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Essential roles for Galpha13 in endothelial cells and neural crest during embryonic development

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ESSENTIAL ROLES FOR $G\alpha_{13}$ IN ENDOTHELIAL CELLS AND NEURAL CREST DURING EMBRYONIC DEVELOPMENT

L. David Willison

Abstract

Toward identifying the G protein pathways that mediate the actions of the thrombin receptor PAR1 and other G protein-coupled receptors in vascular development, we investigated the role of $G\alpha_{13}$ in endothelial cells and other cell types. *lacZ* inserted into $G\alpha_{13}$ exon 1 was highly expressed in endothelial and presumptive neural crest cells in the mouse embryo at midgestation. Endothelium-specific knockout of $G\alpha_{13}$ caused embryonic lethality at E9.5-11.5 that resembled the *Par1* null phenotype. Conversely, restoration of $G\alpha_{13}$ expression in endothelial cells in $G\alpha_{13}$ null embryos by use of a Tie2 promoter/enhancer-driven $G\alpha_{13}$ transgene rescued the E9.5 vascular phenotype in such embryos. Thus, $G\alpha_{13}$ signaling in endothelial cells plays a critical role in vascular development. Roles in other cell types were also uncovered. The Tie2 promoter/enhancer-$G\alpha_{13}$ transgene completely rescued development of endothelium-specific $G\alpha_{13}$ knockout embryos but not $G\alpha_{13}$ global knockouts; transgene-positive $G\alpha_{13}$ null embryos developed for several days beyond their transgene-negative littermates and then manifested a new phenotype that included intracranial bleeding and exencephaly. Knockout of $G\alpha_{13}$ in the neural crest using *Wnt1-Cre* phenocopied these endothelial-rescued $G\alpha_{13}$ nulls. Taken together, our results suggest a critical role for G protein-coupled receptor signaling via $G\alpha_{13}$ in endothelial cells and neural crest during mammalian embryonic development.

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INTRODUCTION

Chapter Ia: Description of vasculogenesis and angiogenesis:

Formation of blood vessels during mammalian embryonic development is a complex and highly regulated process. Angioblasts proliferate, migrate, and differentiate to form primitive vascular structures composed of endothelial cells. These structures remodel by sprouting, branching, growing, and regressing, and mature by the recruitment and differentiation of pericytes and smooth muscle cells. Studies to define the molecular signals that orchestrate vascular development have focused mainly on receptor tyrosine kinases and integrins, and their ligands. Less is known regarding the roles of G protein-coupled receptors (GPCRs) in this process. In this thesis I will present data that advances our understanding of how GPCR signaling, specifically how the heterotrimeric G protein $G\alpha_{13}$, contributes to vascular development. In Chapter 1 I will provide an overview of some of the processes that control vasculogenesis and angiogenesis, and discuss some of the genes and signaling pathways involved.

Blood vessel formation can be divided into two broad categories, vasculogenesis and angiogenesis. Vasculogenesis refers to the *in situ* differentiation of endothelial cells from mesodermal precursors and their subsequent organization into a network, or plexus, of blood vessels. Angiogenesis refers to the processes that occur after this initial vascular plexus is formed and includes sprouting angiogenesis and intussusceptive angiogenesis.[1] Sprouting angiogenesis occurs when new vessels grow out from preexisting ones, and

involves localized proteolytic degradation of the vessel wall and the surrounding extracellular matrix proteins followed by migration and proliferation of endothelial cells as the nascent vessel forms. In the adult, sprouting angiogenesis is thought to coincide with the recruitment of bone marrow derived endothelial stem cells to the site of sprouting, though it is not established whether this also occurs in the embryo.[2-4] Intussusceptive angiogenesis occurs when a preexisting vessel is subdivided into two or more smaller vessels by "transcapillary pillars" and does not require proliferation.[5, 6] In concert these angiogenic processes transform the relatively uniform vascular bed created during vasculogenesis into an expanded, hierarchical tree of vessels. Subsequent vessel maturation involves selective pruning of the vascular tree and the recruitment and differentiation of pericytes and vascular smooth muscle cells to and around the vessel wall, followed by the formation of an extracellular matrix.[7] Without support cells or the extracellular matrix, the vessel is unstable and prone to regression.[8]

Chapter Ib: The Hemangioblast:

The first step in the development of the cardiovascular system is the differentiation of endothelial cells. Accumulating evidence suggests that endothelial cells are derived from a bipotential progenitor cell, the hemangioblast, which has both hematopoietic and endothelial potential. Support for this idea has it roots in classical histological studies conducted nearly a century ago by Florence Sabin.[9] Further evidence of the hemangioblast's existence has come from recent *in vitro* studies in which single cells from mouse embryonic stem cell-derived embryoid bodies gave rise to blast

colonies containing both hematopoietic and endothelial cells.[10, 11] Further work both *in vitro* and *in vivo* suggests that recently gastrulated mesodermal precursor cells defined by the expression of the mesodermal marker Brachyury (*T*) and the vascular endothelial growth factor (VEGF) receptor Flk1[12] or by the expression of the transcrption factor SCL/TAL1 and Flk1[13, 14] are the precursors to the hemangioblast. The application of this model is not restricted to mammals as work in chick embryos has shown that VEGF can direct recently gastrulated SCL/TAL1$^+$ mesoderm cells toward an endothelial cell fate.[15] Other signaling factors have been shown to be important in determining or supporting the murine hemangioblast lineage including fibroblastic growth factors (FGF),[16-18] bone morphogenetic proteins (BMP),[19] and Hedgehog (Hh) ligands.[20]

Pinpointing the timing and location of hemangioblast commitment in the mouse embryo has proved difficult, though one recent study has shown that the commitment to a hemangioblast fate can occur intraembryonically at the primitive streak early during mouse development (around embryonic day E7.0) in cells that co-express Brachyury and Flk1.[21] At this point it is unclear whether these early hemangioblasts are capable of seeding the entire embryo, or if hemangioblasts form independently elsewhere at later stages in the yolk sac. Interestingly, the existence of the hemangioblast, or of hemangioblast-like cells, has also been suggested in the analysis of SCL/TAL1 function in *Danio rerio* and in cell fate studies in *Drosophila melanogaster* where the FGF-Receptor (FGFR) homologue *heartless* is involved in patterning the mesoderm and in maintaining cardiocytes (which are analogous to vertebrate endothelial cells).[22, 23] These

data point to the conserved nature of endothelial cell fate decision across multiple animal phyla.

Historically, the first site of vasculogenesis in the mouse embryo was thought to be the blood islands of the extraembryonic yolk sac beginning at around E6.5-7.0.[24] Blood islands are initially isolated foci of SCL/TAL1$^+$; Flk1$^+$ cells that can be visualized as individual, hollow spheres of endothelial cells surrounding a loose inner mass of hematopoietic precursors.[25] Blood islands are formed by E7.3 and then coalesce into a uniform vascular plexus by E8.5. They then remodel into a branched, arborized tree that connects to the embryo proper via the vitelline vessels by E9.5 to supply the embryo with yolk sac nutrients. The first intraembryonic vascular structures to form are the heart and the great vessels.* At around E7.3 the endocardial primordia are formed as a bilateral cresent of migrating angioblasts in the cranial mesoderm that connects through the midline. Between E8.2-8.5 these bilateral heart fields are brought to the midline and remodeled into endocardial tubes. Simultaneously, at around E7.8 the angioblasts in bilateral aortic primordial become visible in the para-aortic splanchnopleuric mesoderm (known later in development as the aorta-gonad mesonephros or AGM). By E8.5 these have formed a primitive vascular network which is bidirectionally remodeled into the bilateral embryonic aortae.[13] By E8.5 a rudimentary embryonic circulation is established,

* Historically it has been accepted that extraembryonic vasculogenesis does not contribute to intraembryonic vasculogenesis (*i.e.*, that intraembryonic angioblasts differentiate *in situ*) though recent work suggests that blood island-derived extraembryonic cells can contribute to vascular structures within the embryo proper.
[LaRue, A. C., Lansford, R. & Drake, C. J. Circulating blood island-derived cells contribute to vasculogenesis in the embryo proper. Dev Biol 262, 162-72 (2003).]

though the extent to which the intra- and extraembryonic vascular compartments communicate at this developmental stage is still debated.[26, 27]

Chapter Ic: Molecules involved with vessel assembly:

What are the molecular signals that orchestrate the processes just described? VEGF (also known as VEGF-A) was initially discovered in the 1970s based on its ability to cause an increase in vascular permeability in guinea pig skin.[28] It was the first factor discovered that causes an angiogenic effect selectively on endothelial cells. As alluded to in the above discussions of hemangioblast and endothelial cell fate determination, VEGF plays a central role in the control of vascular development. VEGF is a ~40-kilodalton disulfide-linked dimeric glycoprotein that shares homology to platelet derived growth factor-B (PDGF-B) and to other VEGF-related proteins including VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF).[29] VEGF exisits as a variety of splice-variant isoforms that have different signaling characteristics,[30] though in this discussion these distinctions will not be addressed and "VEGF" will refer to any or all VEGF-A isoforms collectively. VEGF binds to three cell surface receptor tyrosine kinases; the fms-like tyrosine kinase (VEGFR1 or Flt1)[31, 32] and fetal liver kinase-1 (VEGFR2 or Flk1)[33] which are both expressed on embryonic vascular endothelium, and VEGFR3 or Flt4 which is expressed on lymphatic endothelium.[34, 35] VEGF can also bind to neuropilin-1 and neuropilin-2 (NP1 and NP2) which have previously described roles in axon guidance as cell surface receptors for class III semaphorins. Though these receptors do not have tyrosine kinase activity and are also expressed on neurons, NP1 enhances the

binding of VEGF to Flk1.[36] Interestingly, mice lacking NP1 suffer from transposition of the great vessels and both *NP1* and *NP1/NP2* double null mice have defects in yolk sac angiogenesis and in neural vascularization in addition to the defects in axon guidance that would be expected due to their interaction with class III semaphorins.[37, 38] Mice lacking NP2 show absence or reduction of small lymphatic vessels and capillaries, but larger lymphatic vessels, arteries, and veins are intact.[39]

Heterozygosity for a deletion of the VEGF gene (*Vegfa*) in mice results in lethality between E11.0 and E12.0 with defects in blood island formation, sprouting angiogenesis, lumen formation, the formation of large vessels, and in plexus remodeling.[40, 41] In addition, using tetraploid aggregation to generate embryos in which the extraembryonic tissues were derived entirely from $Vegfa^{+/-}$ cells while the embryo proper was derived from $Vegfa^{+/+}$ cells resulted in abnormal angiogenesis and death at midgestation. Deletion of *Vegfa* in neonatal mice results in growth arrest and lethality with structural alterations and increased endothelial cell death in the liver vascular bed indicating that VEGF is not only required for proliferation but also endothelial cell survival.[42] Deletion of the genes encoding for the VEGF receptors Flk1 and Flt1 also results in embryonic lethality and severe alterations in the vasculature.

Embryos lacking Flk1, or lacking one specific tyrosine residue in the cyotoplasmic tail of the Flk1 receptor, fail to form blood islands and have widespread defects in vasculogenesis and hematopoiesis.[43, 44] Using chimera analysis it was shown that *Flk1* null cells fail to migrate from the primitive streak into the yolk sac suggesting a

role for VEGF and Flk1 in the chemotaxis/migration of endothelial cells.[45] This hypothesis has been supported by other studies including one in which VEGF/Flk1 signaling was shown to guide the tip of endothelial cell filopodia during angiogenic sprouting in the retina.[46] The regulation of Flk1 transcription/expression is complex and involves many players, some of which have been mentioned above (*e.g.*, Brachyury). As described above, SCL/TAL1 is a transciption factor expressed in hemangioblasts and in endothelial cells, and its deletion in mice results in defects in the yolk sac vasculature as well as in embryonic and adult hematopoiesis.[47-51] SCL/TAL1 apparently acts in concert with other transcription factors like serum response factor, GATA, and ETS to induce Flk1 expression.[52] Another early signal that acts to promote vasculogenesis is FGF produced by the paraxial mesoderm[53-55] and, in the case of FGF2 signaling through FGFR1 acts to upregulate Flk1 expression.[56] The identity of the Zebrafish *cloche* gene is not yet known but it apparently acts cell-autonomously in endothelial cells downstream of SCL/TAL1 and upstream of Flk1 to regulate endothelial cell differentiation.[57-59] Flk1 expression is also regulated by environmental oxygen availability by hypoxia inducible factor-1α (HIF-1α), which will be discussed in more detail later.

Unlike *Flk1* null embryos, those lacking Flt1 are capable of forming blood islands and endothelial cells but have severe defects in vascular organization that results from a disregulation of endothelial cell proliferation. In *Flt1* null embryos excess endothelial cells fill the lumen of vessels in both the extra- and intraembryonic tissues.[60, 61] Although Flt1 is capable of binding VEGF, previous work has not been able to show that the

receptor actually signals in response to VEGF in endothelial cells* and mice lacking the Flt1 cytoplasmic tyrosine kinase domain have no vascular phenotype.[62] It appears that at least in endothelial cells Flt1 functions to tune the threshold for VEGF signaling[63] in a similar, but opposite way as NP1 (see above). VEGFR3, a receptor for the VEGF family member VEGF-C, might also perform a similar role in "tuning" angiogenesis and lymphangiogenesis.[64, 65] Less is known regarding the regulation of Flt1 transcription than there is about the regulation of Flk1 transcription, though possible players include the zinc-finger transcription factor early growth response-1, or Egr1[66, 67] and the endothelial PAS domain protein (which has high homology to HIF-1α).[68] VEGF signaling through Flk1, and PlGF which is thought to signal through Flt1, have been shown to increase expression of Flt1 under certain conditions.[69]

Chapter Id: Molecules involved with vessel stabilization and maturation:

Until this point, I have described the molecular basis to early events in vascular development: the commitment to the hemangioblast lineage followed by the differentiation of endothelial cells and the formation of the first vascular beds. Next, I will discuss some of the processes and molecules involved with vessel remodeling, and stabilization, which are often referred to as vessel maturation or the "resolution phase" of angiogenesis.

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* Previous work *has* suggested that VEGF can signal through Flt1 in monocytes.
[Clauss, M. et al. The vascular endothelial growth factor receptor Flt-1 mediates biological activities. Implications for a functional role of placenta growth factor in monocyte activation and chemotaxis. J Biol Chem 271, 17629-34 (1996).]

Tie1[70, 71] and Tie2 (or *Tek*)[72] define another family of receptor tyrosine kinases that are expressed in endothelial cells in the developing vasculature of mouse embryos and at adult sites of neovascularization.[73-75] *Tie1* null embryos have a normal vessel pattern at E12.5-E13.5 but show reduced survival of endothelial cells and defects in the vascular integrity of endothelial cells that results in edema and hemorrhage that is apparent late in gestation. These animals die at birth with breathing difficulties presumably resulting from defects in the lung vasculature.[74, 76] Two studies in which Tie2 signaling is abrogated or reduced show conflicting results. In the first study, *Tie2* null embryos and embryos in which a dominant negative Tie2 protein is expressed both died *in utero* and showed defects in vascular integrity and a decrease in the number of endothelial cells leading the authors to propose a role for Tie2 in endothelial cell proliferation and survival.[77] The second study showed that *Tie2* null embryos were dead by E10.5 with obvious defects in vascular patterning and angiogenesis by E9.5, including dilated head vessels, a lack of angiogenic sprouting into the neuroectoderm, and a failure of vascular remodeling and maintenance of the vascular capillaries (leading to hemorrhage), but this study did not find defects in endothelial cell survival.[78] The basis for this difference in phenotypes is thought to be in the construction of the groups' respective targeting vectors, and subsequent studies by others have shown that Tie2 is in fact essential for endothelial cell survival and in its absence these cells undergo apoptosis.[75, 76, 79]

Tie2 null embryos have defects in endocardial and heart development. These defects include a decreased complexity of the ventricular endocardium and a collapsed endothelial lining of the atria. Mosaic analysis has shown an absolute requirement for

Tie2 in endocardium. Tie1 and Tie2 are each dispensable for the initial assembly of the embryonic vasculature, however both Tie1 and Tie2 are necessary in the microvasculature later during embryogenesis, and in adults.[80] Tie2 is also required for the association of nascent vessels with perivascular cells and Tie2 appears to negatively regulate intussusceptive angiogenesis.[81] Interestingly, mutation of tyrosine residue 1100 in the intracellular tail of Tie2 recapitulates the endothelial cell and heart defects of global *Tie2* ablation, but does not adversely affect the association of perivascular cells with nascent vessels.[82]

Angiopoietin-1 (Ang1) is a soluble growth factor that is expressed adjacent to both developing and mature blood vessels, and binds to and induces tyrosine phosphorylation of Tie2.[83, 84] Embryos lacking Ang1 exhibit a similar, if less severe, phenotype than *Tie2* null embryos and are dead by E12.5 but do exhibit the prominent endocardial and myocardial defects seen in *Tie2* null embryos. *Ang1* null embryos have a reduction in vascular complexity (*i.e.*, vessel branching and endocardial folding), the endothelial cells are poorly associated with pericytes/vascular smooth muscle cells and with the extracellular matrix, and there are reduced numbers of pericytes associated with endothelial cells.[85] This observation suggests that in addition to supporting angiogenesis, Ang1/Tie2 is involved in the recruitment and/or the stable association of endothelial cells with adjacent pericytes. The fact that mature, stable vessels in the adult consistently express tyrosine phosphorylated Tie2 supports this hypothesis[86] as does the observation that overexpression of Ang-1 results in tissues that are hypervascularized with leakage-resistant blood vessels.[87] Humans that have an activating mutation in Tie2 suffer from

autosomal dominant venous malformations; a condition resulting from hypervascularization.[88]

Ang2 was identified by its sequence homology to Ang1 and it acts as an antagonist for Ang1/Tie2 signaling as it inhibits Ang1-mediated Tie2 tyrosine phosphorylation. Though the mechanism of this effect is not fully understood, Ang2 binds to Tie2 but not Tie1, possibly suggesting a direct inhibitory effect of Ang2 binding to Tie2.[89] Ang2 expression correlates with sites of active, adult vascularization where destabilization of existing vessels is necessary to allow for angiogenic sprouting. Ang2 overexpression in mouse embryos results in discontinuous vessels, separation of the endocardium from the underlying myocardium, absence of the cardinal vein, and poor arborization of vascular beds.[90] Deletion of the mouse gene for Ang2 has no embryonic phenotype but *Ang2* null adults show defects in vessel regression and sprouting in the neonatal eye, a tissue in which postnatal angiogenic remodeling has been well studied.[91] Therefore, Ang2 has a critical function as a destabilizer of vessel architecture.

Stabilization and maturation of blood vessels relies to a large degree on interactions that occur between endothelial cells and the pericytes/VSMC that surround the vessel. The distinction between a pericyte and a VSMC is not absolute, though in general, pericytes are found embedded in the basement membrane on the abluminal side of the vascular endothelial cells and in some instances actually make direct contact with endothelial cells.[92] On the other hand, VSMC are separated from this basement membrane by an additional layer of mesenchymal cells and are found mainly on larger

vessels. One intriguing possibility is that while VSMC are organized to better effect vascular tone, the close proximity of pericytes to endothelial cells serves to integrate intercellular comminication.[93] The heterogeneity of pericyte/VSMC populations is highlighted by the fact that 1) pericytes are able to differentiate into VSMC and *vice versa*, and 2) these cells have multiple embryonic origins depending upon the vessel's location in the embryo where they differentiate. Pericytes are most often thought of as being derived from mesenchyme as is the case with pericytes that develop around the trunk vessels,[94] however the cephalic neural crest has been shown to contribute VSMC/pericytes to the vessels of the face and forebrain[95] and the mural cells in the coronary arteries have been shown to derive (via an epithelial-mesenchymal transformation) from epicardial cells that in turn derive from the splanchnic mesoderm.[96] It has also been proposed that pericytes can transdifferentiate from endothelial cells.[97]

How are pericytes/VSMCs determined and localized around developing vessels in the embryo? One central player in this that has been well studied is transforming growth factor-β (TGFβ). Mice in which the gene for TGFβ1 has been deleted die around 20 days after birth from a multifocal inflammatory response with associated tissue necrosis[98, 99] however, a later study showed that, at least in some genetic backgrounds, 50% of *TGFβ1*$^{-/-}$ and 25% of *TGFβ1*$^{+/-}$ embryos die at around E10.5. These embryos have defects in the yolk sac vasculature including reduced endothelial cell differentiation and vessel number suggesting a role in vasculogenesis.[100] Transgenic expression of TGFβ1 from a vascular smooth muscle promoter results in dilated blood islands and a possible vasculogenesis defect that is restricted to the extraembryonic tissues.[101] In contrast,

animals deficient in either TGFβ2 or TGFβ3 survive to parturition though both result in perinatal death from lung and various other defects.[102-104] Deletion of the gene for TGFβ Receptor II (TGFβRII) results in an embryonic phenotype identical to *TGFβI* knockouts.[105] Analysis of tetraploid embryos in which the extraembryonic tissues, including the yolk sac endothelial cells, lacked TGFβRII showed that TGFβI signaling is not required for endothelial cell differentiation *per se*, but is necessary for the deposition of Fibronectin (Fn) into the extracellular matrix. Reduced Fn in the yolk sac extracellular matrix results in yolk sac instability.[106]

Other TGFβ Receptor knockouts also exhibit vascular phenotypes. Embryos lacking activin-receptor-like kinase-5, or ALK5, a member of the class I TGFβ receptor family have prominent defects in the remodeling and branching of the yolk sac vessels and these embryos die by around E10.5. Endothelial cells from *ALK5*$^{-/-}$ embryos also show increased proliferation and migration in culture, along with decreased Fn synthesis.[107] Embryos deficient in ALK1 (another class I TGFβ receptor family member) have similar defects, and pericytes/VSMC in these embryos show delayed differentiation and fail to be recruited to the vessel walls.[108] *ALK1*$^{-/-}$ embryos also show abnormal fusion of the capillary beds throughout the embryo resulting in large cavernous vessels as well as arteriovenous malformations (AVMs) that form between the cardinal vein and the dorsal aorta. These AVMs are associated with reduced expression of EphrinB2 (see below for a discussion of EphrinB2) suggesting a role for ALK1 in arterial-venous identity.[109] Interestingly, disruption of the Zebrafish ALK1 homologue, *violet beauregarde*, also causes AVMs.[110] Mutations in the human gene for ALK1 cause

hereditay hemorrhagic telangiectasia type 2 (HHT-2), an autosomal dominant disorder characterized by recurrent hemorrhage, especially in the nasal mucosa and the gastrointestinal tract, and dermal and visceral telangiecstases often leading to AVMs in the lungs, brain, and other organs.[111] ALK1 signals through Smad1 and Smad5 and deletion of either of these genes also leads to comparable vascular phenotypes.[19, 112]

Endoglin, or CD105, is a cell surface accessory protein expressed on endothelial cells that binds TGFβ ligands when associated with TGFβRII.[113, 114] Mutations in the human gene for Endoglin cause hereditary hemorrhagic telangiectasia type 1 (HHT-1) which, in comparison to HHT-2 tends to cause more pulmonary AVMs, a later onset of epistaxis, and carries an increased risk for pulmonary hyertension.[115] Vasculogenesis occurs in mice lacking Endoglin (in contrast to *TGFβ1* null embryos) but these animals die by ~E10.5 with defects in yolk sac vessel remodeling, embryonic and extraembryonic hemorrhage, heart defects including absence of the endocardial cushion and failure of the endocardium to undergo epithelial-mesenchymal transformation at the atrioventricular boundary, and a reduction in pericyte/VSMC numbers and their failed recruitment to vessels.[116-118] Similar to *ALK1* null embryos, *Endoglin* null embryos show arteriovenous malformations between the cardinal vein and dorsal aorta.[119]

ALK1 and ALK5 are both expressed on endothelial cells and it appears that TGFβ signaling from each of these receptors has mutually antagonistic roles in endothelial cell behavior. Signaling through TGFβ/ALK5 inhibits endothelial cell proliferation and migration and promotes differentiation of pericytes/VSMC while

TGFβ/ALK1 signaling promotes endothelial cell proliferation and migration and therefore the balance between the relative activity of these signaling pathways determines the effects of TGFβ on endothelial cells.[120] Analysis of immortalized *Endoglin* null endothelial cell lines by two separate groups suggests that Endoglin plays a key role in determining this balance. One study suggests that loss of Endoglin expression leads to an increase in ALK5 expression resulting in the recruitment of more high affinity ALK1 receptors on the cell surface and hence an increase in endothelial cell proliferation.[121] The other seemingly disparate study showed that loss of Endoglin expression decreased ALK5 expression apparently shifting the balance in favor of TGFβ/ALK1 signaling and resulting in decreased endothelial cell proliferation suggesting that Endoglin serves to promote endothelial cell proliferation.[122] Since ALK5 helps recruit ALK1 into TGFβ signaling complexes and is essential for ALK1 activation, one interpretation of these results is that given this requirement of ALK5 for ALK1 signaling, subtleties in the set up of these two experiments or in the relative expression of ALK1/ALK5 in individual cell lines could lead to opposite results.

Using intact yolk sacs from embryos with either a global *Endoglin* knockout or an endothelial cell-specific knockout of *ALK5* it was shown that *Endoglin* null animals have a defect in paracrine TGFβ signaling from endothelial cells to adjacent pericytes that results in the failure of pericyte differentiation and association with endothelial cells. Addition of exogenous TGFβ1 to cultured *Endoglin* null yolk sacs rescues this phenotype.[123] Embryos with endothelial cell-specific knockouts of either *TGFβRII* or *ALK5* develop similar yolk sac defects as the global knockouts suggesting that the effects

of global disruption of these genes is attributable to their function in endothelial cells at this developmental stage. Neural crest cell specific ablation of TGFβRII results in a DiGeorge syndrome-like phenotype characterized, in part, by a failure of neural crest smooth muscle derivatives to differentiate into VSMC in the cardiac outflow tract, thus providing further evidence that TGFβ signaling is crucial within VSMC for their differentiation in some tissues.[124]

A further level of complexity is added to this system when one appreciates that TGFβ is secreted by endothelial cells in an inactive form that associates with the extracellular matrix and must be proteolytically activated for signaling.[125, 126] One player in this process is the gap-junction protein Connexin43 (Cx43). Gap junctions are membrane protein aggregates that connect the cytoplasm of adjacent cells, allowing the passage of second messengers and other signaling molecules.[127, 128] *Cx43* knockout mice die at birth with defects in the patterning of the coronary vessels including decreased complexity of vessel branching and decreased investment of these vessels with VSMC.[129] *In vitro* studies suggest that although *Cx43* null VSMC precursors synthesize latent TGFβ1 and can differentiate upon addition of exogenous activated TGFβ1, they are unable to form gap-junctions with adjacent endothelial cells and this somehow results in an inability to activate latent TGFβ1.[130] Another connexin family member, Connexin45, is expressed predominantly in the embryonic vasculature, and mice in which this gene has been deleted show vascular remodeling defects and die by ~E10.5.[131] It is tempting to propose that the death of *Cx45* null mice is triggered by similar defects in gap-junction communication between endothelial cells and pericytes/VSMC and the subsequent loss of

activation of latent TGFβ, as in *Cx43* knockouts, though this hypothesis has not yet been addressed.

Platelet-derived growth factor (PDGF) receptors are a family of tyrosine kinase receptors that, like TGFβ and Tie2/Angl, are involved with increasing the stability of nascent vessels. Knockout of the genes for PDGF-B and PDGF receptor-β (PDGFRβ) result in perinatal lethality characterized by renal/glomerular defects, cardiovascular defects in which some large arteries are dilated, and hemorrhage.[132, 133] The unifying defect that explains these phenotypes is a defect in signaling within pericytes/VSMC. This is in contrast to other signaling pathways that regulate pericyte/VSMC function such as Tie2/Angl, TGFβ/Endoglin/ALK1/5, and Edgl (see below for a discussion of Edgl) in which the primary signaling defect is in endothelial cells. PDGF-B is expressed in immature capillary endothelial cells during angiogenesis and PDGFRβ is normally expressed on pericyte/VSMC progenitors that surround the vessels. Pericytes in mice lacking either gene show lower rates of proliferation and fail to migrate toward vessel walls resulting in the decreased mechanical stability of the vessel.[134, 135] Analysis of chimeras in which *PDGFRβ* null cells were introduced into wild type embryos showed that *PDGFRβ* null cells were significantly less capable of contributing to pericyte populations (8 fold less capable) than wild type cells.[136]

Endothelial cell-specific ablation of the *PDGF-B* gene recapitulates the pericyte phenotype seen in the global knockout, albeit with less severity, suggesting that this phenotype is a result of loss of endothelial cell secretion of PDGF-B.[137] Unlike the

PDGF-B global knockouts which die perinatally, the endothelial cell-specific knockouts live to adulthood but show persistent pathologies including microhemorrhages and a proliferative retinopathy that resembles that seen in diabetic patients.[138] Interestingly, it appears that *PDGF-B*$^{+/-}$ mice also show proliferative retinopathy.[139] Additional work has shown that correct localization of PDGF-B to heparin sulphate proteoglycans* in the exracellular matrix via a C-terminal retention sequence is required for proper pericyte investment of the microvascular wall during normal development and tumor vascularization.[140, 141] Certain isoforms of VEGF also contain a similar C-terminal retention sequence, and defects in angiogenic sprouting and branching are observed in embryos in which this sequence is deleted.[142, 143]

Additional regulation of PDGF signaling has been shown to depend upon the interaction of PDGFRβ with other membrane bound proteins. The expression of membrane type 1 matrix metalloproteinase (MT1-MMP) in *cis* with PDGFRβ enhances the induction of PDGF-B signaling in VSMCs leading to VSMC proliferation and chemotaxis, possibly by releasing matrix-bound PDGF-B and making it accessible for signaling.[144] MT1-MMP apparently also regulates TGFβ1 signaling. Newly secreted TGFβ1 exists in a latent state by the non-covalent association of its active domain with

* In Zebrafish, the heparin sulphate proteoglycan (HSPG) syndecan-2 is required for sprouting angiogenesis of the intersommitic vessels and potentiates VEGF signaling. Loss of function mutations in *Drosophila* HSPGs result in the disruption of various signaling pathways including FGF, Wingless, and Hedgehog. These results support the argument that localization of secreted signaling factors by the extracellular matrix is essential for normal development.
[Chen, E., Hermanson, S. & Ekker, S. C. Syndecan-2 is essential for angiogenic sprouting during zebrafish development. Blood 103, 1710-9 (2004).] & [Nybakken, K. & Perrimon, N. Heparan sulfate proteoglycan modulation of developmental signaling in Drosophila. Biochim Biophys Acta 1573, 280-91 (2002).]

its N–terminal propeptide termed latency-associated peptide-β1 (LAP-β1). Integrin $\alpha_v\beta_8$ binds to an RGD motif in LAP-β1 allowing its cleavage by MT1-MMP which releases active TGFβ1 that is capable of engaging in autocrine and paracrine signaling.[145] In the embryo, integrin β_8 is expressed on yolk sac endodermal cells and on periventricular cells of the neuroepithelium, and mice in which the β_8 gene is deleted die in two waves: the first group dies at midgestation from deficient vascularization of the yolk sac, and the second group dies perinatally from brain hemorrhage.[146] It is not known whether defects in TGFβ1 signaling are the underlying cause for the defects observed in β_8 null mice or whether loss of β_8 on endothelial cells or some other cell population is responsible for this phenotype, but this is an intriguing possibility.

Some elegant *in vivo* studies have analyzed the contributions of individual signaling components that are downstream of PDGFRβ such as Shc, PI3-kinase, phospholipase-Cγ, and Ras-GAP.[147-149] In these studies, specific tyrosine residues in the cytoplasmic tail of PDGFRβ that allow binding of various downstream signaling modules were deleted. The resulting phenotypes suggest that there is an additive effect of PI3K and PLCγ binding/signaling on the proliferation of pericytes/VSMC in that by decreasing these downstream signals there is a corresponding decrease in pericyte/VSMC proliferation. Ablation of tyrosine residues that mediate Ras-GAP binding produced the opposite effect and led to an *increase* in the population of these cells. Global deletion of *Ras-GAP* in mice causes lethality at midgestation with vascular patterning defects and hemorrhage.[150] Human patients with inactivating mutations in *Ras-GAP* have AVMs,[151]

but whether the mouse or human *Ras-GAP* phenotypes are attributable to defects in endothelial cell or in pericyte/VSMC function is unclear.

Edg, or endothelial differentiation gene, receptors constitute a G protein-coupled receptor family that signals in response to phospholipids and in some instances, as their name implies, are upregulated in differentiating endothelial cells.[152, 153] Edg1, Edg3, Edg5, Egd6, and Edg8 are receptors for the bioactive lipid sphingosine-1 phosphate (S1P) whereas Edg2, Edg4, and Edg7 are receptors for lysophosphatidic acid.[154] Moreover, Edg1, Edg3, and Edg5 are expressed widely, including on embryonic endothelial cells, while Edg6 and Edg8 expression is restricted mainly to immune and nervous systems[155, 156] and Edg7 is expressed in the prostate.[157] Signaling through Edg1 induces the formation of adherens junctions between cells and increases cadherin expression leading to cellular clumping *in vitro*.[154] Deletion of *Edg1* in mice causes embryonic lethality by E14.5, and *Edg1*$^{-/-}$ embryos have intraembryonic hemorrhages and edematous yolk sacs that, while showing apparently normal branched vessels, are filled with fewer blood cells.[158] Edg1 was found to be expressed in the endothelial cells of arteries and capillaries and not in veins. The large, intraembryonic arteries in *Edg1*$^{-/-}$ embryos have a striking defect in which VSMCs are grouped together on the ventral side of the vessel and fail to completely envelop the vessel. This suggests a role for Edg1 in coordinating VSMC migration. Endothelium-specific ablation of the *Edg1* gene, but not VSMC-specific ablation, recapitulates the phenotype of global *Edg1* deletion suggesting that S1P acting on endothelial cells is required for proper VSMC localization/function.[159]

Further work has shown that S1P signaling through Edg1 in endothelial cells is required for endothelial cells to interact and tightly bind to VSMCs. Normally this is accomplished by homotypic interactions between endothelial cell N-cadherin and VSMC N-cadherin. *Edg1* null endothelial cells have a defect in the proper intracellular trafficking and activation of N-cadherin which is the cause of this phenotype.[160] The importance of S1P regulation of N-cadherin may also manifest itself in additional ways. Endothelium-specific deletion of N-cadherin results in vascular defects and embryonic lethality by around E9.5, with a phenotype that is reminiscent of *VE-cadherin* knockout embryos.[161] VE-cadherin is expressed in endothelial cell adherens junctions[162] and is required for preventing the disassembly of embryonic vessels.[163] Endothelium-specific ablation of β-catenin, a protein that signals downstream of VE-cadherin, also results in poorly organized and branched, fragile vessels and embryonic death at midgestation.[164] Surprisingly, *N-cadherin* null embryos showed a reduction in VE-cadherin expression at adherens junctions. Thus N-cadherin acts upstream of VE-cadherin in endothelial cells raising the possibility that loss of S1P signaling in the various Edg knockouts might adversely effect endothelial cell/endothelial cell interactions via VE-cadherin as well as endothelial cell/VSMC interactions via N-cadherin.[165]

The gene that is defective in the Zebrafish mutant, *miles apart*, is a homologue of the S1P receptor Edg5. *Miles apart* embryos have a non-cell autonomous defect in the migration of heart precursor cells.[166] Mice lacking Edg5 or both Edg3/5 do not show embryonic lethality and die perinatally of unknown causes,[167] though this finding may depend upon genetic background or other factors as another group has reported that *Edg5*

null mice exhibit spontaneous seizures.[168] In order to clarify the roles of Edg receptor signaling in mouse embryos the group that generated the *Edg1* knockouts also generated (in a mouse breeding *tour de force*!) *Edg1/5* and *Edg1/3* double knockouts and *Edg1/3/5* triple nulls.[169] While *Edg1/3* null animals showed no change in phenoptye compared to *Edg1* knockouts, *Edg1/5* null animals showed a more severe, earlier embryonic phenotype than single *Edg1* knockouts that is characterized by abnormal and immature branching of vessels in the head, hemorrhage, and lethality by E13.5. The triple knockout displayed an even more severe, but similar phenotype, resulting in death by E11.5. Since Edg1 signals through $G\alpha_i$, and Edg3 and Edg5 signal through $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12/13}$[170] it is possible that the severe triple knockout phenotype is due to loss of redundant downstream signals such as those that emanate from $G\alpha_i$. One important question that remains to be settled is whether, like in *Edg1* nulls, the triple null phenotype results from VSMC dysfunction secondary to loss of S1P signaling in endothelial cells, or whether there are additional requirements for other Edg receptors in endothelial cells and/or VSMC for angiogenesis.

Indeed, there is evidence that S1P signaling has direct effects on endothelial cell function and on angiogenesis. Endothelial cell-specific *Edg1* null mice show defects in limb development including abnormal chondrocyte condensation and digit formation. There was excessive vascularization in these limbs and a coincident upregulation of VEGF and HIF-1α (see below for discussion of HIF-1α).[171] One interesting study showed that S1P (signaling through Edg1 and/or Edg3) potentiates the effects of VEGF on angiogenesis and that S1P increases the maturation of these neovessels as indicated by

their increased production of extracellular matrix and VE-cadherin-containing adherens junctions.[172] By itself, VEGF has been shown to disrupt VE-cadherin aderens junctions and thus it is possible that the balance between the stabilizing effects of S1P signaling and the destabilizing effects of VEGF on these junctions plays an important role in angiogenesis and vascular remodeling.[173] S1P/Edg signaling is also required for EC chemotaxis possibly by playing a role in priming the cortical actin cytoskeleton to correctly respond to additional chemotactic clues.[174] Not to be left out, it appears that S1P also has effects on PDGF signaling. S1P signaling has been found to upregulate PDGF-B expression in VSMCs.[175] S1P treatment of adult rat VSMC inhibits PDGF-mediated chemotaxis[176] and PDGF signaling can, in some instances, increase the production of S1P,[177] though it is not currently known if interactions between the PDGF and S1P signaling pathways account for the observed phenotypes in either Edg or PDGF receptor knockouts. Taken together, these data suggest that S1P signaling through Edg receptors play a central role in coordinating the functions of endothelial cells and VSMCs during angiogenesis at least in part by modulating their responses to other angiogenic factors such as VEGF and PDGF.

Chapter 1e: Additional molecules involved in the regulation of angiogenesis, including G protein-coupled receptors:

The above discussion, though relatively thorough, does neglect some interesting and important players that act primarily in the regulation of angiogenesis. In the next few paragraphs, I will discuss these molecules. I will then end Chapter I with a brief

overview of G protein-coupled receptor signaling, and in particular the role of thrombin signaling through one of its receptors, PAR1, in vascular development,

HIF-1α is a transcription factor that plays a fundamental role in how cells and tissues perceive and regulate oxygen homeostasis. It is therefore not surprising that HIF-1α is a central regulator of angiogenesis. The *Drosophila* tracheal system is in some ways analogous to the vertebrate vascular system. Trachea form when cells on the larval surface invaginate to form tubules which then extend and branch into the larval body to supply target tissues with oxygen. Null mutations in the *Drosophila HIF-1α* gene, *trachealess*, fail to form trachea.[178] The *Drosophila* FGF homologue, *branchless*, has been shown to be downstream of the HIF-1α signal and is thought to be secreted by oxygen-deprived tissues as a chemotactic cue for tracheal tip cells.[179] Mouse embryos lacking HIF-1α exhibit defects in vascular patterning that are visible as early as E8.5 including the absence of cephalic and branchial arch vascularization, perhaps due to the fact that the head vasculature is formed by angiogenic sprouting from vessels in the trunk.[180, 181]

HIF-1α forms active heterodimers with the basic-helix-loop-helix transcription factor arylhydrocarbon-receptor-nuclear translocator, or ARNT. Mice in which ARNT is deleted show a similar phenotype, with the additional observation that the embryonic component of the placenta fails to vascularize.[182, 183] This decrease in placental vascularization is associated with decreased expression of Flk1 and Tie2 in the embryonic placental vasculature. It has been known for some time that reduced oxygen

tension increases VEGF expression[184] and it is now known that HIF-1α mediates this effect by stimulating VEGF gene expression.[185, 186] A closely related hypoxia inducible factor, HIF-2α, which also forms active heterodimers with ARNT is capable of activating transcription of VEGF as well as the genes for Flk1 and Tie2.[187-189] Thus, the association of HIF-1α and/or HIF-2α with ARNT seems to be a crucial regulatory step for multiple stages in angiogenesis.

Integrin signaling is also required at multiple stages of angiogenesis. $\alpha_v\beta_3$ integrin has historically been thought to be crucial for blood vessel formation as it is predominantly expressed on endothelial cells and since blockade of $\alpha_v\beta_3$ with monoclonal antibodies inhibits blood vessel formation.[190] The engagement of $\alpha_v\beta_3$ integrins with extracellular matrix components (*i.e.*, vitronectin) *in vitro* has been shown to enhance, in *cis*, VSMC proliferation and migration, though the mechanism of this is not clear.[191] It also appears that $\alpha_v\beta_3$ surface expression is upregulated by HIF-1α in hypoxic conditions in certain tumors though it is not known whether this regulation is by direct transcriptional activation of the genes for $\alpha_v\beta_3$ by HIF-1α or whether this is important for embryonic angiogenesis.[192] Coexpression of $\alpha_v\beta_3$ with Flk1 has also been shown to increase the ability of VEGF to activate Flk1 and downstream mediators such as PI3-kinase.[193] Conversly, VEGF has been shown to activate $\alpha_v\beta_3$ and other integrins in cancer cells.[194] Coexpression of $\alpha_v\beta_3$ with PDGFRβ also potentiates the signaling activity of PDGF-B.[191] Counterintuitively, ablation of α_v integrins either globally or in endothelial cells has no effect on angiogenesis or on vessel stability, but α_v deletion in glia and neurons using a *Nestin-Cre* transgene leads to brain hemorrhages and axonal

degradation.[195, 196] And in β_3 null and β_3/β_5 double null animals there are no embryonic angiogenesis defects and there are actually increased tumor angiogenic responses to hypoxia and VEGF coincident with increased expression of Flk1 on endothelial cells.[197, 198] Given our current knowledge, it not clear how to resolve the discrepancy between these apparently incongruous effects of $\alpha_v\beta_3$ function in these various systems and assays.

An interesting body of work has emerged in recent years describing another facet of vascular development, the establishment of arteriovenous identity. Arteries and veins are defined by the direction of blood flow and by their morphology and function. It is now clear that arterial and venous endothelial cells also differ in their molecular characteristics. Notch signaling is required nearly ubiquitously in the animal kingdom for its role in determining cell fate decisions in such tissues as muscle, neurons, hematopoietic cells, and epithelium. It is probably not surprising that it also plays a role in cell fate decisions in vascular development. Notch is a cell surface receptor for its cell surface ligands, including Delta and Jagged. Activation of Notch results in its proteolytic cleavage by extra- and intracellular proteases, resulting in the release of an intracellular Notch domain that can then enter the nucleus and directly participate in transcriptional activation of target genes including, in mice, the transcriptional repressors Hey1 and Hey2. Notch expression is thought to be induced on arterial endothelial cells by VEGF binding to Flk1 and NP1.[199, 200] Compound deletion of Notch1/Notch4 from mice results in defects in angiogenic remodeling in the embryo proper, the yolk sac, and the placenta and these embryos fail to form the dorsal aortae and cardinal veins.[201] Embryos with a

gain of function Notch4 also show vascular defects, primarily in the head vessels though the significance of this in terms of the role of Notch signaling in the vasculature is not known.[202]

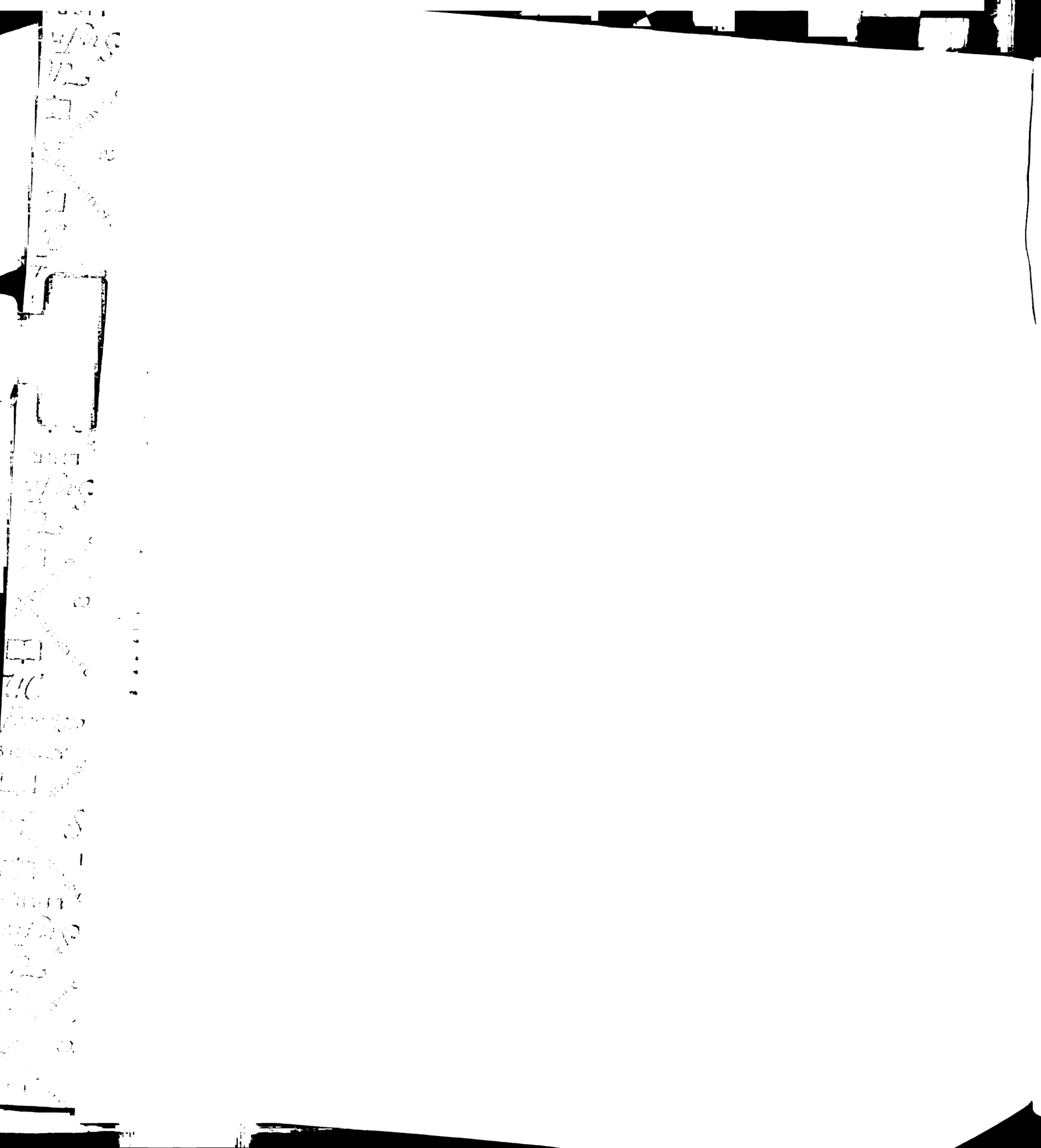
Deletion of the Notch1 ligand Jagged1 in mice also results in vascular defects,* and interestingly the carotid vessels form (in contrast to Notch1 nulls) but they subsequently collapse.[203] The murine Notch ligand Delta-like ligand-4 (Dll4) is expressed primarily in large arteries of the embryos. Haploinsufficiency of Dll4 results in defects in arterial specification and angiogenesis.[204, 205] Double knockout of the downstream transcriptional repressors *Hey1* and *Hey2* results in a similar reduction in arterial specification in the mouse[206] as does reduced expression of *gridlock*, a gene that is downstream of Notch in Zebrafish.[207] The Notch pathway is not active in veins, so how is venous identity determined? Deletion of the orphan nuclear receptor COUP-TII results in defects in vascular development that are associated with an increased expression of Notch1 and Notch signaling mediators.[208, 209] COUP-TFII expression was found to be restricted to veins, and ectopic expression of COUP-TFII throughout the endothelium was found to suppress Notch signaling leading to a suppression of arterial identity. Previous models have proposed that venous identity is, by default, determined by the

* Mutations in human Jagged1 cause Alagille syndrome, an early onset autosomal dominant disease characterized by defects in multiple organ systems. Cardiovascular system defects include congenital heart defects, pulmonary stenosis, and coarctatinon of the aorta.
[Li, L. et al. Alagille syndrome is caused by mutations in human Jagged1, which encodes a ligand for Notch1. Nat Genet 16, 243-51 (1997)] & [Oda, T. et al. Mutations in the human Jagged1 gene are responsible for Alagille syndrome. Nat Genet 16, 235-42 (1997).]

absence of Notch signaling. These results, however, suggest that venous identity is regulated by active repression of Notch by COUP-TFII in venous endothelial cells.

Notch also is involved in VSMC fate determination. Notch-3 has been shown to be required for arterial VSMC determination, but not endothelial cell determination. Arteries in certain vascular beds in *Notch-3* null adults show reduced VSMC numbers.[210] Some mutations in human Notch-3 are associated with an inherited vascular dementia, "cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy" ("CADASIL"), which is characterized by the progressive degeneration of VSMC in small cerebral arteries.[211]

Ephrins and their Eph receptors are cell surface proteins that have, at least initially, been mainly studied as axon guidance cues in the developing nervous system.[212] Ephrin/Eph signaling is also involved in arteriovenous identity, and likely acts downstream of Notch to control the spatial organization of arteries and veins. EphrinB2 and its receptor EphB4 also have been found to have a role in delineating the arteriovenous boundary early in embryogenesis. EphrinB2 is expressed in the endothelial cells of arteries while EphB4 is expressed in endothelial cells of veins and deletion of either gene in mice leads to death by ~E10.0 with widespread defects in angiogenesis throughout the embryonic and extraembryonic tissues.[213-216] The roles of EphrinB2/ EphB4 interactions in vascular development may not be limited to endothelial cells. There is evidence from studies in chick embryos that suggests that EphrinB2/EphB4 signaling may also involve pericyte/VSMC interactions with endothelial cells.[217] In



Xenopus laevis EphrinB2/EphB4 signaling is required for the migration of intersomitic vessels, a defect that was also observed in the mouse knockouts.[218] Interestingly, this study found that in *Xenopus* EphB4 is expressed in endothelial cells while the EphrinB ligands are expressed in the somites *adjacent* to the migratory paths followed by these vessels. Therefore, Ephrin/Eph interactions not only help define arteriovenous boundaries, but are also likely involved in mediating guidance cues to migrating vessels/endothelial cells in a way that is analogous to the role of Ephrin/Eph interactions in guiding axonal migrations.

Chapter 1f: G protein-coupled receptors in vascular development:

With the exception of Edg receptor family signaling, which has already been discussed, significantly less is known regarding the roles of G protein-coupled receptors (GPCRs) in cardiovascular development despite their diversity and ubiquity. In the following section, I will discuss several addional GPCRs that are involved in vascular development.

Endothelins are soluble factors that were discovered by virtue of their powerful vasoconstrictive effects.[219] Three isoforms exist, ET1, ET2, and ET3 and all are synthesized as precursors which are proteolytically cleaved and activated by the membrane-bound metalloproteases, endothelin-converting enzymes (ECE1 and ECE2). ETs signal through two GPCRs, ET_A which preferentially binds ET1, and ET_B which binds all three.[220] Additional roles for ET signaling were revealed in mice lacking the

converting enzymes, the receptors, or the endothelins themselves. ET_B null mice show defects in gut autonomic innervation resulting in aganglionic megacolon which resembles the human condition Hirschprung's disease.[221] ET_A knockouts have defects in neural crest cell (NCC) derivatives resulting in craniofacial and cardiac outflow tract abnormalities.[222] ET-1 is expressed in the pharyngeal arch epithelium, the paraxial mesoderm-derived arch core, and the arch vessel endothelium whereas ET_A is reciprocally expressed in NCCs suggesting that paracrine signaling of ET-1 from multiple tissues is essential in patterning NCC derived cells. Mice in which ECE1 and ECE2 are deleted have comparable defects to ET_A knockouts.[223] In the embryo, each branchial arch artery forms bilaterally but then undergoes assymetrical regression in order to establish correct circulation. The branchial arch arteries in ET_A and ECE-1 null embryos form properly and symmetrically but exhibit an abnormal pattern of asymmetrical regression thus providing an anatomic basis for the observed cardiac outflow tract defects.[224]

The chemokine receptor CXCR4 is a GPCR for stromal-derived factor-1, or SDF1. Embryos that lack CXCR4 or SDF1 die between E15.5 and just after birth with defects in the generation of the large vessels supplying the gastrointestinal tract in addition to defects in neuronal migration and the formation of the ventricular septum of the heart.[225-227] SDF1 and CXCR4 are expressed by endothelial cells, and their expression is increased by VEGF. In an *in vitro* Matrigel-based model of tube formation and branching morphogenesis showed that blockade of SDF1 or CXCR4 with inhibitory antibodies or pertussis toxin (which inhibits $G\alpha_i$) prevents network, and purportedly tube,

formation.[228] Additional studies could determine whether SDF1 and CXCR4 also function in branching morphogenesis and tubulogenesis in embryonic angiogenesis.

The Hedgehog signaling pathway has also been shown to play a role in vascular development. Hedgehog signaling has been shown to be essential for the proper patterning and morphogenesis in many species.[229] In the mouse there are three Hedgehog homologues, Sonic Hedgehog (*Shh*), Desert Hedgehog (*Dhh*), and Indian Hedgehog (*Ihh*). These bind to a 12-pass membrane protein, Patched, which associates with a G protein receptor-*like* membrane protein, Smoothened (*Smo*). Binding of Hedgehog ligands to Patched is thought to release its inhibition on Smoothened thus allowing *Smo* to signal to downstream mediators to effect transcriptional changes. The immediate downstream effectors of Smoothened are unknown though there is some evidence that these are heterotrimeric G proteins.[230] The yolk sacs of $Smo^{-/-}$ embryos contain endothelial tubes that fail to remodel beyond the primary plexus stage and therefore appear stunted at ~E8.5. $Smo^{-/-}$ embryos also fail to form the dorsal aortae, suggesting an early, central role for *Smo* in vessel formation.[231] Yolk sacs from $Ihh^{-/-}$ embryos show a less severe phenotype, in that there is remodeling of the primary vascular plexus, but there are fewer and smaller vessels.[232] *Shh* null Zebrafish embryos also show defects in axial vessels which are even more severe than those seen in *VEGF* null embryos.[233] Hedgehog ligands are secreted from the mouse yolk sac visceral endoderm at early embryonic stages, and it appears that hedgehog signaling is an early step in a signaling cascade that leads to increased VEGF signaling and ultimately Notch signaling which participates in determining arterial fate.[234]

We recently showed that protease-activated receptor-1 (PAR1), a GPCR for thrombin, plays an important role in the proper formation and/or maintenance of blood vessels in mouse embryos.[235, 236] In the embryo, PAR1 is expressed predominantly in the endothelium and endocardium, but is also found to be expressed in circulating blood cells, the mesenchyme of the hindlimbs, and the septum transversum which is the precursor of the diaphragm. About half of *Par1* null embryos die at midgestation with grossly normal embryonic vascular patterning but with defective yolk sac remodeling, hemorrhage in multiple tissues, and dilated pericardium. *Par1* null embryos that do not die *in utero* survive to adulthood with no obvious phenotype. Rescue of these embryonic defects by endothelium-specific expression of PAR1 from a Tie2 promoter/enhancer transgene suggests that the vascular defects observed in *Par1* null embryos result from loss of PAR1 in endothelial cells. Thus, PAR1 function is required at an earlier stage in angiogenesis than any other classical (*i.e.*, not including Patched/Smoothened) GPCR discussed here.

Heterotrimeric G proteins transduce signals from GPCRs and can be divided into four families based on sequence and functional similarity: $G\alpha_i$, $G\alpha_s$, $G\alpha_q$ and $G\alpha_{12/13}$. PAR1 can trigger a host of cellular responses by activating heterotrimeric G proteins of the $G\alpha_{q/11}$, $G\alpha_{i/o}$, and $G\alpha_{12/13}$ families.[237-240] The $G\alpha_{12/13}$ family consists of two proteins, $G\alpha_{12}$ and $G\alpha_{13}$ which share about 70% amino acid identity.[241] Although the signaling pathways that are regulated by $G\alpha_{12}$ and $G\alpha_{13}$ are incompletely understood, studies in cell culture have shown that $G\alpha_{12/13}$ can regulate actin stress fiber formation,[242] focal

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adhesions,[243, 244] Na^+/H^+ exhange,[245] transcription,[246] transformation,[238, 247, 248] and apoptosis.[249, 250] The best characterized downstream mediator of $G\alpha_{13}$ signaling is Lsc (also called p115RhoGEF) which links $G\alpha_{13}$ to Rho[251, 252] to regulate cell shape and chemotaxis.[253]

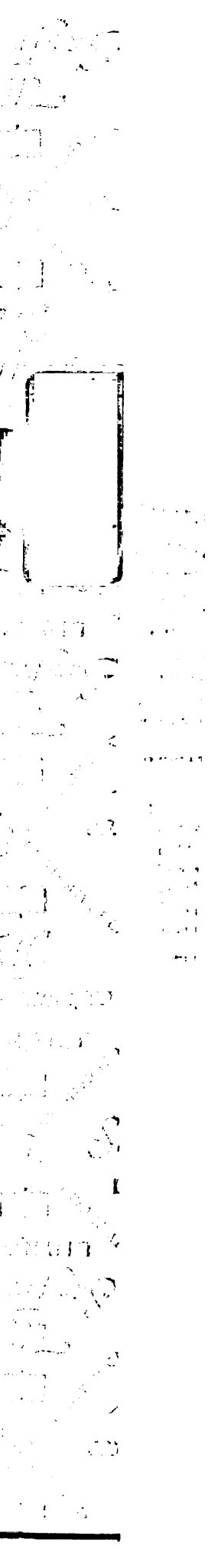
$G\alpha_{13}$-deficient mice fail to form a functional vascular system and die at midgestation.[254] Discontinuities in the endothelial lining, a failure to remodel their yolk sac vascular plexus into a branched vascular tree, and abnormally large and ectopic head vessels were noted in *$G\alpha_{13}$*-deficient embryos. These phenotypes are reminiscent of, but occurred with more penetrance than, those seen in *Par1* deficient embryos. Although $G\alpha_{13}$ is reported to be widely expressed in the embryonic and adult mouse tissues,[254, 255] using a *$G\alpha_{13}^{lacZ}$* knockin allele we observed relatively high expression of $G\alpha_{13}$ in endothelial cells and other specific cell types at midgestation, including cardiac, smooth, and skeletal muscle, and in platelets. Accordingly, we asked whether $G\alpha_{13}$ function downstream of PAR1 or other GPCRs in endothelial cells might play a necessary or sufficient role in vascular development. Our results strongly suggest a key role for GPCR signaling via $G\alpha_{13}$ in endothelial cells in this critical process. In addition, our studies uncovered an important function for $G\alpha_{13}$ in lineages where *Wnt1* is expressed, consistent with a role for GPCR signaling in the development of structures derived from neural crest.

Chapter II:

RESULTS

$G\alpha_{13}$ expression in the mouse embryo and in the adult:

Toward characterizing $G\alpha_{13}$ expression in the mouse embryo, we generated a mouse in which the coding sequence for β-galactosidase (*lacZ*) was inserted into exon 1 of the $G\alpha_{13}$ gene ($G\alpha_{13}^{lacZ}$ allele; Figure 1) such that *lacZ* transcription would mimic that of $G\alpha_{13}$. Whole mount X-gal staining of an E10.5 $G\alpha_{13}^{+/lacZ}$ embryo suggested widespread expression, but analysis of tissue sections from these embryos revealed the staining to be non-uniform (Figure 2B). While faint staining was indeed present throughout the mesenchyme and elsewhere, relatively strong staining was noted in vascular endothelial cells and in the dorsal neuroepithelium; analysis of embryos bearing an endothelium-specific *Tie2-promoter/enhancer-lacZ* transgene showed similar endothelial staining but not neuroepithelial or widespread mesenchymal staining (Figure 2C). $G\alpha_{13}$ expression in endothelial cells was also readily detected by Northern blot using endothelial cells immunopurified from neonatal mice as a source of RNA[256] (data not shown) and by staining for β-galactosidase in such cells from $G\alpha_{13}^{+/lacZ}$ mice (Figure 2B e,f). While these results confirm widespread expression of $G\alpha_{13}$ in the mouse embryo at midgestation[254] they suggest relatively higher expression of $G\alpha_{13}$ in endothelial cells and in the dorsal neuroepithelium in the region where neural crest cells arise and delaminate (Figure 2B g,h). These findings supported our initial focus on defining the importance $G\alpha_{13}$ function in endothelial cells and our subsequent focus on neural crest.



As stated above, $G\alpha_{13}$ expression was found to be non-uniform in embryos at midgestation. To further characterize $G\alpha_{13}$ expression, frozen sections of various tissues from control and $G\alpha_{13}^{+/lacZ}$ mice were stained for β-galactosidase (Figure 3). Indeed $G\alpha_{13}$ is also expressed non-uniformly in adult tissues. Strikingly, $G\alpha_{13}$ appears to be strongly expressed in all muscle lineages as we observed expression in cardiomyocytes and in multiple smooth muscle and skeletal muscle populations (Figure 3 B,C,D,E,G,H,J). Expression was found in vascular smooth muscle cells lining the aorta as well as in other arteries (Figure 3 E,F,G). Smooth muscle lining the trachea and the small bowel expresses $G\alpha_{13}$ (Figure 3 C,D) as does the smooth muscle capsule and trabeculae that surrounds and extends into the mouse spleen (Figure 3 G,H). Skeletal muscle in the trachea and the intercostal muscles also were β-galactosidase positive (Figure 3 C,J). Megakaryocyte expression of $G\alpha_{13}$ was observed in the splenic red pulp (Figure 3 H,I). Using FACS analysis to identify β-galactosidase-expressing cells, $G\alpha_{13}$ was also found to be expressed in all major adult hematpoietic lineages including B and T lymphocytes, macrophages, granulocytes (data not shown). Strong $G\alpha_{13}$ expression was also observed in the epidermis and hair shaft (Figure 3 K). These results suggest that $G\alpha_{13}$ has roles on multiple tissues in the embryo and the adult though analysis of these roles was beyond the scope of this project.

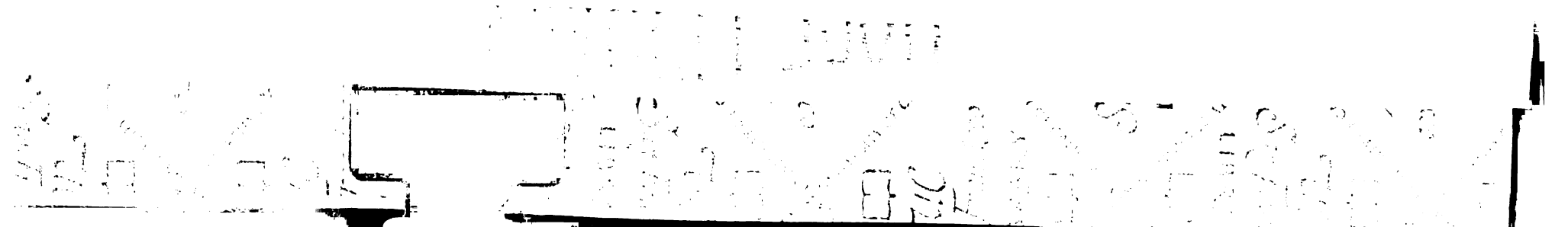


Figure 1: Generation of a $G\alpha_{13}^{lacZ}$ allele

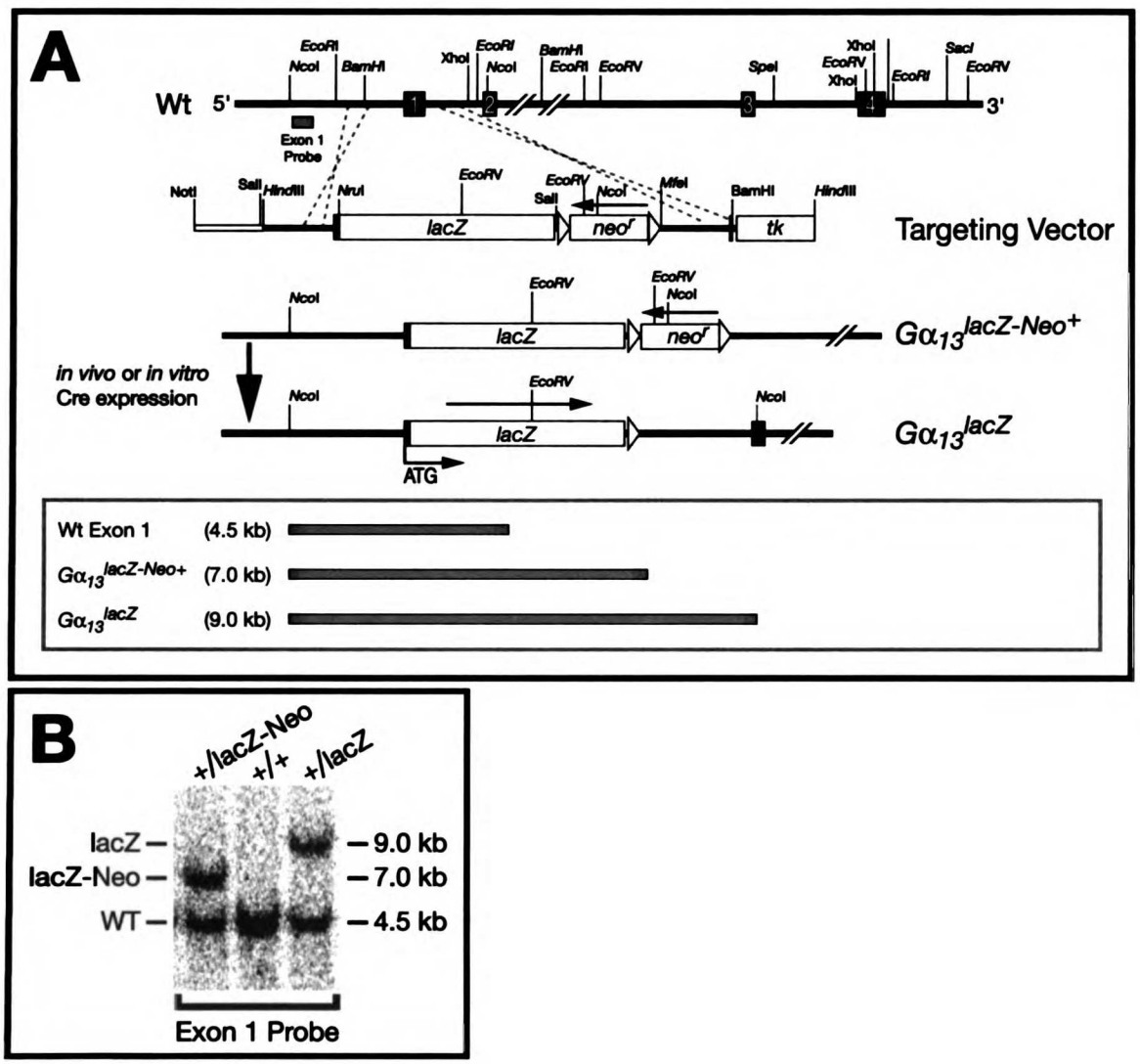


Figure 1 Legend:

A. Diagram of the wild-type (Wt) $G\alpha_{13}$ locus, the $G\alpha_{13}^{lacZ}$ targeting vector, and the recombined locus. Exons (coding in black, untranslated in open boxes), loxP sites (open triangles), and neomycin resistance (*neo*^{r}) and thymidine kinase negative selection (*tk*) cassettes are shown. The targeting vector contains a *lacZ*-Sv40pA cassette fused in frame to the first 45 base pairs of exon 1 (including the native start codon). Homologous recombination in embryonic stem (ES) cells resulted in the replacement of exon 1 with the $G\alpha_{13}$::*lacZ*-Sv40pA fusion sequence, thus generating a null $G\alpha_{13}$ allele. The *neo*^{r} cassette was excised *in vivo* by breeding homozygous $G\alpha_{13}^{lacZ\text{-}Neo^+}$ mice to β-*actin-Cre*$^{Tg/Tg}$ mice. (see **Materials and Methods**)

B. Southern blot analysis of tail DNA from adult mice with the indicated $G\alpha_{13}$ genotypes. Location of the "Exon 1" probe and the expected sizes of DNA fragments detected by this probe after an *Nco*I digest are indicated in (**A**).

Figure 2: Phenotype of $G\alpha_{13}^{lacZ/lacZ}$ embryos and expression of $G\alpha_{13}$-*lacZ* in endothelium and neuroepithelium.

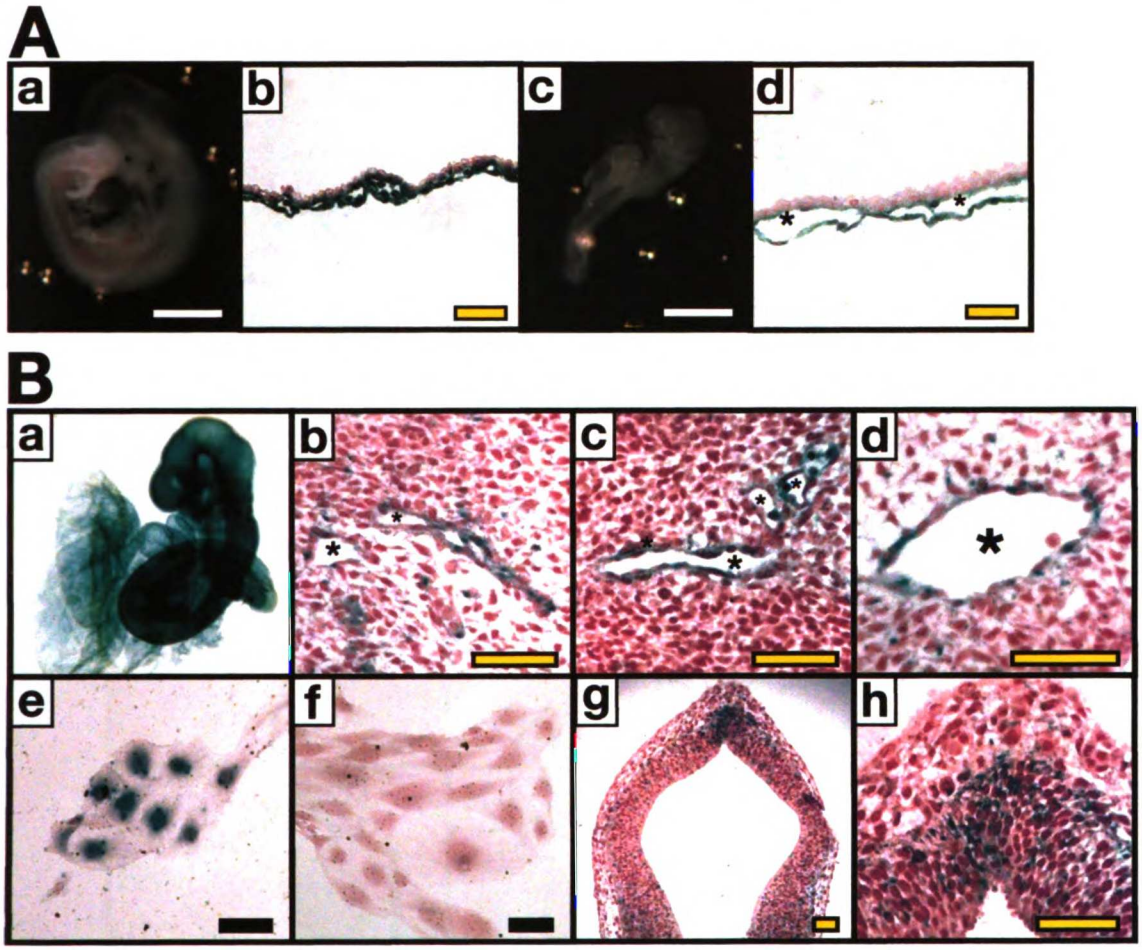


Figure 2 Legend:

A. $G\alpha_{13}^{lacZ/lacZ}$ embryos (**c**) and yolk sacs (**d**) phenocopy $G\alpha_{13}^{-/-}$ emrbyos (see **Figures 7, 8,** and **9** for $G\alpha_{13}^{-/-}$ photos). Compare to littermate control embryo (**a**) and yolk sac (**b**). Additionally, the yolk sacs were stained with X-gal for β-galactosidase activity and reveal $G\alpha_{13}^{lacZ}$ expression in the endothelium. Vessel lumina are indicated with an asterisk in (**d**) and in **Figure 2B**, (**b,c,d**).

B. (**a**) $G\alpha_{13}^{lacZ}$ expression in an X-gal stained E9.5 $G\alpha_{13}^{+/lacZ}$ embryo appears to be ubiquitous in whole mount. (**b,d**) However, analysis of tissue sections from an E10.5 $G\alpha_{13}^{+/lacZ}$ embryo revealed weak staining throughout the mesenchyme and stronger staining in the endothelium of (**b**) intersomitic and (**d**) carotid vessels. (**c**) By comparison, staining of a *Tie2p/e-Cre*$^{Tg/o}$; *ROSA26R* embryo showed endothelial but not mesenchymal staining. (**e,f**) Strong staining of endothelial cells purified from neonatal $G\alpha_{13}^{+/lacZ}$ mice (**e**) compared to those purified from $G\alpha_{13}^{+/+}$ mice (**f**). (**g,h**) Relatively strong X-gal staining was also seen in the dorsal neuroepithelium of E9.5 $G\alpha_{13}^{+/lacZ}$ embryos consistent with $G\alpha_{13}$ expression in neural crest cells. (Yellow bars = 100 μm; White bars = 1 mm; Black bars = 10 μm.)

Figure 3: Expression of $G\alpha_{13}^{lacZ}$ in adult tissues.

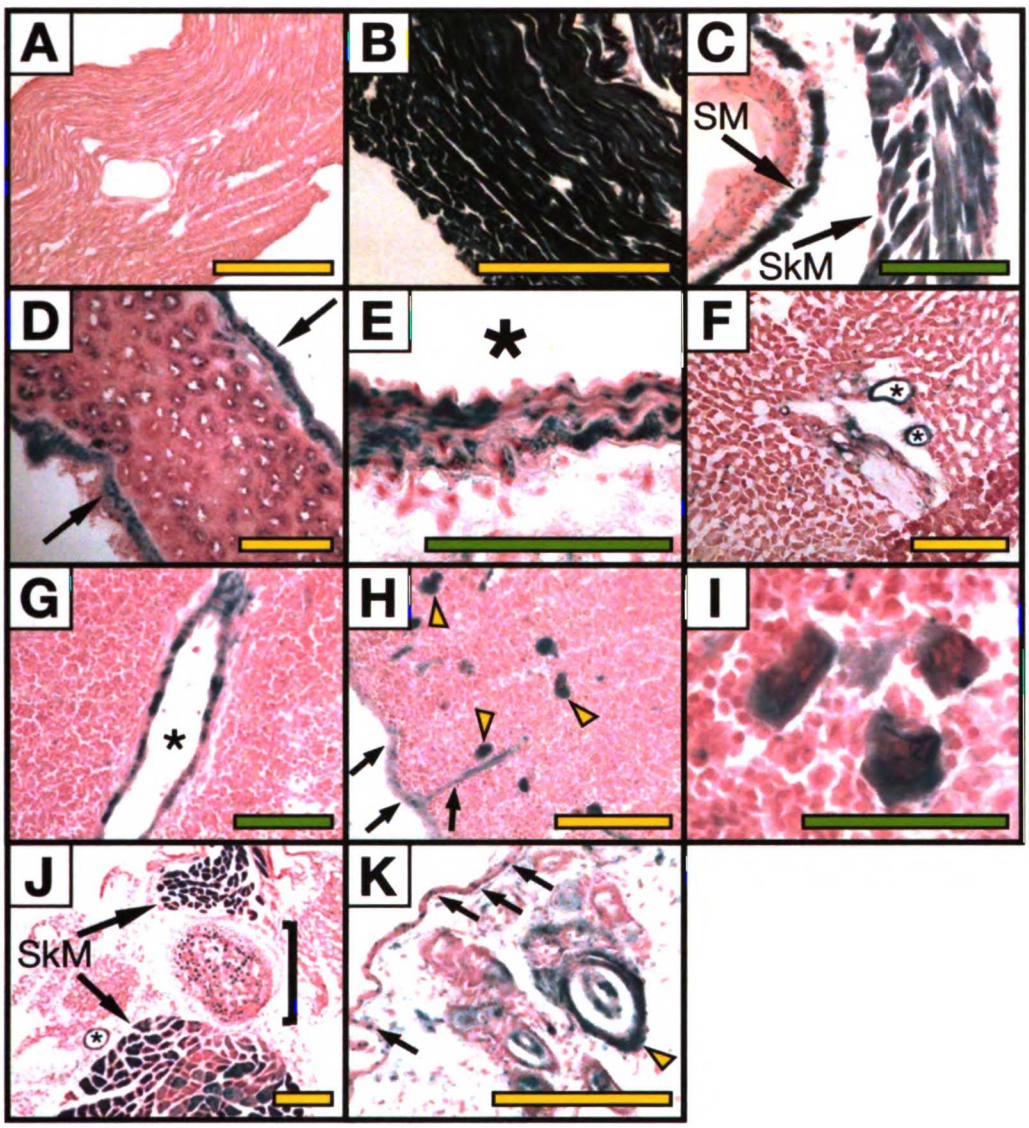


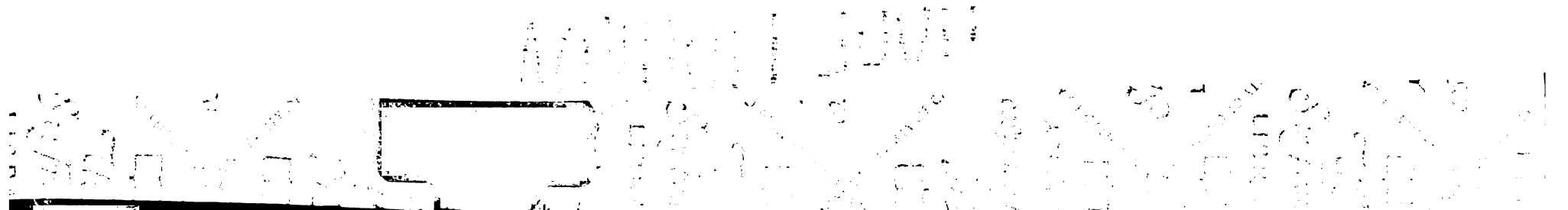
Figure 3 Legend:

X-gal-stained frozen sections from a Wt mouse (**A**), and from $G\alpha_{13}^{lacZ/+}$ mice (**B-K**). (**A**,**B**) Myocardium. (**C**) Trachea showing smooth muscle (SM) and skeletal muscle (SkM) expression. (**D**) Longitudinal small bowel section showing predominately smooth muscle staining (arrows). (**E**) Aorta, showing smooth muscle cell expression. Vessel lumina are indicated with an asterisk in all appropriate panels. (**F**) Liver showing vascular smooth muscle staining, and no detectable hepatocyte staining. (**G**,**H**,**I**) Spleen with vascular smooth muscle staining (**G**), capsular smooth muscle (**H**, arrows), and megakaryocytes (yellow arrowheads in **H**, and magnified in **I**). (**J**) Cross section through rib-cage showing skeletal and arterial smooth muscle staining, as well as staining within the rib (bracket). (**K**) Cross section of skin showing expression predominately in the root and hair sheath (yellow arrowhead), in addition to punctuate staining in keratinocytes (arrows). (Yellow bars = 200 μm; Green bars = 100 μm.)

Chimera analysis:

To begin to address the role of $G\alpha_{13}$ in embryonic development we generated embryonic stem cells (ES cells) homozygous for the targeted $G\alpha_{13}^{lacZ}$ allele. $G\alpha_{13}^{lacZ/lacZ}$ embryos phenocopied $G\alpha_{13}$ null embryos suggesting that our *lacZ* knockin allele is in fact a null allele (Figure 2A). Homozygous $G\alpha_{13}^{lacZ}$ ES cells were generated by selecting for a gene conversion event among heterozygous $G\alpha_{1}^{lacZ}$ ES cells (see Materials and Methods, and reference[257]). Embryonic chimeras were generated by blastocyste injection using 2 homozygous $G\alpha_{13}^{lacZ}$ ES clones ("4A3" and "4C1") and 1 heterozygous $G\alpha_{13}^{lacZ}$ ES clone ("B3"). The neomycin resistance cassette was removed from the targeted $G\alpha_{13}^{lacZ}$ locus in each of these 3 clones. Embryos with 90% or greater chimerism for $G\alpha_{13}^{lacZ/lacZ}$ cells phenocopied global $G\alpha_{13}$ null embryos (data not shown) and analysis was centered on embryos with 20-75% chimerism (Figure 4).

Analysis of 4A3 and 4C1 chimeras suggests that $G\alpha_{13}$ null cells are capable of differentiating into endocardial cells or endothelial cells in the embryonic or extraembryonic tissues. On gross, histological inspection these $G\alpha_{13}$ null endothelial cells were morphologically normal. In fact 4A3 $G\alpha_{13}$ null cells were often observed to constitute the majority of endothelial cells in some vessels (Figure 4 B,C,E,F,H,I). Whether or not this is a generalized feature of $G\alpha_{13}$ null cells is not apparent from this analysis as $G\alpha_{13}$ null cell chimeras from a second ES clone, 4C1, although able to contribute to normal endothelium did so in a reduced capacity compared to 4A3 chimeras (Figure 4 J-L). A likely reason for this is that, among the limited number of chimeric embryos generated with 4C1 cells, there were no high % chimeras (which were routinely



observed in 4A3 chimeras) and as a result there were simply fewer $G\alpha_{13}$ null cells available to contribute to endothelium. Another possibility is that there were phenotypic differences between the 4A3 and 4C1 ES clones. In heterozygous $G\alpha_{13}^{lacZ}$ ES clone ("B3") chimeras we also observed normal incorporation into EC populations, although again the limited number of chimeras obtained with this clone were of low % (Figure 4 M,N).

$G\alpha_{13}$ null and heterozygous ES cells were also able to contribute to various other cell types including the neuroectoderm (Figure 4 D,E,G,H) and mesenchyme (Figure 4 A-L). $G\alpha_{13}$ null ES cells did not appear to contribute to circulating cell populations even in high percentage chimeras (Figure 4 A-C, F,L). β-galaclactosidase-positive rounded cells that resemble hematopoietic cells but are associated with or adherant to the endothelium/endocardium were observed in $G\alpha_{13}$ null ES chimeras (Figure 4 A,J; arrowheads). This raises the possibility that $G\alpha_{13}$ plays a role in embryonic hematopoiesis though our work did not pursue this possibility. In sum, we were unable to find any cell-autonomous or cell-non-autonomous defects in $G\alpha_{13}$ null cells in contributing to normal endothelial cell populations. One possibility is that $G\alpha_{13}$ has functions that are required after the endothelium is already formed and that cannot be discerned by histological analysis alone.

Figure 4: Analysis of the contribution of $G\alpha_{13}^{lacZ/lacZ}$ cells to the endothelium in midgestation embryos.

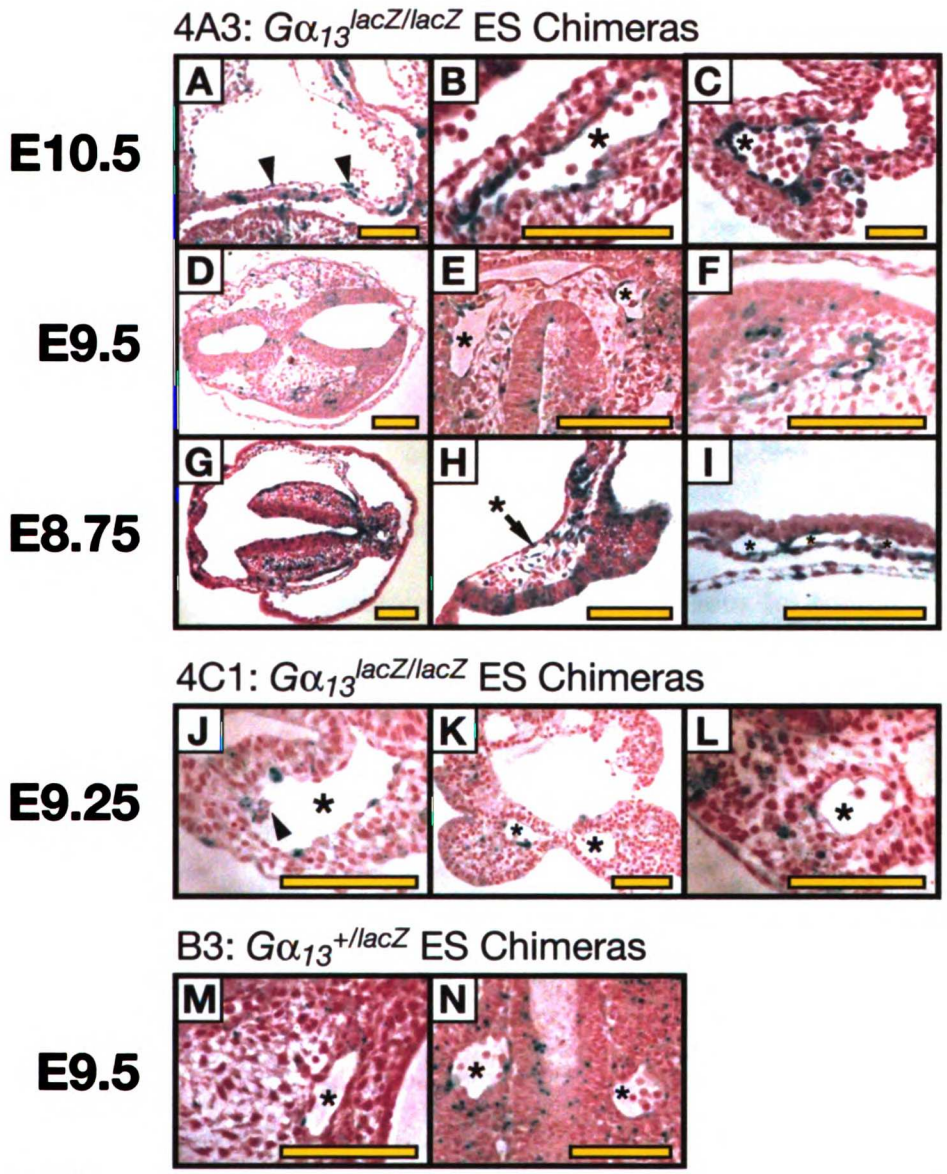


Figure 4 Legend:

Sections of whole-mount β-galactosidase stained $G\alpha_{13}^{lacZ/lacZ}$ (**A-L**) and $G\alpha_{13}^{+/lacZ}$ (**M,N**) embryonic chimeras of the indicated age that were generated by injecting ES cells of the indicated genotypes into Wt blastocysts. $G\alpha_{13}^{lacZ/lacZ}$, and hence $G\alpha_{13}$ null, cells in chimeras derived from ES clone 4A3 (**A-I**) are capable of contributing to normal looking endocardium (**A**, arrowheads) and endothelium (**B,C,E,F,H,I**). Vessels are indicated by an asterisk. $G\alpha_{13}^{lacZ/lacZ}$ null cells also contribute to mesenchyme and neuroepithelium. $G\alpha_{13}^{lacZ/lacZ}$ cells are also able to contribute to the endothelium in chimeras derived from a separate ES clone 4C1 (**J-L**). In control experiments, heterozygous $G\alpha_{13}^{+/lacZ}$ cells in chimeras made from clone B3 ES cells were also observed to incorporate into endothelium (**M,N**). (Yellow bars = 100 μm.)

Characterization of a conditional $G\alpha_{13}$ allele:

We generated mice bearing a $G\alpha_{13}$ allele in which the coding portion of exon 4 was flanked by loxP sites (Figure 5). Mice with one such "floxed" and one wild-type allele ($G\alpha_{13}^{+/flox}$) were mated to each other and to mice heterozygous for the conventional exon 1 knockout $G\alpha_{13}$ allele ($G\alpha_{13}^{+/-}$)[254] to generate $G\alpha_{13}^{flox/flox}$ and $G\alpha_{13}^{flox/-}$ offspring. These were born at or near the expected Mendelian rate and had no obvious abnormalities. Thus, insertion of loxP sites did not appear to disrupt necessary functions of the $G\alpha_{13}$ gene.

To determine whether deletion of exon 4 coding sequence would yield a null allele as predicted, $G\alpha_{13}^{flox/flox}$ mice were mated to mice carrying a β-actin promoter-driven *Cre* recombinase transgene[258] to generate offspring heterozygous for the $G\alpha_{13}$ exon 4 deletion ($G\alpha_{13}^{+/\Delta 4}$). Southern blot and PCR analysis confirmed excision of exon 4, and crosses of $G\alpha_{13}^{+/\Delta 4}$ with $G\alpha_{13}^{+/-}$ mice showed that the $G\alpha_{13}^{\Delta 4/-}$ embryos died at ~E9.5 with a phenotype grossly indistinguishable from that previously reported for embryos homozygous for a $G\alpha_{13}$ allele made by deletion of exon 1 (Figure 6D).[254] $G\alpha_{13}^{\Delta 4/\Delta 4}$ embryos also exhibited the $G\alpha_{13}^{-/-}$ phenotype (not shown). In addition, infection of endothelial cells cultured from $G\alpha_{13}^{flox/flox}$ neonates with an adenovirus that drove expression of *Cre* caused a marked drop in the level of $G\alpha_{13}$ mRNA in the cultures (Figure 6C). Thus, *Cre* can excise the floxed exon 4 resulting in a $G\alpha_{13}$ allele that is functionally null.

Figure 5: Generation of a floxed $G\alpha_{13}$ allele

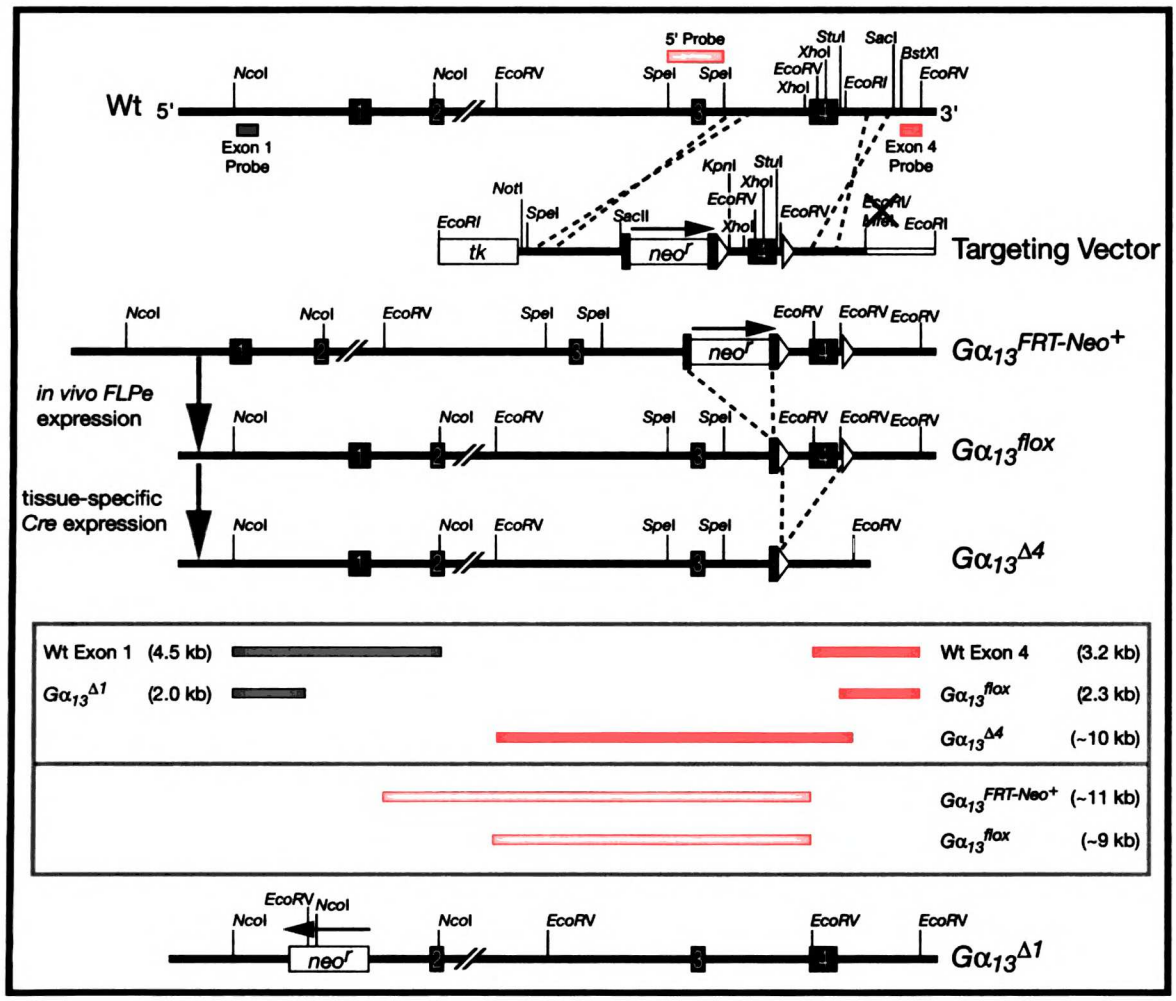


Figure 5 Legend:

Diagram of the Wt $G\alpha_{13}$ locus, targeting vector, and recombined locus. Shaded rectangles indicate FRT sites; other conventions are as in **Figure 1**. *In vivo FLPe*-mediated excision of the *neo*^{r} cassette generated the "$G\alpha_{13}^{flox}$" allele which contains one residual 5' FRT site, and loxP sites flanking exon 4. Cre-mediated excision of the flox allele generated a null $G\alpha_{13}$ allele ("$G\alpha_{13}^{\Delta 4}$"), transcription of which gives rise to an mRNA fragment encoding a truncated $G\alpha_{13}$ protein that is predicted to be inactive and short-lived. Shown at the bottom is a previously described global null $G\alpha_{13}$ allele ("$G\alpha_{13}^{\Delta 1}$", or "$G\alpha_{13}^{-}$" in other figures) in which exon 1 was replaced by a *neo*^{r} cassette through homologous recombination (Offermanns *et al.*, 1997). Sizes of the DNA fragments generated by the various alleles after *NcoI/EcoRV* digestion and Southern hybridization with either Exon 1 (blue) or Exon 4 (red) probe are shown at the bottom. Southern analysis after *EcoRV* digestion and hybridization with the "5' Probe" (yellow) was used to verify *FLPe*-mediated excision of the *neo*^{r} cassette (see **Materials and Methods**).

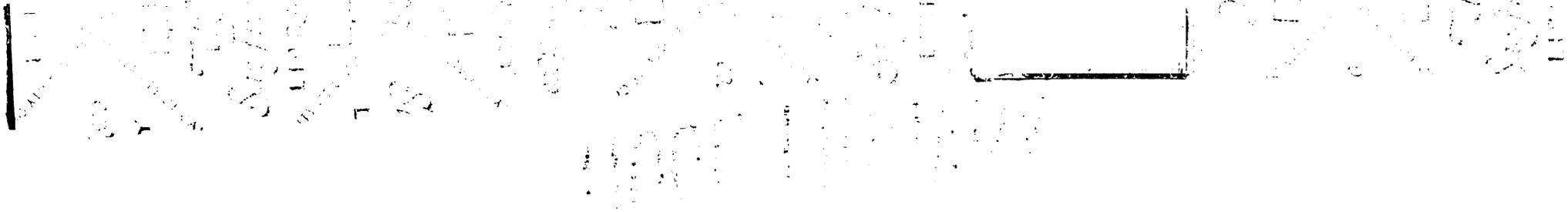


Figure 6: Excision of the floxed $G\alpha_{13}$ allele

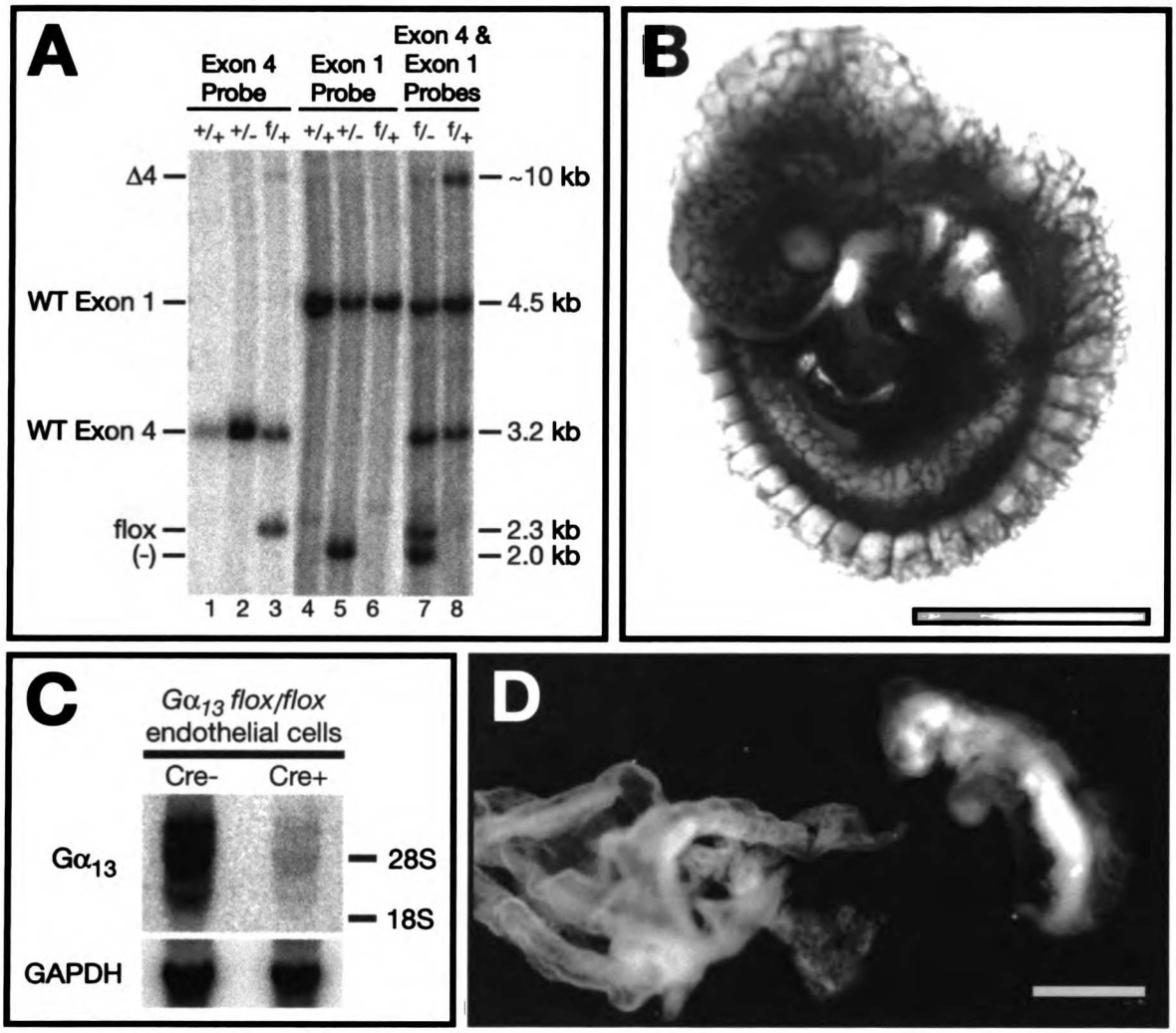


Figure 6 Legend:

A. Southern blot analysis of tail DNA from adult mice with the indicated $G\alpha_{13}$ genotypes: wild-type (+), exon 1 global null (-), flox (f), exon 4 deletion null (Δ4). The probes used are indicated at top (and see **Figure 5**). The mouse from which the DNA in lane 8 was obtained carried one copy of a β-*actin-Cre* transgene such that *Cre* recombinase was expressed globally; note the disappearance of the 2.3-kb band predicted for the $G\alpha_{13}^{flox}$ allele and the appearance of a ~10.0-kb band that corresponds to the $G\alpha_{13}^{\Delta 4}$ allele.

B. Efficiency of endothelium-specific *Cre*-mediated excision by a *Tie2p/e-Cre* transgene (see Kisanuki, *et al.*, 2001). Tie2-Cre transgenic mice were crossed to $ROSA26R^{Tg/Tg}$ lacZ *Cre*-reporter mice; embryos collected at E9.5 were stained for β-galactosidase activity. Virtually all endothelial cells were β-galactosidase positive.

C. Endothelial cells isolated from the lungs of neonatal $Tie2\text{-}Cre^{Tg/o}$; $G\alpha_{13}^{flox/flox}$ mice showed complete excision of the flox allele by Northern analysis and Southern analysis (not shown).

D. $G\alpha_{13}^{\Delta 4/-}$ embryo dissected at ~E10.5 phenocopies $G\alpha_{13}^{-/-}$ embryos (see **Figure 7**) suggesting that the $G\alpha_{13}^{\Delta 4}$ allele is, in fact, a null allele.

(Yellow bars = 1mm.)

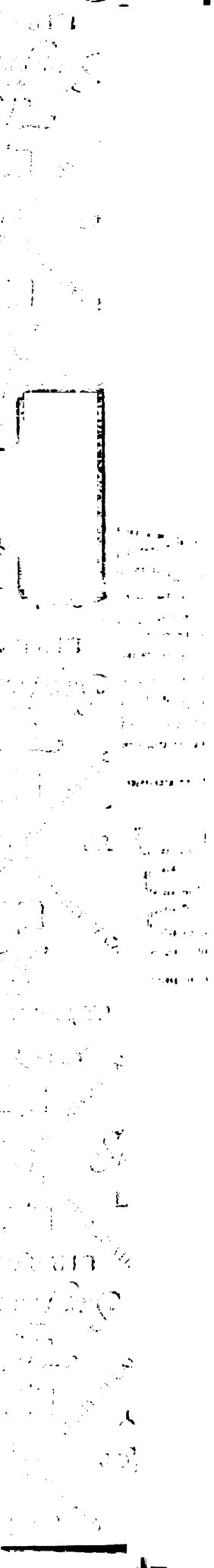
Inactivation of the $G\alpha_{13}$ gene in endothelial cells causes embryonic lethality:

To ablate $G\alpha_{13}$ expression in endothelium, we utilized a transgenic mouse line in which expression of *Cre* recombinase was driven in the vascular endothelium by the Tie2 promoter/enhancer (*Tie2p/e-Cre*).[259, 260] The *Tie2p/e-Cre* transgene yielded efficient gene excision in endothelial cells at midgestation as assessed by *Cre*-dependent activation of β-galactosidase expression by the *ROSA26R Cre*-excision reporter[261] (Figure 6B), consistent with previous reports.[259] $G\alpha_{13}^{flox/-}$ mice were crossed with $G\alpha_{13}^{+/-}$ mice that were hemizygous for the *Tie2p/e-Cre* transgene ($Tie2p/e\text{-}Cre^{Tg/o}$; $G\alpha_{13}^{+/-}$), and offspring were genotyped ~10 days after birth (Table 1). Live $G\alpha_{13}^{flox/+}$ or $G\alpha_{13}^{+/-}$ offspring were produced at similar rates in the presence or absence of the transgene. By contrast, $Tie2p/e\text{-}Cre^{Tg/o}$; $G\alpha_{13}^{flox/-}$ mice were markedly underrepresented compared to their *Cre*-negative counterparts (3 vs. 25; $P < 0.005$), and the few *Cre*-positive $G\alpha_{13}^{flox/-}$ mice that were born were runted. Thus, ablation of $G\alpha_{13}$ function in endothelial cells resulted in a highly penetrant embryonic lethal phenotype.

To characterize this phenotype, embryos from $Tie2p/e\text{-}Cre^{Tg/Tg}$; $G\alpha_{13}^{+/-}$ X $G\alpha_{13}^{flox/-}$ matings were collected at E8.5, E9.5, E10.5, and E11.5 (Table 2). At E8.5, $G\alpha_{13}^{+/-}$, $G\alpha_{13}^{+/flox}$, $G\alpha_{13}^{flox/-}$, and $G\alpha_{13}^{-/-}$ embryos were indistinguishable in gross appearance (not shown). At E9.5, ~half of $G\alpha_{13}^{-/-}$ embryos were dead and all showed delayed or abnormal development. $G\alpha_{13}^{flox/-}$ endothelial-conditional knockout embryos were also severely affected at this time; 36% were dead and 76% were affected. By E10.5, ~90% of $G\alpha_{13}^{-/-}$ and 50% of $G\alpha_{13}^{flox/-}$ embryos were dead; all $G\alpha_{13}^{-/-}$ embryos and 81% of $G\alpha_{13}^{flox/-}$ embryos

were affected (Figure 7). By E11.5, all $G\alpha_{13}^{-/-}$ and 88% of $G\alpha_{13}^{flox/-}$ embryos were dead. Thus, ablation of $G\alpha_{13}$ function by *Tie2p/e-Cre*-mediated excision of the $G\alpha_{13}$ floxed allele resulted in a highly penetrant embryonic lethality at midgestation.

To further characterize the potential causes for lethality in $G\alpha_{13}^{-/-}$ and $G\alpha_{13}^{flox/-}$ embryos we segregated the two most obvious phenotypes, pericardial dilation and hemorrhage, away from lack of heartbeat in affected embryos and tallied the results (Table 3). In the embryo, pericardial dilation is thought to be an indicator of general cardiovascular stress and/or failure. At E9.5 24% of $G\alpha_{13}^{-/-}$ embryos had a dilated pericardium while half, or 12%, of $G\alpha_{13}^{flox/-}$ embryos did. At E10.5, 46% of $G\alpha_{13}^{-/-}$ embryos and 61% of $G\alpha_{13}^{flox/-}$ embryos had a dilated pericardium. Embryonic hemorrhage is a more specific phenotype and suggests defects in the structure and/or stability of embryonic blood vessels. In many previous studies hemorrhage has been shown to result from decreased mechanical stability of endothelial cells[163] or from defective pericyte/VSMC investment of neovessels.[82, 116, 137, 158] At E9.5 67% of $G\alpha_{13}^{-/-}$ embryos showed evidence of hemorrhage, most often in the head, while in $G\alpha_{13}^{flox/-}$ embryos no evidence of hemorrhage was seen. At E10.5, 69% of $G\alpha_{13}^{-/-}$ embryos had hemorrhages while only 23% of $G\alpha_{13}^{flox/-}$ embryos did. The higher observed frequency of dilated pericardium in E10.5 $G\alpha_{13}^{flox/-}$ embryos vs. E10.5 $G\alpha_{13}^{-/-}$ embryos might simply reflect the fact that more $G\alpha_{13}^{flox/-}$ embryos survive intact (*i.e.*, without extensive necrosis) long enough to allow observation of this phenotype, and in any case this phenotype is relatively non-specific. The observation that $G\alpha_{13}^{flox/-}$ embryos show significantly less hemorrhage at E9.5 and E10.5 than $G\alpha_{13}^{-/-}$ littermates is more intriguing. Perhaps the



expression of $G\alpha_{13}$ in a cell type(s) that does not express *Tie2p/e-Cre* (*i.e.*, pericytes/vascular smooth muscle), results in a milder bleeding phenotype of $G\alpha_{13}^{flox/-}$ embryos.

Abnormal vascular development in endothelium-specific $G\alpha_{13}$ knockout embryos.

Endothelium-specific $G\alpha_{13}$ knockout embryos showed a gross phenotype that was similar to, but less severe than, that reported for the conventional $G\alpha_{13}$ knockout.[254] Both showed wrinkled yolk sacs with a paucity of blood-filled vessels as well as pale and delayed embryos with pericardial swelling and variable amounts of bleeding into cavities and tissues (Figures 7 and 9). The onset and penetrance of developmental delay and pericardial dilatation was similar in $G\alpha_{13}^{-/-}$ embryos and $G\alpha_{13}^{flox/-}$ endothelium-specific knockout embryos, but gross hemorrhage was more evident in the former (Figure 7, and Table 3).

The structure of the yolk sacs of *Tie2p/e-Cre*$^{Tg/o}$; $G\alpha_{13}^{flox/-}$ embryos was strikingly abnormal. At E9.5 and E10.5, wild-type yolk sacs contain an arborized vasculature with large and small vessels evident by gross examination and by PECAM staining. By contrast, large vessels were lacking from *Tie2p/e-Cre*$^{Tg/o}$; $G\alpha_{13}^{flox/-}$ yolk sacs, and unusually large vascular spaces were apparent on cross section (Figure 9 E-H). This was even more obvious in $G\alpha_{13}^{-/-}$ yolk sacs (Figure 9 I-L). Whole mount β-galactosidase staining of E9.5 *Tie2p/e-Cre*$^{Tg/o}$; $G\alpha_{13}^{flox/-}$ embryos that carried the *ROSA26R Cre*-excision reporter revealed a grossly normal vascular pattern in the trunk (see intersomitic vessels,

branchial arch vessels, and endocardium in Figure 8). However, head vessels were delayed and disorganized compared to those seen in littermate controls (Figure 8 C,F,I). Thus, endothelial cell differentiation occurred and early vasculogenesis and patterning were relatively normal in embryos that lacked $G\alpha_{13}$ function in endothelial cells, but subsequent remodeling of the vasculature was markedly impaired.

Table 1. Impaired viability of endothelium-specific $G\alpha_{13}$ knockout mice.

	+/flox	+/-	-/-	flox/-
Tie2p/e-Cre$^{Tg/o}$	38	29	0	3
Tie2p/e-Cre$^{o/o}$	40	33	0	25

Genoptypes of 168 progeny from ♀ *Tie2p/e-Cre$^{Tg/o}$; $G\alpha_{13}^{+/-}$* X ♂ *$G\alpha_{13}^{flox/-}$* intercrosses alive at ~postnatal day 10.

Table 2. Genoptype and gross phenotype of embryos from *Tie2p/e-Cre$^{Tg/Tg}$; $G\alpha_{13}^{+/-}$* X *$G\alpha_{13}^{flox/-}$* intercrosses.

Age	Total	+/flox	+/-	-/-	flox/-
E9.5	102	27/32 (13%)	29/34 (9%)	11/21 (100%)	16/25 (76%)
E10.5	80	21/23 (9%)	20/22 (9%)	2/19 (100%)	8/16 (81%)
E11.5	86	17/20 (15%)	27/29 (10%)	0/13 (100%)	3/24 (92%)

Alive/total number of embryos recovered and the percent of abnormal embryos for each genotype at the indicated gestational age are shown. Alive is defines as having a heartbeat. Abnormal included embryos showing pericardial dilation, hemorrhage, developmental delay, or absence of a heartbeat. collected at the indicated day of gestation. The percentage of "affected" embryos, including those without a heartbeat is indicated in parentheses.

Table 3. Frequency of hemorrhage and pericardial dilation in *Tie2-Cre$^{Tg/o}$; $G\alpha_{13}^{-/-}$*, *Tie2-Cre$^{Tg/o}$; $G\alpha_{13}^{flox/-}$*, and *Tie2-Cre$^{Tg/o}$; $G\alpha_{13}^{flox/+}$* embryos.

	-/-; Tie2-Cre$^{Tg/o}$		flox/-; Tie2-Cre$^{Tg/o}$		flox/+; Tie2-Cre$^{Tg/o}$	
	E9.5	E10.5*	E9.5	E10.5*	E9.5	E10.5
Hemorrhage	67% (14/21)	69% (9/13)	0% (0/25)	23% (3/13)	0% (0/35)	0% (0/23)
Pericardial Dilation	24% (5/21)	46% (6/13)	12% (3/21)	61% (8/13)	0% (0/35)	0% (0/35)

* Necrotic E10.5 embryos are excluded from the data in these columns.

Figure 7: Gross phenotype of $G\alpha_{13}$ endothelium-specific and $G\alpha_{13}^{-/-}$ knockouts

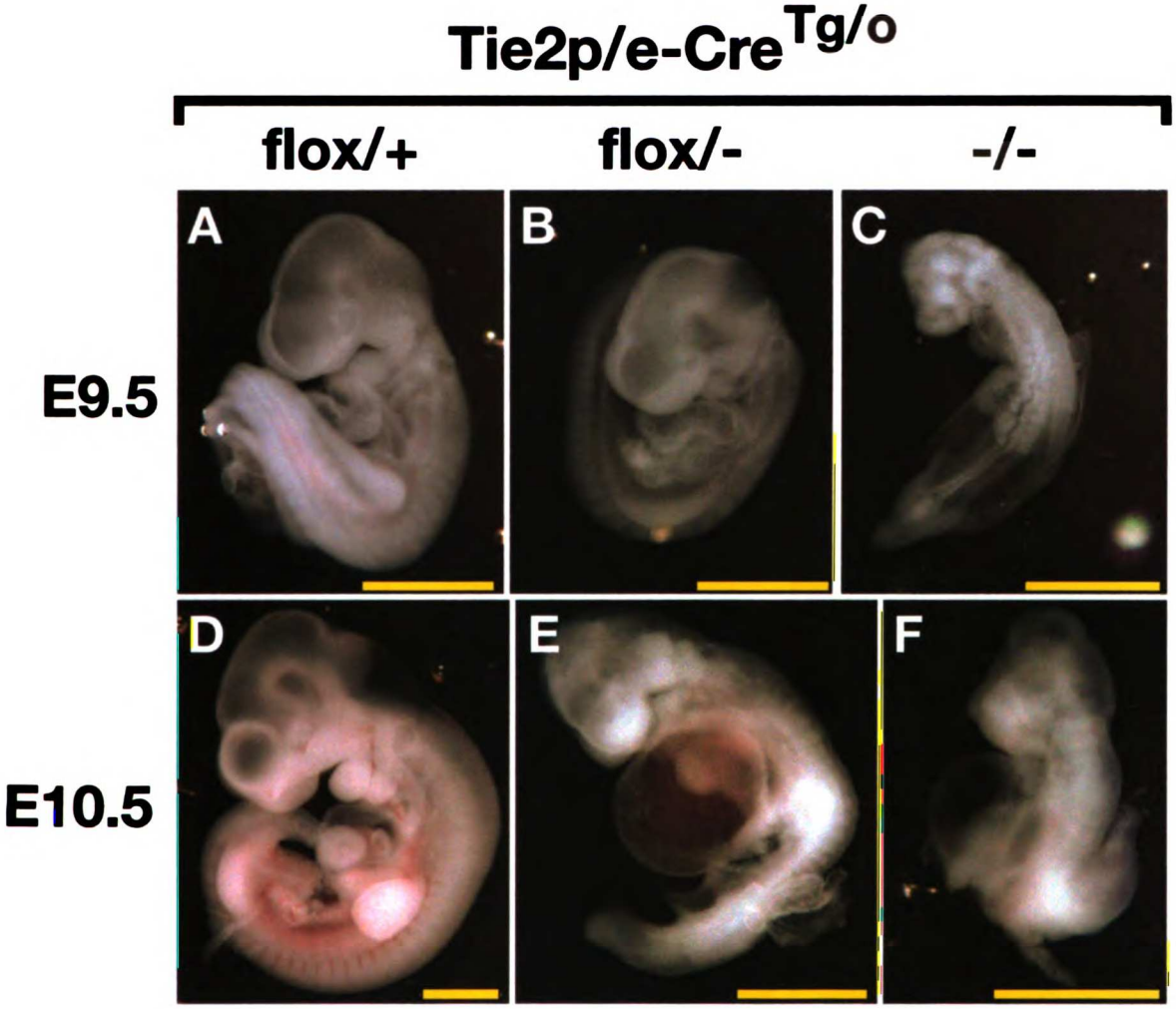


Figure 7 Legend:

Phenotype of $G\alpha_{13}$ endothelium-specific knockout and $G\alpha_{13}^{-/-}$ embryos. $G\alpha_{13}^{flox/+}$ embryos (**A,D**), $G\alpha_{13}^{flox/-}$ embryos (**B,E**), and $G\alpha_{13}^{-/-}$ embryos (**C,F**). All embryos (**A-F**) are *Tie2p/e-Cre*$^{Tg/o}$ and had a heartbeat at the time of photography. At E9.5, $G\alpha_{13}^{-/-}$ embryos (**C**) were significantly delayed compared to control embryos (**A**); the time of developmental arrest appeared to be ~E8.5 as previously reported (Offermanns, *et al.*, 1997). At E9.5, $G\alpha_{13}^{flox/-}$ embryos (**B**) had turned but appeared arrested at ~E9.0. By E10.5, >80% of $G\alpha_{13}^{flox/-}$ embryos (**E**) were either dead or morphologically abnormal, and all $G\alpha_{13}^{-/-}$ embryos are dead and/or grossly abnormal. Dilated pericardial sacs, consistent with cardiovascular failure, were a prominent feature in both $G\alpha_{13}^{flox/-}$ and $G\alpha_{13}^{-/-}$ embryos (also see **Table 3**). (Yellow bars = 1 mm.)

en

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Figure 8: Vascular phenotype of $G\alpha_{13}$ endothelium-specific and $G\alpha_{13}^{-/-}$ knockouts

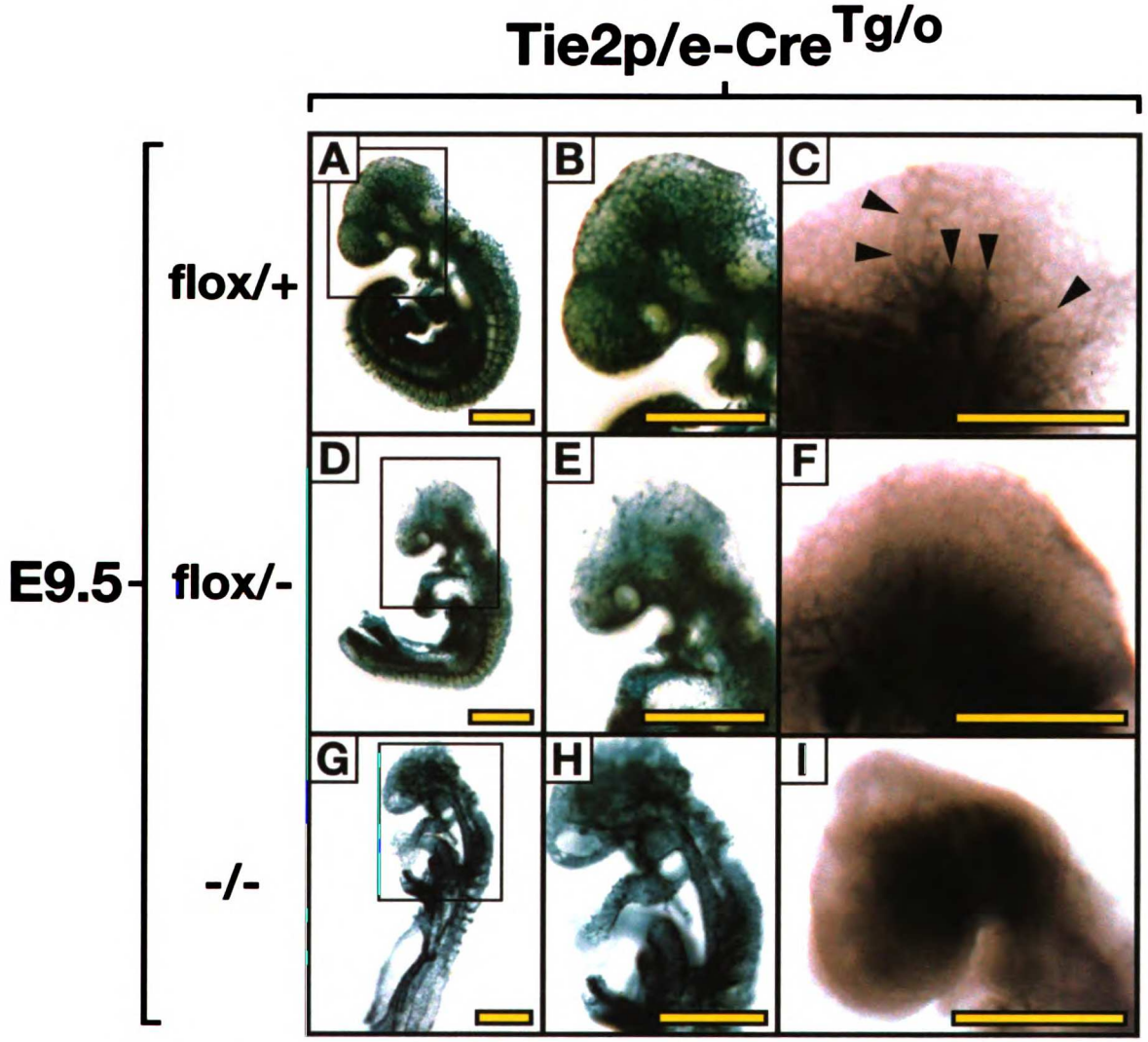


Figure 8 Legend:

Whole mount β-galactosidase staining of *Tie2p/e-Cre*$^{Tg/o}$; *ROSA26R* embryos (**A,B,D,E,G,H**), or CD105 (*a.k.a.*, Endoglin) immunostained *Tie2p/e-Cre*$^{Tg/o}$ embryos (**C,F,I**) with the indicated $G\alpha_{13}$ genotypes collected at E9.5. Control embryos (**A,B,C**) show an extensive vascular network in the head. Note the arborization of large vessels (**C**, arrowheads). In contrast, endothelium-specific null embryos (**D,E,F**) and global $G\alpha_{13}$ nulls (**G,H,I**) displayed a more primitive vascular plexus in the head compared to controls. No difference in the vascular anatomy of control and $G\alpha_{13}^{flox/-}$ embryos was observed in the truncal regions. (Yellow bars = 1 mm.)

Figure 9: Yolk sac vascular phenotype of $G\alpha_{13}$ endothelium-specific and $G\alpha_{13}^{-/-}$ knockouts

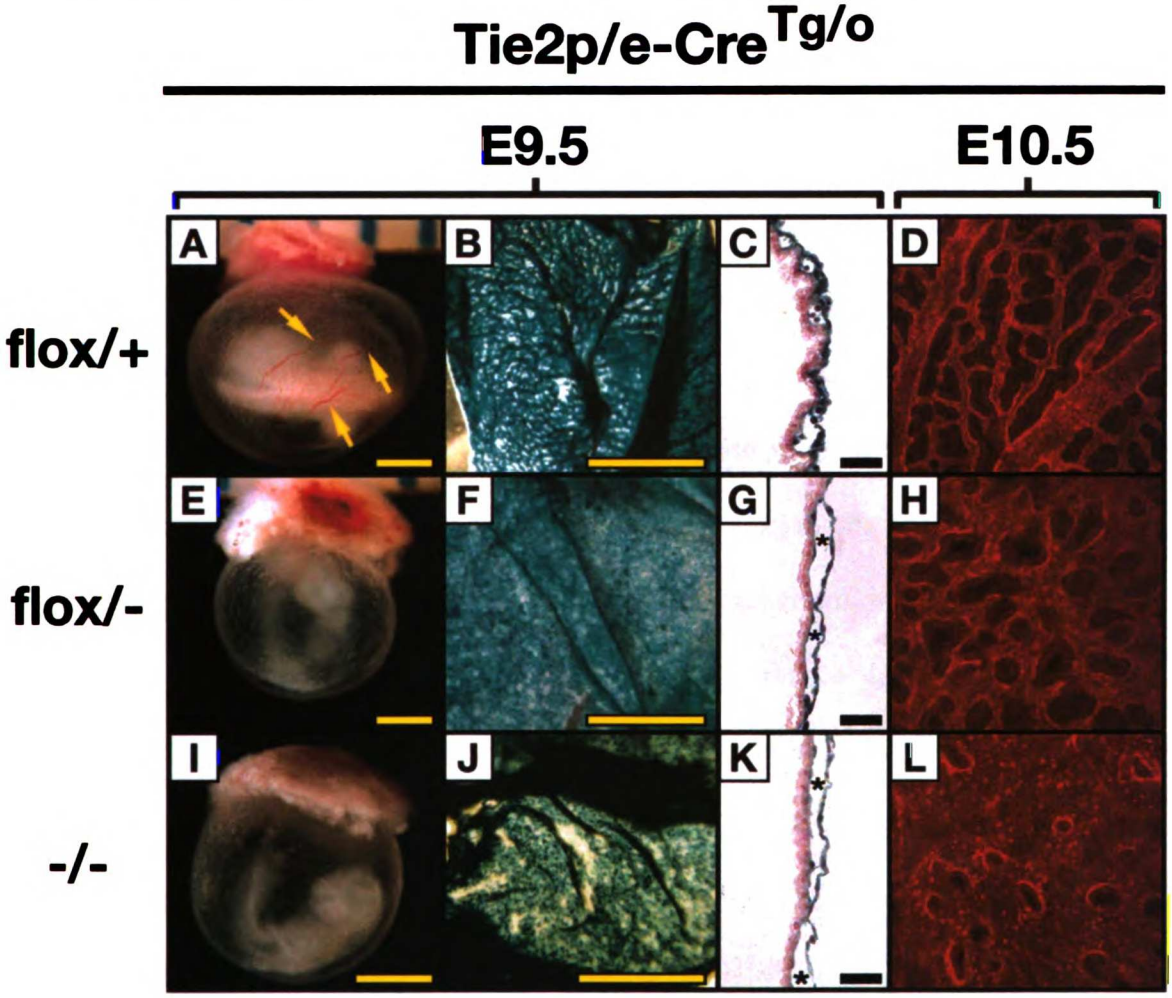
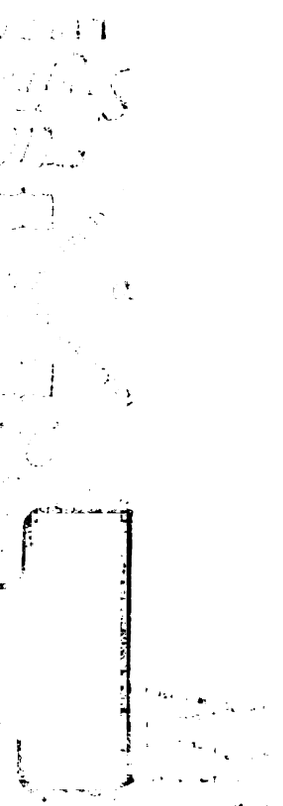


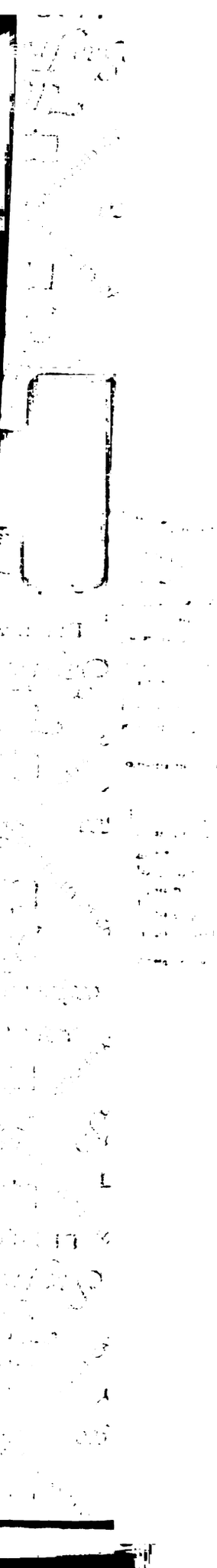
Figure 9 Legend:

Phenotype of $G\alpha_{13}$ endothelium-specific knockout and $G\alpha_{13}^{-/-}$ yolk sacs. $G\alpha_{13}$ genotypes were $G\alpha_{13}^{flox/+}$ (**A-D**), $G\alpha_{13}^{flox/-}$ (**E-H**), and $G\alpha_{13}^{-/-}$ (**I-L**). At E9.5 (left), large blood-filled vessels (arrows) were seen in control yolk sacs (**A**) but not in $G\alpha_{13}^{flox/-}$ (**E**) or $G\alpha_{13}^{-/-}$ (**I**) yolk sacs, which were pale and dimpled. X-gal staining of yolk sacs also carrying one copy of the *Tie2p/e-Cre* transgene and one copy of the *ROSA26R lacZ* reporter allele (**B,C,F,G**), or one copy of the *Tie2p/e-lacZ* transgene (**J,K**) revealed an arborized structure with large and small vessels in control yolk sacs (**B**) while $G\alpha_{13}^{flox/-}$ (**F**) and $G\alpha_{13}^{-/-}$ (**J**) yolk sacs exhibited a more plexus-like structure that lacked large vessels. Cross sections of such yolk sacs (**C,G,K**) showed blood-filled vessels spaced at regular intervals and lined with blue-stained endothelial cells in controls (**C**) but enlarged and often bloodless vascular spaces in $G\alpha_{13}^{flox/-}$ (**G**) and $G\alpha_{13}^{-/-}$ (**K**) yolk sacs (*). Immunofluorescence staining of E10.5 yolk sacs for the endothelial marker PECAM1 (CD31), shown at right, provides a higher resolution view of these phenotypes. (Yellow bars = 1 mm; Black bars = 100 μm.)



Generation of transgenic mice expressing $G\alpha_{13}$ in endothelial cells:

Although relatively high expression of $G\alpha_{13}$ was detected in endothelial cells during embryonic development, expression was also detected in mesenchyme, dorsal neural tube, foregut and elsewhere (Figure 2B). The results described above strongly suggest that $G\alpha_{13}$ expression in endothelial cells is required for normal vascular development and for survival in mouse embryos, but these results do not preclude a vital role for $G\alpha_{13}$ signaling in other cell types involved in vascular development or other processes. Indeed, the greater severity of the complete vs. endothelium-specific $G\alpha_{13}$ knockout phenotypes might reflect a necessary role for $G\alpha_{13}$ in other cell types. To probe for necessary roles for $G\alpha_{13}$ in cells types other than endothelium, we asked whether $G\alpha_{13}$ expression in endothelial cells might be sufficient to rescue development of $G\alpha_{13}^{-/-}$ embryos. To direct expression of $G\alpha_{13}$ to the endothelium, we used the Tie2 promoter/enhancer[262] to drive transcription of a *$G\alpha_{13}$-IRES-lacZ* cassette in transgenic mice (Figure 10A). Whole-mount β-galactosidase staining of embryos from two *Tie2p/e-$G\alpha_{13}$-IRES-lacZ* (subsequently abbreviated as *Tie2p/e-$G\alpha_{13}$*) transgenic lines confirmed expression of the transgene in vascular structures from E8.5 onward. Staining was restricted to endothelium and endocardium and to a fraction of hematopoietic cells suggesting that transgene expression was appropriately cell-type specific (Figure 10B a,b).



Endothelium-specific expression of $G\alpha_{13}$ rescues early lethality of $G\alpha_{13}^{-/-}$ embryos and reveals a new phenotype:

$G\alpha_{13}^{+/-}$ mice hemizygous for the endothelium-specific $G\alpha_{13}$ transgene (*Tie2p/e-*$G\alpha_{13}^{Tg/o}$; $G\alpha_{13}^{+/-}$) were crossed to $G\alpha_{13}^{+/-}$ mice and embryos collected at various times. As expected, approximately half of the transgene-negative $G\alpha_{13}^{-/-}$ embryos recovered at E9.5 were dead and the remainder were delayed and/or otherwise abnormal (Table 4 and Figure 10B d). In striking contrast, transgene-positive $G\alpha_{13}^{-/-}$ embryos were recovered at the expected Mendelian rate at E9.5, alive and indistinguishable from their $G\alpha_{13}^{+/+}$ and $G\alpha_{13}^{+/-}$ littermates (Table 4 and Figure 10B c,e). Analysis of $G\alpha_{13}^{-/-}$ yolks sacs at E9.5 also revealed a striking difference in the presence or absence of the transgene. In the absence of endothelial $G\alpha_{13}$ expression, $G\alpha_{13}^{-/-}$ yolk sacs showed a grossly abnormal vascular plexus (see Figure 9 J,K,L), but in the presence of the transgene, $G\alpha_{13}^{-/-}$ yolk sacs displayed a pattern of branching vessels indistinguishable from that seen in wild-type littermates (Figure 10B f,g,h,i). Thus, expression of $G\alpha_{13}$ in endothelial cells was sufficient to prevent vascular defects and death of $G\alpha_{13}^{-/-}$ embryos at E9.5.

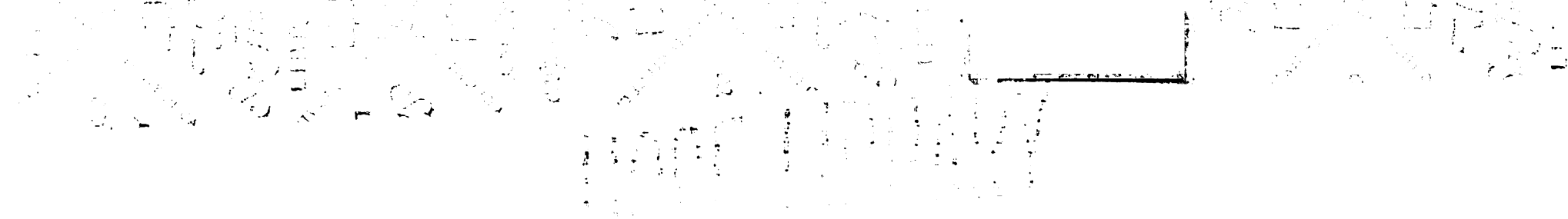
$G\alpha_{13}^{-/-}$ embryos carrying the *Tie2p/e-*$G\alpha_{13}$ transgene survived for several days beyond their transgene-negative counterparts and then developed a second phenotype. By E11.5, all transgene-negative $G\alpha_{13}^{-/-}$ embryos were dead and partially resorbed, but 13 of 14 transgene-positive $G\alpha_{13}^{-/-}$ embryos were alive. Most of these had exencephaly (11/14) and/or obvious hemorrhage within the head mesenchyme or ventricles (6/14) (Figure 12 A,C and Table 4). By E12.5, no transgene-negative $G\alpha_{13}^{-/-}$ embryos were recovered in any form, while transgene-positive $G\alpha_{13}^{-/-}$ embryos were recovered at only

slightly less than the expected rate (Table 4). Most of these lacked a heartbeat and all showed exencephaly and/or hemorrhage (Figure 12 E,G and data not shown).

These results suggested that $G\alpha_{13}$ expression in endothelial cells is sufficient for proper embryonic development through E9.5-10.5. However, they did not distinguish whether the inability of the *Tie2p/e-Gα_{13}* transgene to completely rescue development of *G$\alpha_{13}^{-/-}$* embryos was due to a requirement for $G\alpha_{13}$ function in another cell type vs. failure of the transgene to adequately reproduce the normal level or temporal and spatial pattern of $G\alpha_{13}$ expression in endothelial cells. To address this issue, we asked whether the *Tie2p/e-Gα_{13}* transgene would completely rescue the endothelium-specific *Gα_{13}* knockout.

Tie2p/e-Gα13 transgene rescues development of the endothelium-specific _Gα13_ knockout:

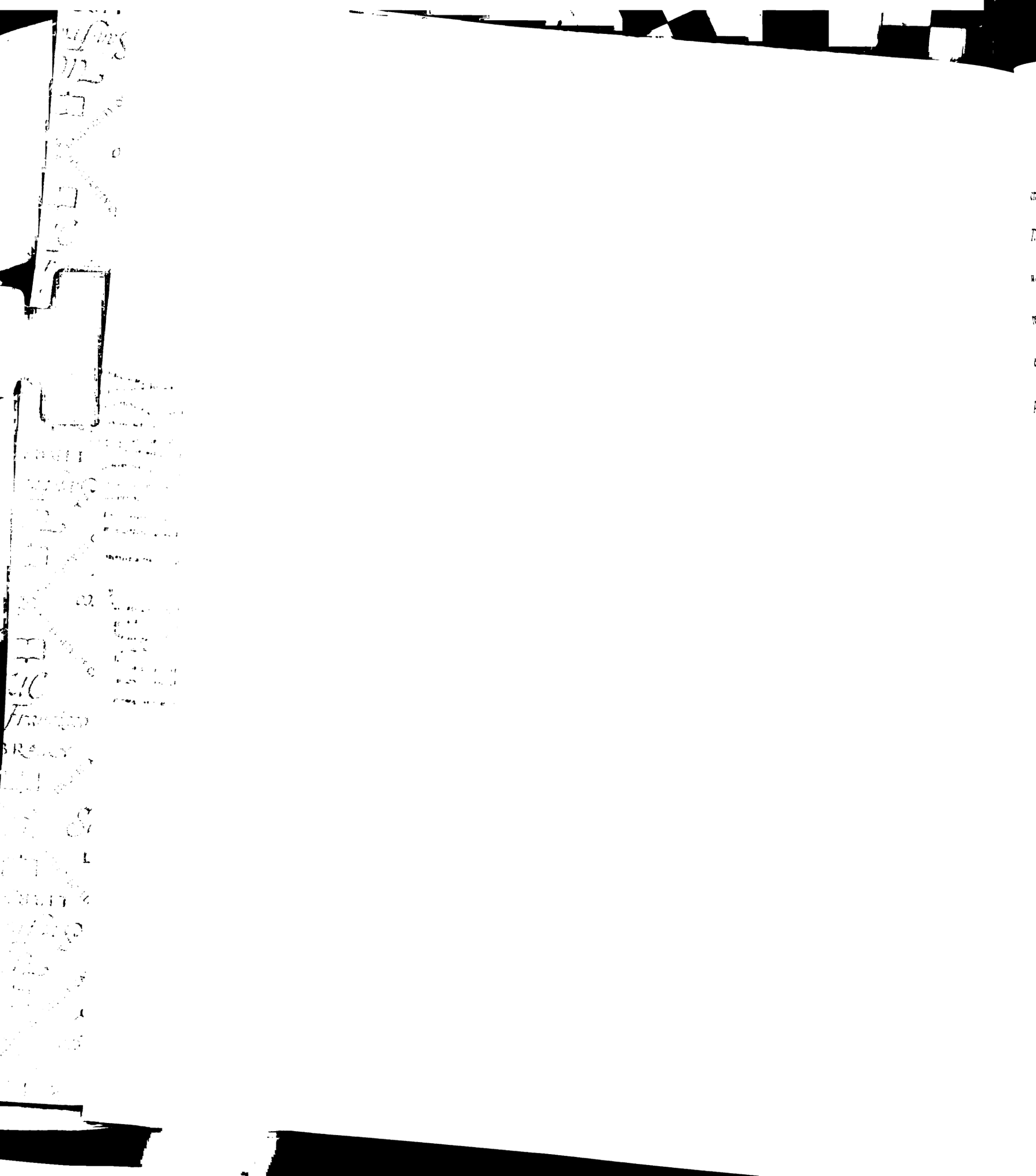
Mice hemizygous for the endothelium-specific *Gα_{13}* transgene and bearing one floxed and one null *Gα_{13}* allele (*Tie2p/e-G$\alpha_{13}^{Tg/o}$; G$\alpha_{13}^{flox/-}$*) were crossed to *G$\alpha_{13}^{+/-}$* mice homozygous for the *Tie2p/e-Cre* transgene (*Tie2p/e-Cre$^{Tg/Tg}$; G$\alpha_{13}^{+/-}$*). Offspring were genotyped 10-15 days after birth (Table 5). No *G$\alpha_{13}^{-/-}$* mice were recovered even when the endothelium-specific *Gα_{13}* transgene was present. Strikingly, however, while no *Tie2p/e-Cre$^{Tg/o}$; G$\alpha_{13}^{flox/-}$* pups were recovered in the absence of the transgene, *Tie2p/e-G$\alpha_{13}^{Tg/o}$; Tie2p/e-Cre$^{Tg/o}$; G$\alpha_{13}^{flox/-}$* mice were recovered at the expected Mendelian rate. These results strongly suggest that the *Tie2p/e-Gα_{13}* transgene drives $G\alpha_{13}$ expression in a



manner adequate to support the $G\alpha_{13}$ functions in endothelial cells that are necessary for embryonic development. Thus, the contrast between the phenotypes of endothelium-specific vs. conventional $G\alpha_{13}$ knockouts carrying the *Tie2p/e-*$G\alpha_{13}$ transgene is likely due to a necessary role for $G\alpha_{13}$ in a cell type(s) other than endothelial cells – a role that becomes apparent only when $G\alpha_{13}^{-/-}$ embryos are supported beyond E9.5 by the endothelial transgene.

Ablation of $G\alpha_{13}$ function in neural crest recapitulates the late phenotype of endothelium-rescued $G\alpha_{13}^{-/-}$ embryos:

The relatively high level of $G\alpha_{13}$ expression in the region of the neural tube that gives rise to neural crest (Figure 2 g,h) raised the possibility of an important function of $G\alpha_{13}$ in this tissue. The phenotypes seen in endothelium-rescued $G\alpha_{13}^{-/-}$ embryos were consistent with this hypothesis (see Discussion). To probe for necessary functions of $G\alpha_{13}$ in neural crest cells or their derivatives, we utilized *Wnt1-Cre* transgenic mice.[263] *Wnt1-Cre* functions in the neural crest and the dorsal neural tube beginning at E8.5 (Figure 11C) and has been used to conditionally inactivate several genes in neural crest-derived tissues.[264-267] When $G\alpha_{13}^{flox/flox}$ mice were crossed to $G\alpha_{13}^{+/-}$ mice hemizygous for the *Wnt1-Cre* transgene, only 6 *Wnt1-Cre*$^{Tg/o}$; $G\alpha_{13}^{flox/-}$ pups were recovered among 150 live offspring; 37 would be expected by Mendelian inheritance ($P < 0.001$ by chi-square). Thus, loss of $G\alpha_{13}$ function in *Wnt1-Cre* trangene-expressing tissues resulted in embryonic lethality ($P < .001$) with >80% penetrance.



Analysis of *Wnt1-Cre*$^{Tg/o}$; *G*$\alpha_{13}$$^{flox/-}$ embryos collected at E12.5 revealed both craniofacial hemorrhage and neural tube closure defects – an apparent phenocopy of the *Tie2p/e-G*$\alpha_{13}$$^{Tg/o}$; *G*$\alpha_{13}$$^{-/-}$ embryos (Figure 12 F,H). All *Wnt1-Cre*$^{Tg/o}$; *G*$\alpha_{13}$$^{flox/-}$ embryos were affected at E12.5 and ~30% lacked a heartbeat, and by E14.5, only necrotic and resorbing *Wnt1-Cre*$^{Tg/o}$; *G*$\alpha_{13}$$^{flox/-}$ embryos were recovered (not shown), suggesting that embryos lacking Gα_{13} function in Wnt1-expressing cells die ~E12.5-14.5. Thus, Gα_{13} plays a necessary role in *Wnt1-Cre* expressing lineages.

Wnt1-Cre can excise floxed alleles in neural crest cells but also in anterior neural tube and other cells in the developing central nervous system. To assess the necessary role of Gα_{13} in the latter, *G*α_{13} was inactivated in neuronal and glial precursor cells using a *Nestin-Cre* transgenic line.[268] At postnatal day 10-15, *G*$\alpha_{13}$$^{flox/-}$ mice bearing the *Nestin-Cre* transgene were recovered at the expected Mendelian rate and were grossly normal (not shown). Southern analysis of brains from these mice confirmed virtually complete excision of *G*α_{13} exon 4 (Figure 11A). *Wnt1-Cre*–driven excision of the *ROSA26R Cre*-reporter was readily detected in the anterior and dorsal region neural tube at E8.5 and became more widely distributed by E9.5 (Figure 11C). *Nestin-Cre*–driven excision in neural tube was nearly absent at E8.5 and 9.5 but by E10.5 was detected throughout the CNS but not in neural crest derivatives (Figure 11B and not shown). Thus, we cannot exclude differences in the time of onset of excision as the basis for the different phenotypes of the between the *Wnt1-Cre*–driven and *Nestin-Cre*–driven *G*α_{13} conditional knockouts. Overall, however, our data suggest that Gα_{13} function in neural crest-derived cells, and not in CNS neurons or glia, is necessary for proper embryonic development.

Table 4. Endothelial $G\alpha_{13}$ transgene rescue of early $G\alpha_{13}^{-/-}$ lethality. Genotype and gross phenotype of embryos from *Tie2p/e-*$G\alpha_{13}^{Tg/o}$; $G\alpha_{13}^{+/-}$ X $G\alpha_{13}^{+/-}$ intercrosses.

		$G\alpha_{13}^{+/+}$		$G\alpha_{13}^{+/-}$		$G\alpha_{13}^{-/-}$	
	Total	Tg^+	Tg^-	Tg^+	Tg^-	Tg^+	Tg^-
E9.5	90	11/11 (0%)	9/9 (0%)	23/23 (0%)	23/23 (0%)	11/11 (0%)	6/13 (100%)
E11.5	104	15/15 (0%)	12/12 (0%)	28/28 (0%)	25/25 (0%)	13/14 100%)	0/10 (100%)
E12.5	151	21/22 (5%)	16/16 (0%)	49/49 (0%)	44/46 (4%)	2/18 (100%)	0/0 (N/A)

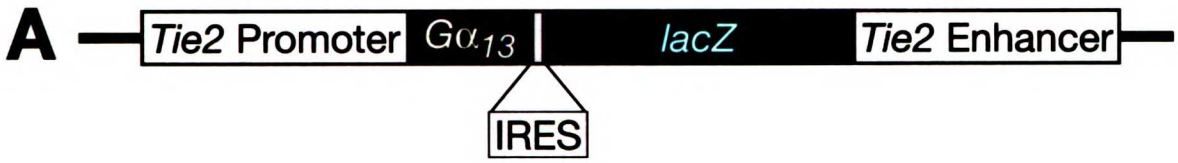
Number of alive/total recovered embryos and percent of embryos that were abnormal. Abnormal includes embryos showing hemorrhage, pericardial dilation, small size, developmental delay, and/or a neural tube defect in addition to those lacking a heartbeat. Note that the transgene yielded complete rescue of $G\alpha_{13}^{-/-}$ embryos through E9.5 and nearly complete rescue at the level of viability through E11.5, at which time the rescued embryos manifested a new phenotype (see text and **Figures 11** and **12**). NA, not applicable.

Table 5. *Tie2p/e-*$G\alpha_{13}$ transgene rescues development of endothelium-specific $G\alpha_{13}$ knockout mice.

	flox/+	+/-	-/-	flox/-
Tie2p/e-$G\alpha_{13}^{Tg/o}$	19	20	0	21
Tie2p/e-$G\alpha_{13}^{o/o}$	25	18	0	0

Genoptypes of 103 progeny from *Tie2p/e-*$G\alpha_{13}^{Tg/o}$; $G\alpha_{13}^{flox/-}$ X *Tie2p/e-*$Cre^{Tg/Tg}$; $G\alpha_{13}^{+/-}$ intercrosses were determined at postnatal days 10-15. All carry the Tie2p/e-Cre transgene such that all $G\alpha_{13}^{flox/-}$ are endothelium-specific knockouts. Note the complete rescue of the endothelium-specific knockouts by the transgene.

Figure 10: Rescue of early $G\alpha_{13}^{-/-}$ lethality with an endothelium-specific $G\alpha_{13}$ transgene



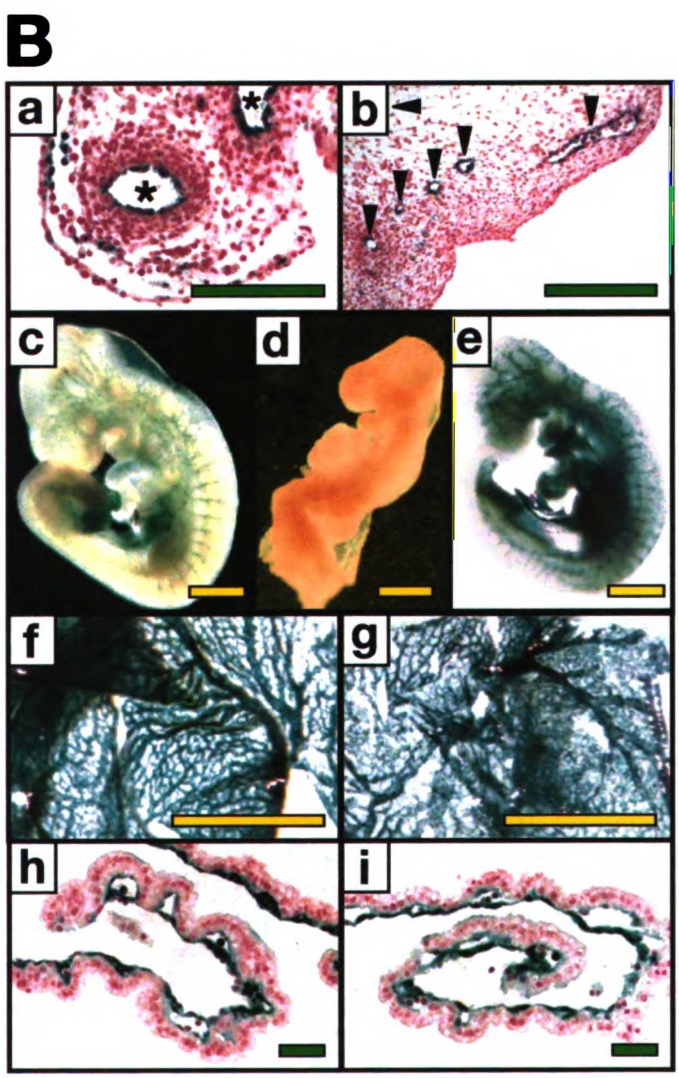


Figure 10 Legend:

A. Diagram of the *Tie2p/e-Gα$_{13}$-IRES-lacZ* construct (*Tie2p/e-Gα$_{13}$*) used to generate mice in which *Gα$_{13}$* is expressed in endothelial cells.

B. X-gal staining of an E10.5 *Tie2p/e-Gα$_{13}^{Tg/o}$* embryo showing staining of endothelium in (**a**) umbilical artery and vein (*) and (**b**) head vessels (arrowheads). (**c-e**) Whole mount X-gal staining of E9.5 embryos. In the presence of the *Tie2p/e-Gα$_{13}$* transgene, both *Gα$_{13}^{+/+}$* (**c**) and *Gα$_{13}^{-/-}$* (**e**) embryos appear grossly normal and show endothelium-specific vascular staining. *Gα$_{13}^{-/-}$* embryos that lacked the transgene (**d**) were all grossly abnormal at this time. Whole mount (**f,g**) and cross section (**h,i**) of X-gal stained E9.5 yolk sacs. *Tie2p/e-Gα$_{13}^{Tg/o}$; Gα$_{13}^{+/-}$* mice were mated to *Gα$_{13}^{+/-}$* mice that were homozygous for the *Tie2p/e-lacZ* transgene to generate embryos in which endothelial cells exhibited strong X-gal expression. The yolk sac vasculature from both *Gα$_{13}^{+/+}$* (**f,h**) and *Gα$_{13}^{-/-}$* (**g,i**) embryos that carried the *Tie2p/e-Gα$_{13}$* transgene appeared normal. Note contrast to *Gα$_{13}^{-/-}$* yolk sacs that lacked the transgene (**Figure 9, J and K**).

(Green bars = 100 μm; Yellow bars = 1 mm.)

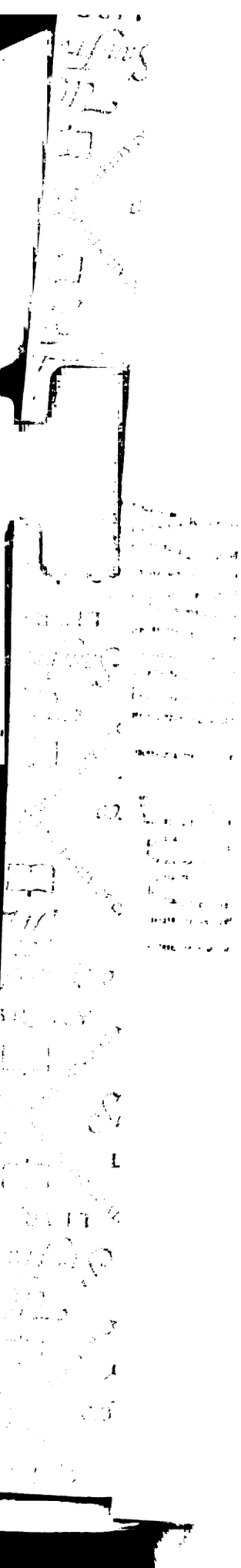


Figure 11: Deletion of $G\alpha_{13}$ in neuronal cell populations with *Nestin-Cre* and *Wnt1-Cre*

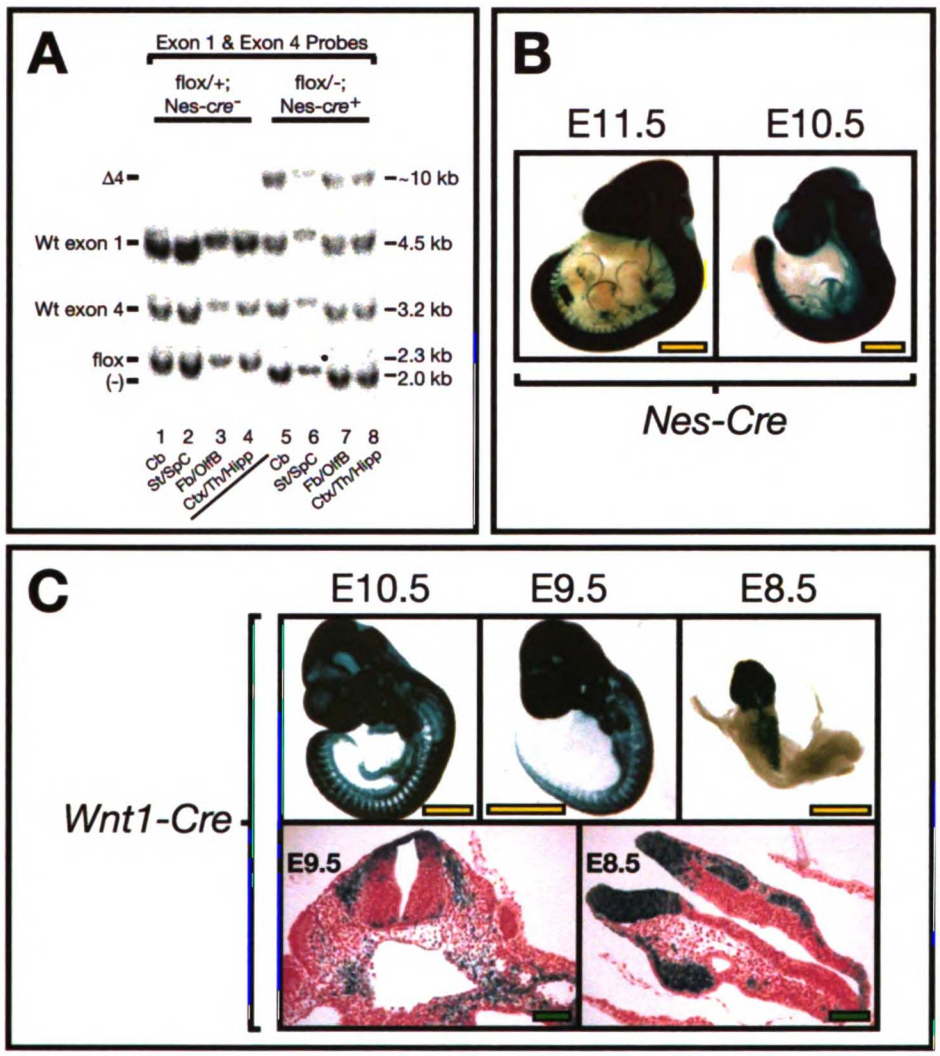


Figure 11 Legend:

A. Southern analysis of the indicated regions of mouse brain from $G\alpha_{13}^{flox/+}$ (lanes 1-4) and *Nestin-Cre*$^{Tg/o}$; $G\alpha_{13}^{flox/-}$ (lanes 5-8) adult animals to estimate the efficiency of excision by the *Nestin-Cre* transgene. In lanes 5, 7, and 8 there appears to be nearly 100% excision of the flox allele as judged by loss of the 2.3 kb band and appearence of the ~10 kb band. The excision of the flox allele in lane 6 appears to be less efficient (see black dot in lane 6, and **Figure 5** and **Figure 6** for details). (**Cb** = cerebellum; **St/SpC** = brain stem and spinal cord; **Fb/OlfB** = forebrain and olfactory bulb; **Ctx/Th/Hipp** = cortex, thalamus, and hippocampus).

B. β-galactosidase staining of *Nestin-Cre*$^{Tg/o}$; *ROSA26R* embryos at the indicated gestational age indicates that *Nestin-Cre* is expressed by E10.5 in neuronal tissues.

C. β-galactosidase staining of *Wnt1-Cre*$^{Tg/o}$; *ROSA26R* embryos at the indicated gestational age suggests that *Wnt1-Cre* is expressed earlier and its expression is more resticted to neural crest cells than *Nestin-Cre*. (Yellow bars = 1 mm; Green bars = 100 μm.)

Figure 12: Exencephaly and hemorrhage in embryos in which $G\alpha_{13}$ is absent in neural crest cell populations.

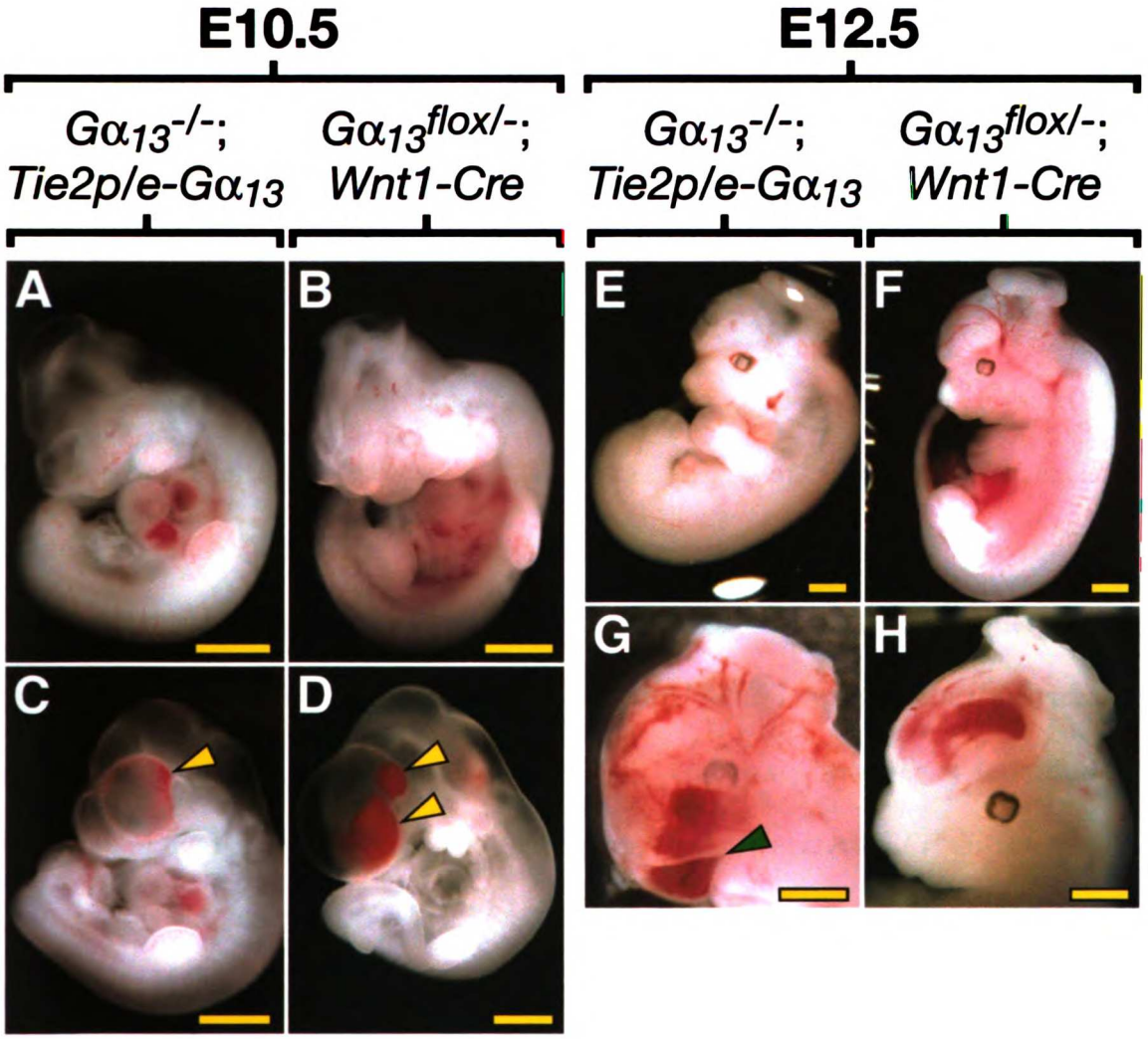


Figure 12 Legend:

Conditional excision of $G\alpha_{13}$ in Wnt1-expressing tissue recapitulates the phenotype of endothelium-rescued $G\alpha_{13}^{-/-}$ embryos. *Tie2p/e-*$G\alpha_{13}^{Tg/o}$; $G\alpha_{13}^{+/-}$ (**A,C,E,G**) and *Wnt1-*$Cre^{Tg/o}$; $G\alpha_{13}^{flox/-}$ (**B,D,F,H**) embryos exhibit similar defects. At E10.5 (**A-D**), there is evidence of neural tube closure defects (**A,B**) and bleeding into the telencephalon (**C,D**) in both genotypes (yellow arrowheads). By E12.5 (**E-H**), embryos of both genotypes exhibit variable exencephaly and craniofacial hemorrhage. Occasional embryos also exhibited craniofacial abnormalities, such as the midline facial cleft seen in **G** (green arrowhead).

(Yellow bars = 1 mm.)

$G\alpha_{13}^{-/-}$ endothelial cells are defective in network formation in vitro:

Our results strongly suggest that $G\alpha_{13}$ signaling in endothelial cells plays a critical role in vascular development. What function(s) might it serve in this context? $G\alpha_{13}$ links G protein-coupled receptors to Rho activation and perhaps to other effector pathways. Toward exploring the effect of $G\alpha_{13}$ deficiency on endothelial cell responses and behaviors, we ablated $G\alpha_{13}$ function in cultured mouse microvascular endothelial cells. Cells were immunopurified from the skin of $G\alpha_{13}^{flox/flox}$ neonatal mice and infected with adenovirus directing expression of *Cre* plus green fluorescent protein (*GFP*), or *GFP* alone.[269] Southern and Northern blot analysis of such cultures suggested an efficiency of >95% for $G\alpha_{13}$ excision (see Figure 6 C, and data not shown). Curiously, $G\alpha_{13}$ excision had no detectable effect on Rho activation or changes in endothelial cell shape or stress fiber formation in response to PAR1 agonist. In addition, no effect was detected in assays of endothelial cell movement on coverslips, adhesion on various matrices or contraction of collagen gels (data not shown). There was, however, a reproducible difference in the behavior of cells cultured on Matrigel. When Ad-GFP^+ infected wild-type or Ad-GFP^+ infected $G\alpha_{13}^{flox/flox}$ endothelial cells were plated on Matrigel, they formed a monolayer that remodeled to become a "network" of cords and tubes (Figure 13 a,c). When Ad-Cre^+-GFP^+ $G\alpha_{13}^{flox/flox}$ endothelial cells were plated on Matrigel at the same density, the monolayer formed normally but showed decreased remodeling (Figure 13b). The different remodeling behaviors depended upon the $G\alpha_{13}$ genotype in that no differences were seen between wild-type cells infected with the Ad-Cre^+-GFP^+ vs. Ad-

Figure 13: $G\alpha_{13}$-deficient endothelial cells fail to form a network on Matrigel

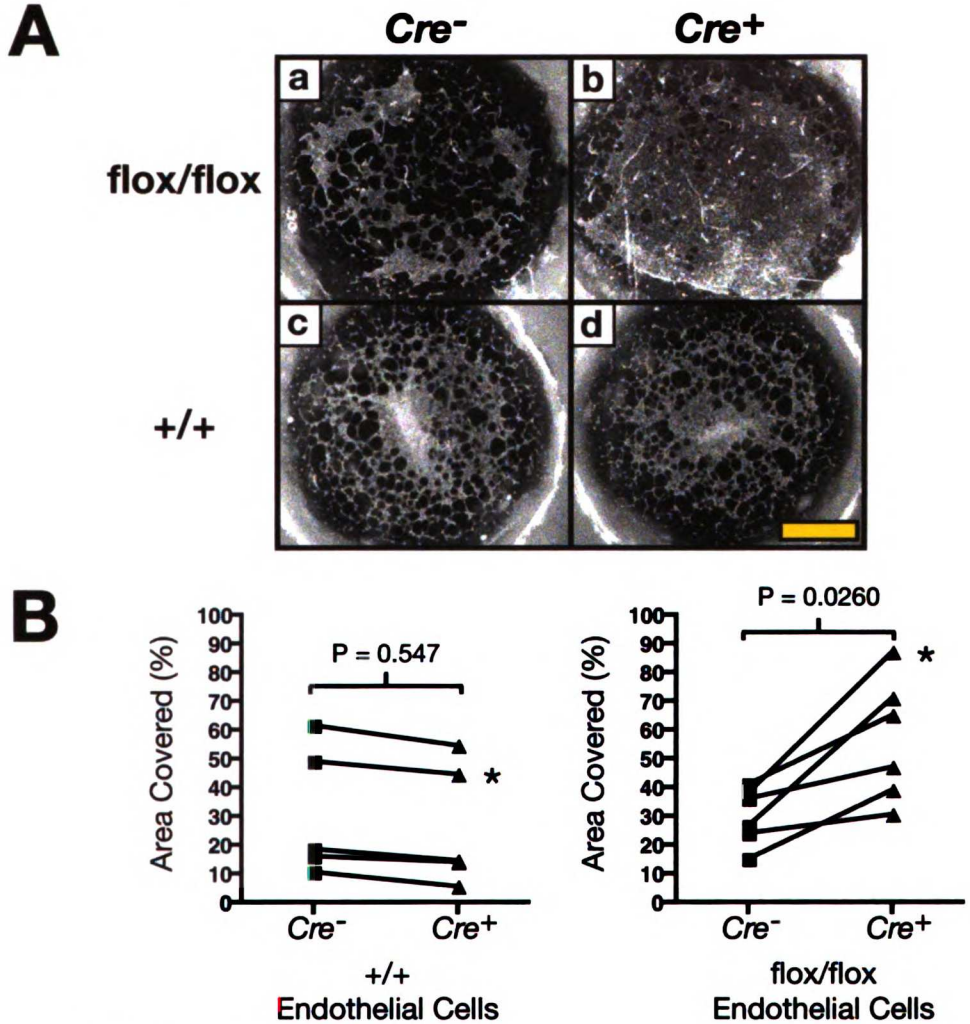


Figure 13 Legend:

A. $G\alpha_{13}^{flox/flox}$ endothelial cells infected with *GFP*$^+$-adenovirus (*Cre*$^-$) (**a**) formed an interconnected network of cords and tubes after plating on Matrigel, whereas endothelial cells infected with a *GFP*$^+$-Cre$^+$-adenovirus (*Cre*$^+$) (**b**) remained primarily as a sheet of cells (see **Figure 6C** for the efficiency of adenoviral *Cre* excision in $G\alpha_{13}^{flox/flox}$ endothelial cells). In parallel control experiments, endothelial cells isolated from $G\alpha_{13}^{+/+}$ mice formed similar networks whether infected with the *Cre*$^-$ (**c**) or *Cre*$^+$ adenovirus (**d**). (Bar = 4.5 mm.)

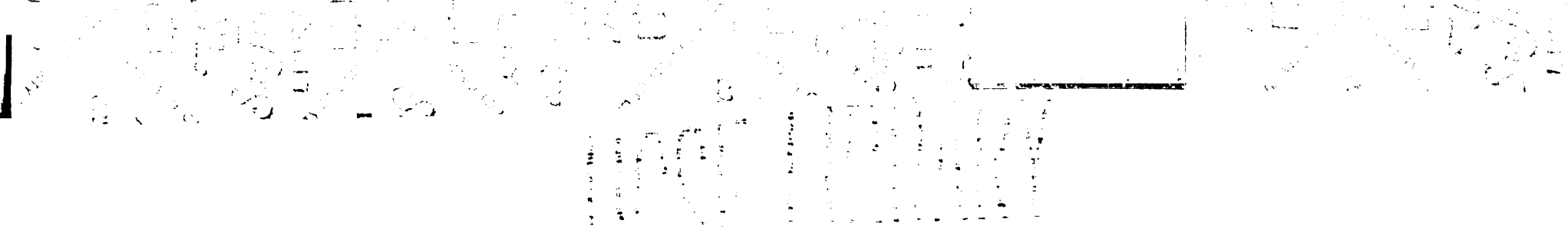
B. The percent of the Matrigel surface covered with cells was calculated for 5 $G\alpha_{13}^{+/+}$ and 6 $G\alpha_{13}^{flox/flox}$ experiments (a total of 10 $G\alpha_{13}^{+/+}$ and 12 $G\alpha_{13}^{flox/flox}$ samples) and is expressed as "Area Covered (%)". Connected points indicate paired samples from a single endothelial cell preparation one half of which was infected with *Cre*$^+$ virus and other half infected with *Cre*$^-$ virus. Asterisks mark the samples shown in (**A**). Note that network formation decreased (and hence the area of Matrigel surface covered by cells increased) in association with *Cre* expression in $G\alpha_{13}^{flox/flox}$ but not $G\alpha_{13}^{+/+}$ endothelial cells. P values were calculated with the Mann-Whitney test.

Chapter III:

DISCUSSION

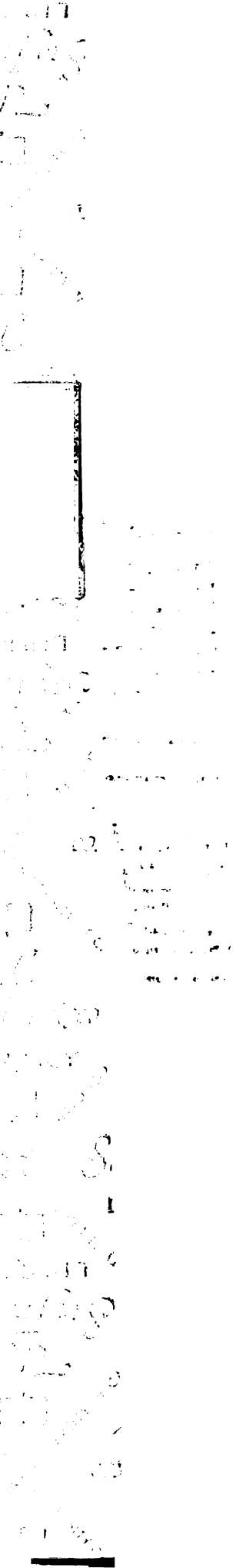
While there are over 300 non-odorant G protein-coupled receptors in mammals, they signal via only four classes of heterotrimeric G proteins: $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$. The role of G protein-coupled receptors in embryonic development is relatively unexplored, and the absolute number of these receptors and the partially redundant functions of some family members make a systematic study of the necessary roles of GPCRs in development a daunting proposition. Probing for roles of GPCR signaling by ablation of their common G protein signaling pathways is a more tractable alternative.[254, 270-272] This study focused on $G\alpha_{13}$, and our results imply a key role for G protein-coupled receptor signaling via $G\alpha_{13}$ in several distinct cell types and developmental processes.

The observation that *Tie2p/e-Cre*-mediated excision of the floxed $G\alpha_{13}$ allele caused abnormal vascular structures and embryonic death beginning at E9.5 strongly suggests a key role for $G\alpha_{13}$ in a *Tie2p/e-Cre*-expressing lineage. *Tie2p/e-Cre* can mediate excision of floxed alleles in both endothelium and in hematopoietic lineages (see reference[273] and data not shown). However, several observations point to an endothelial rather than hematopoietic defect as the cause of embryonic death in the *Tie2p/e-Cre*$^{Tg/o}$; $G\alpha_{13}^{flox/-}$ embryos. While the majority of endothelial cells in $G\alpha_{13}^{+/lacZ}$ knockin embryos showed β-galactosidase staining at E9.5-10.5 (Figure 2), less the 2% circulating embryonic blood cells were β-galactosidase-positive at this time (data not shown). Thus,



$G\alpha_{13}$ may not be expressed in most embryonic blood cells. It is formally possible that $G\alpha_{13}$ is important in stem cells that represent only a small fraction circulating cells, and that loss of $G\alpha_{13}$ from such cells would have a profound effect. However, while defects in hematopoiesis can cause death of the embryo between E10.5 and 12.5, typically such affected embryos are morphologically indistinguishable from their wild-type littermates except for pallor.[49, 274-278] Specifically, these embryos do not exhibit the abnormal vascular structures, failed remodeling of blood vessels and pericardial dilatation that were already present in most *Tie2p/e-Cre*$^{Tg/o}$; $G\alpha_{13}^{flox/-}$ embryos at E9.5. One notable exception is SCL/TAL1, loss of which was shown to cause developmental arrest at E9.5 with both failed yolk sac hematopoiesis and abnormal vasculature.[47, 278] The observed vascular phenotype was latter shown to be due to a role for SCL/TAL1 in endothelial cells since expression of SCL/TAL1 in hematopoietic cells from a GATA1 promoter-driven transgene rescued hematopoiesis but did not prevent vascular abnormalities or death.[49] Thus, our results are most consistent with the hypothesis that $G\alpha_{13}$ function in endothelial cells is necessary for proper vascular development.

The observation that *Wnt1-Cre* mediated excision of $G\alpha_{13}$ caused intracranial hemorrhage and exencephaly and at least grossly phenocopied $G\alpha_{13}^{-/-}$ embryos that were rescued by the *Tie2p/e-*$G\alpha_{13}$ transgene strongly suggests a necessary role for $G\alpha_{13}$ in neural crest or its derivatives. *Wnt1-Cre* mediates excision of floxed alleles in both neural crest cells and in the developing CNS. *Nestin-Cre* is not active in neural crest. *Nestin-Cre* caused virtually complete excision of the floxed $G\alpha_{13}$ allele in the brains of $G\alpha_{13}^{flox/-}$ mice without causing any obvious developmental abnormality, but the onset of



Nestin-Cre–mediated excision was later than that driven by *Wnt1-Cre*. While we cannot exclude the possibility that the difference between the *Wnt1-Cre* and *Nestin-Cre* phenotypes might be due to the earlier loss of $G\alpha_{13}$ function in *Wnt1*-expressing cells, several considerations suggest that the *Wnt1-Cre* conditional phenotype is due to loss of $G\alpha_{13}$ function specifically in neural crest and its derivatives. Analysis of $G\alpha_{13}^{lacZ}$ knockin embryos revealed relatively high expression of $G\alpha_{13}$ in the dorsal neural tube in presumptive neural crest precursor cells, and the details of the $Wnt1\text{-}Cre^{Tg/o}$; $G\alpha_{13}^{flox/-}$ phenotype are consistent with a role for $G\alpha_{13}$ signaling in neural crest. Failed migration of non-neuronal neural crest cells in other mutant mouse strains such as *PDGFRα* knockout mice and *Patch* mutant mice (in which there is, among other genetic abnormalities, a deletion in the *PDGFRα* gene)* is associated with cranial cystic malformations/blebs, hemorrhage, and craniofacial malformations[279] like those seen in $Wnt1\text{-}Cre^{Tg/o}$; $G\alpha_{13}^{flox/-}$ embryos. Alterations in neural crest cell function can also be associated with neural tube closure defects[264, 279, 280] and this is the likely cause of the exencephaly seen in $Wnt1\text{-}Cre^{Tg/o}$; $G\alpha_{13}^{flox/-}$ embryos.

Exactly how $G\alpha_{13}$ functions in vascular endothelial and neural crest cells relate to the observed phenotypes is unknown. $G\alpha_{13}$ contributes to regulation of the small GTPase

* *N.B.*: The genetic defect in *Patch* mutant mice (which is on chromosome 5) is completely distinct from the *Patched* gene (which is on chromosome 13) that is involved in Hedgehog signaling. Interestingly, the *Patch* mutation is a large genomic deletion that includes the *PDGFRα*, *Kit*, and *Flk1* loci. The name "Patch" derives from the observation that mice harboring this mutation have a white patch on their rump that results from the defective migration of a subset of melanocytes.
[Brunkow, M. E., Nagle, D. L., Bernstein, A., Bucan, M. A 1.8-Mb YAC contig spanning three members of the receptor tyrosine kinase gene family (Pdgfrα, Kit, and Flk1) on mouse chromosome 5, Genomics 25, 421-32 (1995).]

Rho by G protein-coupled receptors via Lsc/p115RhoGEF,[251, 252] and Rho plays a key role in regulation of the actin cytoskeleton.[282, 283] Additionally, the Rho family member RhoA has been shown to be involved in regulating the assembly of endothelial cells into vessels in the adult mouse[284] and RhoB has been found to support endothelial cell survival and sprouting morphogenesis in the adult mouse retina.[285] However, despite extensive studies of $G\alpha_{13}$-null endothelial cells in culture, we were unable to detect gross defects in Rho activation, or in agonist-induced changes in cell shape or movement (data not shown).

The heterotrimeric G proteins $G\alpha_{12}$ and $G\alpha_{q/11}$ are also capable of regulating Rho[286-289] therefore it is possible that partial redundancy with these or other G proteins masked effects in these assays. Indeed, while $G\alpha_{12}^{-/-}$ and compound $G\alpha_{12}^{+/-}$; $G\alpha_{13}^{+/-}$ embryos are phenotypically normal, $G\alpha_{12}^{-/-}$; $G\alpha_{13}^{-/-}$ embryos show a more severe, earlier vascular phenotype than global $G\alpha_{13}^{-/-}$ embryos; and $G\alpha_{12}^{-/-}$; $G\alpha_{13}^{+/-}$ embryos show a less severe vascular phenotype than global $G\alpha_{13}^{-/-}$ embryos and are able to develop about a day longer than $G\alpha_{13}^{-/-}$ embryos. These results suggest redundancy in $G\alpha_{12}$ and $G\alpha_{13}$ signaling pathways in vascular development.[288] Redundancy of $G\alpha_{12}$ and $G\alpha_{13}$ signaling pathways is not observed in all cell types or all processes since $G\alpha_{13}$ but not $G\alpha_{12}$ signaling in platelets is required for platelet activation.[290] $G\alpha_q^{-/-}$ mice survive to adulthood but have a bleeding diathesis resulting from defective platelet activation.[270] $G\alpha_{12}^{-/-}$; $G\alpha_q^{-/-}$ embryos exhibit clear defects by E13.5 including defects in digitation of the foot and hand plates but show no obvious vascular defects. $G\alpha_{12}$ and $G\alpha_q$ were expressed by the endothelial cells in our endothelial cell cultures (data not shown) and perhaps they show partial redundancy with $G\alpha_{13}$ functions in endothelial cells.

A reproducible effect of $G\alpha_{13}$-deficiency was seen only when endothelial cells were examined for a more complex behavior: the ability to form networks on Matrigel. $G\alpha_{13}$ null endothelial cells tended to remain organized as sheets rather than reorienting to form networks of cords and tubes. Thus, the signaling defect in endothelial cells lacking only $G\alpha_{13}$ may be relatively subtle and involve cell-cell and/or cell-matrix interactions. In this regard, it is interesting to consider analogies between $G\alpha_{13}$ phenotypes and the *concertina* gastrulation defect. *Concertina* is the *Drosophila* homolog of vertebrate $G\alpha_{13}$.[291] Gastrulation in fly embryos creates mesoderm and endoderm from a uniform blastula, and furrow formation and inward migration of cells occurs at sites along the length of the embryo rather than through a single blastopore as in mouse.[292] *Concertina* mutant embryos begin furrow formation by forming a zone of tightly apposed cells, as do wild-type embryos, and constrict the apices of some of these cells - though ultimately not enough cells constrict to form an organized groove. Thus, *concertina*/$G\alpha_{13}$ is not necessary for the initiation of gastrulation in *Drosophila* but plays a necessary role in coordinating or propagating changes in cell shape and movement. Furthermore, loss of function of DRhoGEF (a *Drosophila* Lsc/p115RhoGEF homologue) and *Drosophila* Rho signal downstream of *concertina* and loss of any member of this signaling cascade results in similar gastrulation defects.[293, 294] This appears to be similar to the endothelial defects seen in the studies presented here; endothelial cells form an initial plexus but fail to properly reorganize it. Detailed studies of the cytoskeletal rearrangements in endothelial cells and their movement during vertebrate vasculogenesis and angiogenesis are needed.

Studies of the signaling molecules that orchestrate the behavior of endothelial cells during blood vessel development have focused mainly on growth factors and receptor tyrosine kinases. Our results emphasize a role for G protein-coupled receptors in this process. Our group previously showed that knockout of *Par1*, a G protein-coupled receptor for thrombin, caused a partially penetrant embryonic lethality at midgestation that could be attributed to a role for PAR1 in endothelial cells.[235, 236] This phenotype was similar in time of onset and general features to that observed in *Tie2p/e-Cre*$^{Tg/o}$; *G*$\alpha_{13}^{flox/-}$ embryos in the present study, but the *Par1*$^{-/-}$ phenotype was less penetrant. Similarly, knockout of *Edg1*, a Gα_i-coupled receptor for sphingosine-1 phosphate,[158] caused lethality that began at ~E12.5 and was attributable to a necessary function in endothelial cells.[159] Combined-deficiency of this receptor along with knockouts of *Edg3* and *Egd5*, which couple to Gα_i, Gα_q, and G$\alpha_{12/13}$, yielded earlier phenotypes.[169, 170] Interestingly, combined deficiency of the G$\alpha_{12/13}$-coupled Edg3 and Edg5 receptors resulted in a partially penetrant vascular phenotype observed after E13.5. Thus, multiple GPCRs and heterotrimeric G proteins play both unique and partially redundant roles in helping to orchestrate endothelial cell function during blood vessel development, and signaling via Gα_{13} plays a key role in this process.

How might Gα_{13} signaling regulate vascular processes? G$\alpha_{12/13}$ has been shown to interact with the cytoplamic tail of N-cadherin and acts to upregulate β-catenin–mediated transcriptional activation by destabilizing β-catenin binding to cadherins.[295, 296] This interaction appears to also down-regulate cadherin-mediated cell adhesion.[297] Thrombin activation of PAR1 has been shown to induce the endocytosis of the TGFβ1 binding

membrane protein Endoglin thus leading to a reduction in TGFβ1 signaling in endothelial cells, though it is not yet known whether $G\alpha_{13}$ is required for this. These results suggest that $G\alpha_{12/13}$ could be involved in EC/VSMC and/or EC/EC association (see the Introduction for a discussion of this). There are also reports that $G\alpha_{12/13}$ signaling through Sonic Hedgehog/Smoothened to RhoA activation and down-stream transcriptional changes in neuronal cells is responsible for smoothened-induced inhibition of neurite outgrowth.[298] Due to the severe vascular defects seen in embryos with inactivating mutations in Hedgehog signaling pathways it is formally possible that this Hedgehog/Smoothened/$G\alpha_{12/13}$ signaling axis has implications in understanding the observed phenotypes or in the defects we observe in *Wnt1-Cre*$^{Tg/o}$; *Gα*$_{13}^{flox/-}$ embryos. These possibilities are speculative but might suggest future experiments to better define the function of $G\alpha_{13}$ in the vasculature.

Our studies also suggest a key role for $G\alpha_{13}$ signaling in neural crest cells. Defects in neural crest cell function in mice lacking the $G\alpha_{13}$–coupled Endothelin Receptors ET_A and ET_B (and in related knockouts) have been reported (see the Introduction for details) and have been attributed, at least in part, to loss of $G\alpha_{q/11}$ activation by these receptors.[272, 299] These knockouts survived to birth with phenotypes quite distinct from the embryonic phenotypes seen with knockout of $G\alpha_{13}$ function in Wnt1-expressing cells. Mice in which $G\alpha_{12/13}$ is deleted in neural crest cells using a different *Cre*-expressing transgene, *P0-Cre*, do not have neuronal or craniofacial defects but rather defects in the cardiac outflow tract.[272] The *P0-Cre* transgene drives expression in neural crest populations that give rise to the spinal dorsal root ganglia, the sympathetic

and enteric nervous systems, and in the ventral craniofacial mesenchyme. These results suggest that Endothelin Receptors do not signal through $G\alpha_{12/13}$ in neural crest cells that contribute to certain craniofacial structures. Our results with the *Wnt1-Cre* transgene imply that $G\alpha_{12/13}$ signaling is, however, required in other neural crest cell populations. Intriguingly, RhoB is selectively expressed in delaminating neural crest cells in chick embryos.[300] It would be interesting to explore whether defective $G\alpha_{13}$ signaling to RhoB in mouse neural crest cells is responsible for the defects we observed in *Wnt1-Cre*$^{Tg/o}$; $G\alpha_{13}^{flox/-}$ embryos. Thus, our results suggest a broader role for GPCR signaling in the development of neural crest-derived tissues and will stimulate efforts to identify the receptors and ligands involved.

MATERIALS AND METHODS

Mutant Mouse Strains

Generation of the $G\alpha_{13}^{lacZ}$ allele:

BAC-125h06 containing the $G\alpha_{13}$ gene (also known as *gna13*) was obtained by screening a mouse 129/SvJae genomic library with two PCR primer pairs (F1/R1 and F2/R2 primer pairs; see Table 5 for all oligonucleotides and primer pairs described here). The F1/R1 pair was designed to amplify sequences in $G\alpha_{13}$ exon 1 and the F2/R2 pair was designed to amplify sequences in $G\alpha_{13}$ exon 4. To facilitate construction of the targeting vector (Figure 1A), two $G\alpha_{13}$ gene fragments from BAC-125h06 were subcloned into pBC-SK (Stratagene) for sequencing and restriction site mapping. The first was a *BamH*I fragment containing ~0.2-kb of 5' untranslated sequence, exons 1 and 2, and ~6.0-kb of 3' intronic sequence. The second was an *EcoR*I/*Xho*I fragment containing ~3.0-kb of 5' untranslated sequence, exon 1, and a portion of intronic sequence downstream of exon 1. To construct the targeting vector portions of these subcloned fragments were PCR amplified and introduced into pNTK,[301] which contains a loxP-flanked neomycin-resistance cassette (floxed-*neo*') and a thymidine kinase cassette (*tk*). The 5' homologous arm of the vector contained 1.7-kb of 5' untranslated sequence and the first 45 base pairs of exon 1, including the start codon, and was amplified by PCR (Primers: F3/R3) from BAC-125h06 with *Pfx* proofreading polymerase and cloned into pCRII-TOPO-Blunt (Invitrogen). A plasmid containing a *lacZ*-Sv40pA cassette (in which all the *EcoR*I and *Xba*I sites in the MCS and the Sv40 polyadenylation sequence had been deleted) was cut with *Hind*III and *Nru*I and the similarly cut 5' homologous arm was inserted such that the

3' end of the arm (*i.e.*, the 45-bp of $G\alpha_{13}$ coding sequence) was fused in frame to the *lacZ* gene via the *Nru*I site. The 3' homologous arm included the last 120-bp of exon 1, 2.5-kb of intron 1, and the first 29-bp of exon 2. It was PCR amplified (Primers: F4/R4) from BAC-125h06 and subcloned in the same manner as the 5' homologous arm. It was then excised from pCRII-TOPO-Blunt with *BamH*I and *Mfe*I and inserted between the floxed-*neo*^{r} and *tk* cassettes in similarly cut pNTK. Finally, the 5' homologous arm *lacZ*-Sv40pA fusion was released from its plasmid backbone with *Sal*I and inserted 5' of the floxed-*neo*^{r} cassette in the similarly cut 3' homologous arm pNTK construct. The linearized vector was electroporated into RF8 ES cells, which were selected as described.[302, 303] 463 ES clones were screened by Southern hybridization; 3 were correctly targeted at both the 5' and 3' ends. Mice generated from one of these, clone 51, were used for the studies presented. The loxP-flanked *neo*^{r} cassette was excised *in vivo* by crossing mice heterozygous for the $G\alpha_{13}^{lacZ\text{-}Neo+}$ allele to mice carrying a β-actin promoter-*Cre* transgene (see Figure 1B).[304]

Generation of a floxed $G\alpha_{13}$ allele:

BAC-125h06 was again used as a source of $G\alpha_{13}$ gene fragments. Two additional fragments were subcloned from BAC-125h06 into pBC-SK. The fist was an *Eco*RV fragment containing most of exon 4 and 2.6-kb of 3' untranslated sequence. The second was an *Eco*RI fragment containing exons 3 and 4, 7.9-kb of intronic sequence upstream of exon 3, and 290-bp of 3' untranslated sequence. The targeting vector (Figure 5) contained a 5' homologous arm corresponding to a 2.3-kb *Spe*I/*Xho*I fragment in intron 3, exon 4 (0.57-kb), and a 3' homologous arm corresponding to 2.0-kb of 3' untranslated

sequence. The 2.3-kb 5' homologous arm was excised from the *EcoR*I BAC-125h06 subclone with *Spe*I and *Xho*I and inserted into a similarly cut *modified* pBC-SK plasmid (in which the native multi-cloning site (MCS) had been replaced by an oligonucleotide linker (F5/R5) containing the following sites: 5'-*Not*I-*Spe*I-*Xho*I-*Kpn*I-*EcoR*I-3'). Next, another oligonucleotide linker (F6/R6) was inserted into the *Xho*I and *Kpn*I sites at the 3' end of the 5' homologous arm (*i.e*, the *Xho*I and *Kpn*I sites in the new MCS created by F5/R5, above) thus destroying the *Xho*I and *Kpn*I sites and adding the following sites: 5'-*Sac*II-*Kpn*I-*EcoR*V-*Xho*I-3'. A *Sac*II/*Kpn*I fragment from pK11 (pBS-SK-FRT-PGKNeo-FRT-LoxP; G. Martin, UCSF) containing an FRT-flanked *neo*' cassette with a single 3' loxP site was inserted into the 3' end of the 5' homologous arm sequence with *Sac*II and *Kpn*I (in the same orientation as the native $G\alpha_{13}$ gene). To generate the 3' homologous arm, a 2.7-kb *Xho*I/*Sac*I fragment containing the 3' end of exon 4 and 1.7-kb of 3' untranslated sequence was excised from the *EcoR*V BAC125h06 subclone and inserted into similarly cut pBC-SK. An oligonucleotide linker containing a loxP sequence and an associated *EcoR*V site (F7/R7) was then inserted into the *Stu*I/*EcoR*I sites 290-bp downstream of the stop codon, destroying the *EcoR*I site. To complete the 3' homologous arm, another oligonucleotide linker (F8/R8) was inserted into the *Sac*I site at the 3' end of the 3' homologous arm loxP construct, destroying the *Sac*I site and introducing an *EcoR*I site. The 3' homologous arm/loxP contruct was cut with *Xho*I and *EcoR*I and inserted into the similarly cut 5' homologous arm FRT-*neo*'-loxP construct (described above). A 0.45-kb *Xho*I fragment spanning the 3' end of the intron and 5' end of exon 4 was excised from the *EcoR*I BAC-125h06 subclone and inserted into the *Xho*I site at the 5' end of the 3' homologous arm. To assemble the final vector, a modified

pNTK plasmid was first generated in which the *Not*I site in the linearization MCS was deleted by *Not*I digest followed by fill-in with Klenow fragment polymerase and religation. Next, the floxed-*neo*^{r} cassette was deleted by digestion with *Xho*I and *Spe*I, and insertion of an oligonucleotide linker (F9/R9) that also destroyed the *Xho*I and *Spe*I sites and inserted *Mfe*I and *Not*I sites. In order to introduce a *tk* selection cassette, all relevant sequences that had been assembled in pBC-SK (*i.e.*, everything above) were excised as a single unit with *EcoR*I and *Not*I and inserted into the *Mfe*I/*Not*I sites in the modified pNTK plasmid thus destroying the insert-derived *EcoR*I site and allowing for linearization with *EcoR*I. All $G\alpha_{13}$ coding sequences and fragment junctions in the final targeting vector were sequenced. The linearized vector was electroporated into RF8 ES cells and 42 ES clones were selected and screened. Two were correctly targeted at both the 5' and 3' ends. Mice generated from one of these, clone 9C3, were used in the studies shown. The FRT-flanked *neo*^{r} cassette was removed by crossing mice heterozygous for the $G\alpha_{13}^{flox\text{-}Neo+}$ allele to mice bearing a transgene in which a modified *FLP*-recombinase ("*FLPe*") gene is driven by a β-actin promoter.[304-306] Excision of the *neo*^{r} cassette was confirmed by *EcoR*V digest and Southern analysis with a *Spe*1 fragment termed "5' Probe" (see Figure 5). Studies were done in mixed C57BL/6:129SvJae or C57BL/6:129SvJae:ICR backgrounds and used littermate controls.

Generation of *Tie2p/e-*$G\alpha_{13}$ transgenic mice:

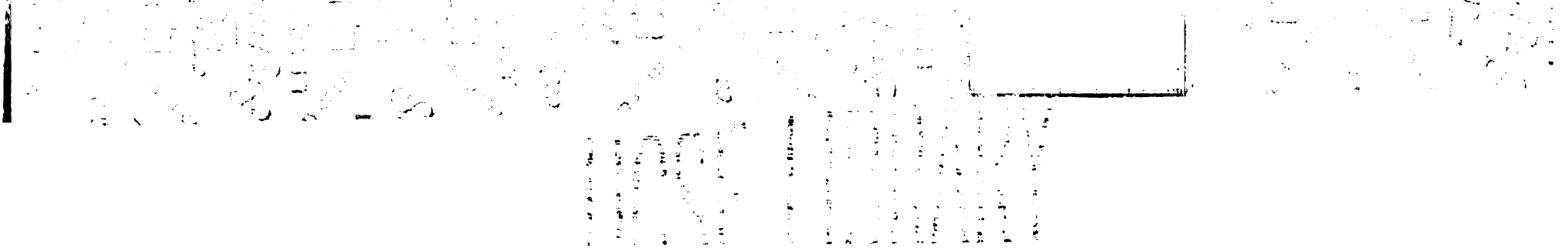
A transgene to direct $G\alpha_{13}$ expression to endothelium was generated in a manner analogous to the *Tie2p/e-Par1* transgene previously described.[236] Briefly, $G\alpha_{13}$ coding sequence from pCDNA1-$G\alpha_{13}$ (provided by Dr. Henry Bourne, UCSF) was ligated, along

with an internal ribosome entry sequence[307] (from Dr. Marc Tessier-Lavigne, UCSF), into pBS-*Tie2p/e-lacZ* (from Dr. Thomas Sato, UT Southwestern) such that the $G\alpha_{13}$-*IRES* cassette was 3' of the Tie2 promoter sequence and 5' of the β-galactosidase coding sequence, polyA, and Tie2 enhancer. A restriction fragment containing the *Tie2p/e-*$G\alpha_{13}$-*IRES-lacZ* insert was purified and introduced by pronuclear injection into fertilized oocytes from ICR strain mice. Two independent transgenic lines that showed endothelium-specific β-galactosidase staining in E8.5-E11.5 embryos were selected for study.

Other mouse strains used: *Tie2p/e-Cre* transgenic mice were a gift of Dr. M. Yanagisawa (University of Texas, Southwestern).[259] *Wnt1-Cre* mice were a gift of Dr. A. McMahon (Harvard University).[266, 267] *Tie2p/e-lacZ* transgenic mice were a gift of Dr. T. Sato (University of Texas, Southwestern).[260] *β-actin-Cre* transgenic mice and *FLPe*-recombinase transgenic mice were from J. Miyazaki (Osaka University Medical School)[258] and S.M. Dymecki (Carnegie Institute of Washington),[306] respectively. The *ROSA26R* Cre reporter strain[308] and *Nestin-Cre* transgenic mice[268] were from Jackson Laboratories. $G\alpha_{13}^{+/-}$ (exon 1 deletion) mice were a gift of Dr. M. Simon (California Institute of Technology).[254]

Genotyping of mutant mouse strains:

Genomic DNA from ES cell clones, embryonic tissue, or mouse tail samples was isolated as described.[236] $G\alpha_{13}^{lacZ}$ and $G\alpha_{13}^{lacZ\text{-}Neo+}$ alleles were genotyped by *Nco*I digestion and Southern hybridization with the Exon 1 probe (see Figure 1). $G\alpha_{13}^{flox}$, $G\alpha_{13}^{FRT\text{-}Neo+}$, and



$G\alpha_{13}$ alleles were genotyped by digestion with *EcoR*V and *Nco*I and Southern hybridization with Exon 1 and Exon 4 probes (see Figure 5 and Figure 6A). These probes were PCR-amplified from BAC-125h06 DNA (see Table 6 for PCR primer sequences). The *Tie2p/e-*$G\alpha_{13}$ and *Tie2p/e-lacZ* transgenes were detected by Southern blot analysis using a *lacZ* cDNA probe. The *Tie2p/e-Cre*, *Wnt1-Cre*, *Nestin-Cre*, and *β-actin-Cre* transgenes were detected either by PCR or by Southern blot using the *Cre* sequence amplified by the same primer pair (see Table 6 for the *Cre* PCR primer sequences).

Generation of homozygous $G\alpha_{13}^{lacZ}$ *ES cells and chimeric embryos:*

The heterozygous $G\alpha_{13}^{lacZ}$ Neo ES cell clone #51 was grown on STO feeder cells in 2.5mg/ml G418 for 10 days to promote gene conversion. 144 ES clones were screened by Southern hybridization to Exon 1 probe, and 29 (20.1%) were homozygous for the $G\alpha_{13}^{lacZ\text{-}Neo+}$ allele. To remove the loxP-flanked *neo*^{r} cassette, clone 4A2 was electroporated with a plasmid containing both a puromycin-resistance cassette and a *Cre*-expression cassette (pCAGGS-NLS-CRE-PGKPuro/cg; a gift from Dr. Gail Martin, UCSF), and were grown on puromycin-resistant STO cells. Approximately 50% of the puromycin resistant clones showed excision of the floxed *neo*^{r} cassette from both $G\alpha_{13}^{lacZ\text{-}Neo+}$ alleles. Two of these, "4A3" and "4C1", were used for the production of embryonic chimeras. A heterozygous $G\alpha_{13}^{lacZ}$ ES clone, "B3", was generated by electroporation of ES clone #51 with pCAGGS-NLS-CRE-PGKPuro/cg followed by puromycin selection as above. Chimeric embryos were generated by micro-injection of ES cells into Wt E3.5 blastocysts using standard techniques.[302]

Embryo dissection, immunostaining and X-gal staining:

Noon of the day a plug was observed was defined as E0.5. After euthanasia, the uterine horns of the pregnant female were removed and embryonic and extraembryonic tissues were dissected free. The yolk sac was photographed and its properties noted, then the embryo was dissected. The presence of a heartbeat and other phenotypic characteristics were recorded before dissecting the next embryo. Whole-mount X-gal staining for β-galactosidase activity was performed as described.[262] X-gal-stained yolk sacs and embryos were embedded in paraffin or plastic, sectioned, and counter-stained with nuclear fast red. For analysis of β-galactosidase activity in the adult, tissues were dissected and fixed as above except on ice for 30 to 60 minutes, then rinsed in PBS and equilibrated in 18% sucrose overnight at 4°C before processing for frozen sectioning. Frozen sections were then stained with X-gal at 37°C for 1-2 days. Immunostaining for PECAM1 used rat anti-mouse CD31 (Pharmingen) and Cy3-conjugated goat anti-rat secondary antibody (Jackson Immunoresearch) while immunostaining for Endoglin used rat anti-mouse CD105 (Pharmingen) and an HRP-conjugated goat anti-rat secondary antibody.

Southern and Northern analysis

For Northern analysis of $G\alpha_{13}$ expression in purified adenoviral-infected mouse endothelial cells, total RNA was prepared using Trizol (Invitrogen) according to the manufacturer. 10 μg of RNA was separated in 1% agarose gels and transferred to a

Hybond-N membrane. Samples were hybridized in Sigma Perfect-Hyb to a DNA probe for $G\alpha_{13}$ exon 4 (corresponding to nucleotides 703-1144 of the $G\alpha_{13}$ coding squence). All Southern and Northern probes were labeled with ^{32}P-dCTP using random primer labeling kits according to manufacturer's instructions (Amersham Rediprime II); hybridization was detected by scanning Southern and Northern membranes using a Storm PhosphorImager (Molecular Dynamics).

Isolation and adenoviral infection of neonatal endothelial cells

Microvascular endothelial cells were isolated from the skin of $G\alpha_{13}^{flox/flox}$ mice as described.[256] Over 95% of cells in these preparations expressed the endothelial markers PECAM1 and ICAM2.[256] Immediately after the second round of immunopurification, cells were subjected to 2 successive rounds of infection with ~100 IFU/ml of $Cre^{+}GFP^{+}$ or control (GFP^{+} only) adenovirus (a gift from Dr. Hilary Beggs, UCSF) for 24 hr each.[269, 309] One to 6 days after the second infection, the cells were used for experiments.

Matrigel assay

400-500 µl of Matrigel (Beckton Dickinson, Catalogue #354234) was gelled in 18mm cylindrical holes in an agarose gel for 1-2 hr at 37°C. 1 X 10^5 endothelial cells that had been infected with the appropriate adenovirus, were plated onto the Matrigel surface and incubated 14-16 hr at 37°C in DME with 20% fetal bovine serum and 50 µg/mL endothelial cell growth supplement (BTI, Stoughton, MA). Planimetry to calculate Network Area % was calculated using Adobe Photoshop 7.0.

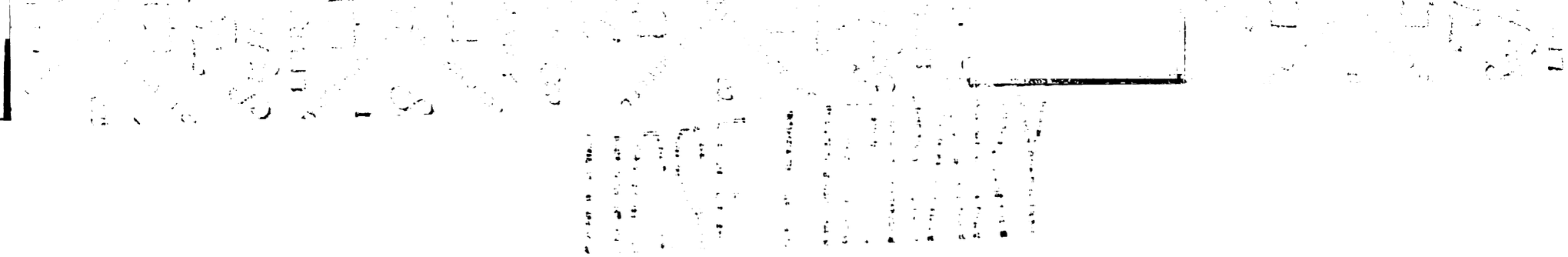


Table 6. *Oligonucleotides used for the generation and genotyping of mice. Each is shown in a 5'-3' direction. Internal restriction endonuclease sites are shown in bold; overhangs are marked with a dotted line and the restriction enzyme that generates the compatible overhang is shown.*

F1 AGCAGCGCAAGTCCAAGGAGATCG
R1 GATCTCGTTGCTGTAGATGGTGGG

F2 AATCGGGTTTTCAGCAACGT CTC
R2 CTGCAGCATGAGCTGCTTCAGG

F3 **AAGCTT**TATTGTTTACTGCAGCCAGTGTAGTGAGTGAGTC
HindIII
R3 **TCGCGA**GCGAAGCACACGGACAGCACGGAGCG
NruI

F4 **CAATTG**AATAGATAGAGCGGCAAGTCCACCTTCC
MfeI
R4 **GGATCC**TCTCTCGGGCATCTACCAGCAC
BamHI

KpnI
F5 **GCGGCCGC-ACTAGT**CGG**CTCGAG**ATT**GGTACC-GAATTC**GTAC
NotI *SpeI* *XhoI* *KpnI* *EcoRI*
R5 GAATTCGGTACCAATCTCGAGCCGACTAGTGCGGCCGCAGCT
SacI

XhoI *KpnI*
F6 TCGA**CCGCGG**GGA**GGTACC-GATATC-CTCGAG**CGTAC
SacII *KpnI* *EcoRV* *XhoI*
R6 GCTCGAGGATATCGGTACCTCCCCGCGG

F7 CCT**GATATC**ATAACTTCGTATAGCATACATTATACGAAGTTAT
EcoRV *loxP*
R7 AATTATAACTTCGTATAATGTATGCTATACGAAGTTATGATATCAGG
EcoRI

SacI
F8 TGGGC**GAATTC**GGGAAGCT
EcoRI
R8 TCCCGAATTCGCCCAAGCT
SacI

XhoI
F9 TCGA**CAATTG**AAGGAAAAAA**GCGGCCGC**
MfeI *NotI*
R9 GATCGCGGCCGCTTTTTTCCTTCAATTG
BamHI

5' Exon 1 Probe GTTTTTGCCTGAGCAGCGTC
3' Exon 1 Probe TCCTGTTACATCCACTGAGACCG

5' Exon 4 Probe TTACAGACAGATATCTGGGTGGCA
3' Exon 4 Probe GATTACTTACATGCCGTCTCCACA

5' *Cre* PCR Primer GATATCTCACGTACTGACGGT
3' *Cre* PCR Primer GCCGGCTAATCGCCATCTTC

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