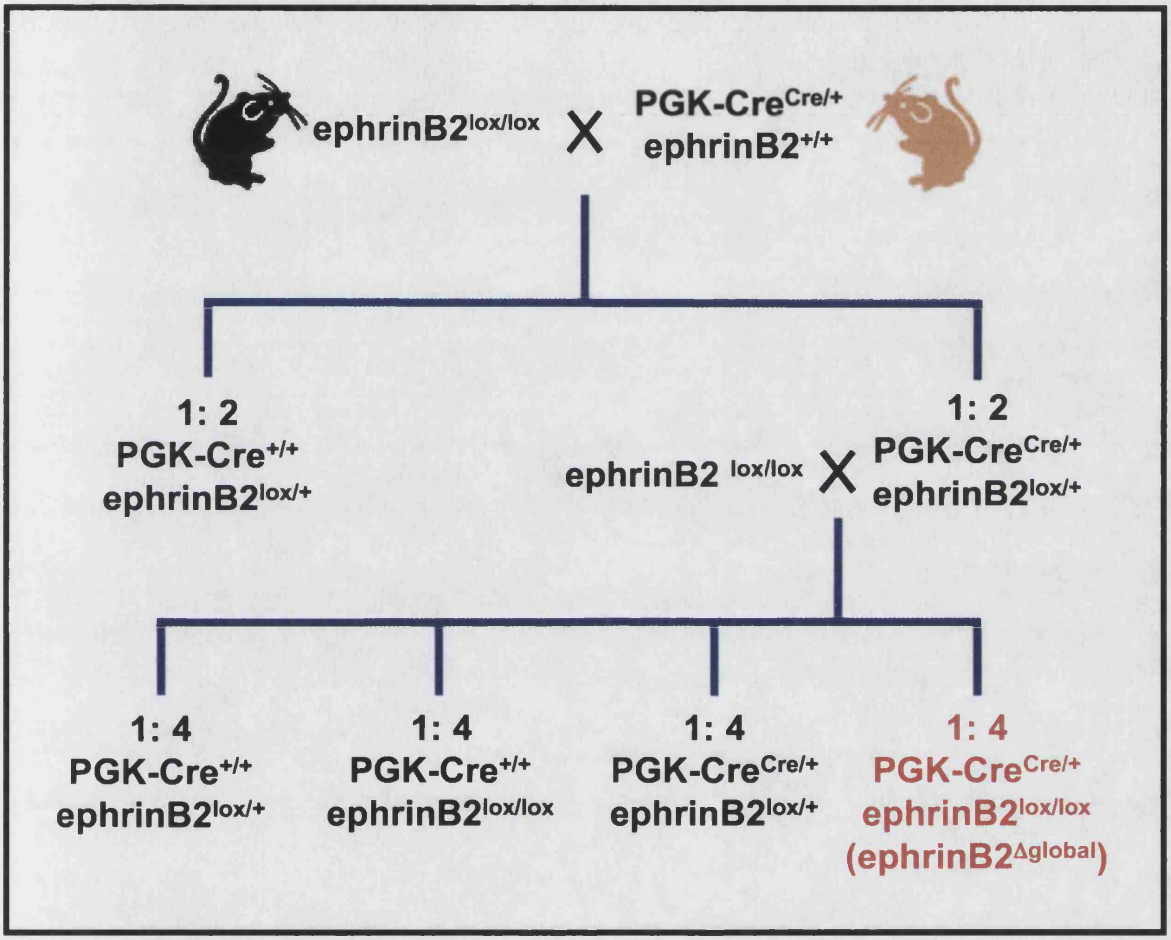


Figure 3.4: Breeding scheme for the generation of ephrinB2$^{\Delta global}$

The scheme illustrates the breeding steps required to generate ephrinB2$^{\Delta global}$ embryos. A similar breeding scheme was used for the generation of tissue-specific ephrinB2 mutants with various transgenic lines.

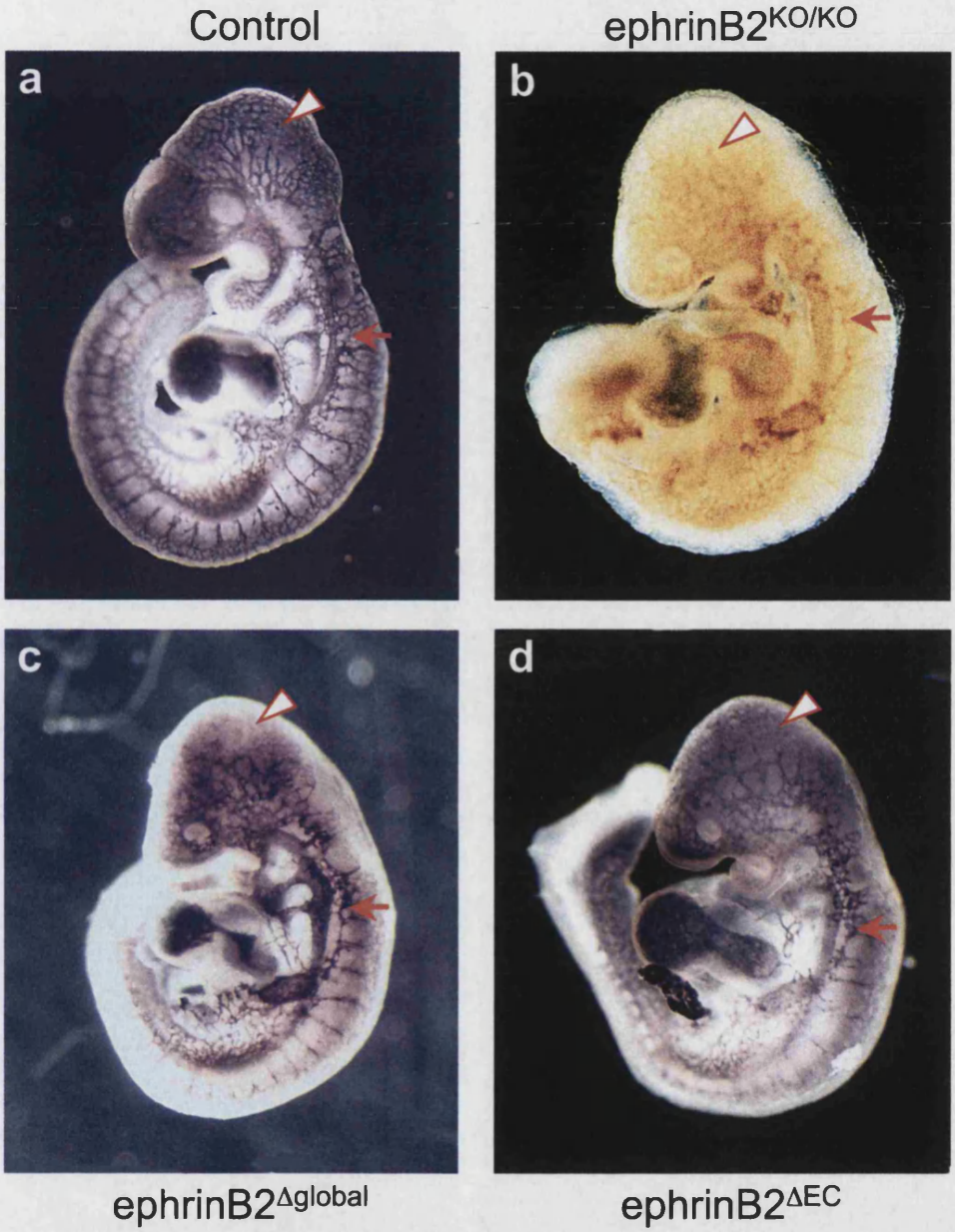


Figure 3.5: Global and endothelial cell-specific deletion of ephrinB2 leads to vascular defects and lethality by E11

PECAM-1 stained whole embryos at E9.5 revealing the different vascular morphology of ephrinB2 mutants. Blood vessel morphology is severely disrupted in ephrinB2$^{\Delta global}$ (c) and ephrinB2$^{\Delta EC}$ (d) embryos compared to littermate controls (a). These defects are similar to those displayed by ephrinB2 knockout mice (b) and include defects in remodelling of blood vessels in the head regions (arrowheads) and formation of posterior cardinal veins (arrows).

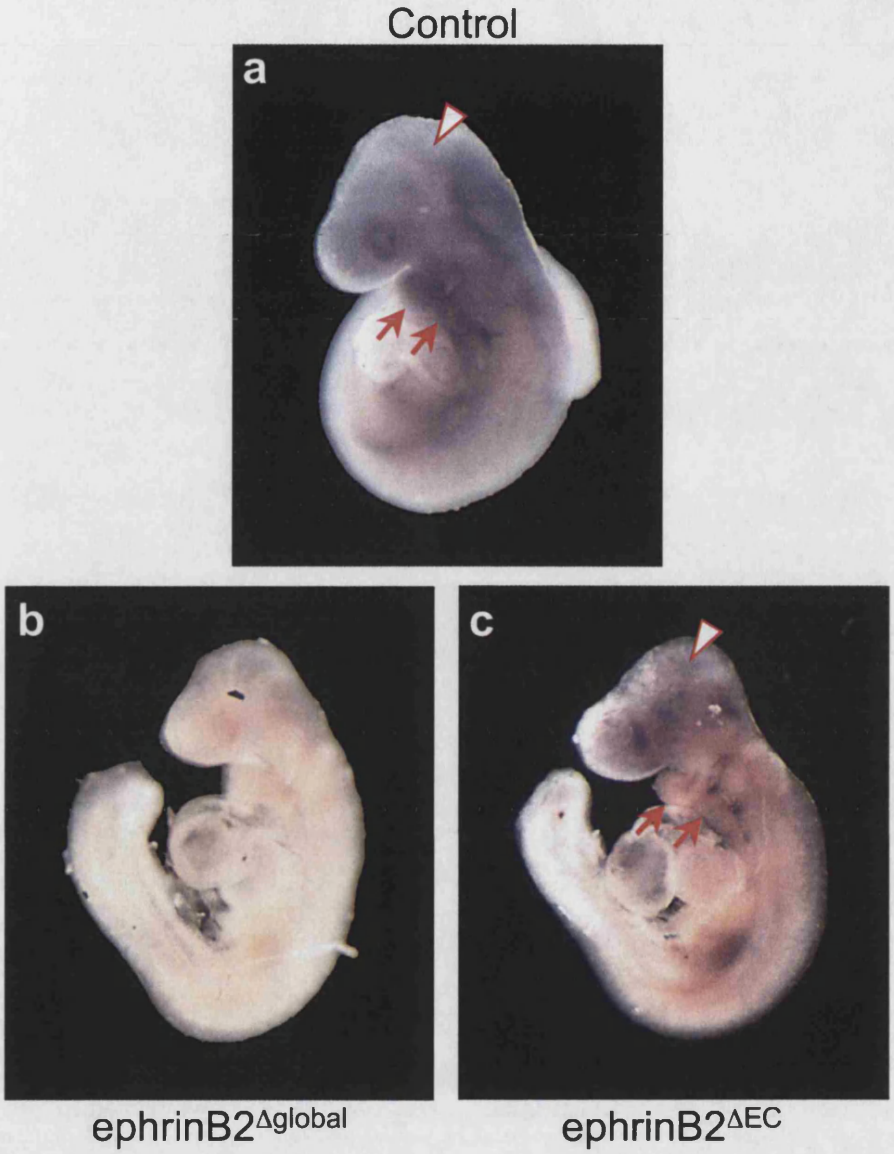


Figure 3.6: Absence of ephrinB2 mRNA transcripts in ephrinB2$^{\Delta global}$ mutants and a partial decrease in ephrinB2$^{\Delta EC}$ mutant

In situ hybridisation of whole embryos at E9.5 for the detection of ephrinB2 mRNA transcripts. No detectable staining was obtained for ephrinB2$^{\Delta global}$ mutants (b), while ephrinB2$^{\Delta EC}$ embryos displayed reduced ephrinB2 expression (c) in comparison to controls (a). EphrinB2 mRNA (efnb2) was absent in the ephrinB2$^{\Delta EC}$ vasculature but not in the branchial arches (arrows) and head regions (arrowheads). Overall, transcription levels in ephrinB2$^{\Delta EC}$ mice appeared reduced, presumably due to growth retardation at E9.5.

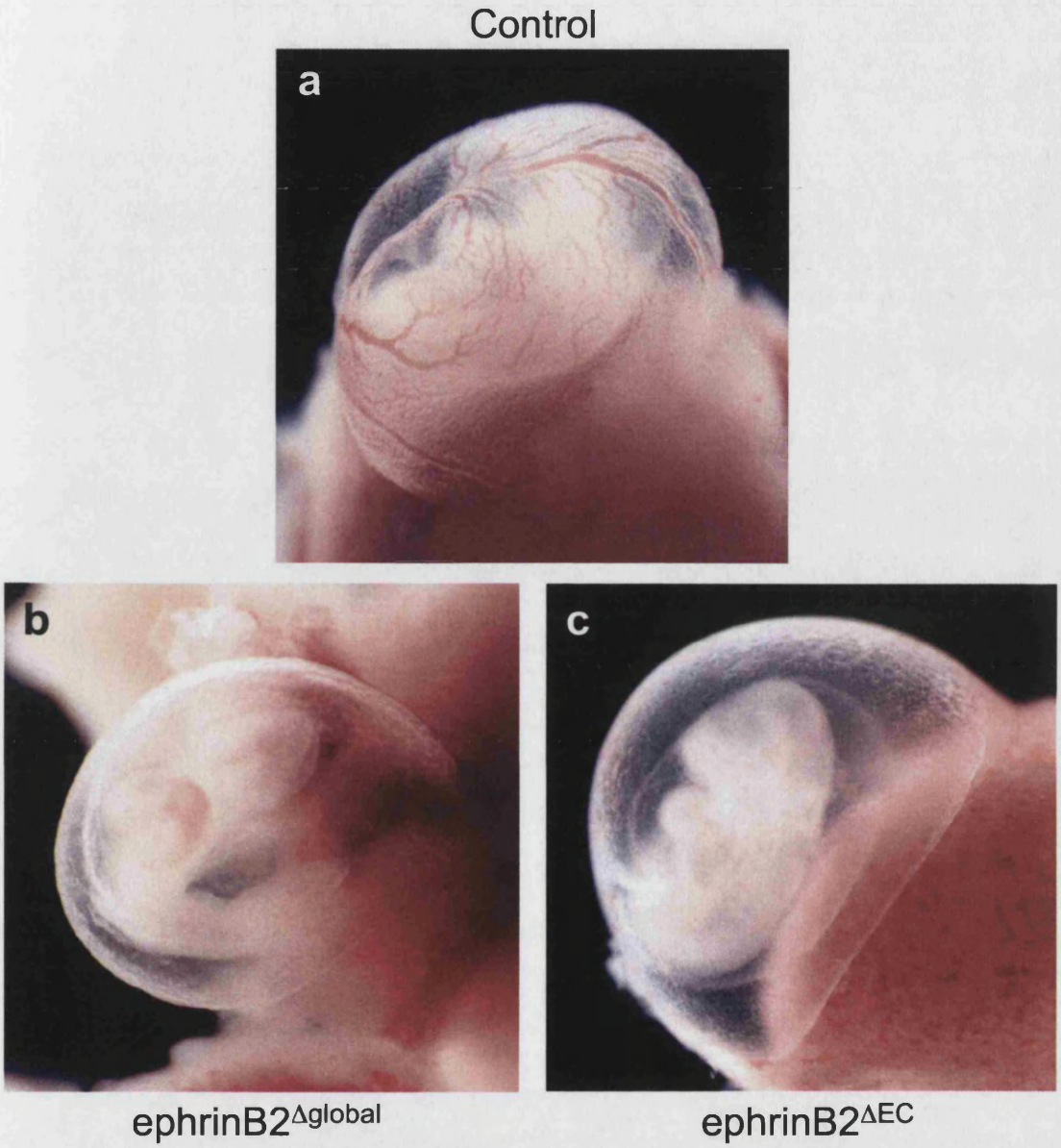


Figure 3.7: Blood vessel defects in the yolk sacs of ephrinB2$^{\Delta global}$ and ephrinB2$^{\Delta EC}$ mutants

Images taken from freshly dissected E9.5 embryos with intact yolk sacs. In both ephrinB2$^{\Delta global}$ (b) and ephrinB2$^{\Delta EC}$ (c) mutants, the vitelline vessels and associated branches were absent. A control is shown in (a).

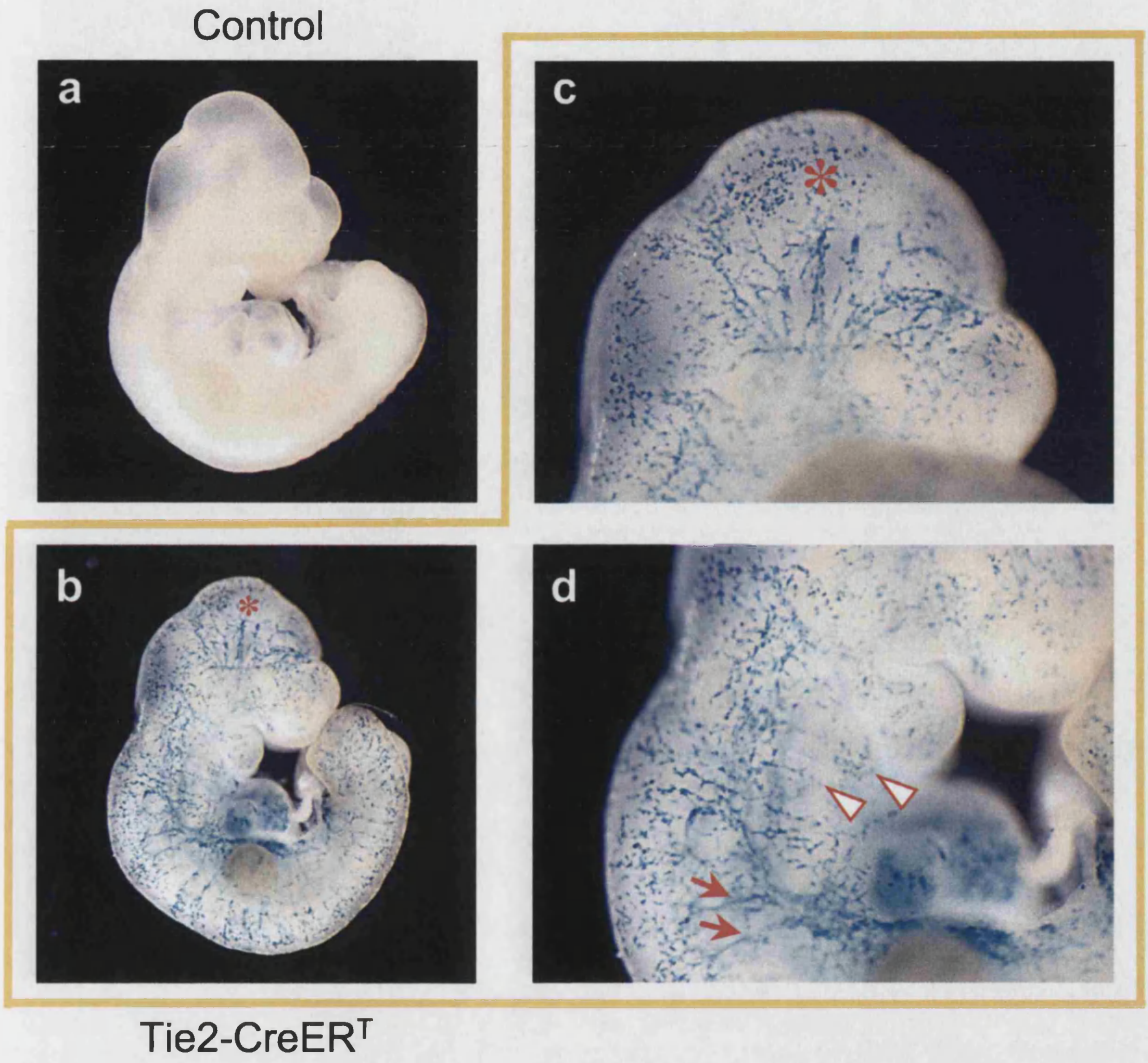


Figure 3.8: LacZ staining of Tie2-CreER^T/Rosa26R double heterozygous embryos reveal blood vessel structures upon 4OH-TM administration

Pregnant females were subjected to the triple-injection regime of 4OH-TM. LacZ staining on Tie2-CreER^T/Rosa26R double heterozygous E11 embryo revealed different vascular structures including the vascular branching in the head region (b,c- asterisks), the vessels in the branchial arches (d- arrowhead) and the intersomitic vessels (d- arrows). Blue staining was absent in littermate controls (a).

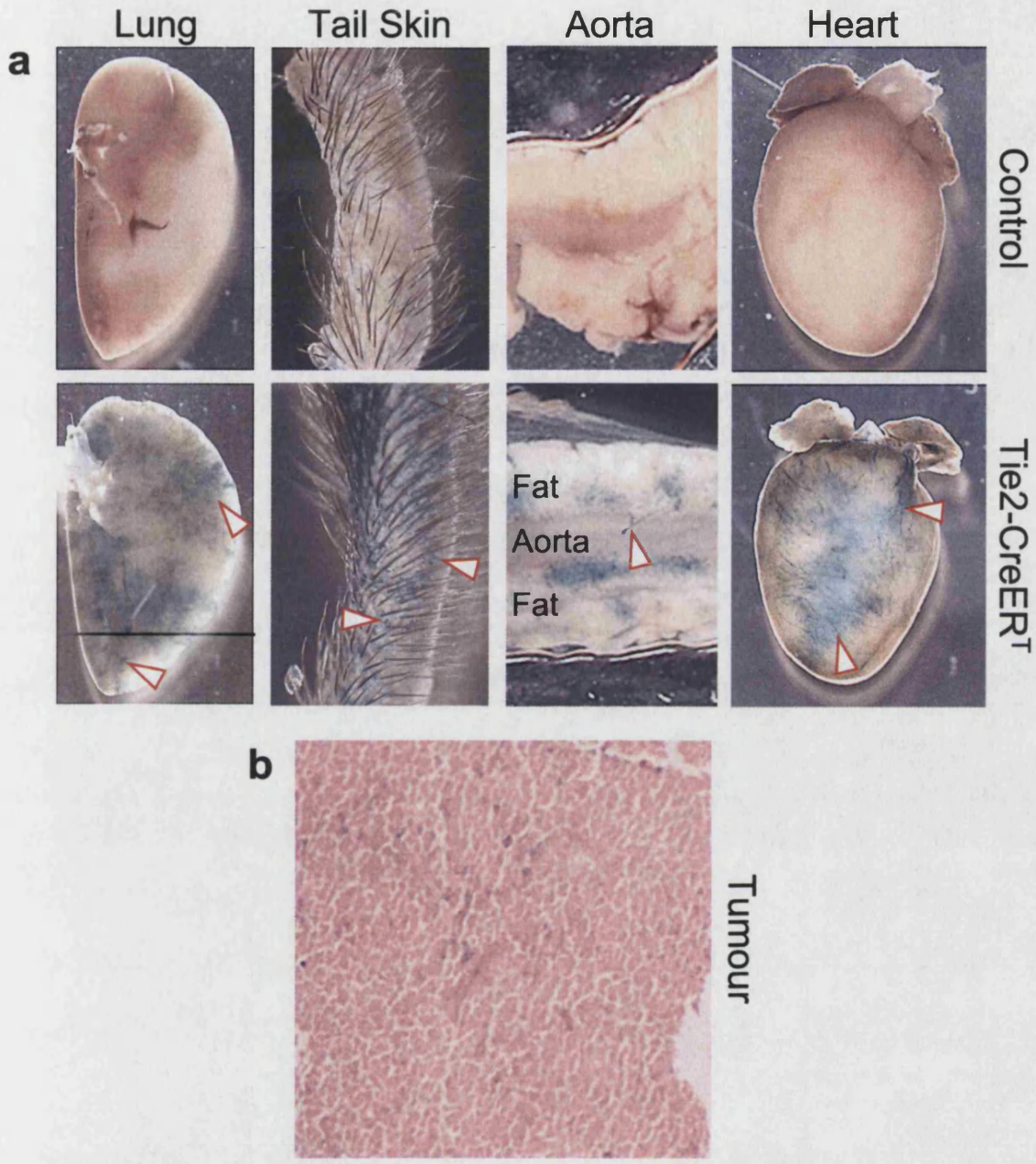


Figure 3.9: Induction of Tie2-CreER^T in adult organs, tissues and tumours
Tie2-CreER^T adults in a Rosa26R background were implanted with tamoxifen timed release pellets. LacZ expression in a range of harvested tissues and organs revealed induction of CreER^T activity in several vascular structures (a, arrowheads). Blue staining was absent in the controls. LacZ staining of Lewis Lung Carcinoma tumour cells in Tie2-CreER^T/Rosa26R transgenic mice, followed by pellet implantation, led to blue staining in structures that may correspond to small intratumour vessels and capillaries (b). Sections (7μm) have been counterstained with eosin.

Chapter4 | Characterisation of SMMHC-Cre mice, generation and characterisation of mural cells specific Cre transgenic line

4.1 Introduction

Mural cells are involved in complex interactions with both the endothelial lining of blood vessels and the surrounding extracellular matrix. They play important roles during embryonic development, which range from the stabilisation of nascent vessels, the inhibition of EC proliferation and angiogenesis, to the modulation of the blood flow (Carmeliet, 2000).

Shin and co-workers (Shin et al., 2001) have demonstrated that ephrinB2 expression extends from the arterial endothelium to vSMCs during late embryonic development and in adults. However, the function and requirement of ephrinB2 in mural cell populations has yet to be elucidated. To enable the inactivation of ephrinB2 in mural cell populations with the Cre-loxP approach, a transgenic line expressing Cre selectively in vSMC/pericyte populations was required. Many investigators have experienced great difficulties in generating such a line, largely due to the fact that many smooth muscle promoters are only transiently active during development or are used by other cell types, for example, in the heart and somites (Edelberg et al., 1998; Hirota et al., 1999). These problems have previously limited the generation of suitable tools for SMC-specific inactivation studies *in vivo*.

4.2 Results

4.2.1 Characterisation of SMMHC-Cre mice

Regan and co-workers (Regan et al., 2000) reported the generation of a transgenic line expressing Cre under the smooth muscle myosin heavy chain promoter (SMMHC-Cre). With the help of Rosa26R Cre reporter mice, they showed that their line expressed Cre

as early as E12.5 in aortic and airway SMCs. In later stages of embryonic development, expression progressively increased in smaller vessels and other SMC-containing tissues such as the intestine and heart. They reported that SMC-restricted expression persists in adult mice and includes blood vessels in skeletal muscle, the carotid arteries and numerous other organs such as the oesophagus, trachea and ureter.

Initial experiments were designed to reproduce the published data on the SMMHC-Cre line and in particular, the induction of Cre expression in SMC populations during embryonic development. The line was bred into the Rosa26R background and resulting embryos were assayed for β-galactosidase activity. The analysis at various embryonic stages ranging from E12.5 to E17.5 did not reproduce the reported staining patterns (data not shown). Consequently, it was concluded that this line could not be utilised to elucidate the role of ephrinB2 in vSMCs during embryonic development.

4.2.2 Generation of the PDGFRβ-LacZ and PDGFRβ-Cre line and determining their expression patterns

The difficulties with the SMMC-Cre line described above prompted the attempt to generate a new SMC-specific Cre line. Cre expression under the control of a PDGFRβ promoter fragment was decided upon because this receptor is a common marker for SMCs, pericytes and their progenitors (Hellstrom et al., 1999). To evaluate whether this genomic fragment that was 13Kb in size and covered exons 3 to 6 of the PDGFRβ gene, contained the necessary regulatory elements for expression in SMC populations, a PDGFRβ-LacZ reporter construct was generated. An internal ribosomal entry site (IRES) was fused to a LacZ cDNA (IRES-LacZ), followed by the SV40T polyadenylation signal and inserted into exon 4 of the PDGFRβ genomic fragment (Figure 4.1). When transient transgenic experiments by pronuclear injections into mouse embryos indicated that the PDGFRβ gene fragment permitted β-galactosidase expression in blood vessels (Figure 4.2), several founder lines were generated and tested in greater detail. Of the 12 lines tested, two exhibited staining correlating to vSMCs. The line with better expression, referred to as PDGFRβ-lacZ, showed high level of staining in blood vessels at E12 and E13.5 (Figure 4.2) as well as in later stages of embryonic development. At E18.5, vascular structures in various vascular beds,

including the mesenteric vessels of the intestine, blood vessels in meninges and skin, and the mesangial cells of the kidney glomeruli, were stained (Figure 4.3). Very little lacZ expression was visible outside the vascular system although PDGFRβ expression has been reported in the trachea and ureter (data not shown).

The analysis on the PDGFRβ-LacZ line validated the suitability of the PDGFRβ genomic fragment for SMC/pericyte specific expression. Subsequently, a PDGFRβ-Cre construct was generated by inserting an IRES-Cre cassette into the PDGFRβ genomic fragment as described before. A total of 13 transgenic founder lines were generated and tested in combination with the Rosa26R reporter line to assess Cre activity in embryos. Two functional lines were identified and the better of those, referred to as the PDGFRβ-Cre, was used for all subsequent experiments. Stainings of E11.5 and E13.5 PDGFRβ-Cre/Rosa26R double transgenic embryos revealed high β-galactosidase expression in a wide range of vessels, similar to what has been seen for PDGFRβ-LacZ mice (data not shown). Stained tissues and organs at E18.5 included the mesenteric vessels in the intestine, vascular beds in the skin, and blood vessels and glomerular mesangial cells in the kidney (Figure 4.4). Some visceral SMC populations were also stained, such as the smooth muscle lining the stomach and the trachea (Figure 4.4).

4.2.3 Cell-type specificity of PDGFRβ-Cre mice

The experiments described below were designed to determine whether PDGFRβ-Cre expression is restricted to mural cell populations and excluded from the neighbouring endothelium.

4.2.3.1 Staining of the aorta

Aortas isolated from E18.5 embryos derived from timed matings between PDGFRβ-Cre and Rosa26R mice, were assayed for β-gal activity. The stained tissue fragments were embedded in paraffin and sectioned. Cross-sections of the aorta revealed that the SMC layer was strongly lacZ positive while the monolayer of endothelial cells lining the lumen were devoid of blue staining (Figure 4.5a).

Antibody stainings on aortic sections from an eight-week-old mouse that carried PDGFRβ-Cre and Rosa26R alleles showed that α-Smooth Muscle Actin (α-SMA) antibody and β-galactosidase antibody were overlapping (Figure 4.5b). The vast majority of α-SMA cells express Cre recombinase.

4.2.3.2 RT-PCR analysis of isolated Cre-positive cells from skin

The gene expression profile of Cre-expressing cells from the PDGFRβ-Cre mice were investigated by breeding to the RosaEYFP reporter line. This reporter leads to expression of Yellow Fluorescent Protein (YFP) as a result of Cre-mediated recombination (Srinivas et al., 2001). The RosaEYFP reporter enables easy visual monitoring of Cre activity without the need to stain. Embryos at E18.5 that contained both the PDGFRβ-Cre and Rosa26EYFP alleles were identified under the ultraviolet light of the microscope by the yellow fluorescence in the skin. Fluorescent cells from the skin were isolated by Fluorescence-Activated Cell Sorting (FACS) and total RNA was extracted for the synthesis of cDNA and analysis by PCR. The RT-PCR indicated the presence of gene transcripts for the mural markers desmin and PDGFRβ (Figure 4,5c). While the endothelial marker PECAM-1 was absent from these cells, the receptors EphB2, EphB3 and EphB4 were also detected.

4.2.3.3 Genotyping on isolated endothelial cells from skin

Another approach used to determine whether the PDGFRβ-Cre allele led to Cre expression in the endothelium was to look for evidence of recombination events in these cells. Aortas from eight-week-old adult PDGFRβ-Cre mice, in an ephrinB2$^{lox/+}$ heterozygous background, were isolated and cultured on collagen-coated dishes to induce sprouting. After 14 days, endothelial cells were isolated using Dynal beads coated with anti-PECAM-1 antibody and genotyped. This approach verified the presence of the intact loxP-flanked gene area and thereby the absence of Cre activity in the endothelium (Figure 4.5d).

4.3 Discussion

Previous studies have shown ephrinB2 expression in vSMC of embryonic and adult mice (Shin et al., 2001). To investigate the role of ephrinB2 in these cells, and circumvent the early lethality of global embryos, mutants were generated using the Cre/loxP approach. For Cre expression in mural cells, SMMHC-Cre mice (Regan et al., 2000) were characterised but this line showed only poor Cre expression in the embryo and was not suitable for further experiments. More recently, Frutkin and colleagues have reported similar problems with the SMMHC-Cre line, which in their hands, induced recombination in the parental germline but not in vSMCs (Frutkin et al., 2004). Therefore a new vSMC-specific Cre transgenic line was generated, in which expression of the recombinase is controlled by a fragment of the PDGFRβ gene, which encodes a growth factor receptor found in SMCs and pericytes. With this strategy, several founders expressing Cre were generated and characterised by breeding to Rosa26R reporter mice. Specific staining in vSMCs at various embryonic stages and in adults indicated that the genomic PDGFRβ fragment used for the generation of the transgenic line contained the regulatory elements required for expression in the vascular system. This is further supported by the fact that the PDGFRβ-lacZ line displayed a similar expression pattern and argues against a major influence of the site at which the transgene was integrated into the genome.

As PDGFRβ has been described to be present in SMC and pericytes and not in the endothelium *in vivo*, it appeared likely that the staining of blood vessels represented expression in mural cells. However, this requires further verification because the PDGFRβ genomic fragment and the integration of the transgenic construct may have led to additional expression domains. Data presented here confirmed specific activity of the PDGFRβ-Cre in mural and not endothelial cells as: a) only SMCs were positive in sections of stained aorta, b) immunofluorescent signals obtained with anti-β-galactosidase and anti-α-SMA antibodies were largely overlapping, c) RT-PCR on YFP-positive cells from skin contained transcripts for desmin and PDGFRβ but not PECAM-1, and d) endothelial cells isolated from PDGFRβ-Cre ephrinB2$^{lox/+}$ double heterozygotes contained an intact loxP-flanked allele. Although each of these experiments may have its own limitations, it is evident that PDGFRβ-Cre expression in

the vascular system is restricted to mural cell populations and absent in the endothelium. Next, this line was used in combination with conditional ephrinB2 mice to address the role of the ligand in pericytes and vascular smooth muscle cells.

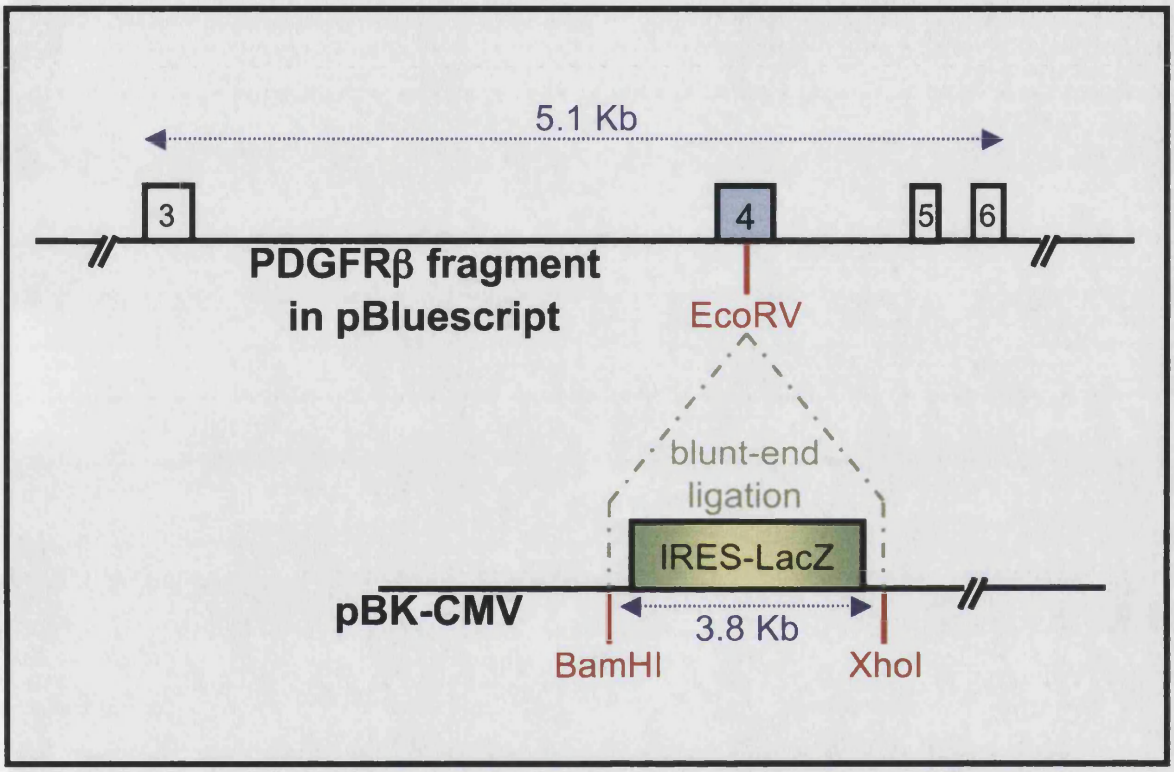


Figure 4.1: Generation of the PDGFRβ-LacZ construct

A schematic diagram showing the cloning of the PDGFRβ-LacZ construct. The IRES-LacZ cassette, also containing the SV40 pA signal at the 3' end, was inserted into exon 4 of the PDGFRβ gene fragment, which included exons 3 to 6, as well as 6.2kb of upstream and 2.2kb of downstream intronic sequence.

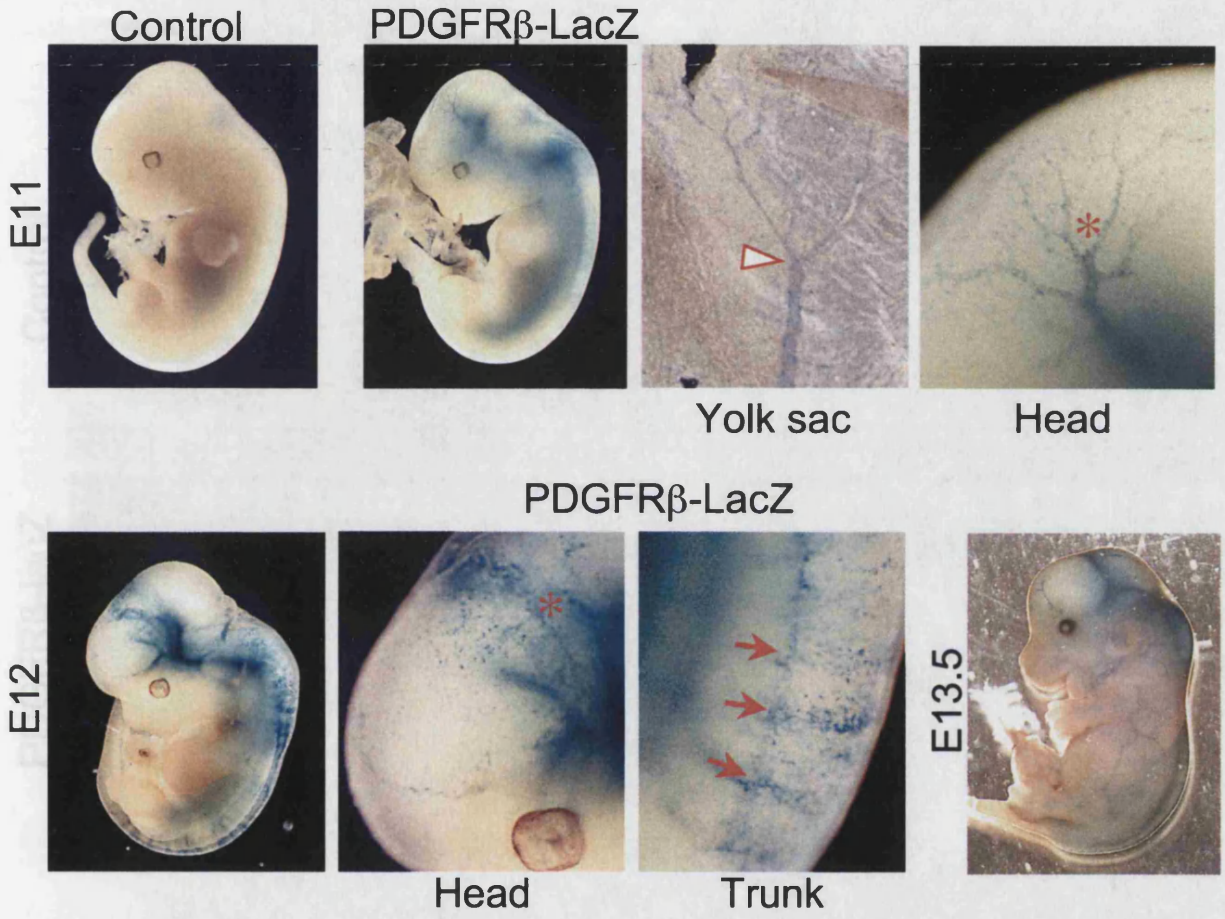


Figure 4.2: PDGFRβ-LacZ expression in embryos reveal staining in vascular structures

The embryo at E11 was from transient transgenic experiments while the ones at E12 and E13.5 were generated from the stable line. β-galactosidas activity was detected in a wide range of vascular beds, such as the large vitelline vessel in the yolk sac (arrowhead), the anterior cardinal vein (asterisks), and intersomitic vessels (arrows).

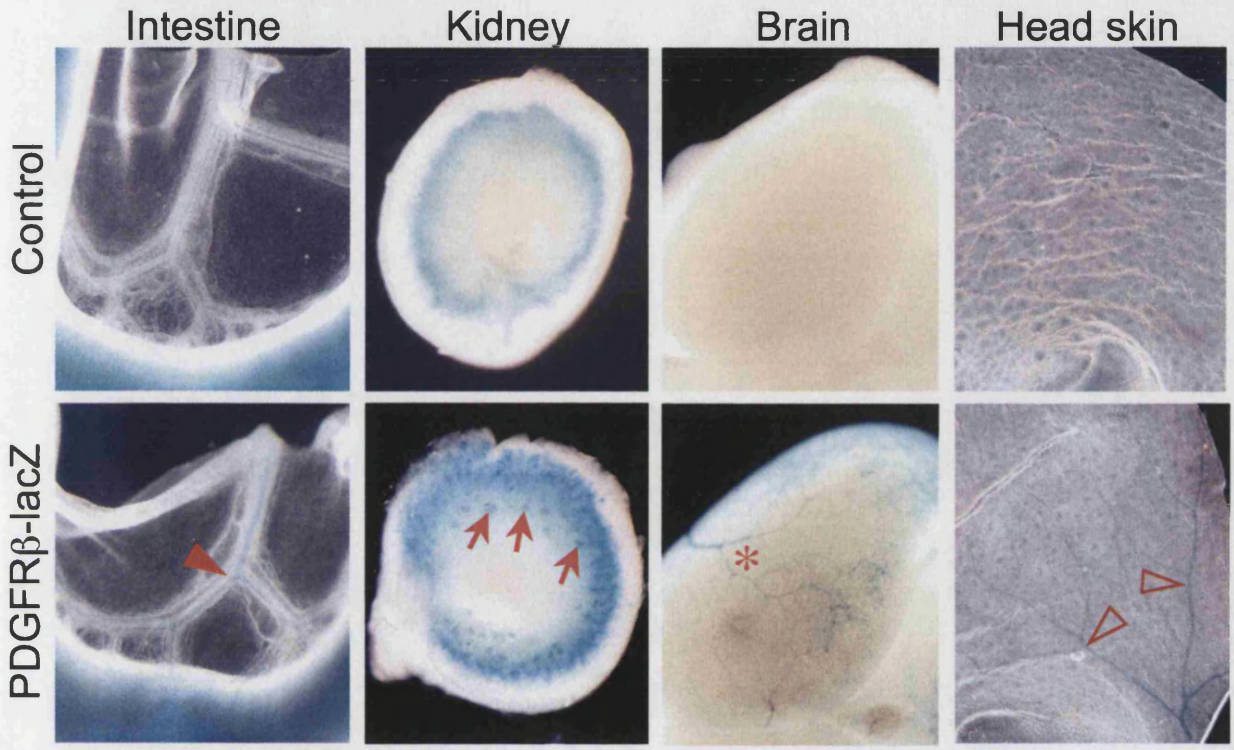


Figure 4.3: LacZ staining of blood vessel structures in different tissues/organs of PDGFRβ-LacZ embryos at E18.5

β-galactosidase activity was evident in multiple tissues and organs of PDGFRβ-LacZ embryos at E18.5. These included the mesenteric vessels leading into the intestine (arrowhead), glomeruli of the kidney (arrows), the vascular network in the meninges (asterisk) and vessel in the skin (open arrowheads). Specific staining was absent in the control littermates.

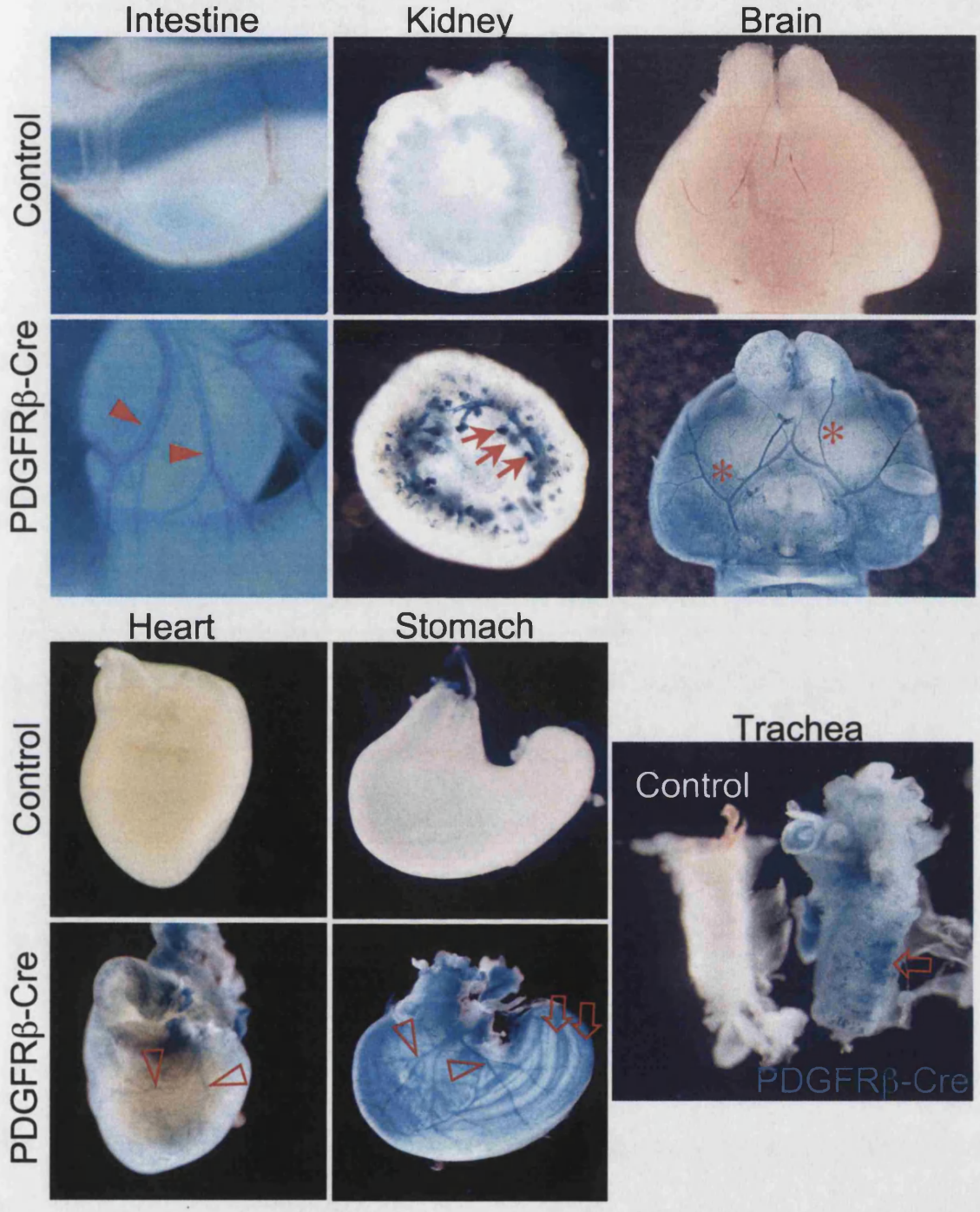


Figure 4.4: LacZ staining of different tissues and organs from E18.5 PDGFRβ-Cre and Rosa26R double transgenic embryos

Cre activity was detected in the vasculature of multiple tissues and organs including the mesenteric vessels leading into the intestine (arrowheads), kidney glomeruli (arrows), the vascular network in the meninges (asterisks), and the vasculature of the heart and stomach (open arrowheads). Visceral SMCs lining the stomach and trachea were also positive (open arrows). No staining was observed in the control tissues.

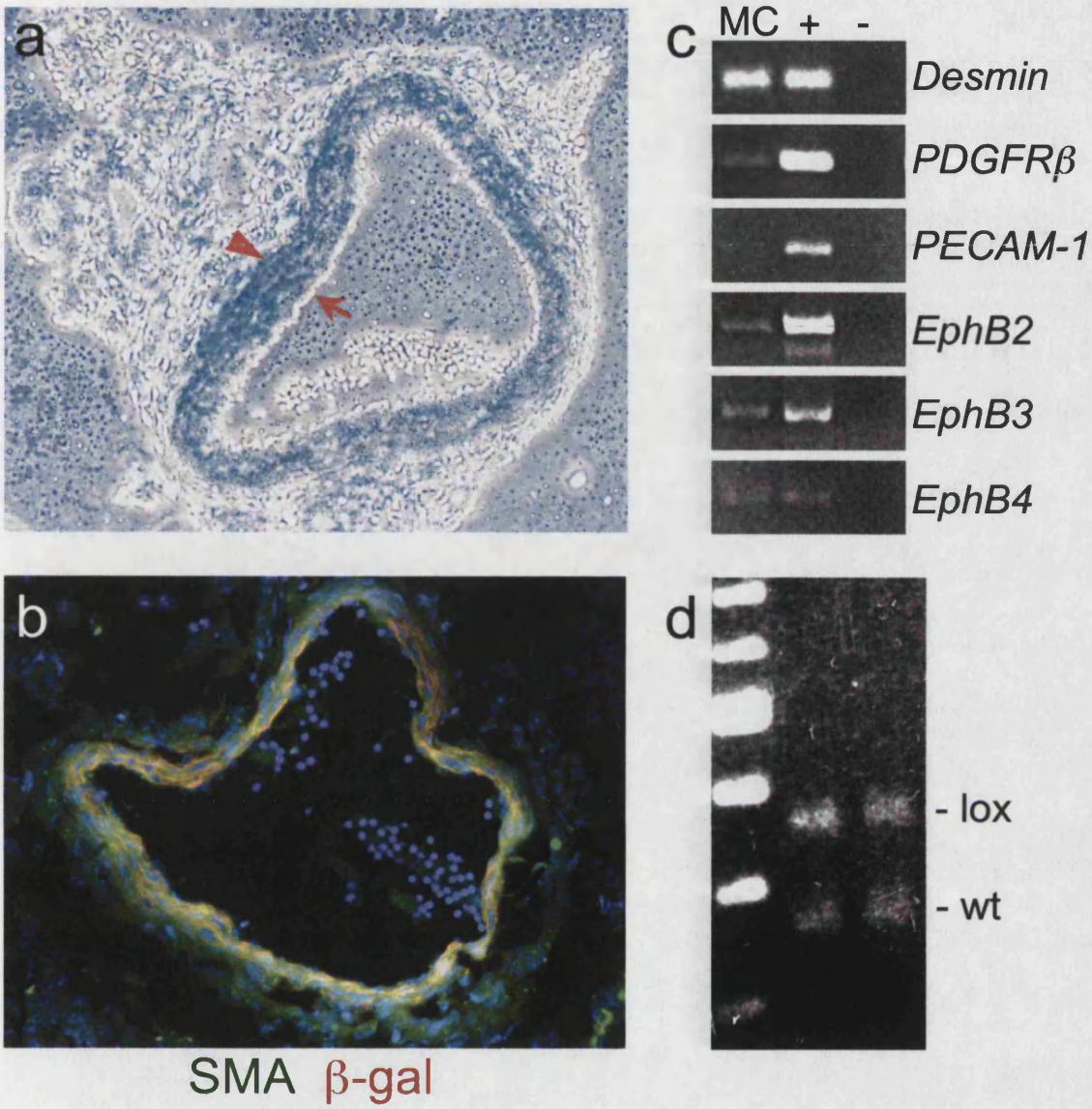


Figure 4.5: PDGFRβ-Cre transgenic express Cre selectively in mural cells Staining of cross-sections through the aorta of PDGFRβ-Cre mice in the Rosa26R background. β-galactosidase staining (a) was restricted to SMC layers (arrowhead) and absent from the endothelium lining the lumen (arrow). Double fluorescent labelling using α-SMA and β-galactosidase antibodies (b) reveal that lacZ expressing cells were also α-SMA positive. RT-PCR of Cre-positive cells from E18.5 skin (MC) showing desmin and PDGFRβ transcripts and absence of PECAM-1 mRNA (c). The isolated cells were also positive for the receptors EphB2, EphB3 and EphB4. Positive control (+) reactions were performed on whole embryo cDNA. PCR on genomic DNA from two independent samples of endothelial cells isolated from aortic sprouts, revealed the presence of the intact ephrinB2^{lox} allele (lox), i.e. the absence of Cre-mediated gene deletion (d), as well as the wildtype allele (wt).

Chapter5 | Investigating the role of ephrinB2 in mural cells

5.1 Introduction

The results presented in the previous chapter and work by Gerety *et al.* (Gerety and Anderson, 2002) have shown that expression of ephrinB2 in endothelial cells is essential for normal blood vessel morphogenesis and angiogenic remodelling. In addition to the endothelium, mural cells in the embryonic and adult organism are another site of ephrinB2 expression at later stages of embryogenesis that persists into adulthood. Generation of PDGFRβ-Cre transgenics allowed the function of ephrinB2 in these cells to be addressed.

5.2 Results

5.2.1 Deletion of ephrinB2 in mural cells

Breeding of the PDGFβ-Cre mice into the ephrinB2 conditional background led to the generation of ephrinB2$^{\Delta MC}$ mutants, that is, mice lacking the ligand in pericytes and vSMCs. These mutants developed to term but died shortly after birth, probably due to respiratory failure. EphrinB2$^{\Delta MC}$ mutants exhibited oedema in the back region and widespread superficial haemorrhaging in the skin, which was particularly evident in the limb, head and dorsal area (Figure 5.1a-c). This phenotype is reminiscent of the appearance of PDGFRβ (Figure 5.1d,e) and PDGF-B knockouts, but perhaps less severe. The excessive haemorrhaging in the ephrinB2$^{\Delta MC}$ skin was detected as early as E15.5 (data not shown).

Analysis of haematoxylin and eosin stained skin sections showed blood-filled vascular structures near the mutant epidermis (Figure 5.2a,b). Immunohistochemistry with the

anti-PECAM-1 antibody, a marker for endothelial cells, revealed that capillaries in the ephrinB2$^{\Delta MC}$ skin were dilated (Figure 5.2c,d).

EphrinB2$^{\Delta MC}$ mutants display additional defects in the vascular beds of other organs. Freshly dissected intestines were pale and mesenteric lymphatic vessels were filled with erythrocytes, indicative of haemorrhaging (Figure 5.3a,b). Furthermore, sections revealed the presence of blood-filled lymphatics in the intestinal wall (Figure 5.3c,d). In mutant kidneys, "comma" and "S" shaped stages of the developing nephrons were normal. However, the appearance of more mature glomeruli was morphologically abnormal as indicated by dilated capillary tufts (Figure 5.3e,f). Alveoli in lungs were filled with blood cells (Figure 5.3g,h), which may explain the lethality of newborns.

5.2.2 Determining the expression of ephrinB2 and its receptor in the vasculature

Immunohistochemical staining of skin sections with anti-ephrinB2 antibody confirmed the absence of the ligand in arterial smooth muscle cells of large vessels in ephrinB2$^{\Delta MC}$ mutants. Unexpectedly, ephrinB2 protein was undetectable in the adjacent arterial endothelial cells (Figure5.4a,b). Stainings with anti-EphB4 antibody revealed the venous endothelium in both mutants and controls (Figure 5.4c,d). Since the previous characterisation of the PDGFRβ-Cre line showed no indication of Cre activity in endothelial cells, it is plausible that the inactivation of the gene in mural cells leads to the downregulated expression of ephrinB2 in the endothelium.

As shown in Figure 4.5c, several receptors for ephrinB2 are expressed in isolated mural cells. The spatial expression pattern of these molecules in the skin vasculature was characterised by antibody staining to identify potential sites of ephrinB2-EphB receptor interaction and signalling. EphrinB2 protein labelled the endothelium and the SMC layer of the large arterioles (Figure 5.5a). EphrinB2 levels were below the detection limit in smaller arterioles and in capillaries (Figure 5.5b,c). EphB4 signal was present in endothelial cells of both larger and smaller venules but absent in capillaries (Figure 5.5d-f). Antibodies against EphB2 and EphB3 stained the mesenchyme surrounding both larger arterioles and venules as well as some vSMC (Figure5.5g,j). Both EphB2

and EphB3 were absent in SMC-coated vessels of smaller calibre and in capillary beds (Figure 5.5h,i,k,l).

Although microvessels in the skin are very sensitive to the effects of ephrinB2 deletion, immunohistochemistry failed to show expression of ephrinB2 or its receptors in the small calibre vasculature. Whole-mount lacZ staining was performed on various organs of E18.5 ephrinB2taulacZ embryos (Shin et al., 2001) to visualise the pattern of ephrinB2 promoter activity. This approach showed weak but specific staining in smaller vessels and capillary plexi of the meninges, skin and intestine, revealing the presence of ephrinB2 in these structures (Figure 5.6a-d). However, lacZ expression increased with vessel diameter and was most prominent on larger arterioles and arteries.

5.2.3 Normal patterning of the mutant vascular network

Inactivation of the ephrinB2 gene in the mural cells resulted in downregulated expression in the endothelium. Because endothelial ephrinB2 is required for angiogenesis in the early embryo, whether this reduction impairs the vascular architecture was investigated. EphrinB2$^{\Delta MC}$ mutants were bred into the Tie2lacZ background, a reporter expressing β-galactosidase in endothelial cells (Schlaeger et al., 1997). Surprisingly, staining of the vascular network in the mesenteric vessels and skin of mutants at E18.5, revealed no appreciable differences to controls (Figure 5.7a-d).

5.2.4 Normal mural cell numbers and distribution pattern in the larger vessel of mutants

To investigate whether the disruption of vascular integrity and haemorrhaging in the ephrinB2$^{\Delta MC}$ mutants was caused by insufficient mural cell coverage, as is the case in mutant mice with a disrupted PDGFRβ pathway, the line was bred into the X-lacZ background. This reporter line expresses lacZ in pericytes (Tidhar et al., 2001). As β-galactosidase staining in these mice is confined to the nucleus, stained cells can be readily quantified. Analysis of different tissues including skin, mesentery, and the meninges showed no obvious differences between ephrinB2$^{\Delta MC}$ mutants and littermate

controls (Figure 5.8a-j). Quantification of stained cells in the vasculature in the meninges and skin indicated that the loss of the ephrinB2 gene has no impact on the number of X-lacZ positive cells in these tissues (Figure 5.9).

5.2.5 EphrinB2 is required for normal microvessel architecture

The data presented above shows that ephrinB2 ablation in mural cells does not affect their number and did not provide an explanation for the vascular defects in ephrinB2$^{\Delta MC}$ mutants. Therefore, the microvessel architecture was investigated by whole-mount double immunofluorescence on skin. At the lower magnification (10x), staining with antibodies detecting PECAM-1 and α-SMA showed no obvious difference in the number of mutant vSMCs. Prominent α-SMA staining covered large arteries and veins of ephrinB2$^{\Delta MC}$ mutants (Figure 5.10a,b). This was in strong contrast to the situation in PDGFRβ knockouts, in which vSMC coverage was sparse on large vessels and absent in the microvascular network (Figure 5.10c). The analysis of ephrinB2$^{\Delta MC}$ microvessels at the high magnification (63x) revealed that the spatial distribution of vSMCs was discontinuous and incomplete (Figure 5.10d,e). Moreover, many α-SMA-positive cells were rounded and poorly associated with the endothelium (Figure 5.10f,g). Double staining for PECAM-1 and the pericyte marker, desmin, showed that pericytes in control embryos were tightly wrapped around the abluminal surface of endothelial cells. In contrast, ephrinB2-deficient pericytes were poorly associated with the endothelium (Figure 5.11a-d) and appeared to protrude cellular processes away from vessels (Figure 5.11d,e). Other pericytes displayed an abnormally round morphology and failed to envelope the capillaries (Figure 5.11f,g).

These findings were also confirmed by results at the ultrastructural level. Electron micrographs of skin microvessels confirmed that mutant pericytes made, were poorly associated with the endothelium (Figure 5.12a-d). An excessive number of collagen fibrils were seen in the extracellular matrix surrounding the mutant vessels and pericytes. Microvessels were dilated and some released erythrocytes due to ruptures in the endothelium (Figure 5.12e).

5.2.6 Abnormal smooth muscle recruitment to ephrinB2$^{\Delta MC}$ lymphatic capillaries

Anti-PECAM-1 immunofluorescence allowed the visualisation of endothelial cells of lymphatics and blood vessels. Both vessel types can be distinguished in embryonic skin as the former have a significantly larger lumen. Lymphatic capillaries are normally devoid of smooth muscle cells whereas the lymphatic collecting ducts are covered to facilitate the transport of excess interstitial fluids and extravasated molecules into the venous circulation. Unexpectedly, lymphatic capillaries in ephrinB2$^{\Delta MC}$ skin displayed abnormal recruitment of SMCs (Figure 5.13a-d). It was also observed that a subset of mutant vSMCs appeared to bridge blood vessels and lymphatics suggesting that they may move between vessels of a different type (Figure5.13e,f). Double staining with α-SMA and the lymphatic marker LYVE-1 verified that lymphatics had abnormal vSMC coverage (Figure 5.14a). These observations were confirmed by stainings of skin sections (Figure 5.14b,c). Due to the macroscopic similarities between PDGFRβ knockout (PDGFRβ-KO) and ephrinB2$^{\Delta MC}$ mice, PDGFRβ-KO skin samples were analysed by double stainings with α-SMA and LYVE-1 antibodies. This demonstrated that the inactivation of the PDGFRβ gene also resulted in ectopic SMC recruitment to lymphatics (Figure 5.14d,e).

5.2.7 Isolation and immortalisation of vSMCs for *in vitro* studies

To study the effects of ephrinB2 inactivation in cultured cells, mouse smooth muscle cells were isolated and immortalised. This was achieved with the help of tsA58 in "immorto" mice, in which the expression of a temperature-sensitive version of the SV40 T-antigen can be induced by γ-interferon. SMCs from cultured adult aorta fragments were isolated and immortalised and the identity of these cells was confirmed by α-SMA immunofluorescence (Figure 5.15a).

Lysates from immortalised SMCs were selected for glycoproteins with wheat germ agglutinin and the resultant solutes were loaded on SDS acrylamide gels for western

blotting. The results confirmed the presence of ephrinB2 and its receptors EphB2, EphB3 and EphB4 in the cultured cells (Figure 5.15b,c).

5.2.8 Sprouting from ephrinB2$^{\Delta MC}$ mice explants

To investigate whether the ablation of ephrinB2 in mural cells affects SMC sprouting and migration in response to PDGF-B, sprouting assays were performed. Aorta pieces were isolated from ephrinB2$^{\Delta MC}$ mutants and cultured on collagen I-coated plates in medium supplemented with 20ng/ml of PDGF-B. Photographs taken after 48 hours showed that both control and mutant pieces cultured in the absence of PDGF-B displayed no SMC sprouting (Figure 5.16a and data not shown). Sprouting was only observed in a subset of mutant and control aorta fragments (Figure 5.16b,c). Subsequent analysis on the number of explants with sprouts revealed that ephrinB2$^{\Delta MC}$ had lower success rates (15.4%) than controls (54.5%)(Figure 5.16d). This suggests that the loss of ephrinB2 may affect cell migration and sprouting.

5.3 Discussions

The generation of a mural cell-specific Cre transgenic line permitted the investigation of the role of ephrinB2 in pericytes and vSMCs. EphrinB2$^{\Delta MC}$ mutants were generated by breeding the PDGFRβ-Cre line into the ephrinB2 conditional background. These mutants survived to term but died within minutes after birth. Embryos at E18.5 displayed oedema and widespread haemorrhaging in the skin that macroscopically resembled knockout mice lacking PDGF-B or PDGFRβ. Further similarities with these mutants were locally dilated microvessels (microaneurysm) in the skin and the accumulation of blood in the lymphatic vessels of the mesentery. In ephrinB2$^{\Delta MC}$ mice kidneys, glomeruli with abnormally dilated capillary tufts were observed. Similar defects were seen in the glomeruli of PDGF-B and PDGFRβ knockouts due to the reduced numbers of mesangial cells (Leveen et al., 1994; Soriano, 1994; Lindahl et al., 1998; Hellstrom et al., 1999). However, unlike PDGF-B and PDGFRβ knockouts, ephrinB2$^{\Delta MC}$ mutants have normal numbers of pericytes and vSMCs in the affected tissues. More detailed analysis confirmed that ephrinB2-deficient mural cells are

impaired in their ability to associate with microvessels. Therefore, ephrinB2 does not play an obvious role in mural cell proliferation or survival but is a mediator of cell-cell interactions and adhesion.

Attachment and investment defects are also characteristic of microvessels in the PDGF-B retention mutants, which express a truncated form of PDGF-B that is unable to bind matrix and form surface gradients (Abramsson et al., 2003). The diffusion of PDGF-B alters its spatial presentation so that the signal cannot be properly interpreted by mural cells. Although PDGF-B retention mutants are hypomorphic and mural cells are reduced due to lower PDGF-B levels, it is plausible that ephrinB2 could be in some way required for the cellular response to PDGF-B. This would also be consistent with the ectopic SMC coverage on skin lymphatics in both ephrinB2$^{\Delta MC}$ and PDGFRβ-KO mice. PDGFRβ signalling, in response to PDGF-B secreted by blood vessels, may prevent the ability of SMCs to colonise lymphatics. The ephrinB2$^{\Delta MC}$ phenotype may well indicate a role of Eph-ephrin interactions in this process.

Based on the expression analysis described above, ephrinB2 is most likely to engage EphB receptors on vessels of large diameter, i.e. those vessels that have the most extensive mural cell coverage. As ephrinB2 and EphB levels decrease with vessel diameter, one would predict only low, yet potentially important, interactions within the microvasculature. The mutant phenotype suggests that such interactions would presumably enhance cell-cell contacts and attachment. Alternatively, gradients of Eph/ephrin molecules along vessels may promote cell migration and ensure the correct spacing of mural cells. This scenario may involve repulsive sorting signals.

The ablation of ephrinB2 in mural cells also resulted in downregulated expression in arterial endothelial cells. The mechanism underlying these changes is unclear but may involve direct regulation by Eph-ephrin signalling. Alternatively, other signals, provided by secreted factors or matrix, may control the expression of Eph-ephrin molecules in the vasculature. Whatever the mechanism, it is remarkable that the reduction of endothelial ephrinB2 expression remains without consequences for angiogenic remodelling. This finding suggests that the function of ephrinB2 shifts from angiogenesis to mural cell recruitment during development.

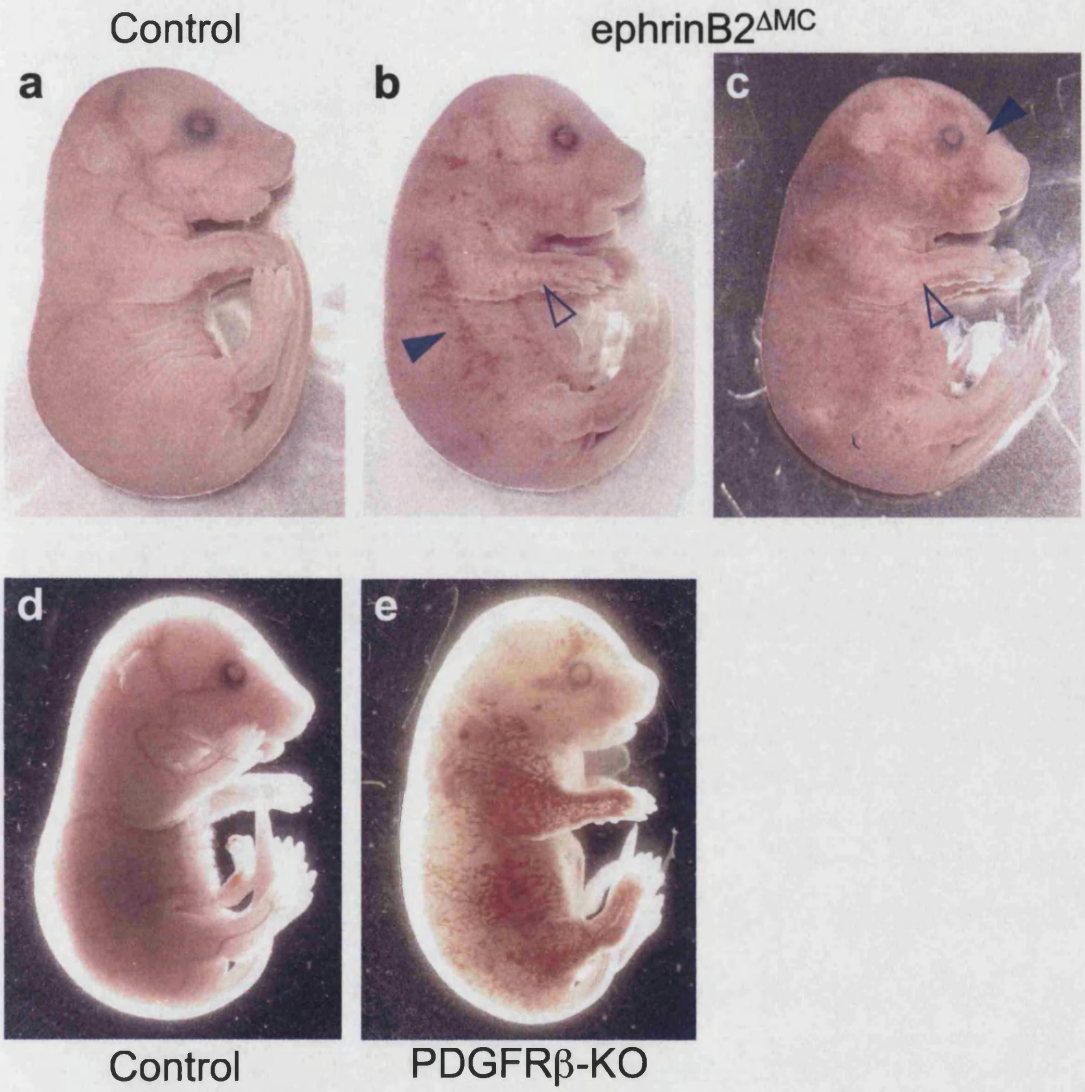


Figure 5.1: Vascular defects in ephrinB2$^{\Delta MC}$ mutants at E18.5

Freshly dissected E18.5 ephrinB2$^{\Delta MC}$ embryos from two different litters show severe haemorrhaging in skin of the limbs (b,c, open arrowheads), back (b, arrowhead) and head (c, arrowhead) which was absent in littermate controls (a). Haemorrhaging is also a prominent feature in PDGFRβ knockout embryos at the same stage (d,e).

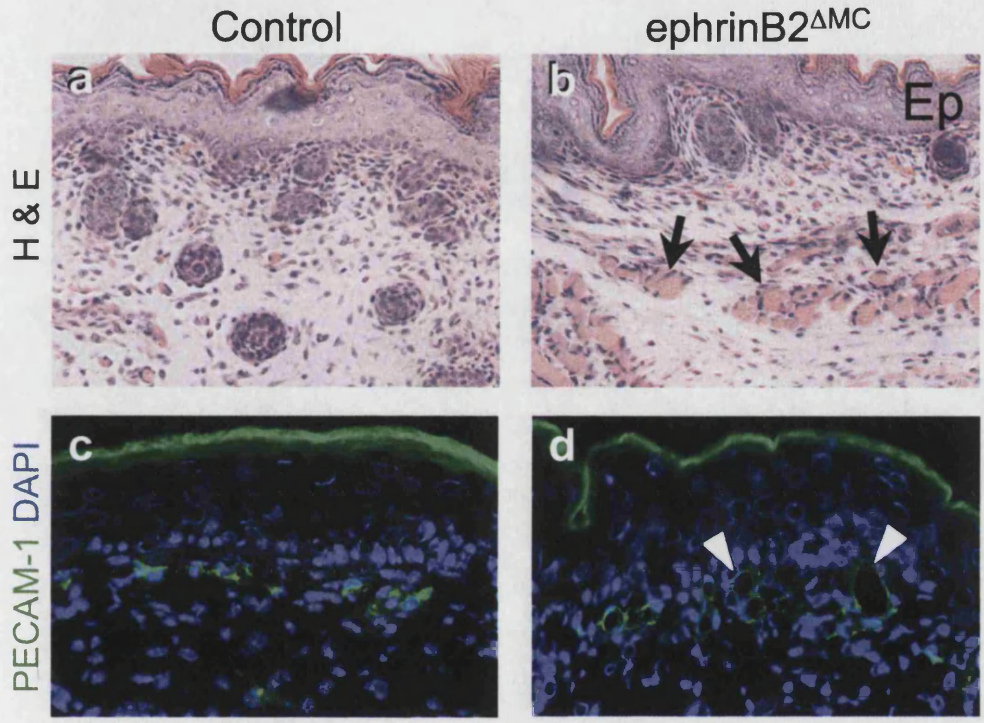


Figure 5.2: Ablation of ephrinB2 in mural cells leads to microaneurysms in the skin

Haematoxylin and eosin (H & E) staining of ephrinB2$^{\Delta MC}$ skin sections at E18.5 showing that vascular structures near the epidermis (Ep) were severely dilated and filled with blood cells (b, arrows). Capillaries, which are labelled for the EC marker PECAM-1, are dilated in ephrinB2$^{\Delta MC}$ skin (d, arrowheads). The nuclei were counter-stained with 4,6-diamidino-2-phenylindole (DAPI). Control sections are shown in (a) and (c).

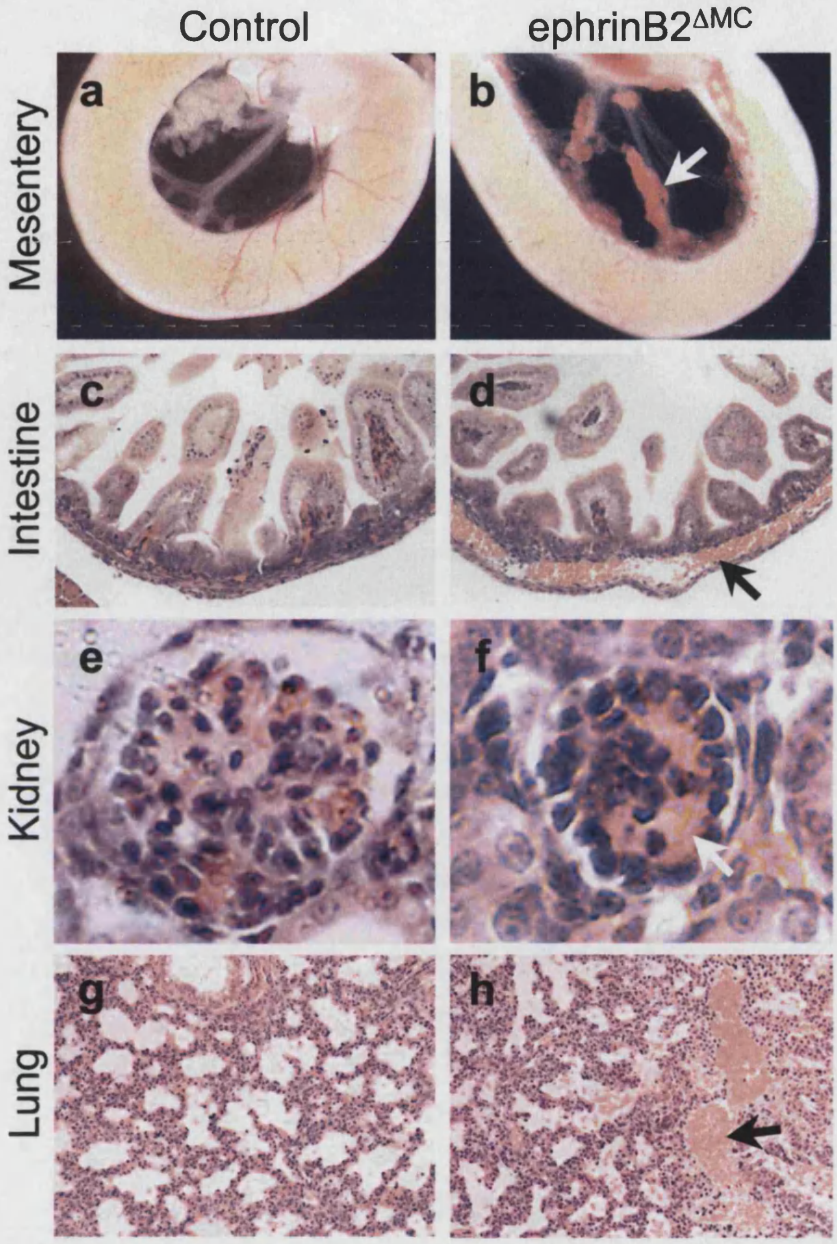


Figure 5.3: Vascular defects in ephrinB2$^{\Delta MC}$ mutants

Freshly dissected intestine of mutants were pale and the mesenteric lymphatics were filled with blood (b, arrow). H & E stainings of ephrinB2$^{\Delta MC}$ intestinal sections showed large blood-filled vascular structures (d, arrow). Mutant glomeruli displayed abnormal dilation of the capillary tuft (f, arrow). Blood occupied the alveolar spaces in the mutant lung. These defects were not seen in the littermates control samples (a, c, e and f).

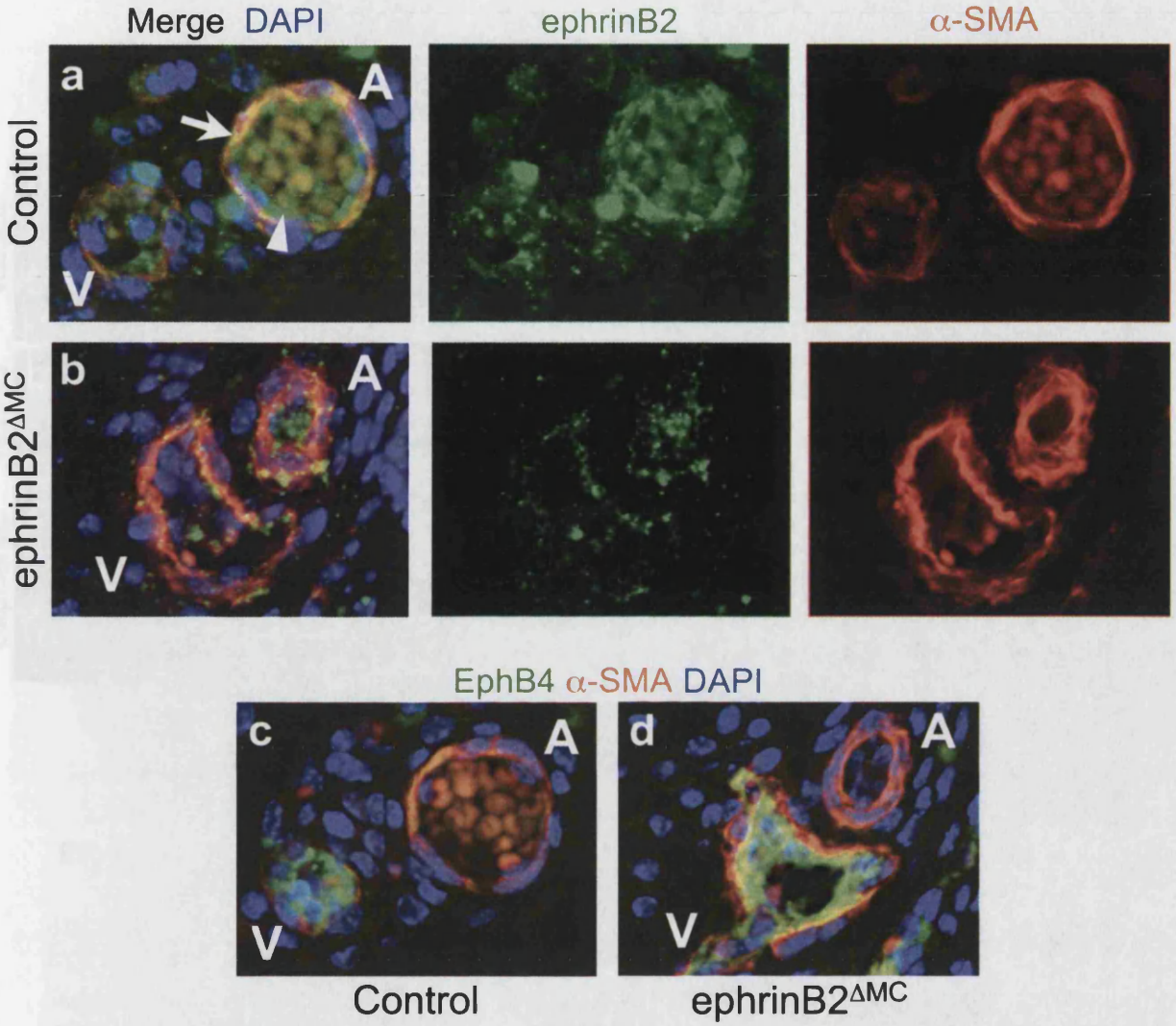


Figure 5.4: The deletion of ephrinB2 in mural cells causes the loss of the ligand in ECs

Double immunofluorescence with antibody against PECAM-1 and α-SMC on embryonic skin sections confirming the presence of ephrinB2 in both arterial SMCs (a, arrow) and ECs (a, arrowhead) of controls. In the ephrinB2$^{\Delta MC}$ mutants, ephrinB2 was absent in both cell types as the remaining green fluorescence is background signal (b). Green and red channels and merged images are shown. EphB4 was expressed in venous ECs of both controls and mutants (c,d). Nuclei staining (DAPI) is shown in blue.

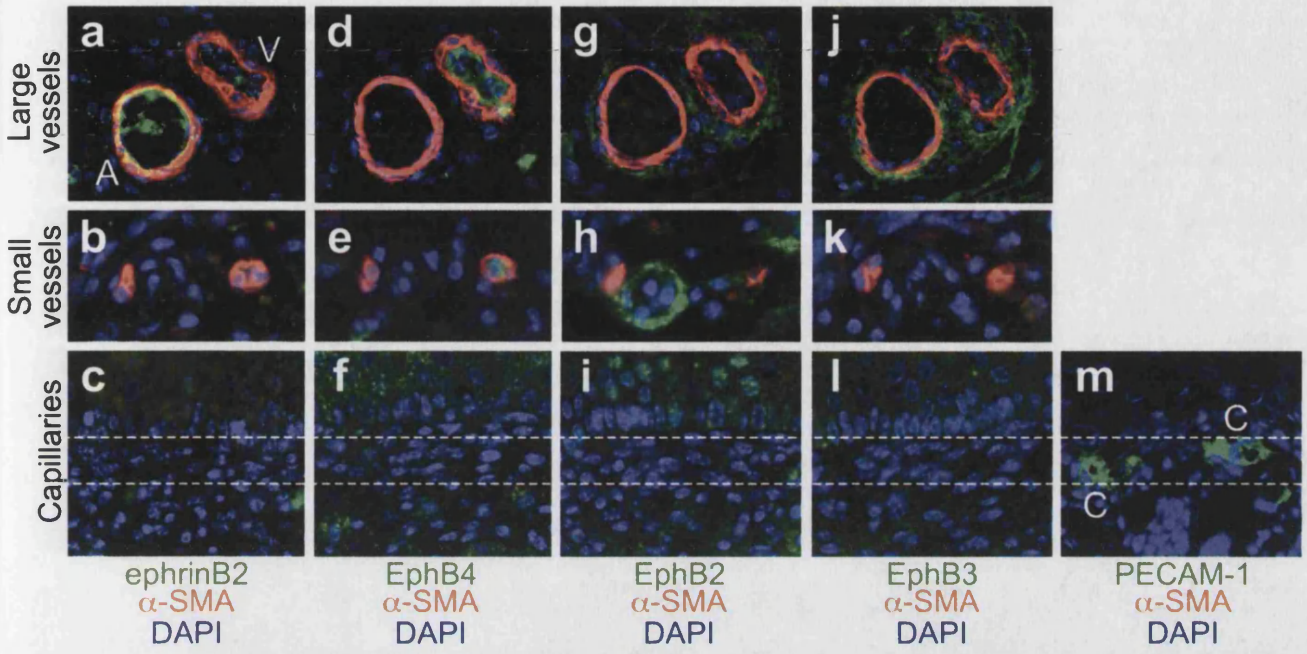


Figure 5.5: Expression of ephrinB2 and EphB receptors in blood vessels of the skin

Immunofluorescence with specific antibodies directed against ephrinB2, EphB4, EphB2, and EphB3 (green) in combination with α-SMA stainings (red) on consecutive sections from E18.5 skin. Arteries are denoted by (A) and veins by (V), while PECAM-1 (green) was used to stain capillaries (C) in (m). In larger vessels, ephrinB2 signal labels the endothelium and SMCs of the artery, EphB4 the venous endothelium, EphB2 and EphB3 the mesenchyme surrounding the vessels and a subset of the SMCs (a,d,g,j respectively). In the smaller vessels ephrinB2, EphB2 and EphB3 (b,h,k) are undetectable, while EphB4 is visible in the vein (e). Capillaries were negative for ephrinB2 and the three receptors (c,f,i,l). Nuclei are shown in blue (DAPI).

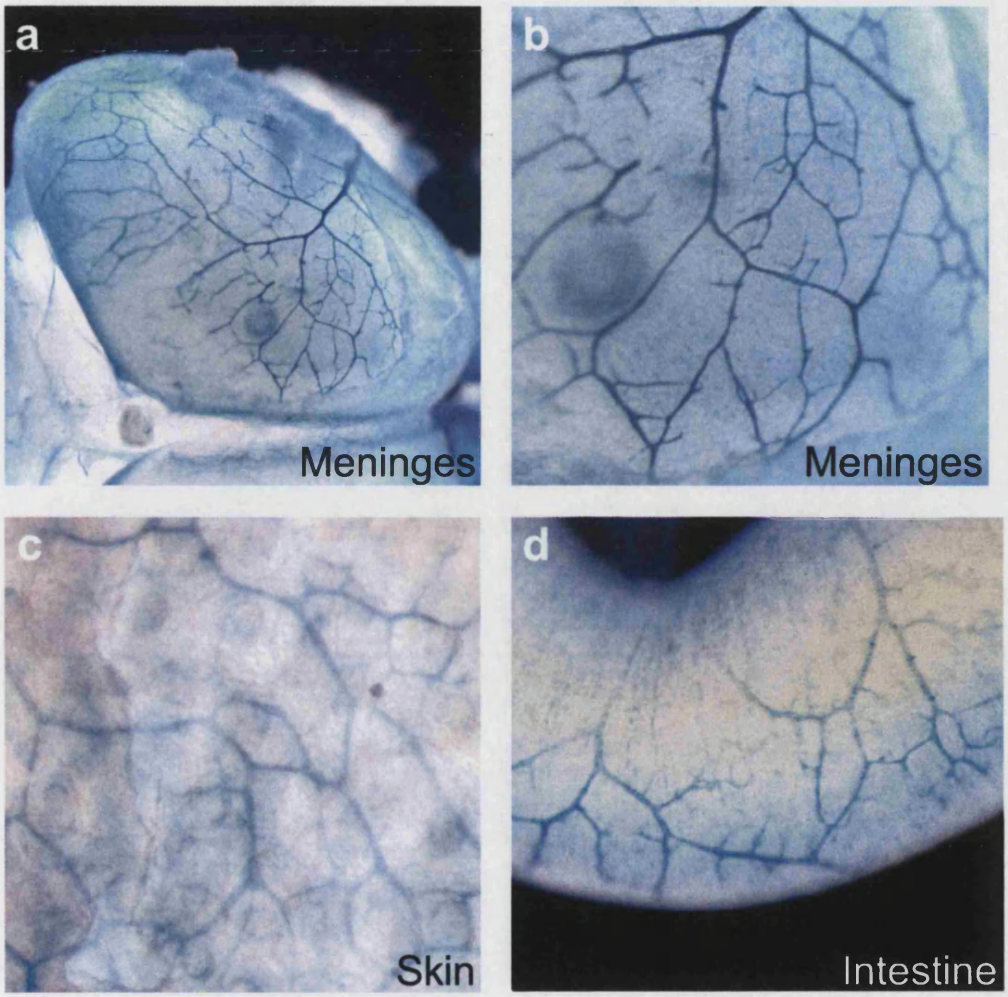


Figure 5.6: EphrinB2 expression in smaller vessels and capillaries
LacZ stainings of tissues from ephrinB2taulacZ heterozygous embryos at E18.5. β-galactosidase is most prominent in larger vessels but weaker signals extend into the microvessels.

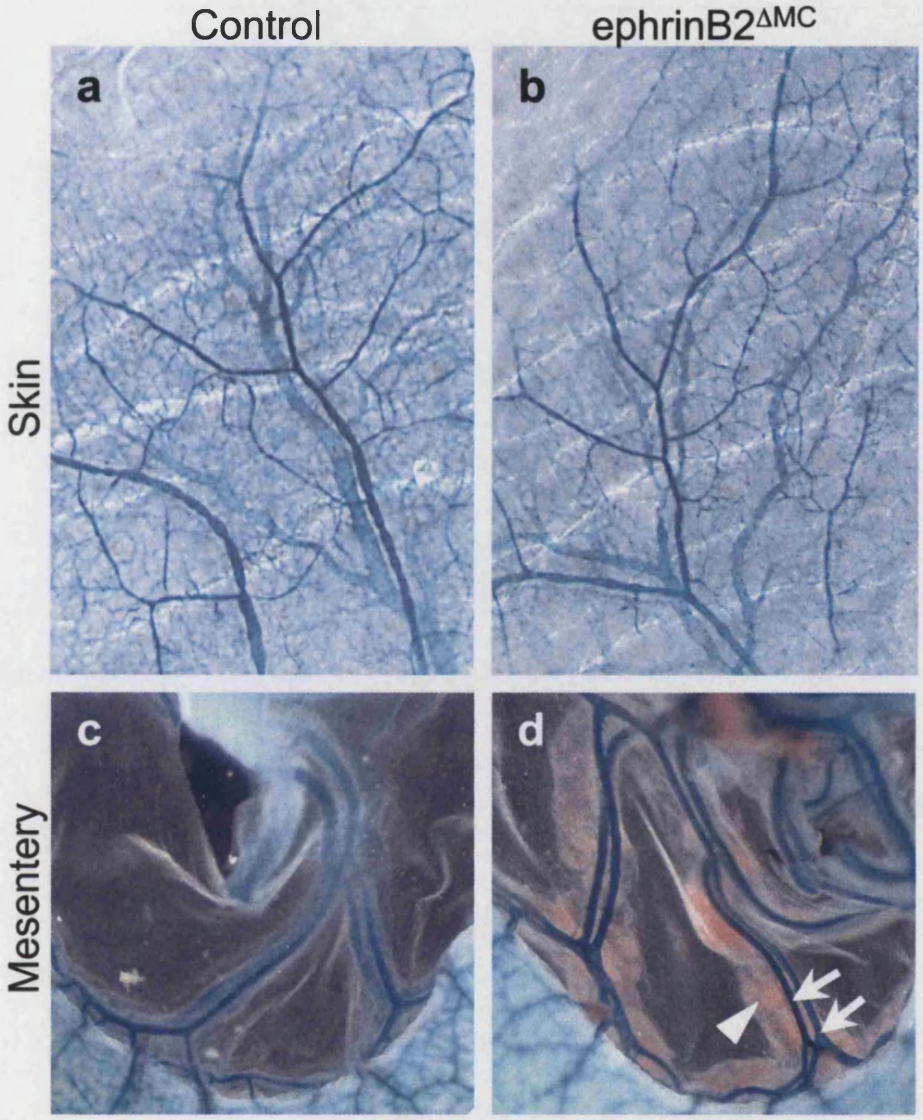


Figure 5.7: Normal patterning of the ephrinB2$^{\Delta MC}$ endothelium Freshly dissected tissues from E18.5 embryos in a Tie2lacZ background were assayed for β-galactosidase activity. Staining revealed no appreciable differences between mutant and control vasculature in skin (a,b) and mesentery (c,d). Mesenteric blood vessel are indicated by arrows and accumulated blood cells in mutant lymphatics by an arrowhead (d).

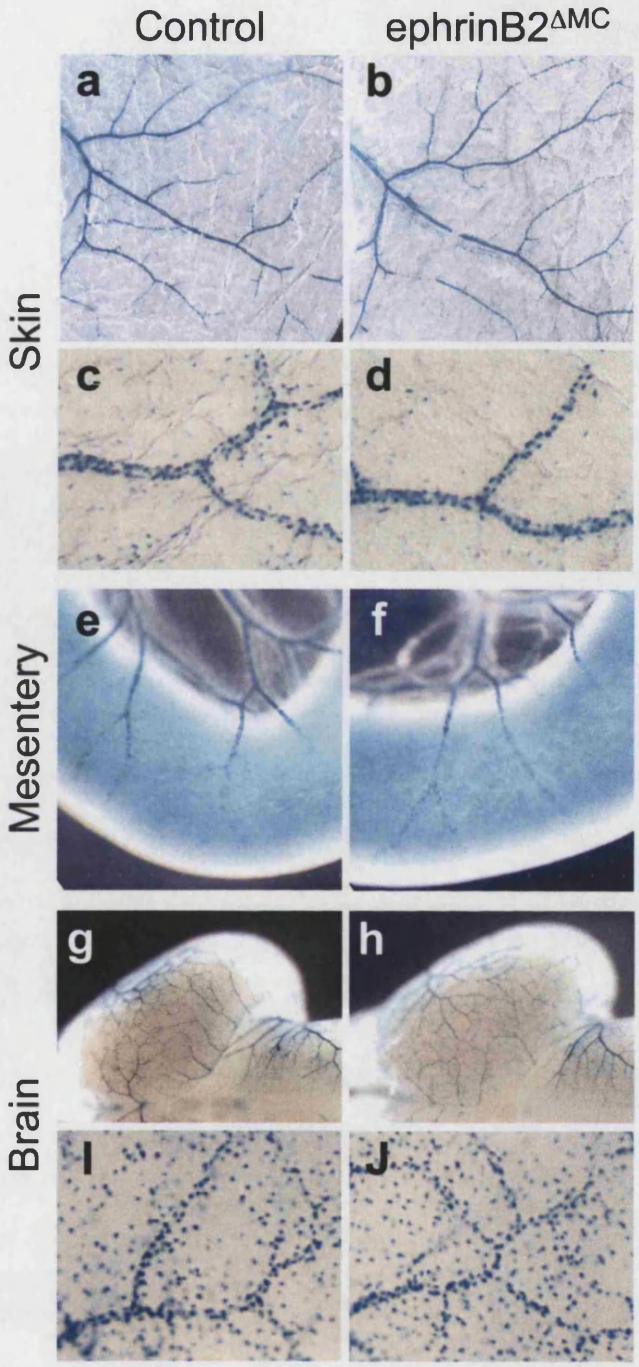


Figure 5.8: Normal mural cell numbers and in distribution pattern in the larger vessels of mutants

Freshly dissected tissues and organs from E18.5 embryos in a X-lacZ4 background were assayed for β-gal activity labelling pericytes. The number and spatial distribution of the X-lacZ4 positive mural cells was similar in the vascular beds of the skin of the skin (a-d), intestinal mesentery (e,f), and meninges (g-j).

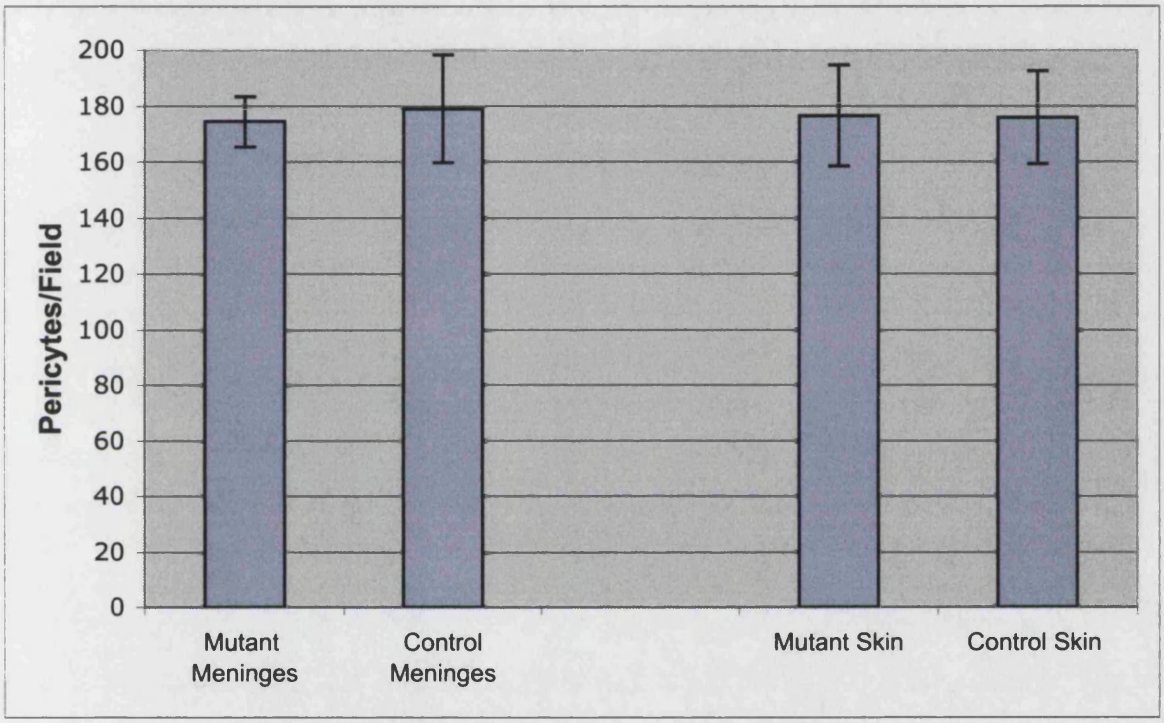


Figure 5.9: Quantification of mural cell numbers in mutants and controls

The number of mural cells were quantified in four fields of each sample type. The result revealed no significant differences between the mutant and control samples. Standard of deviations are indicated by error bars.

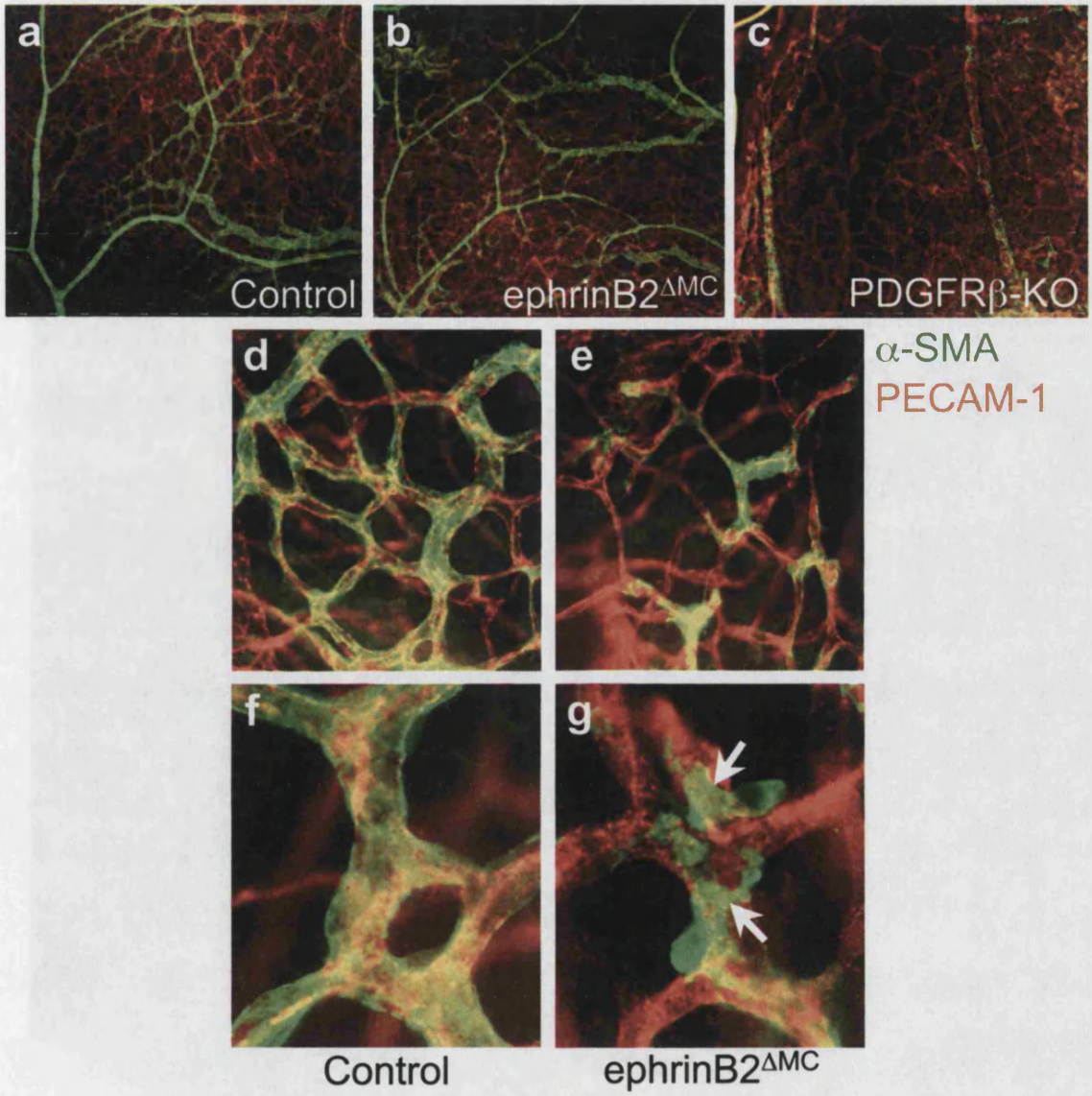


Figure 5.10: The ablation of ephrinB2 in mural cells results in abnormal microvessel architecture

Whole-mount immunofluorescence with antibodies against α-SMA (green) and PECAM-1 (red) on skin of E18.5 embryos. At low magnification, the overall organisation of the mutant vasculature appeared comparable to controls and large vessels had robust SMC coverage (a,b). In PDGFRβ-KO skin, vSMCs was sparse even on larger vessels (c). At high magnification, vSMC distribution in the mutant microvasculature appeared patchy and incomplete (d,e). Stained cells had abnormally round shapes and did not ensheath the endothelium (f,g, arrows).

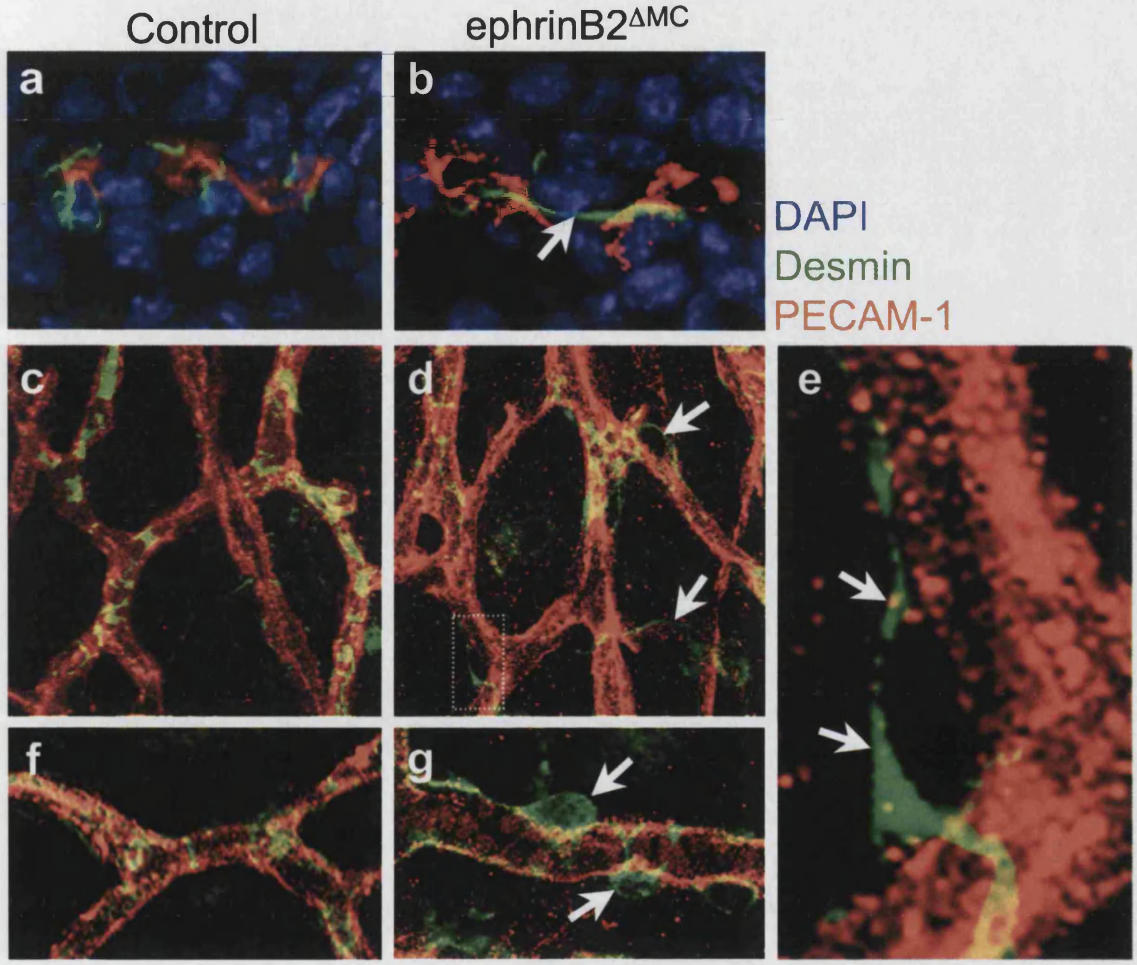


Figure 5.11: Pericytes failed to envelope the vessels and make proper associations with the endothelium in mutants Immunofluorescence on skin paraffin sections (a,b) and whole-mounts (c-g) with desmin (green) and PECAM-1 (red) antibodies. Images at 63x magnification revealed that mutant pericytes were loosely connected to the endothelium (a-d, arrows) and were stretching away from vessels (d,e, arrows). Other pericytes were abnormally round and failed to wrap around the PECAM-1 positive endothelium (f,g, arrows).

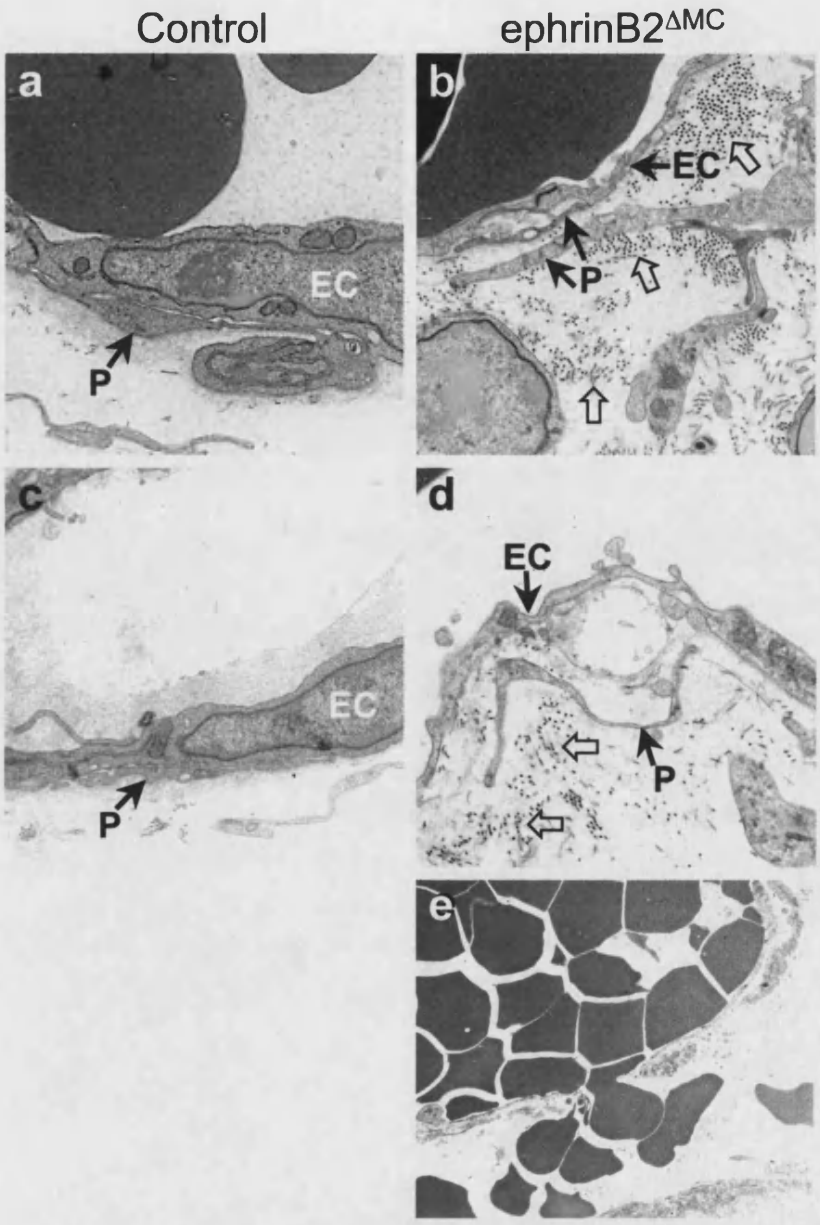


Figure 5.12: Mutant pericytes failed to form proper associations with the endothelium

Electron micrographs showing impaired interaction between pericytes (P) and endothelial cells (EC) of mutants (b,d) in contrast to controls (a,c). Note that the extracellular matrix surrounding ephrinB2$^{\Delta MC}$ vessels contains more collagen fibres (open arrows). Poor pericyte association leads to vessel dilation, rupture and the release of blood cells into the surrounding tissue (e).

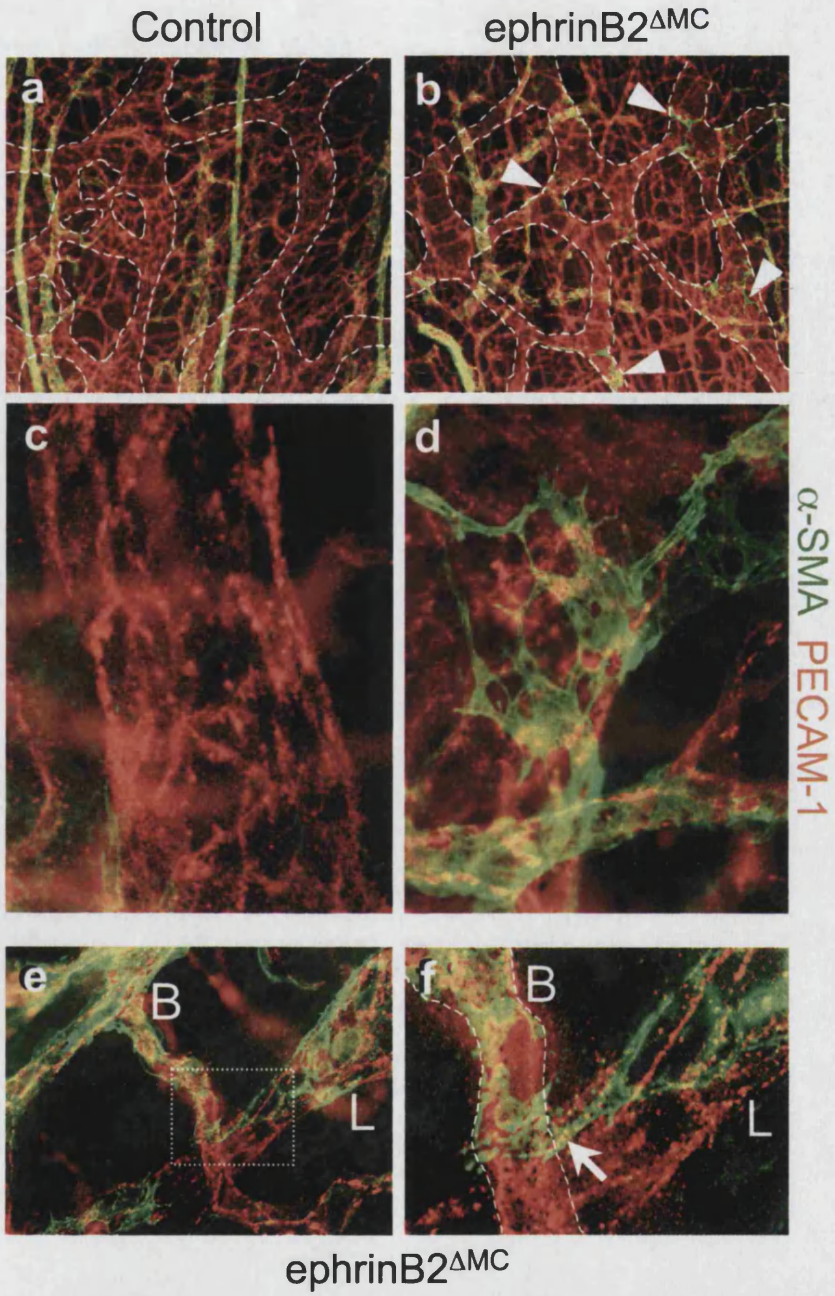


Figure 5.13: Abnormal SMC recruitment to capillary lymphatic vessels in ephrinB2$^{\Delta MC}$ mutants

Whole-mount immunofluorescent staining of E18.5 skin with α-SMA (green) and PECAM-1 (red) antibodies. Blood vessels are denoted by (B) and lymphatics by (L). Images taken at a magnification of 10x showed ectopic SMC coverage of lymphatic capillaries in mutants (b, arrowheads) but not in controls (a). Ectopic SMC coverage was extensive in some regions (c,d). Some α-SMA-positive cells seemed to cross from blood vessels to adjacent lymphatics (e,f, arrow).

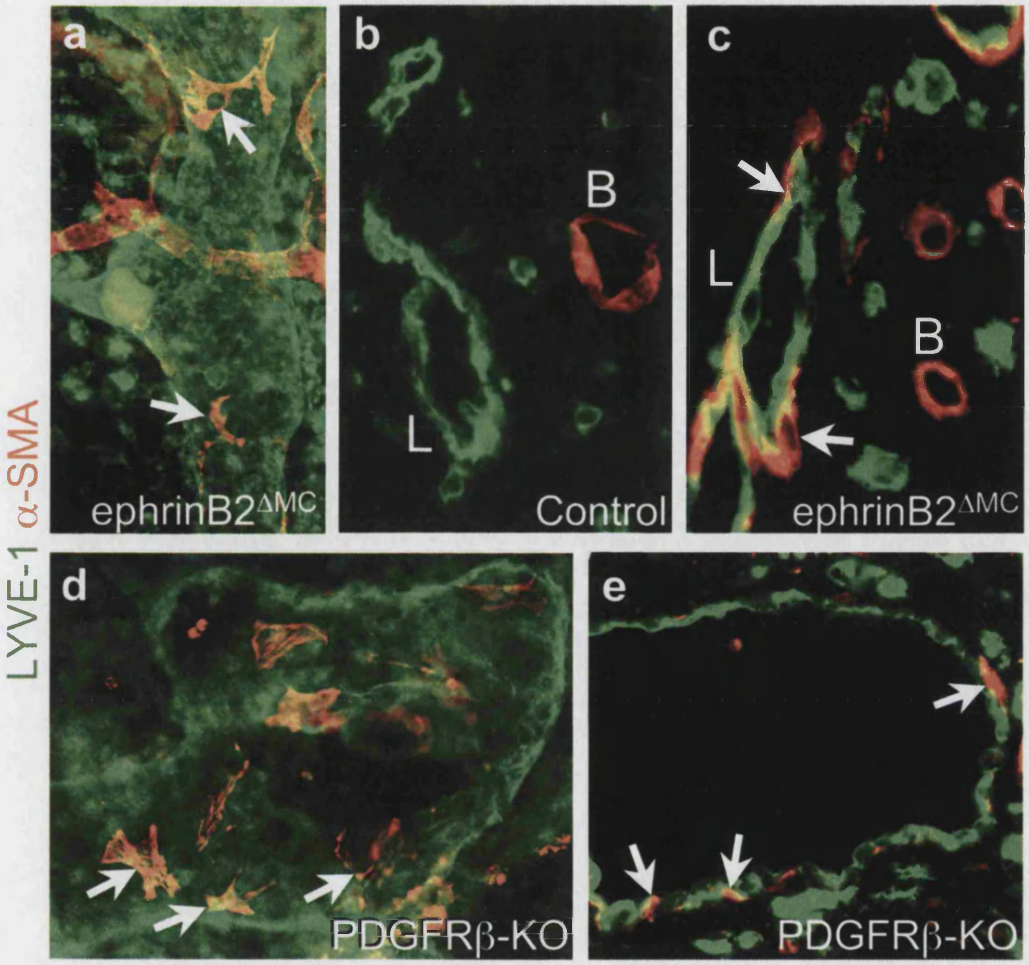


Figure 5.14: Abnormal SMC recruitment to lymphatic capillaries in ephrinB2$^{\Delta MC}$ mutants and PDGFRβ null mice

Immunofluorescence of E18.5 skin whole-mounts (a,d) and paraffin sections (b,c,e). Images were taken at 40x magnification. Co-stainings of LYVE-1 (green) and α-SMA (red) confirmed that SMCs covered lymphatic capillaries in the ephrinB2$^{\Delta MC}$ mutant (a, arrows). SMC recruitment to lymphatics (arrow) was also visible in skin sections (c, arrows) and absent in control (b). Similar ectopic SMC coverage of lymphatics (arrows) was evident in PDGFRβ-KO skin samples (d,e, arrows).

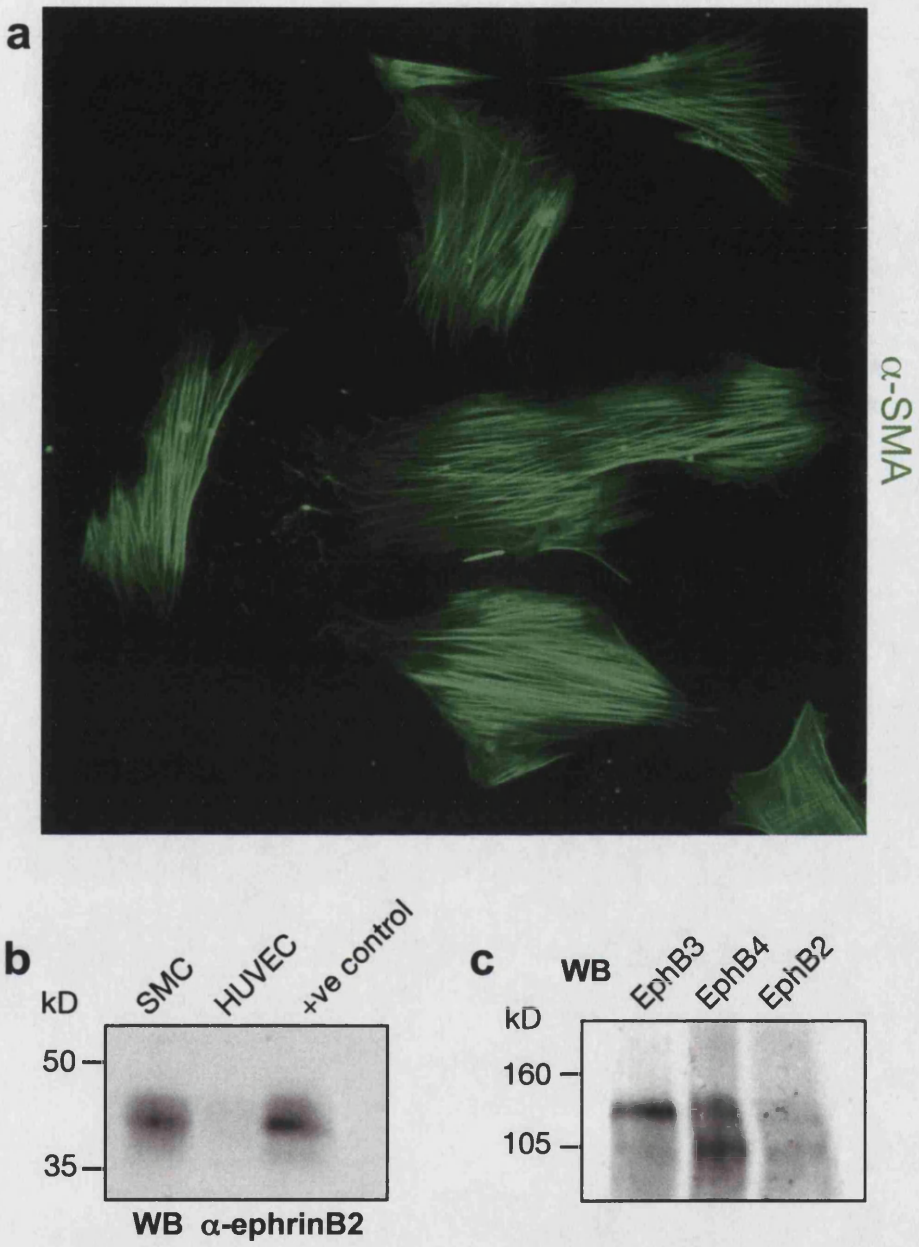
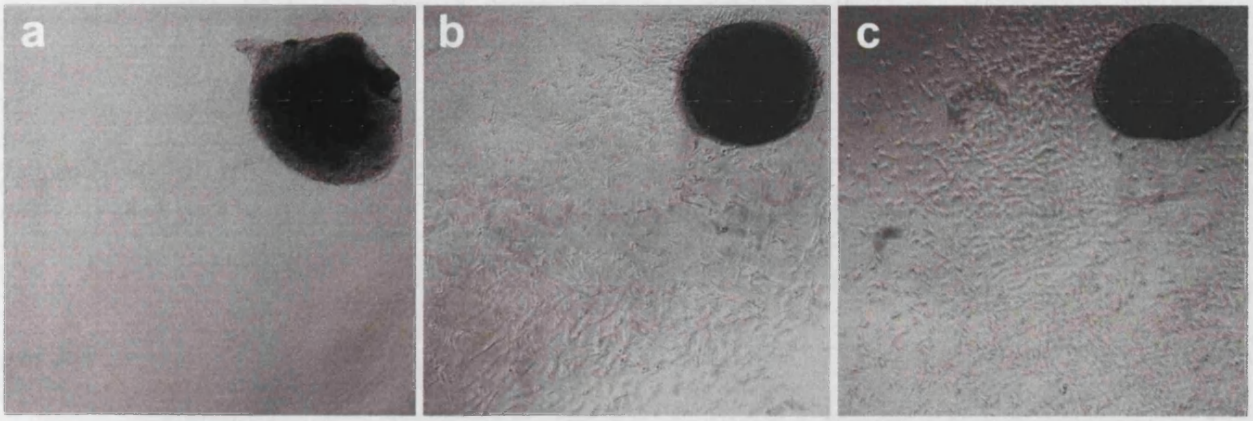


Figure 5.15: Immortalised vSMCs express ephrinB2 and EphB receptors

α-SMA-positive cultured cells that were isolated from from aortic fragments (a). Western Blot (WB) showing expression of ephrinB2 (b) as well as EphB2, EphB3 and EphB4 receptors (c), in immortalised SMCs. Embryo lysates was used as positive control (b).



	Control	EphrinB2$^{\Delta MC}$
Number of aortic pieces that sprouted in the presence of PDGF-B	12	2
Total number of aortic pieces	22	13
	54.5%	**15.4%**

Figure 5.16: Aortic pieces from ephrinB2$^{\Delta MC}$ showed less sprouting in the presence of PDGF-B

Images taken of aortic pieces in culture after 48 hours, in the absence/presence of PDGF-B (20ng/ml) as indicated. In the absence of PDGF-B, no sprouting from the aortic explants was observed (a). In the presence of PDGF-B, a fraction of both mutant and control samples displayed sprouting from explants (b,c). However, the number of sprouting mutant explants was lower in comparison to controls (d).

Chapter6 | Discussion & concluding remarks

6.1 General Overview

The vascular network is the first functional system to develop in the embryo and ensures that adequate blood flow provides tissues with a sufficient supply of nutrients and oxygen and removes waste products and carbon dioxide. The formation of this functional blood vessel network is initiated by "vasculogenesis", a process in which angioblasts proliferate and coalesce into a primitive network of vessels known as the primitive capillary plexus (Carmeliet et al., 1998). The endothelial cell lattice created by vasculogenesis then serves as a scaffold for angiogenesis, which is the remodelling of the network into a hierarchically organised vasculature. This process involves extensive changes in the cell-cell and cell-ECM interactions in response to signals by precisely balanced angiogenic stimulators and inhibitors (Carmeliet, 2000). Angiogenesis occurs mostly during embryonic development, to address the increasing demands of tissues and organs. In contrast, the adult vasculature is stable and there is very little turnover of endothelial cells. Angiogenesis in adult organisms is confined to physiological wound healing processes and the menstruation cycle but is also a hallmark of many pathological conditions such as proliferative diabetic retinopathy and tumour neovascularisation (Papetti and Herman, 2002).

Over the past few years, several studies have demonstrated the essential role of ephrinB2 in the developing vascular system, where its early expression is restricted to the endothelial cells of the arterial, but not venous blood vessels (Wang et al., 1998). EphrinB2 null mutant mice are lethal at midgestation due to severe malformation of the vasculature including the lack of angiogenic remodelling. Similar defects occurred in EphB4 deficient mice and in a subset of mice lacking the two receptors EphB2 and EphB3 (Adams et al., 1999; Gerety et al., 1999). As these are binding partners for ephrinB2, it suggests that the ephrinB2 activation of reciprocal receptor-ligand interactions and the downstream signal transduction cascades appear to be essential for normal blood vessel formation. Although the role of the ephrinB2 cytoplasmic domain, in the process of vascular morphogenesis, is controversial, several studies have been

shown that ephrins have active, receptor-like signalling functions (Bruckner et al., 1999; Lu et al., 2001; Song, 2003). The cytoplasmic domain may be also involved in conformational alterations, protein stability, intracellular translocation, membrane presentation, internalisation and recycling, but such functions remain to be addressed.

Two studies have shown that ephrinB2 expression progressively extends from the arterial endothelium to surrounding arterial smooth muscle cells and pericytes, during late embryogenesis (Gale et al., 2001; Shin et al., 2001). This arterial pattern persists into adults but the ligand is also upregulated in settings of physiological and pathological angiogenic processes. The observation that ephrinB2 is essential for angiogenesis in the early embryo and expression patterns in the adult suggest that the ligand may modulate blood vessel remodelling in a wide range of settings. Moreover, the expression of ephrinB2 in SMCs and pericytes hint at an involvement in endothelial-mural cell interactions.

6.2 The role of ephrinB2 in endothelial cells

The early lethality of ephrinB2 null mutants is a major obstacle for functional studies during later stages of vascular morphogenesis. Moreover, it has not been possible to address the role of ephrinB2 in specific cell types. A genetic approach in mice, using the Cre-loxP system, has been employed to overcome these limitations. To determine the role of ephrinB2 in endothelial cells, the transgenic Tie1-Cre line was bred into a background of ephrinB2^{lox} mice. The resulting ephrinB2$^{\Delta EC}$ mutants exhibited defects in angiogenic remodelling, cardiac malformations and lethality reminiscent of conventional ephrinB2 knockouts. These findings indicate that ephrinB2 expression in endothelial and endocardial cells is essential for the principle steps of cardiovascular development. EphrinB2 in other cell populations or other B-class ephrins are unable to compensate for these defects. As the Tie1-Cre is expressed in both endothelial and endocardial cell lineages, the question remains open whether some of the vascular defects in ephrinB2$^{\Delta EC}$ mutants are caused by cardiac malfunction and the resulting haemodynamic changes.

The early lethality of the ephrinB2$^{\Delta EC}$ mutants did not permit and functional studies during late embryogenesis or in adults. To control the time course of EC-specific gene inactivation, the Tie2-Cre^{ERT} transgenic was characterised to explore its suitability for functional studies. A first series of experiments suggested the line permitted only poor Cre-mediated recombination in embryos, adults and tumours treated with tamoxifen. These problems were probably caused by technical difficulties in the solubilisation of tamoxifen and 4OH-TM and recent results have shown significant improvements when applying a modified protocol. This may now permit EC-specific inactivation of ephrinB2 during various stages of development. Alternatively, other tamoxifen-controlled Cre lines or different inducible systems may be employed for the same purpose.

6.3 EphrinB2 function in mural cells

The successful generation and characterisation of PDGFRβ-Cre mice permitted the generation of mural cell-specific ephrinB2 mutants, ephrinB2$^{\Delta MC}$. These mice develop to term but died shortly after birth, presumably due to respiratory failure. EphrinB2$^{\Delta MC}$ embryos exhibited oedema and widespread haemorrhaging in the skin, which was particularly evident in the limb, back and head regions, reminiscent of the PDGF-B and PDGFRβ null phenotypes (Soriano, 1994; Hellstrom et al., 1999). Other defects included haemorrhaging in the lymphatics of the mesentery and intestine, dilation of the capillary tufts in the glomeruli and blood-filled alveolar spaces in the lungs.

Immunohistochemistry on mutant skin showed that the ablation of ephrinB2 in mural cells led to downregulated ephrinB2 expression in the endothelium of larger arterioles in the skin. It remains unclear whether a similar downregulation occurs in the endothelium of smaller arterioles and in microvessels, because neither the expression of ephrinB2 nor EphB receptors were detectable by antibody stainings in these structures. This could be due to the detection limitations of the antibodies as well as low or absent ephrinB2 protein. Nevertheless, lacZ staining of multiple tissues from ephrinB2^{tauLacZ} mice has indicated at least some ephrinB2 expression in the small calibre vessels. Cross-breeding of ephrinB2^{tauLacZ} mice and PDGFRβ-Cre/ephrinB2$^{lox/+}$ double

heterozygotes will allow the study of the regulation of ephrinB2 expression in a more conclusive manner.

Despite the downregulation of endothelial ephrinB2 in mutant arterioles, it is remarkable that embryos survive until birth. Moreover, β-galactosidase staining under the control of the Tie2lacZ reporter, indicated that the overall organisation of the mutant blood vessel network was surprisingly normal. This observation is in stark contrast to the severe vascular defects seen in ephrinB2 and EphB4 null mice, as well as in ephrinB2$^{\Delta EC}$ mutants. Thus, ephrinB2 expression in the endothelium may be only essential during the early stages of vascular morphogenesis and later critical functions may shift from the endothelium to mural cells. This coincides with progressively increasing ephrinB2 expression in these cell types.

Quantification of mural cells in a X-lacZ4 reporter background and antibody staining indicated that the number of mural cells in mutants was comparable to control embryos. However, characterisation of the mutant microvasculature indicated that the association of SMCs and pericytes with the endothelium was compromised. Mural coverage of the microvessels was discontinuous and incomplete. Furthermore, mutant pericytes failed to attach properly to the endothelium. Because Eph receptors and ephrins can mediate adhesion as well as repulsive interactions during development (Murai and Pasquale, 2003), the loss of ephrinB2 may lead to adhesion defects without appreciable consequences for mural cell proliferation and survival. However, the expression levels for ephrinB2 protein and its receptor are highest on vessels of larger calibre and decreases with vessel diameter. This suggests that a gradient of ephrinB2 may help to establish a stable vessel wall, perhaps by controlling mural cell guidance and/or adhesion. Previous work has also shown that the co-expression of ephrin ligands can mask Eph receptors in the same cells and thereby modulate cell-cell interactions and signalling (Hornberger et al., 1999). The characterisation of isolated vSMCs suggests that ephrinB2 may mask the co-expressed receptors EphB2, EphB3 and EphB4 in a similar fashion. Preliminary results suggest that the binding of soluble ephrinB2/Fc protein to cultured ephrinB2-deficient SMCs is increased, i.e. that more receptors have become available for interaction with other cells (data not shown). In this setting, mural

ephrinB2 may modulate repulsive signals, that is, Eph receptor activation by ligands presented by endothelial cells and adjacent pericytes/vSMCs.

Irrespective of the adhesive or repulsive nature of the underlying mechanism, mutant microvessels are not sufficiently supported by mural cells, which lead to their dilation and eventual rupture. The presence of blood-filled lymphatics in skin and intestine may be secondary to the haemorrhaging seen in these tissues.

The data provided here, introduces the conundrum of why the association of mural cells to the wall of larger and intermediate-size vessels appears to be normal, while only the microvessels are affected in the mutants. In contrast to the defect in mutant microvessels, larger vessels manage to maintain their stability and it is plausible that other molecules are able to compensate for the loss of ephrinB2 in these pericytes and vSMC populations. Mature vessels with robust SMC coating may not be susceptible to perturbations caused by the loss of ephrinB2. In the microvasculature, where angiogenic sprouting is still taking place and mural cell coverage is incomplete, vessels are more prone to destabilisation and may therefore require intact Eph-ephrin interactions. The fact that the rupture of blood vessels was only seen to have occurred at later stages of embryonic development indicates that ephrinB2 may not have the same essential role in earlier steps of vascular morphogenesis.

To acquire a more complete understanding of the role of ephrinB2 in mural cells, several issues need to be addressed. It will be important to understand whether the engagement and activation of Eph receptors is required for the stabilisation of microvessels. Furthermore, ligand cleavage or the internalisation of the ligand-receptor complex may play a critical role. Conditional ephrinB2 mice could be also used to study the origin of SMCs in different vascular beds. Such work is limited by a lack of markers for the precursors of pericytes and vSMCs. One possible experiment may be to breed the ephrinB2 conditional line into the Wnt1-Cre background. As the Wnt1-Cre is neural crest cell (NCC) specific (Danielian et al., 1998; Brewer et al., 2004), the ephrinB2 gene would be inactivated in SMCs above the thoracic, i.e. in those cells that are derived from NCC progenitors. Consequently, these mutants may develop vascular defects and haemorrhaging in the head region.

EphrinB2$^{\Delta MC}$ mice display phenotypic similarities to mutants in the PDGF-B/PDGFRβ pathway (Soriano, 1994; Hellstrom et al., 1999). In particular, ephrinB2$^{\Delta MC}$ mutants and PDGF-B retention mutants both showed impairment of pericyte investment in the microvessel wall (Lindblom et al., 2003). As the PDGF-B retention mutants express a truncated form of PDGF-B that cannot be retained by binding to the extracellular matrix, this impairs its normal presentation sites and disrupts gradients essential for the proper recruitment of mural cells. Overexpression of PDGF-B in tumours grown in PDGF-B retention mutants led to an increase in pericyte numbers but failed to rescue recruitment and investment defects (Abramsson et al., 2003).Thus, the precise presentation of the PDGF-B signal is essential to establish functional mural-endothelial interactions. The levels of PDGFRβ expression in ephrinB2$^{\Delta MC}$ vSMCs appear normal (data not shown) and preliminary work from the laboratory suggests that ephrinB2 may modulate the cellular response to PDGF-B. Previous work has shown that activated PDGF receptor can induce the phosphorylation of tyrosine residues in the cytoplasmic domains of the B-class ephrins (Bruckner et al., 1997). Future work will address the biological relevance of this observation and explore the possibility of a direct link between the two pathways.

6.4 Ectopic recruitment of SMCs to lymphatics capillaries

Another feature of the ephrinB2$^{\Delta MC}$ mutants is the ectopic recruitment of SMCs to the lymphatic capillary beds of the skin. Lymphatics remove excess interstitial liquid and extravasate molecules and drain into veins. This function requires that lymphatic endothelial cells can open intercellular gaps as well as the absence of mural cells. Abnormal SMC coverage and constriction of lymphatics in FOXC2 mutant mice and in human patients suffering from lymphedema distichiasis, leads to lymphedema (Petrova et al., 2004). It has been shown that this SMC recruitment is caused by ectopic expression of PDGF-B in lymphatic capillaries. Indicative of some sort of link of unknown nature between ephrinB2 and the PDGFRβ pathway, PDGFRβ knockout mice also showed SMCs on skin lymphatics despite the dramatic reduction of vascular smooth muscle coverage. Surprisingly, PDGF-B retention mutants or PDGF-B nulls did not phenocopy this defect and their lymphatic capillaries remained free of vSMCs. This indicates that other factor, perhaps the related PDGF-D molecule, might compensate for

the loss of PDGF-B, although to a limited extent. It also shows that SMC recruitment to distal lymphatics is not a secondary response to oedema but is caused by specific molecular defects. The relevant molecular signals remain to be investigated, but ectopic PDGF-B secretion by lymphatic capillaries in ephrinB2$^{\Delta MC}$ mutants may attract mural cells, like in the FOXC2-deficient mice. Alternatively, the absence of ephrinB2 in mural cells may eliminate repulsive signals that prevent attachment to EphB4-expressing lymphatics (data not shown). To address the precise mechanism of how ephrinB2 mediates proper assembly of the mural cells to the microvessels as well as ectopic SMC coverage in the lymphatics in its absence, it will be important to understand the repulsive or attractive nature of EphB-ephrinB2 interactions in the vascular system.

6.5 Which pathways may be involved in the ephrinB2$^{\Delta MC}$ phenotype?

Besides the possible role of PDGFβ discussed above, other molecular signals may be directly linked to the ephrinB2 function in pericytes and vSMCs. Electron micrographs from ephrinB2$^{\Delta MC}$ mutant skin show excessive collagen fibrils around microvessels. Thus, ECM remodelling appears to be directly or indirectly involved , as a consequence of impaired mural cell-EC association in ephrinB2$^{\Delta MC}$ mutants. Metalloproteases are key mediators of ECM remodelling, with functions ranging from the proteolytic cleavage of growth factors, ECM degradation, to the cleavage of cell-surface proteins (Chang and Werb, 2001). At this point, it is conceivable that the function of matrix metalloproteases (MMPs), metalloprotease-disintegrins (ADAMs) or natural tissue inhibitors of metalloproteases (TIMPS), may be altered in ephrinB2$^{\Delta MC}$ mutants.

Previous studies have linked ephrins to integrin-mediated cell migration and adhesion. EphrinB1 can transduce signals that activate integrin-mediated migration, attachment and angiogenesis in Chinese hamster ovary (CHO) cells (Huynh-Do et al., 2002). EphrinA1 can block integrin-mediated vascular smooth muscle cell spreading via the Rac/PAK pathway (Deroanne et al., 2003). Integrins regulate cell-matrix and cell-cell interactions as well as motility function, roles that may contribute to the ephrinB2$^{\Delta MC}$ phenotype.

TGF-β signalling controls a range of cellular responses in endothelial cells and mural cells that affect their function (Goumans et al., 2003). These signals can be either stimulatory or inhibitory depending on the presence of additional cues. Several members of the TGF-β family have been implicated in the regulation of angiogenesis (Pepper, 1997; Goumans et al., 2003). Nevertheless, a connection of TGF-β molecules to Eph/ephrin signalling remains to be established.

Members of the Sphingosine-1 phosphate (S1P) family are also regulators of embryonic angiogenesis and blood vessel maturation, through the generation of knockouts (Kono et al., 2004). It has been suggested that ephrinA1 can act in synergy with S1P to destabilise blood vessels in early steps of angiogenesis (Deroanne et al., 2003). A role of S1P-induced signalling pathways in ephrinB2$^{\Delta MC}$ mutants is currently not supported by direct evidence.

A challenge in the identification of the molecular mechanism underlying the defects in the ephrinB2$^{\Delta MC}$ mutants, will be the separation of primary factors from secondary processes caused by haemorrhaging and oedema.

6.6 Concluding remarks

The work presented here demonstrates that endothelial ephrinB2 expression is essential for angiogenesis and blood vessel development. Endothelial cell-specific ephrinB2 deficiency leads to defects in angiogenic remodelling and cardiac malformations. This shows that endothelial ephrinB2 is indispensable for angiogenesis in early embryonic development and it will be important to investigate similar roles in a number of physiological and pathological settings.

In addition, my work shows that ephrinB2 is not only a critical regulator of angiogenesis but also involved in mural cell recruitment and vessel wall assembly. It has been previously shown that ephrinB2 expression progressively extends from the arterial endothelium to surrounding SMCs and pericytes (Gale et al., 2001; Shin et al., 2001), but the functional significance of this process has remained unclear. Mural cell-specific deletion of ephrinB2, leads to impaired mural-endothelial interaction, loss of blood vessel integrity and, consequently, haemorrhaging and oedema.

Future work will address whether ephrinB2 is involved in human disease conditions with similar vascular defects, for example diabetic retinopathy, or in the disorganised morphology of tumour blood vessels. This will also be an important step in determining whether ephrinB2 might be a target for pro- or anti-angiogenic therapies or relevant in cardiovascular disorders.

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