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I, Jieqing Fan, hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Molecular & Developmental Biology.

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Crim1 Maintains Retinal Vascular Stability during Development by Regulating Endothelial Cell Vegfa Autocrine Signaling

Student’s name: Jieqing Fan

This work and its defense approved by:

Committee chair: Richard Lang, Ph.D.

Committee member: Michael Robinson, Ph.D.

Committee member: Vladimir Kalinichenko, M.D., Ph.D.

Committee member: Saulius Sumanas, Ph.D.

Committee member: James Wells, Ph.D.
Crim1 maintains retinal vascular stability during development by regulating endothelial cell Vegfa autocrine signaling

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Jieqing Fan
B.S. Tsinghua University, China (2007)

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Committee chair: Richard A. Lang, Ph.D.
ABSTRACT

Angiogenesis defines the process in which new vessels grow from existing vessels. Revealing the molecular mechanisms involved in this process will help deepen our understanding of cancer, diabetes, and many other diseases, and will direct avenues for future development of pro-angiogenesis or anti-angiogenesis therapies.

In this study, using murine retinal angiogenesis as a model system, we show that Crim1 (Cysteine-rich motor neuron 1), a type 1 trans-membrane protein, is highly expressed in angiogenic endothelial cells. Deletion of the Crim1 gene in endothelial cells causes defective retinal angiogenesis characterized by delayed vessel extension and reduced vessel density. We demonstrated that endothelial Crim1 prevents ectopic vessel regression. We showed that Vegfa autocrine signaling is required for angiogenesis and we provide in vivo and in vitro data consistent with a model in which Crim1 enhances the autocrine signaling activity of Vegfa in VECs in part via Vegfr2.
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SECTION 1.1 Angiogenesis: a significant biological process

Blood vessels transport blood, which carries oxygen, nutrients and immune cells, throughout the body (Figure 1.1). This hierarchically branched network of endothelial tubes is involved in the growth, functioning and homeostasis of almost all of the organs in the body. Blood vessels are formed through at least two distinct mechanisms: vasculogenesis and angiogenesis (Figure 1.2) (Carmeliet, 2003). Vasculogenesis is the process in which local mesoderm-derived cells differentiate into endothelial cells (ECs) which then assemble into primitive tubes (Figure 1.1; Figure 1.2A). Vasculogenesis

![Figure 1.1 Formation of a vascular network.](image)

Figure 1.1 Formation of a vascular network. Endothelial progenitor cells differentiate into endothelial cells, which then assemble into the large axial vessels and primitive capillary plexus. Vessels then undergo sprouting angiogenesis to complete the vasculature. HSCs influence vessel formation. MΦ: macrophages. See text for more detail about the molecules. Image adapted from (Carmeliet, 2003)
appears to be largely confined to the formation of the first primitive vascular structures as well as the large axial vessels in the early embryo (Flamme et al., 1997). By contrast, ‘angiogenesis’ defines the process in which new blood vessels grow from existing vessel beds (Figure 1.2B). ECs sprout, branch and connect to expand vascular network to support blood flow into new area (Geudens and Gerhardt, 2011).

Figure 1.2 Schematic diagrams of stages in vasculogenesis and angiogenesis. Images adapted from (Chinoy, 2003)

Angiogenesis is not only an important development process, but also a driving force in pathogenesis of a series of diseases (Carmeliet, 2003) including cancer, ocular diseases, atherosclerosis, etc. (Bikfalvi, 2006). In the past decade, we have seen explosive number of scientists get involved in angiogenesis research in development and diseases, which greatly deepened our knowledge of angiogenesis at genetic,
molecular and cellular levels. In addition, angiogenesis is revealing to be an effective therapeutic target (Carmeliet and Jain, 2011a).

Section 1.1.1 Angiogenesis in normal organ development

To guarantee that organs undergoing development are adequately oxygenized, blood vessel growth is synchronized with organogenesis and this growth is precisely orchestrated in time and location to make sure precise number and size of vessels are formed (Carmeliet, 2003). One remarkable feature of angiogenesis is that nascent network is constantly connected to existing vessel beds (Figure 1.2) while the normal perfusion within existing vessels is not affected; new vessels are immediately remodeled as functional conduits once formed (Geudens and Gerhardt, 2011).

One can find numerous examples of how angiogenesis is implicated in organogenesis. Adult human brain receives about 20% cardiac output, and this central nervous system vasculature is formed through angiogenesis. Vascular endothelial growth factor (VEGF) plays a critical role in initiating this process. During embryogenesis, vessels from pia mater invade centripetally towards the ventricles; some of these invading vessels form secondary branches to surround the ventricles whereas others extend centrifugally back towards the pia mater. This special structure makes the ventricles extremely sensitive to perinatal ischemia (Greenberg and Jin, 2005). Vessels in the brain are also distinctive in that they have high level of tight junction molecules between endothelial cells and high coverage of pericytes. These contribute to the blood-brain-barrier, which, if disrupted, will cause edema, threatening
the neurons, causing high intracranial pressure and may ultimately resulted in death (Liebner et al., 2008; Paolinelli et al., 2011).

As part of the partial nervous system, neural retina also has a vasculature formed through angiogenesis and the whole process has been shown to be strictly regulated. In mouse, on postnatal day 1, vessels start to sprout from the central vein within the optic stalk and then extend and branch toward the periphery of the retina (Gerhardt et al., 2003) (Figure 1.3). Elongation and branching all happen on a preformed astrocyte template, which is believed to be not only the major source of growth factor VEGF-A (Fruttiger, 2002), but also provider of extracellular matrix for endothelial cells to migrate on (Stenzel et al., 2011), thus the primary plexus grows within the ganglion cell layer and, parallel to the out-limiting membrane, and is completely formed within the first postnatal week in mouse. Vessels then sprout vertically into the depth between outer-nuclear-layer and photoreceptor layer and then turn and branch horizontally to form a deep plexus (Figure 1.3). A third layer of network in outer-plexiform-layer is formed subsequently so that by 4 weeks old, the mouse acquires the vasculature composed of three parallel network within the retina (Gariano and Gardner, 2005). Given the

Figure 1.3 Schematic of mouse retinal vascular development. Blood vessels (green) progress postnatally forming the superficial plexus by P6 to P8 depending on background. Descending sprouts meet deep layer myeloid cells (red arrows), turn, branch, and form the deep plexus by P18 (image courtesy of Richard Lang and James Stefater).
simplicity in analyzing the network and accessibility of the eye by genetic manipulation and pharmaceutical approaches, mouse retina vasculature has emerged as a powerful model system in angiogenesis research (Geudens and Gerhardt, 2011).

Out of the nervous system, the interplay between angiogenesis and growth and differentiation of other cell types has been implicated in other organ development. For example, at the onset of pancreas morphogenesis, endothelial cells begin to be recruited to the epithelial bud. Later on, signals from endothelial cells were shown to repress the expression and activity of Ptf1a, and thus repress acinar differentiation; endothelial cells are also required to maintain pool of endocrine progenitors and derivatives (Cerf, 2011). Osteogenesis is also closely coupled with angiogenesis. During endochondral bone development, before replacement of the cartilaginous matrix with bone, invasion of blood vessels is a critical step. Blood vessels may not only bring oxygen and nutrients; progenitors of osteoblasts have been found in the wall of bone marrow microvasculature as well (Schipani et al., 2009).

Another example resides in the hematopoietic system. The hematopoietic microenvironment is critical for the self-renewal, proliferation, and differentiation of pluripotent hematopoietic stem cells. Within the hematopoietic microenvironment, whether it is embryonic yolk sac, fetal liver, or adult bone marrow, microvascular endothelium not only acts as a gatekeeper controlling the trafficking and homing of hematopoietic progenitors, but also provides cellular contact and secretes cytokines that allow for the preservation of the steady state hematopoiesis (Kopp et al., 2005; Schipani et al., 2013).
In conclusion, blood vessels are critical components of almost all organs. It not only provides oxygen and nutrients, the interface between endothelial cells and epithelial cells and other cells may also be critical for functionality of organs, as in kidney, liver and lung (Compernolle et al., 2002). Vasculature, especially microvasculature in majority of the organs develops through angiogenesis, and angiogenesis is closely and precisely coupled with every step during organogenesis.

Section 1.1.2 Pathological angiogenesis

Given the importance of vasculature in multiple organs, it is easy to understand that inadequate or excessive angiogenesis will cause dysfunction of these organs later on. Indeed, there has been expanding evidence implicating angiogenesis in progression of multiple diseases, many of which are life-threatening.

Cancer

Tumors smaller than 2 mm² are usually avascular and grow slowly. When tumors grow even bigger, the tumor cells and stromal cells undergo a molecular switch inside the cells called ‘angiogenic-switch’ when pro-angiogenic factors are secreted (Hanahan and Folkman, 1996). As in normal development and ischemic tissue, hypoxia is the driving force for this switch, in addition, oncogenic switch in tumor cells is also involved; activation of Ras causes production of VEGF from tumor cells and down regulates anti-angiogenic thrombospondin-1 secretion (Figure 1.4A). This effect may be expanded by an autocrine positive loop (Kushner and Bautch, 2013). In addition, transformed tumor
cells will further activate quiescent stromal cells such as fibroblast cells and myeloid cells (Moserle and Casanovas, 2013).

Blood vessels support tumor and eventual metastasis of tumors. Solid tumors have much higher density of vessels than non-transformed tissue indicating that tumors rely on blood vessels recruitment for growth. However, blood vessels in the tumors are different from those in normal organs that they bear lower level of adhesion junctions and tight junctions between endothelial cells and may have inadequate coverage of mural cells such as pericytes (Kushner and Bautch, 2013) (Figure 1.4B). As a result, vessels in the tumors are often leaky, thus actually cannot effectively alleviate hypoxia; they appear winding and lose vessel hierarchy, insistent to vascular remodeling and normalization. These characteristics are both the result and cause of complexity of pro-

Figure 1.4 Tumor angiogenesis. A) ‘Angiogenic switch’ of cells in requirement for neovascularization. This cell can be epithelial cell, myenchymal cell or other cell types in developmental normal organ or tumors. B) Tumor microvasculature (and microvasculature in many other pathogenesis) is often uneven, tortuous, lacking cell-cell adhesion, lacking intact cytoskeleton and full coverage of mural cells, as compared to microvasculature in normal organs. Adapted from (Bikfalvi, 2006; Kushner and Bautch, 2013)
and anti-angiogenic factors in tumor microenvironment and reflect the difficulty to synchronize angiogenic signals (Carmeliet and Jain, 2011b).

Consequently, the impacts that blood vessels exert on tumor progression are complex. Intriguingly, with leaky vessels, tumor cells can become tolerant to low oxygenation. The connection between hypoxia and more aggressive/invasive phenotype of tumor cells has been established. The transition is largely mediated by hypoxia-inducible factor 1 (HIF1), and the molecular switches include deregulation of adhesion junction molecules, basement membrane modification molecules and signaling molecule c-Met in tumor cells (Kushner and Bautch, 2013). Based on these observations and the fact that pure anti-angiogenic therapies showed the mixed results, therapies involve short-term tumor vasculature normalization have been proposed (Carmeliet and Jain, 2011b; Welti et al., 2013); yet given the abnormal phenotypes of endothelial cells in tumor vasculature than those in normal vessels, the efficacy needs further validation. Nevertheless, we predict that with more knowledge in factors regulating endothelial behavior in tumors and their effects or tumor and stromal cells, effectiveness of cancer therapies can be brought to a new level.

**Atherosclerosis**

Atherosclerosis is an important cause of coronary or peripheral artery disease. The association of atherosclerosis and angiogenesis has been established for a long time. Capillary growth within the walls of large arteries may contribute to the establishment of a proliferative lesion and invasion into the intima. Neovascularization of the lesions promotes their progression to rupture and causes subsequent thrombotic
events. Since plaque vessels are immature, leaky and poorly covered by mural cells, thus they facilitate the release of red blood cells and proteases into the lesion (Ho-Tin-Noe and Michel, 2011). Blood cells increase plaque lipid content and provide microenvironment for plaque cell proliferation; proteases degrade extracellular matrix of fibrous cap; both contribute to necrotic core of the plaque. It has been reported that inhibition of plaque neovascularization reduces the accumulation of macrophages and progression of the plaque to advanced atherosclerotic lesions. VEGF and other pro-angiogenic factors are thought to be secreted by the lesion to induce neovascularization upon HIF activation, although when hypoxia occurs in lesions is still largely unknown (Hristov and Weber, 2011; Moreno et al., 2004).

**Ocular disease**

Excessive vessel growth in the eye is related to many ocular diseases, including retinopathy of prematurity (ROP), diabetic retinopathy, age-related macular degeneration (AMD), and choroidal neovascularization (Carmeliet, 2003; Gariano and Gardner, 2005). Though causes of neovascularization vary between diseases, the angiogenesis process is largely mediated by VEGF signaling. For example, in premature infants blood vessels in the retinal are far from maturation thus is very sensitive to environmental oxygen. Exposure to oxygen treatment as is often used on pre-term babies causes retinal blood vessel constriction and subsequent hypoxia in the eye. This will induce an excessive angiogenesis, although other researchers do not think oxygen treatment as a main risk factor for ROP (Shastry, 2010). These vessels usually abnormally invade into the vitreous humors; the fibrous tissue associated may
cause retinal detachment and in severe cases, cause blindness; and patients with ROP are more prompted to development other ocular disease in late time (Gariano and Gardner, 2005).

Microvasculature – as is in the eye – is very sensitive to poor blood sugar control. In diabetes patients, hyperglycemia causes pericyte apoptosis, breaks down retina-blood barrier, and makes these small vessels very leaky. This leads to edema in the retina and obstruct vision; sometimes, the edema is not resolvable (Klaassen et al., 2013). When diabetic retinopathy progresses to proliferative stage, tissue ischemia causes excessive neovascularization along the retina and into the vitreous, causing cloudy vision and hemorrhaging in the eye and may eventually harm the retinal neurons and result in retinal detachment (Petrovic, 2013). Note that diabetic retinopathy is not the only vascular disease that patients with diabetes suffer: impaired angiogenesis in the limbs also causes ischemia (Bikfalvi, 2006; Carmeliet, 2003).

**Arthritis and psoriasis**

Chronic inflammatory disorders such as chronic polyarthritis are also angiogenesis-dependent. In the early phase, this disease is characterized by a proliferative lesion of synoviocytes in the synovia. The neovessels in rheumatoid joints are dysfunctional, contributing to a hypoxic environment (Akhavani et al., 2009; Taylor and Sivakumar, 2005).

Within the inflamed synovia, the number and quality of microvessels are also altered. VEGF and integrin $\alpha_V\beta_3$ seem to play an essential role since blocking their activity in animal models results in disease improvement. Recent data suggest that
tumor necrosis factor-α blockade may modify angiogenesis in rheumatoid arthritis by acting possibly on integrin function in endothelial cells (Ranieri et al., 2005).

Psoriasis is also considered as an immune-mediated disorder that is closely related to blood vessel formation. The histological hallmarks at onset of the disease include high-density dermal vascularization and infiltration and accumulation of immune cells, mainly Th1 and Th17 cells, which will subsequently induce hyperproliferation of keratinocytes (Heidenreich et al., 2009).

**Obesity**

The prevalence of obesity in United States is very obvious. In 2009-2010 35.7% of U.S. adults and 16.9% of U.S. children and adolescents were obese (Body Mass Index, BMI>=30) (from website of Centers for Disease Control and Prevention). Obesity increase the risk of many health conditions including hypertension, cardiovascular diseases, vascular disease in nervous system, type 2 diabetes, several types of cancer and inflammatory diseases (Lijnen, 2008). Not surprisingly, neovascularization supplies adipogenesis with oxygen and nutrients. The hallmark for fat cell development is the emergence of fat cell clusters, which are vascular structures in the adipose tissue with few or no fat cells (Lijnen, 2008). Later each fat cell is in contact with at least one vessel; growing adipose tissue trigger blood vessel formation, and in turn endothelial cells promote adipose differentiation (Daquinag et al., 2011; Pang et al., 2008).

VEGF signaling is accounted for most of the pro-angiogenic events in adipogenesis, although many other molecules have been added to the list of factors affecting angiogenesis in adipose tissue including PDGF, PIGF, FGF-2, ANG2, ANG4,
leptin, TSP-1, etc. (Lijnen, 2008). Interestingly, PPARgamma activators stimulate adipocyte differentiation and increase adipose tissue mass; however, it also inhibits endothelial cell growth in vitro. It was found that VEGF and ANGPTL4 secretion was elevated in adipose tissue treated with PPARgamma activators; ANGPTL4 not only promotes angiogenesis, but also moderates the effects of PPARgamma only on endothelial cells independent of VEGF (Gealekman et al., 2008).
SECTION 1.2 Coordinated cell behaviors in angiogenesis

Blood vessels are polarized tubular structure which has a luminal (apical) side and basal side, thus neovascularization cannot be achieved by mere proliferation of homogeneous endothelial cells (Lizama and Zovein, 2013). In order to form a new perfuseable vessel segment while maintaining the normal function of existing vessels, angiogenesis requires coordination of cell behaviors, including a series of morphological changes, of neighbouring endothelial cells (Figure 1.5) (Adams and Eichmann, 2010; Geudens and Gerhardt, 2011). In this section I seek to introduce in more details and discuss the role of molecular players, especially those involved in regulating cytoskeleton, cell-cell adhesion and cell-matrix adhesion, in these changes.

Figure 1.5 Schematic of steps in sprouting angiogenesis. Endothelial sprouting requires dynamic changes of inter-endothelial junctions and the sub-endothelial basement membrane. Adapted from (Eilken and Adams, 2010)
Section 1.2.1 Tip cell selection and sprout initiation

Scientists have established the ‘tip-stalk cells’ concept in angiogenic sprouting. Tip cells are positioned at the very end of sprouts, followed by stalk cells (Gerhardt et al., 2003). Morphologically, tip cells extend numerous filopodia into avascularized area; directionality of the filopodia depends on gradient of environmental cues. The polarized tip cells decide the directional migration and elongation of the whole sprouts. Stalk cells proliferate to support the elongation of the vessels sprouts (Gerhardt et al., 2003); they also contribute to the nascent lumen of the sprouts (Zovein et al., 2010).

When nutrition and oxygen demands exceeds the capacity of existing vessels, local tissues will secrete pro-angiogenic cues, predominantly vascular endothelial growth factors (VEGF), to activate the originally quiescent vessels. Though all cells receive the pro-angiogenic signals, cells within the activated endothelium are specified into tip cells and stalk cells in a ‘salt and pepper’ fashion and acquire different gene-expression profile, morphology and work share in angiogenic process (Phng and Gerhardt, 2009). Recently many groups found that tip cell selection was resulted from a feedback loop between VEGF and Notch/Dll4 signaling (Hellstrom et al., 2007b; Siekmann and Lawson, 2007; Suchting et al., 2007); the mechanism mimicked Notch-mediated lateral-inhibition process in other circumstances. Small random differences in VEGF concentration and responsiveness to VEGF signaling lead to small imbalance of DLL4 expression between two adjacent cells. The cells responding more to VEGF signaling and thus expressing more DLL4 will signal to the adjacent cells, leads to further down-regulation of VEGF receptors and consequently, DLL4, in the adjacent cells. This imbalance is gradually enlarged and reinforces the phenotypic differences
between tip cells and stalk cells (Hellstrom et al., 2007a). Other ligands that can signal through Notch1 also take part in balancing tip cell-stalk cell ratio. For example Jagged1 elevates vessel density by antagonizing Dll4-Notch signaling in cells expressing Fringe family glycosyltransferases (Benedito et al., 2009).

Figure 1.6 Tip cell fate specification. Left: Image of sprouting ECs in the postnatal retina. New sprouting (yellow arrow), establish sprouts with distal tip cells (green arrows), ECs forming new connections (orange arrow), and perfused vessels (red asterisks). Right: Diagram of tip cell selection and fate enforcement by Notch signaling. (Adams and Eichmann, 2010)

A recent report showed that tip cell and stalk cell fate are interchangeable; cells within a sprout are always competing for the dominant tip-cell position (Jakobsson et al., 2010). Thus cells are constantly evaluating levels of VEGF and other pro-angiogenic cues and ensure that the cell with highest responsiveness to these cues leads the sprouts so that the new vessels can be patterned closest to the demand of the tissue. Thus it becomes interesting to investigate: 1) at what threshold of Notch signaling a tip cell fate is stabilized; and 2) how do signals that fine-tuning Dll4/Notch signaling, such as Vegfc-Vegfr3 and Sema3E-PlexinD1 (discussed below) involved in shifting of cells.
Section 1.2.2 Sprouts elongation

Tip cells guide the directional growth of the vessels sprouts in response to gradient of environmental cues, yet tip cells themselves rarely proliferate (Gerhardt et al., 2003), although division of leading cells in zebrafish intersomitic vessels (ISVs) were observed. The elongation of the sprouts is largely supported by proliferation of stalk cells and collective migration of tip cells and stalk cells. Interestingly, the division plane of two daughter cells is usually perpendicular to the long axis of the sprout (Zeng et al., 2007). This is consistent with the fact that capillaries are mostly wrapped by only one layer of endothelial cells and this mechanism ensure most efficient elongation of the sprouts (Geudens and Gerhardt, 2011). The molecular mechanisms deciding the division axis is still largely unknown. In Drosophila trachea, it has been shown that the leading tip cell exert pulling force on stalk cells which is critical for the intercalation of these cells into growing sprouts (Caussinus et al., 2008). Similarly, pulling force from the vascular tip cells, which are polarized and contact with surrounding VEGF gradient may also help to establish the division direction of vascular stalk cells (Lizama and Zovein, 2013).

Section 1.2.3 Lumen formation

At least two mechanisms have been proposed in lumen formation during vascular tube development: 1) Coalescence of intracellular vacuoles, which ultimately fuse across the sprouts. 2) Formation and enlargement of intercellular space between two endothelial cells. Both are supported by experimental evidence (Iruela-Arispe, 2011). In the latter case, endothelial cells need to define the apical facet and the apical (luminal) space is formed by apical exocytosis of vacuoles. Integrinβ1 binding to the basement membrane
and adhesion junction molecule VE-Cadherin are required in setting up the apical-basal polarity. Par3 is responsible for the apical transportation of apical/luminal molecules and vacuoles, in which small RhoGTPases Rac1 and Cdc42 probably also play a role (Iruela-Arispe, 2011; Zovein et al., 2010).

Recently by investigating the lumen formation of mouse and zebrafish aorta, people found that the extracellular lumenization process can also be achieved by apically locating CD34-sialomucins, the negative charges of which will cause repulsion of the two endothelial cells and thus initiate lumenization. Contraction of apically localized F-actin also contributes to the shape change of the endothelial cells which further help the intercellular spacing (Strilic et al., 2009). Whether this cell-repulsion mechanism is also used in microvasculature lumen formation is worth further investigation.
Section 1.2.4 Anastomosis: fusion of sprouts

In order to form a closed circulatory system, when two sprouts grow and meet each other, the endothelial cells as well as the luminal space within each sprouts need to be connected and this process is called anastomosis. It has been found that cell-cell adhesion molecules including VE-cadherin are not only expressed at lateral side of stalk cells; it is also transported to the very end of filopoquia of the tip cells and may initiate the adhesion of two cells (Almagro et al., 2010; Blum et al., 2008; Montero-Balaguer et al., 2009). When two cells migrate towards each other, the VE-cadherin contacts also expand. The ‘ring-like’ distribution of adhesion molecules also defines the apical-basolateral boundary, which is critical for subsequent lumen formation (Blum et al., 2008; Zovein et al., 2010). The connection of two sprouts may require macrophages as a ‘bridging cell’ (Fantin et al., 2010), although macrophage-independent fusion of two sprouts also exists.

**Figure 1.7 Anastomosis of two angiogenic sprouts.** (A–C) Images of two zebrafish intersomitic vessels (labeled by EGFP) fusing to form DLAV. Note the distribution of adhesion molecules (in this case, ZO-1). (A’–C’) Schematic of (A–C), cell 1 (green) meets cell 2 (red), adhesion is initiated (A’) and enlarged (B’) and finally redistributed to the boundary between apical and basolateral side thus exhibits a ring shape. (Blum et al., 2008)
Section 1.2.5 Vessel remodeling and maturation

Once the nascent network is formed, vessels undergo extensive remodeling to form the mature vasculature. While the initial density of the vessel network is dependent on tip cell number (Hellstrom et al., 2007a), the regression process ensures that the final vessel number closely meets the requirement of the tissue (Sun et al., 2005).

Vessel regression can be largely attributed to endothelial cell apoptosis, in which Akt activity plays a critical role (Niu et al., 2004; Sun et al., 2005). Many signaling pathways have been demonstrated involved in vessel regression, including Vegf signaling, angiopoietin -tie receptors pathways (Lobov et al., 2002; Rao et al., 2007), Wnt pathways (Lobov et al., 2005; Phng et al., 2009) and etc. When tissue is vascularized, increased oxygen level will decrease the activity of pro-survival signals, and finally cause vessel pruning: some branches are stabilized whereas others regress.

Recently scientists showed that lumen restriction and loss of endothelial cell adhesion precede vessel branch regression and destruction of vessel branches may depend on endothelial cell retraction to adjacent vessel beds in addition to cell death (Gerhardt lab, unpublished data). No matter what mechanisms are involved, blood flow is critical in maintaining the stability of vessel branches (Lobov et al., 2011; Wang et al., 2010a).
SECTION 1.3 Endothelial signaling pathways regulating angiogenesis

Angiogenesis is the process where vascular system is constantly adapting to new oxygen as well as nutrient needs. A great number of signaling pathways have been identified involved in pro- or anti-angiogenesis, the interplay between these signaling pathways is implicated in context-dependent angiogenesis. Here I focus on VEGF signaling pathway which is key to angiogenesis and discuss how some newly identified pathways get into the teamwork.

Section 1.3.1 Vascular endothelial growth factor (VEGF)

As clearly mentioned above, production of vascular endothelial growth factor (VEGF, or VEGFA) is the limiting step in almost all angiogenesis events, no matter in normal development or in pathogenesis (Carmeliet, 2003). VEGFA is secreted growth factor which has strongest affinity to two receptor tyrosine kinases (RTK): VEGFR2 (also known as FLK-1 or KDR) and VEGFR1 (also known as FLT-1) (Ferrara et al., 2003). Though these receptors are also found in bone marrow derived cells and some neurons, the VEGFA signaling is regarded as the mere most important pathway promoting endothelial cell survival, proliferation and migration in vitro and in vivo (Gerber et al., 1999); loss of only one allele of Vegfa gene in mice cause embryonic lethality between E11.5 and E12.5 (Ferrara et al., 1996). These mice have impaired angiogenesis and blood-island formation. VEGFA has three major isoforms VEGFA\(_{121}\), VEGFA\(_{165}\) and VEGFA\(_{189}\) (in mouse: Vegfa\(_{120}\), Vegfa\(_{164}\), Vegfa\(_{188}\), lower-cased numbers are numbers of amino acids.) different in C-terminus truncation. As the matrix binding property of VEGFA resides in the C-terminus of the ligand, the longest isoform has the
highest affinity to glycoproteins in extracellular matrix whereas the shortest isoform is most diffusible (Stenzel et al., 2011; Stringer, 2006). VEGFA$_{165}$ is the most common form and has intermediate stickiness on matrix. This characteristic is critical in angiogenesis because it helps to set up a high to low gradient of the ligand along the avascular- to-vascular axis and ensures that the proliferation and collective migration of endothelial sprouts are towards avascularized area.

$Flt1^{-/-}$ mice died at E8.5 to E9.5 (Fong et al., 1995; Fong et al., 1999), but different from $Vegfa$ deletion these mice exhibited excessive proliferation of the blood island. Thus FLT-1 is considered as an inhibitory receptor probably because it avidly binds VEGFA yet causes weak downstream signaling cascades (de Vries et al., 1992), unlike FLK-1. This hypothesis is confirmed by studies on mice of late stages (Kappas et al., 2008). In addition, alternative splicing of $Flt-1$ can make another soluble form of the protein sFlt-1, which is not membrane bound but released to extracellular space. sFLT-1 is secreted by stalk cells in the angiogenic sprouts to limit the orientation of the sprouts thus promote more effective expansion of the network (Kappas et al., 2008); in some contexts it also can be secreted by myeloid cells to down-regulate vessel density (Stefater et al., 2011; Stefater et al., 2013).

VEGFR2 is the major mediator of the mitogenic, angiogenic and permeability-enhancing properties of VEGFA (Ferrara et al., 2003; Terman et al., 1992). $Flk1$-null mice also died at E8.5 lacking blood islands and organized blood vessels (Shalaby et al., 1995). Binding of VEGFA to VEGFR2 leads to receptor dimerization and autophosphorylation, as well as phosphorylation of downstream effectors including phospholipase C-γ, PI3K, Ras GTPase-activating protein and Src family (Ferrara et al.,
The RAF-MEK-ERK pathway drives endothelial cell proliferation (Takahashi et al., 1999) and the PI3K-AKT branch serves as pro-survival signal by up-regulating BCL-2 and A1 (Gerber et al., 1998a). The pro-survival effect in vivo is more evident in newly formed vessels rather than mature vessels: the dependence on VEGF signaling for survival is mitigated after mural cell coverage (Benjamin et al., 1999). In certain situations, such as in arterial-venous specification, the preference of one branch of the pathway is critical, and this may be related to AKT’s inhibition of RAF1 (Deng et al., 2013). How the two signaling cascades are coordinated within endothelial cells is still unclear.

VEGFR2 is constantly transported between plasma membrane and Golgi apparatus (Horowitz and Seerapu, 2012; Tiwari et al., 2013). Endocytosis of the receptor not only adjusts available receptors at the cell membrane, it is also found to be limiting step in initiating intracellular signaling cascade (Gourlaouen et al., 2013; Lampugnani et al., 2006; von Zastrow and Sorkin, 2007). Mutations in molecules involved in internalization of receptor and trafficking of the endosomes, including EphrinB2 (Sawamiphak et al., 2010; Wang et al., 2010b), Synectin and Myosin VI (Lanahan et al., 2010), and Nrp1 (Lanahan et al., 2013) result in impaired Vegf signaling and compromised angiogenesis and arterial morphogenesis.

VEGFA belongs to a protein family that also includes placental growth factor (PIGF), VEGFB, VEGFC, and VEGFD. VEGFC binds to receptor VEGFR3 and is thought to be majorly involved in promoting lymphangiogenesis (Simons and Eichmann, 2013). However, recent studies showed that VEGFR3 is also expressed in vascular endothelial cells and that VEGFC-VEGFR3 signaling is required in angiogenesis.
especially when VEGFA-VEGFR2 signaling is missing (Tammela et al., 2008). Researchers pointed out that Rspo1-Wnt-VegfC-Vegfr3 pathway may serve as an endothelial cell autonomous permissive cue in developmental angiogenesis (Gore et al., 2011). Another group found that Vegfc secreted by macrophages signals to Vegfr3 to regulate vessel density through regulation of tip-stalk cell specification (Tammela et al., 2011b).

Though VEGFA-VEGFR2 signaling is generally regarded as a paracrine signaling pathway where the receptor is expressed in vascular endothelial cells and ligand expressed by surrounding cells, emerging evidence showed that endothelial cell derived Vegfa also played a crucial role in maintaining vascular homeostasis (Lee et al., 2007). Though embryonic development appeared normal, mice with Vegfa removed from endothelial cells showed a lower birth rate than predicted by mendelian ratio. These mice showed gradually increased endothelial cell apoptosis in all major organs and half of them suffered sudden death by the age of 6 months. The sudden death may be resulted from cardiac ischemic caused by coagulant dead endothelial cells. Researchers found that the ligand-receptor signaling was cell autonomous and happened within the endothelial cells thus they proposed that the endothelial cell derived Vegfa induced phosphorylation of internalized Vegfr2 and downstream signaling cascade; whether this signaling cascade was different from that downstream of paracrine signaling is still unknown.

**Section 1.3.2 Axon guidance molecules**
Tip cells of the angiogenic sprouts exhibit features of growth cones of path-finding axons such as extended actin-rich filopodia which can response to environmental attractive or repulsive cues (Adams and Eichmann, 2010). Indeed a list of receptors for axon guidance molecules have been found expressed on angiogenic endothelial cells including Eph family receptor tyrosine kinases, Nrps, PlexinD1, Robo4, and UNC5B (Larrivee et al., 2009). Axon guidance molecules can directly induce sprouting and/or affect angiogenesis by regulating Vegf signaling.

*Slits and Roundabouts*

Robo proteins (Robo1-4) are receptors for Slits (Slit1-3), although since Robo4 is structurally distinct from other Robos its binding ability to Slits remains controversial. Both Robo1 and Robo4 are found expressed on endothelial cells. The strongest effect of germline deletion of Robo4 gene was during pathological retinal angiogenesis, with Robo4−/− mice showing elevated Vegfa induced vessel permeability and hypervascularization during oxygen-induced retinopathy (Jones et al., 2008). The mechanisms resided in Robo4’s ability to directly or indirectly counteract Vegfa induced phosphorylation of Src. Intriguingly, ROBO4 in zebrafish functioned in the opposite way; it seemed to promote intersegmental vasculature (ISV) angiogenesis (Bedell et al., 2005) by activating Cdc42 and Rac1 and increasing endothelial cell motility (Kaur et al., 2006). In vitro studies showed that Robo1 formed heterodimer with Robo4 and was involved in up-regulating endothelial cell motility by initiating actin polymerization (Kaur et al., 2006). In vivo function of Robo1 remains unclear (Gore et al., 2011). Deletion of Slit3 resulted in reduced vascularization in mouse embryonic diaphragm.
**Semaphorins, Plexins and Neuropillins**

Membrane-associated Semaphorins bind to Plexins, whereas secreted class III Semaphorins (Sema3A to G) bind to Nrps, which do not signal themselves but function as coreceptors for Plexin signaling (Bagri et al., 2009). PlexinD1 is predominantly expressed in endothelial cells and endothelial PlexinD1 knockouts exhibited neonatal lethality as well as defects of the vasculature, heart outflow tract and skeleton (Gu et al., 2005). Sema3E expressed in developing somites acts as a repulsive cue to restrict vessel growth and branching in the intersomitic region. In zebrafish, this Vegf antagonizing effect is mediated by overexpression of sFlt1 (Lamont et al., 2009). Whether it is conserved in mouse trunk segments is unknown. In mouse retinas Sema3E is expressed in ganglion cells and PlexinD1 is expressed in angiogenic front endothelial cells. Deletion of either the ligand or the receptor caused irregular angiogenic front and an overall reduced vessel density correlated with Dll4 overexpression and lower percentage of tip cell versus stalk cells. Thus in this case Sema3E-PlexinD1 negatively regulated Vegfa signaling though not in a broad way, in which case we would have seen a vessel over growth (Kim et al., 2011).

Sema-Plexin signaling regulates tumor angiogenesis too. In several cancer models, Sema3A (and Sema3F) was identified as an endogenous angiogenic inhibitor lost in tumor progression. Re-expression Sema3A prevents angiogenesis, and promotes normalization of the originally tortuous and leaky tumor vasculature to alleviate hypoxia. This could be very beneficial in anti-cancer therapy (Moriya et al., 2010).
Neuropilins (Nrp1, Nrp2) bind to structurally and functionally unrelated ligands, the best studied of which are secreted class III Semaphorins and VEGF family members. Semas and Vegfs bind to different sites of the extracellular domain of the receptor. The preference of downstream signaling cascade depends on ligand availability and receptor endocytosis mechanisms (Gerhardt et al., 2004; Salikhova et al., 2008). Vegf signaling mediated by Nrp promotes tip cell elongation and vessel sprouting. Nrp1 null mice exhibit embryonic lethality because of defective heart development as well as compromised vessel branching and enlarged vessels, due to reduced endothelial cell motility (Gerhardt et al., 2004).

**ephrins and Ephs**

Eph receptor tyrosine kinases (RTKs), a large family of transmembrane proteins with a single cytoplasmic kinase domain, and their binding partners, the ephrins, regulate morphogenesis in many different tissues and species (Larrivee et al., 2009). In addition to their role as Eph-activating ligands (termed “forward signaling”), ephrins also have receptor-like signal transduction capability, so-called “reverse signaling” (Pasquale, 2005). EphA2 and ephrinA1 are detected in tumor cells and vasculature in tumor xenografts, and are related to Vegf signaling and endothelial cell migration (Brantley-Sieders et al., 2006; Ogawa et al., 2000).

Both EphB4 and ephrinB2 are required for vascular development, and they are expressed in veins and arteries, respectively (Adams et al., 1999). In zebrafish, EphB4 and ephrinB2 has been shown involved in selective sprouting of the common precursor vessel which determines the preference of ephrinB2 expression endothelial cells.
segregation into dorsal aorta rather than cardinal vein. This process requires interplay between Notch, Vegf and Eph-ephrin signaling (Herbert et al., 2009; Lin et al., 2007). This interplay also balances caliber of the developing dorsal aorta and cardinal vein in mice (Kim et al., 2008). It remains unclear if such mechanism is applied to arterial-venous specification during microvasculature formation in mammals.

Recently, scientist used two genetic approaches to demonstrate that ephrinB2 expressed on endothelial cells promotes filopodia elongation and directional sprouting. The reverse signaling mediated by ephrinB2 is required in endocytosis of Vegfr2 which precedes subsequent activation of Rac1, Akt and Erk (Sawamiphak et al., 2010; Wang et al., 2010b). Ephrin-B2 is also expressed on vascular smooth muscle cells and is required cell-autonomously in migration and adhesion of mural cells to nascent vessels and thus the stability of the microvasculature (Foo et al., 2006).

Section 1.3.3 Other important signaling pathways

Transforming growth factor β family members

Transforming growth factor (TGF-β), the activins and bone morphogenetic proteins (BMPs) form a large family of secreted pleiotropic growth factors that are important in developmental and function of multiple organs. Several endothelial pathologies are associated with mutations in the TGF-β pathway, underlining its importance in vascular biology. For example, hereditary hemorrhagic telangiectasia (HHT) is characterized by the development of mucocutaneous telangiectasia and arteriovenous malformations in the lungs, brain, liver, and gastrointestinal tract (Jakobsson and van Meeteren, 2013). Interestingly, three genes identified related to HTT are Eng, Alk1 and Smad4, which are
all key players in TGF-β signaling pathways. Recent in vivo studies in mouse have been focused on knocking-out receptors or Smad proteins which mediate transcription activation/repression downstream of TGFβ and BMP signaling, specifically in endothelial cells (Geng et al., 2013; Larrivee et al., 2012).

Phenotypes of Alk1, Alk5 or Tgfbr2 deletion using two Alk1-driven cre lines depend on the onset of the cre activity (Jakobsson and van Meeteren, 2013). When the cre is active by E9.5, both Alk5 and Tgfbr2 deletion caused blood vessel morphogenic defects and intracerebral hemorrhage. With another cre line turned on at E14.5, neither Alk5 nor Tgfbr2 deletion showed any phenotype at embryonic stages, while Alk1 deletion demonstrated blood vessel morphogenic defects and intracerebral hemorrhage. These results indicated that Alk5 and Tgfbr2 were more related to angiogenesis rather than vessel stabilization. Inducible endothelial deletion of Tgfbr2 also caused hemorrhages in the brain and retina; however contradict evidence left the question whether it promotes or inhibits angiogenesis. On the other hand, combinatorial deletion of Smad1 and Smad5 in endothelial cells resulted in excessive endothelial cell filopodia formation and sprouting during mid-gestation (Moya et al., 2012). Researchers proposed that BMP signaling coordinates with Notch signaling to strengthen the stalk-cell phenotype, as Id protein, effector of Smads, helped to stabilize Hes1, a Notch signaling target gene itself and alsoDll4 and Dll1 transcription suppressor (Moya et al., 2012). In zebrafish, however, the formation of ventral veins seemed to be dependent on BMP2B and more surprisingly, independent of VEGFA (Wiley et al., 2011).

*Wnt signaling pathway*
In canonical Wnt pathway, β-catenin translocates in to the nuclei when the cytoplasmic sequestering complex degraded after ligand/receptor binding, and works with Lef proteins and Tcf proteins to activate or suppress target genes (Clevers and Nusse, 2012). Wnt pathway has been implicated in endothelial cell differentiation, heart valve development and endothelial cell mobilization during tumor angiogenesis. Recent studies showed that canonical Wnt pathway has an exclusively important role in regulation central nervous system angiogenesis and establishment of the blood-brain-barrier (BBB). Wnt7a and Wnt7b, two canonical pathway ligands, are expressed by neural progenitor cells along the dorsal-ventral axis in the presumptive spinal cord and forebrain (Daneman et al., 2009). Combinational deletion of the two ligands caused severe CNS angiogenesis defects, and this is phenocopied by endothelial inactivation of β-catenin (Liebner et al., 2008). In addition, it has been found that BBB specific genes including Claudin3, Claudin5 and Glut1 could be Wnt pathway targets both in vitro and in vivo.

Recent studies suggest strong interplay between Notch signaling pathway and Wnt signaling pathway (Corada et al., 2010). Nrarp, a Notch target, not only regulated sprouting activity of endothelial cells, but also functioned to stabilize β-catenin co-effector Lef1 in nuclei of endothelial cells, thus strengthening the canonical Wnt pathway (Phng et al., 2009). Mice deficient in Nrarp showed precautious vessel regression probably as a consequence of reduced EC proliferation, since CyclinD1 was identified as a target gene for canonical Wnt pathway. Indeed, in the same report researchers showed that deletion of the receptor Lrp5 or Lef1 had the same effect. Secondly, Wnt/β-catenin pathway can induce expression of Dll4 directly and thus
promoting Notch signaling. This not only promoted the quiescence and stability of the angiogenic vessel, but also affected artery-vein specification, where Notch pathway is also actively involved (Corada et al., 2010). Wnt receptors and \( \beta \)-catenin can also be activated by non-Wnt ligands, such as Norrin ligand, which has very close relation to Norrie disease. Genetic abrogation of Norrin, Lrp5, Frizzled4 or Tspan12 which binds to the ligand-receptor complex and strengthens \( \beta \)-catenin mediated signaling, all cause defected retinal angiogenesis and failure of formation of deep plexus, probably through down-regulation of Sox17 expression in the endothelial cells (Junge et al., 2009; Ye et al., 2009).

There also has been increasing evidence showing involvement of non-canonical Wnt pathway in angiogenesis. Endothelial Frizzled4 loss-of-function also caused mitigated proliferation and branching of kidney vasculature, yet this effect was mediated by Wnt/PCP (planar cell polarity) pathway rather than canonical Wnt/\( \beta \)-catenin (Descamps et al., 2012). Ex vivo studies showed that ligand binding caused activation of Dvl3 and Dvl1 and subsequent localization of MTOC (microtubule organizing center) and Golgi apparatus to the leading edge of the cells (Descamps et al., 2012). A work by our group showed that myeloid cell deletion of Wntless, a protein that is critical for production of functional Wnt ligands, caused significantly elevated angiogenesis in the retina, and this effect was majorly attributed to non-canonical pathway. Although we proposed a mechanism in which regulation of sFlt expression was dependent on a myeloid cell autonomous non-canonical Wnt pathway, it is very possible that change in the availability of Wnt ligands exerts a direct effect on endothelial cells (Stefater et al., 2011; Stefater et al., 2013). It was also found that endothelial cells also express ligands
specific for canonical and non-canonical pathways, and that disruption of the ligand production from EC also caused increased vessel density due to defects in vessel regression, and this effect was mediated by NFAT.

New players
Other molecules known to regulate microvasculature formation include growth factor FGFs, EGFs, angiopoietin proteins with Tie receptors (Carmeliet, 2003), chemokines CXCs (Vandercappellen et al., 2008), calcium channels (Munaron et al., 2013), orphan G protein-coupled receptor (Kuhnert et al., 2010), etc., which are not discussed in detail here. Bioactive lipid sphingosine-1-phosphate (S1P) in the circulating blood has been found involved in restricting angiogenesis and maintaining vessel stability recently. S1P through receptor S1pr1 inhibits Vegfa induced signaling and vessel permeability, and stabilizes VE-Cadherin at cell-cell junctions (Gaengel et al., 2012).
SECTION 1.4 Research Objectives

We have been seeking to use retinal vasculature as a model system to study development angiogenesis and use mouse genetic manipulations to characterize functions of novel molecules involved in regulating angiogenesis. In this dissertation, I focused on the discussion of a transmembrane protein Crim1 (Cystein-rich motor neuron 1), which is expressed in endothelial cells of microvasculature. Since in previous studies we found that Crim1 binds to and stabilizes Cadherin-catenin complexes at cell-cell junctions in neuroepithelium (Ponferrada et al., 2012), we originally hypothesized that it functions in vascular system also in regulating cell-cell adhesion. We found that Crim1 promoted angiogenesis by ensuring nascent vessel stability during sprouting angiogenesis. While Crim1’s function in regulating VE-Cadherin in endothelial cells was still quite inconclusive, we ended up demonstrating that Vegfa-Vegfr2 private-loop signaling within endothelial cells, which was considered critical in maintaining stability of mature vessels, was also important in sprouting angiogenesis. We provide in vivo evidence showing that Crim1 functions synergistically with Vegfa in promoting angiogenesis and in vitro data supporting the model that Crim1 enhances the autocrine signaling of Vegfa via Vegfr2.
CHAPTER TWO

Crim1 maintains retinal vascular stability during development by regulating endothelial cell Vegfa autocrine signaling

(Published data)
SECTION 2.1 Abstract

Angiogenesis defines the process in which new vessels grow from existing vessels. Using the mouse retina as a model system, we show that Crim1 (Cysteine-rich motor neuron 1), a type 1 trans-membrane protein, is highly expressed in angiogenic endothelial cells. Conditional deletion of the Crim1 gene in vascular endothelial cells (VECs) causes delayed vessel expansion and reduced vessel density. Based on Vegfa binding by Crim1 (Wilkinson et al., 2007) and Crim1 expression in retinal vasculature where angiogenesis is Vegfa-dependent (Fruttiger, 2007), we tested the hypothesis that Crim1 might be involved in the regulation of Vegfa signaling. Consistent with this hypothesis we showed that VEC-specific conditional compound heterozygotes for Crim1 and Vegfa showed a phenotype more severe than each single heterozygote and indistinguishable from the conditional homozygotes. We further showed that Crim1 knockdown in cultured VECs resulted in diminished phosphorylation of Vegfr2, but only when VECs are asked to rely on an autocrine source of Vegfa. The effect of CRIM1 knockdown on reducing VEGFR2 phosphorylation was enhanced when VEGFA was also knocked down. Finally, the effect of CRIM1 knockdown on VEGFR2 phosphorylation was not further diminished by an anti-VEGFA antibody, but was completely suppressed by SU5416, a small molecule VEGFR2 kinase inhibitor. These data are consistent with a model in which Crim1 enhances the autocrine signaling activity of Vegfa in VECs at least in part via Vegfr2.

SECTION 2.2 Introduction
Angiogenesis defines the process in which new vessels grow from existing vessels through branching morphogenesis (Geudens and Gerhardt, 2011; Phng and Gerhardt, 2009). During angiogenesis, some vascular endothelial cells (VECs) in the originally quiescent vessels will be induced to become ‘tip cells’. These cells are polarized and extend filopodia to probe microenvironmental cues, and migrate to lead the elongation of new vessel branches (Gerhardt et al., 2003). The VECs adjacent to tip cells will become ‘stalk cells’, which proliferate and contribute to lumen formation (Gerhardt et al., 2003). Fusion of new sprouts in a process called anastomosis contributes to vascular network formation (Geudens and Gerhardt, 2011). This draft network then undergoes extensive remodeling to become functional (Potente et al., 2011). Deregulated angiogenesis occurs in many pathological processes, including cancer (Gasparini et al., 2005), diabetic retinopathy (Crawford et al., 2009), retinopathy of prematurity (Flynn and Chan-Ling, 2006), and age-related macular degeneration (AMD) (Jager et al., 2008), where anti-angiogenic therapy can be valuable (Gasparini et al., 2005).

The vascular endothelial growth factor (Vegf) family members are critical regulators of vessel development and homeostasis. Vegfa is an indispensable pro-angiogenic factor in almost all non-pathological and pathological angiogenesis (Carmeliet and Jain, 2011a). Vegfa signals via the vascular endothelial growth factor receptor 2 (Vegfr2), a conventional tyrosine kinase receptor. Vegfr1 is an inhibitory receptor because it binds Vegfa avidly but does not signal with high activity (Shibuya, 2001). There is also a soluble isoform of Vegfr1 that inhibits Vegfa through sequestration (Shibuya, 2001). During angiogenesis, Vegfa signals to VECs to promote tip cell formation (by enhancing expression of Dll4 (Geudens and Gerhardt, 2011; Phng
and Gerhardt, 2009)), tip cell migration (Gerhardt et al., 2003), stalk cell proliferation
(Gerhardt et al., 2003) and VEC survival via Akt (Gerber et al., 1998b). Vegfr3, a
receptor for Vegfc, is restricted to the lymphatic vessels in the adult, but is upregulated
during developmental angiogenesis in tip cells and during pathological angiogenesis
(Tammela et al., 2011a). It has recently been shown that VECs themselves are a
source of Vegfa and that the private-loop of signaling via Vegfr2 within the VECs is
critical (Lee et al., 2007; Segarra et al., 2012). Mice in which Vegfa was deleted
specifically in VECs showed postnatal mortality associated with vascular degeneration
(Lee et al., 2007) suggesting a role for autocrine Vegfa in vascular homeostasis.
Although it has been shown that endothelial cells up-regulate Vegfa production under
stress conditions such as hypoxia (Lee et al., 2007; Namiki et al., 1995), other
molecules involved in regulation of the ligand and downstream effectors of this pathway
are largely unknown.

Cysteine-rich Motor Neuron 1 (Crim1) is a type I trans-membrane protein that
has N-terminal homology to insulin-like growth factor binding domains (IGFBP) and six
cysteine-rich von Willebrand factor C (vWC) repeats which are similar to those of
Chordin, a BMP antagonist (Kolle et al., 2000). Crim1 is expressed in multiple tissues
and cell types including vertebrate CNS (Kolle et al., 2003; Pennisi et al., 2007), kidney
(Wilkinson et al., 2007), eyes (including lens (Lovicu et al., 2000)) and the vascular
system (Glienke et al., 2002; Pennisi et al., 2007; Wilkinson et al., 2007). It has been
suggested that Crim1 has a role in vascular tube formation in vitro (Glienke et al., 2002).
It is localized in endoplasmic reticulum (ER) and accumulates at cell-cell contacts upon
stimulation of endothelial cells (Glienke et al., 2002). Mice homozygous for a gene-trap
mutant allele (*Crim1KST264*) or germ-line mutants (Chiu et al., 2012) display perinatal lethality with defects in multiple organs including hemorrhagic necrosis and enlarged glomerular capillary lumens (Wilkinson et al., 2007). The molecular function of Crim1 has been somewhat enigmatic, but it is known to form complexes with N-cadherin and β-catenin (Ponferrada et al., 2012) and it is also known to bind growth factors including Vegfa (Wilkinson et al., 2007; Wilkinson et al., 2009). Analysis suggests that in the glomerulus of the kidney, Crim1 on the cell surface of podocytes regulates the delivery of Vegfa from podocytes to endothelial cells (Wilkinson et al., 2007; Wilkinson et al., 2009).

In the current study, based on Vegfa binding by Crim1 (Wilkinson et al., 2007) and expression of Crim1 in retinal vasculature, we tested the hypothesis that Crim1 might be involved in the autocrine activity of Vegfa. Consistent with this, VEC-specific conditional compound heterozygotes for *Crim1* and *Vegfa* showed a phenotype more severe than each single heterozygote and indistinguishable from the conditional homozygotes. Crim1 knockdown in cultured VECs resulted in diminished phosphorylation of Vegfr2, but only when VECs are asked to rely on an autocrine source of Vegfa. VEGFA knockdown enhanced the effect of CRIM1 knockdown on reducing VEGFR2 phosphorylation. Finally, the effect of CRIM1 knockdown on VEGFR2 phosphorylation was not further diminished by an anti-VEGFA antibody, but was completely suppressed by SU5416, a small molecule VEGFR2 kinase inhibitor. These data are consistent with a model in which Crim1 enhances the autocrine signaling activity of Vegfa in VECs at least in part via Vegfr2.
Section 2.3 Results

Section 2.3.1 Crim1 is expressed in both endothelial cells and pericytes.

Crim1 is expressed in vascular endothelial cells (VECs) in vitro and in vivo (Glienke et al., 2002). To examine the expression pattern of Crim1 in angiogenic vasculature, we analyzed flat-mounted preps of mouse embryonic hindbrain and postnatal retinas from a Crim1:GFP mouse line (MGI: 4846966). In vasculature of both organs, GFP was expressed in VECs marked by Isolectin-IB4 (Figure 2.1 A-I). Notably, in the center of the retinal vascular plexus, GFP intensity was lower in VECs but also present in smooth muscle cells marked by NG2 labeling (Figure 2.1 E, F arrowheads). We also isolated CD31⁺CD45⁻ VECs from wild type P7 mouse retinas using FACS (Figure 2.1 J). We confirmed cell identity by end-point RT-PCR detecting the endothelial cell marker Cd31 and the pericyte marker Pdgfrb (Figure 2.1 K). Crim1 transcripts were detected in retinal VECs using two different sets of primers (Figure 2.1 K). Crim1 protein was also labeled using immunofluorescence in P6 and P10 wild type retinal sections with a newly-developed antiserum. High immuno-reactivity was observed in VECs labeled by Isolectin IB4 (Figure 2.1 M, N, P, Q) as well as cells associated with the vasculature, probably pericytes (Figure 2.1 P, arrowheads). The expression of Crim1 in VECs indicated it might have a role in vascular development.

Figure 2.1 Crim1 is expressed in both endothelial cells and pericytes in angiogenic vasculature. (A-F) Flat-mounted P6 Crim1:GFP mouse retina labeled with isoelectin IB4 and NG2 antibody. Enlarged images (C-F) showing co-localization of the GFP expression in isoelectin labeled endothelial cells and NG2 labeled pericytes/smooth muscle cells (white arrowheads). (G-I) GFP signal was also detected in hindbrain vasculature of E12.0 reporter mice embryo. (J) Representative FACS chart showing endothelial cell population sorted from retina. (K) End-point RT-PCR in sorted endothelial cells and whole retina. For each primer set, PCR products were amplified with similar amount of cDNA and same cycle number. RT: reverse transcribed. (L-Q) Cross section of P6 and P10 retina labeled with Crim1 antiserum. No primary antibody was added in (L, O). (O-Q) are higher magnification images. Arrows: Crim1 expression in endothelial cells. Arrowheads: Crim1 expression in other cell type.
Figure 2.1 Crim1 is expressed in both endothelial cells and pericytes in angiogenic vasculature. (Figure legend see previous page)
Section 2.3.2 Validation of the Crim1<sup>flox</sup> allele

Crim1<sup>flox</sup> mice were crossed with the germ-line expressed Ella-Cre line to generate the Crim1<sup>Δflox</sup> allele. Mice homozygous for this allele did not show lethality until E17.5 (Figure 2.3 A) but postnatally we identified only 3 live pups from over 20 litters, indicating that homozygotes died perinatally. Crim1<sup>Δflox/Δflox</sup> embryos exhibited anomalies that included peridermal-blebbing, edema, hemorrhage (especially in the CNS), eye hypoplasia, and syndactyly (Figure 2.3 B-T). The changes phenocopy defects described in mice homozygotes for the hypomorphic allele Crim1<sup>KST264</sup> (Pennisi et al., 2007) and another germ-line null allele (Chiu et al., 2012) but occur at higher penetrance and severity (Figure 2.3 B). These data validate Crim1<sup>flox</sup> as a conditional loss-of-function allele.

Figure 2.2 Crim<sup>flox</sup> allele design and inducing VEC specific cre activity in Pdgfb-iCre mouse line. (A) Diagram of Crim<sup>flox</sup> allele. (B) Genomic DNA PCR could distinguish mice with or without Crim<sup>flox</sup> allele. In Crim<sup>flox/flox</sup>;Pdgfb-iCreER and Crim<sup>flox/+</sup>;Pdgfb-iCreER mice injected with tamoxifen, a ‘deletion’ band showing recombination of the genomic DNA can also be detected using the tail tip genomic DNA. Lane1: Crim<sup>flox/+</sup>. Lane2: Crim<sup>flox/+</sup>;Pdgfb-iCreER. Lane3: Crim<sup>flox/flox</sup>. Lane4: Crim<sup>flox/flox</sup>;Pdgfb-iCreER. flox: Crim1<sup>flox</sup>; WT: wild type; iCre: Pdgfb-iCreER; Δflox: Crim1<sup>(EC)Δflox</sup>; bp: basepairs. (C) Tamoxifen injection plan of experiments in this paper. In most experiments, tamoxifen was injected daily to pups from date-of-birth (P1) to the day of analysis (P5, P6, P7). For experiment shown in supplemental figure 2, tamoxifen was injected starting from P7. (D) Specificity and efficiency of Pdgfb-iCreER line upon injection of tamoxifen. Cre activity was visualized by labeling of X-Gal in P5 retina preps from injected Pdgfb-iCreER;ROSA26ER pups. (E) Detection of DNA recombination of targeted Crim1<sup>flox</sup> allele in tamoxifen injected, Pdgfb-iCreER positive pups.
Figure 2.3 Germ line Crim1 null mice exhibited multiple developmental defects. (A) Percentage of different genotypes in embryonic litters from intercrosses between Crim1Δflox/+ mice at different stages. Numbers in the bars represent total embryos collected. (B) Frequency of anomalous phenotypes observed in Crim1Δflox/Δflox embryos collected at different stages. ND: not (old enough) to define a defect. (C-T) Representative images showing developmental defects in Crim1Δflox/Δflox embryos. Stages and phenotypes are indicated. Hollow arrowheads: peridermal blebbing; filled arrowheads: hemorrhage or hematoma; yellow arrow: widespread edema. (I, J, M, N) Syndactyly in germ line Crim1 null mice often happen between digits 3 and 4. (O-P) Eye hypoplasia. (Q-R) Kidneys were mildly smaller in Crim1 null mice. Scale bars in (O-R): 500 μm.

Section 2.3.3 Deletion of Crim1 in VECs resulted in defective vascular development in the retina

We combined the Pdgfb-iCreER allele with Crim1flox and used daily intraperitoneal injection of tamoxifen starting at P1 or P7 (Figure 2.2 C) to elicit cre recombinase activity. Pdgfb-iCreER mice, upon tamoxifen administration, give specific cre activity in VECs including tip cells (Claxton et al., 2008) (Figure 2.2 D). The Crim1flox allele was effectively recombined in retinal VECs as shown by genotyping PCR of DNA from
FACS-sorted P7 endothelial cells (Figure 2.2 E); the recombined $Crim1^{\text{flox}}$ allele was detected in tail DNA only when mice had the $Pdgfb-iCreER$ allele (Figure 2.2 B).

<table>
<thead>
<tr>
<th>Isolectin IB4</th>
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<tr>
<td>Control</td>
<td>$Crim1^{\text{flox/flox}}, Pdgfb-iCreER$</td>
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![Figure 2.4](image)

Figure 2.4 Loss of Crim1 from endothelial cells causes reduced vessel growth in the primary plexus of retinal vasculature. (A, B, D, E) Flat-mounted P6 mouse retina labeled with isolectin IB4. VEC conditional mutant mice showed reduced vessel expansion to the periphery as quantified in (C). (F) Vessel density measured by counting branchpoints number in randomly selected 200 $\mu$m X 200 $\mu$m fields (5 fields per retina). M: $Crim1^{\text{flox/flox}}, Pdgfb-iCreER$ pups. C: Control littermates. Error bars represent s.e.m.

In the first postnatal week, a primary vascular plexus grows within the ganglion cell layer of the mouse retina through sprouting and anastomosis, expanding from the optic stalk and reaching the periphery by about P8 (Fruttiger, 2007). Tamoxifen injected $Crim1^{\text{flox/flox}}, Pdgfb-iCreER$ pups showed delayed radial expansion of vasculature from center to periphery over a P5-P7 time-course compared with Cre negative control littermates ($Crim1^{\text{flox/flox}}$ or $Crim1^{\text{flox/+}}$) (Figure 2.4 A-C). More distinctively, vessel density
quantified by branch-point number was also reduced in both the angiogenic front and remodeling plexus area behind the front (Figure 2.4 D-F).

**Figure 2.5** Phenotype of deep layer retinal vasculature of Crim1 VEC conditional mutant mice. (A-D) Flat-Mounted P10 retinas labeled with isolectin IB4 showing deepest layer retinal vasculature (which is between out-plexus-layer and photo receptor layer) showed delayed vascularization of the retina. Tamoxifen was injected starting from P7 when vessel just started to sprout into the deep layer. (E-G) Quantitations of vertical sprouts, branchpoints in deep layer vasculature and vessel length in deep layer vasculature.
Starting at P7-P8, vessels sprout into the outer plexiform layer (OPL) and then turn, sprout and connect to form the deep vascular layer that resides between the OPL and the photoreceptors (Fruttiger, 2007). \textit{Crim1}^{\text{flax/flax}}, \textit{Pdgfb-iCreER} pups injected with tamoxifen from P7 to P10 showed a delayed invasion of vasculature into the deepest layer (Figure 2.5 A-D), as shown by fewer vertical sprouts, reduced branch-point number and reduced total vessel length (Figure 2.5 E-G). Combined, these data showed that endothelial Crim1 plays a role throughout retinal vascular development.

**Section 2.3.4 Loss of Crim1 from endothelial cells caused abnormal vessel morphology and compromised VEC proliferation**

The initial density of the sprouting vasculature can be affected by tip cell number (Hellstrom et al., 2007b; Larrivee et al., 2012; Phng et al., 2009). The angiogenic front in \textit{Crim1} VEC conditional mutants was hypocellular compared with wild type littermates, as indicated by Erg1/2/3 antibody labeling of the VEC nuclei (Figure 2.6 A-C). In addition, a much higher occurrence of vessel segments with small caliber was observed in \textit{Crim1} VEC conditional mutant mice (Figure 2.4 E; Figure 2.6 B).

Two-hour BrdU incorporation showed a reduced number of S-phase VECs in the conditional mutant pups (Figure 2.6 D-F). Tip cells of the \textit{Crim1} VEC conditional mutant mice exhibit a mildly reduced density of filopodia but these filopodia still attached closely to the underlying astrocytes (Figure 2.6 G-K). Expression of the Notch pathway target \textit{Vegfr3} was normal in VEC conditional mutants (data not shown), suggesting that loss of Crim1 from VECs was unlikely to disrupt the Notch signaling pathway.
Section 2.3.5 Endothelial loss of Crim1 resulted in defective cell adhesion molecule distribution and precocious vessel regression

Previous studies from our lab indicated that Crim1 has an active role in promoting neuroepithelium cell adhesion through regulation of cadherin-catenin complexes (Ponferrada et al., 2012). Other investigators also showed that Crim1 accumulated at endothelial cell-cell contacts under certain conditions (Glienke et al., 2002). Given the important role that the adhesion molecule VE-cadherin plays in initiating anastomosis (Hoelzle and Svitkina, 2012) and maintaining vessel stability (Dejana et al., 2009; Phng
et al., 2009), we assessed the possibility of a cell adhesion anomaly in $\text{Crim1}^{\text{flox/flox}}; Pdgfb-$iCreER mice retina vasculature.

**Figure 2.7 Abnormal adhesion junctions distribution in Crim1 VEC conditional mutants.** (A-L) Antibody labeling in flat-mounted retina preps showed VE-cadherin distribution in P6 angiogenic front (A-H) and P7 remodeling plexus areas (I-L). White and pink arrowheads point to vessel segments without continuous VE-Cadherin distribution. (M) Quantitation of vessel segments without continuous junctional VE-Cadherin signal (normalized to total Isolectin IB4 labeling positive segments). M: $\text{Crim1}^{\text{flox/flox}}; Pdgfb-$iCreER pups. C: Control littermates. Error bars represent s.e.m.
We observed an increase in vessel segments with discontinuous VE-Cadherin labeling in *Crim1* conditional mutants (Figure 2.7 A-L). (One vessel segment is defined as an isolectin labeled connection between two branch-points). This change was more significant at the angiogenic front (Figure 2.7 M). VE-Cadherin negative segments usually had reduced caliber. In addition, Podocalyxin labeling, marking the apical/luminal side of the vessels, was missing in VE-Cadherin negative regions (Figure 2.8 A-H, arrowheads). In summary, VEC conditional mutant mice had a higher percentage of thin, cadherin-deficient, non-lumenized vessel segments.

Normally, vessel pruning occurs mostly around arteries where oxygen level is high (Adini et al., 2003; Phng et al., 2009). Regressed vessel segments appear as ‘vessel ghosts’, which are labeled for vascular basement membrane markers such as Collagen IV but lack isolectin-positive VECs (Phng et al., 2009). In *Crim1*^flox/flox^*, Pdgfb-iCreER*
Figure 2.9 Depletion of Crim1 from endothelial cells caused precocious vessel regression. (A-H) Flat-mounted retinas labeled with Isolectin IB4 and Collagen IV antibody. Collagen IV positive and Isolectin IB4 labeling negative vessel segments represented regressed capillaries, e.g. vessel ghosts (filled arrowheads) and retracted sprouts (empty arrowheads). Increase of vessel ghosts was more obvious at angiogenic front (A-D) than in remodeling plexus (E-H). Several cases of long retracted sprouts (I, J) or endothelial cells detaching from vessel bed (K, L) was observed. (M) Quantification of vessel ghosts. (continued on next page)
pups, there was a significant increase in vessel ghosts at the angiogenic front (Figure 2.9 A-D, M) but no significant increase within the remodeling plexus (Figure 2.9 E-H, M). We observed several cases of retracted sprouts (Figure 2.9 I, J) and vessel fragments completely disconnected from the vessel bed (Figure 2.9 K, L).

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**Figure 2.10** Mural cell recruitment in Crim1 endothelial cell conditional mutant mice was not affected. (A-L) Flat-mounted P7 mice retina preps labeled with Isolectin IB4, desmin antibody (pericyte marker) and Smooth-muscle-actin antibody (smooth muscle cells marker).

**Figure 2.9** Depletion of Crim1 from endothelial cells caused precocious vessel regression (continued). (N-Q) Active-caspase-3 labeling of the retinas. Vessel segments with positive active-caspase-3 labeled endothelial cells increase in Crim1 conditional mutant pups (O white arrows). Active-caspase-3 labeling coincided with DNA fragmentation (P-Q white arrows.) (R) Quantification of vessel segments with positive active-caspase-3 labeled endothelial cells. (S) Occurrence of active-caspase-3 positive vessel segments in different distance range from center of the retina (relative to vessel expansion); *: p<0.05. (M, R, S) M: Crim1\textsuperscript{flox/flox}\textsubscript{:Pdgfb-iCreER} pups. C: Control littermates. Error bars represent s.e.m.
We also labeled active caspase-3, an early marker for apoptosis (Figure 2.9 N-Q). The VEC apoptosis index was significantly higher in *Crim1*^{flox/flox}; *Pdgfb-iCreER* retinas than that of control retinas (Figure 2.9 N, O, R). We measured the distance of dying vessel fragments from the center of the retina and found that the most apparent increase of apoptosis in the VEC conditional mutant mice was within the region between 60% to 70% of the distance to the edge of the angiogenic front. This corresponds to the more angiogenic active vessel front (Figure 2.9 S). Mural cells recruited to newly formed capillaries are also critical for vessel stability (Gerhardt and Betsholtz, 2003). In *Crim1*^{flox/flox}; *Pdgfb-iCreER* mutant pups, the attachment of desmin^{+} (Figure 2.10) or NG2^{+} pericytes (data not shown) to VECs at the angiogenic front was not compromised, although Smooth muscle actin (SMA) showed mildly reduced labeling around arteries (Figure 2.10 E-H). This suggested that depletion of Crim1 from VECs did not have a significant effect on mural cells. Combined, these data suggest that loss of Crim1 from VECs caused instability of newly formed vessels, resulting in vessel fragmentation and precocious regression.
Section 2.3.6 Crim1 and Vegfa function cooperatively in retinal vasculature development

Previous studies showed that Crim1, via its cysteine-rich-motif, is able to physically bind ligands bearing a cysteine-knot motif, including several BMPs, PDGF and VEGFA, before their secretion (Wilkinson et al., 2007; Wilkinson et al., 2003). This and the Vegfa-dependence of retinal angiogenesis raised the possibility that Crim1 might regulate Vegfa activity. In the retina, in the first postnatal week, Vegfa transcript is found in astrocytes and neurons (Fruttiger, 2007; Rao et al., 2013). Vegfa protein becomes tethered to the extracellular matrix of astrocytes in avascularized regions ahead of the angiogenic front (Gerhardt et al., 2003; Stenzel et al., 2011). In addition to paracrine Vegfa, VEC-derived, autocrine Vegfa signaling has been implicated in vessel homeostasis (Lee et al., 2007).

To examine whether autocrine signaling was also required for angiogenesis in vivo, we generated Vegfa VEC-conditional knockout mice by crossing Pdgfb-iCreER line to the Vegfa^{loxP} line (Gerber et al., 1999) and induced Cre activity using tamoxifen injection. Compared with Cre negative control littermates, conditional heterozygotes and homozygotes showed reduced vascularized area (Figure 2.11 A-C) and dose-dependent density (Figure 2.11 A, B, D). This phenotype also has many similarities to the Crim1, VEC-conditional phenotype. In particular, Vegfa conditional deletion results in many more Collagen IV+, isoelectin negative vessels ghosts (Figure 2.12) and vessel segments that show discontinuous VE-cadherin labeling (Figure 2.12). Like the Crim1 conditional mutant, in the Vegfa conditional mutant, the discontinuous VE-cadherin labeling which correlates with mislabeled Podocalyxin suggesting the absence of lumen.
Combined, these data confirm that VECs are a biologically important source of Vegfa (Lee et al., 2007) and indicate that the Crim1 and Vegfa VEC loss-of-function phenotypes are very similar.

One means to assess the possibility that Crim1 might regulate Vegfa function in vivo is to generate compound conditional mutants and perform a quantitative assessment of the phenotype. In this way, it is possible to decide whether two molecules contribute to the same biological process. Thus, we combined the Vegfa\textsuperscript{loxP}, Crim1\textsuperscript{floX} and Pdgfb-iCreER alleles and found that both Vegfa\textsuperscript{loxP/+}, Pdgfb-iCreER and Crim1\textsuperscript{floX/+}; Pdgfb-iCreER heterozygote mutants showed mildly reduced expansion of the vasculature as well as vessel density (Figure 2.13 D-F compared to Figure 2.13 A-C). Interestingly, the Vegfa\textsuperscript{loxP/+}; Crim1\textsuperscript{floX/+}; Pdgfb-iCreER double heterozygote mutants

Figure 2.11 VEC Vegfa conditional knockout mice exhibited compromised retinal angiogenesis. (A and B) Flat-mounted P6 retina preps labeled with Isolectin IB4 showing slightly reduced vessel expansion to the periphery to the retina and greatly decreased vessel density in Vegfa VEC conditional mutant pups. (C, D) Quantitation of vascularized area (normalized to retina sizes) and vessel density, measured by branchpoints number in randomly selected fields. *: p<0.05; **: p<0.01; ***: p<0.005; ****: p<0.001; NS: not significant. Scale bars: 250 μm. Error bars represent s.e.m.
exhibited much more severely defective angiogenesis than either of the single heterozygotes (Figure 2.12 D-F) and recapitulated the retinal vascular phenotypes of

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Figure 2.12 Vegfa VEC conditional mutant mice exhibited similar precocious regression phenotypes as Crim1 VEC conditional mutant mice. (A-H) Isolectin IB4, VE-Cadherin and Collagen IV labeling of angiogenic front vasculature in control (A-D) and Vegfa<sup>loxP/loxP</sup>;Pdgfb<iCreER</i> (E-H) littermates. Hollow arrowheads in (A, B, E, F) point to vessel ‘ghosts’, indicating vessel regression and filled arrowheads in (C, D, G, H) point to VE-Cadherin labeling discontinuous regions. (I-P) Isolectin IB4, VE-Cadherin and podocalyxin labeling of angiogenic front vasculature in control (I-L) and Vegfa<sup>loxP/loxP</sup>;Pdgfb<iCreER</i> (M-P) littermates. Filled arrowheads point to VE-Cadherin-labeling discontinuous regions where vessels also lack luminal marker labeling.
either Crim1 or Vegfa VEC-specific knockouts (Figure 2.13 G, H). This synthetic phenotype provides suggestive evidence that Crim1 and Vegfa function in the same pathway.

Figure 2.13 Endothelial derived Crim1 and Vegfa function cooperatively in retinal vasculature development in vivo. (A-F) Flat-mounted P6 retina preps of indicated genotypes labeled with Isolectin IB4. (G, H) Quantitation of vascularized area (normalized to retina sizes) and vessel density of retinal vasculature from mice with indicated genotypes. Numbers of Crim1<sup>loxP/loxP</sup>;Pdgfb-iCreER and Vegfa<sup>loxP/loxP</sup>;Pdgfb-iCreER retinas were obtained from other crosses (see Figure 2.5C, 2.6F, 2.12C, 2.12D) and reused here. *: p<0.05; **: p<0.01; ***: p<0.005; ****: p<0.001; NS: not significant. Scale bars: 250 µm.
Section 2.3.7 CRIM1 promotes autocrine VEGFA-VEGFR2 signaling within endothelial cells

In order to examine directly the possibility that Crim1 might regulate Vegfa signaling, we established lentiviral shRNA-mediated knock-down of CRIM1 in human umbilical vein endothelial cells (HUVECs). Two different shRNAs were able to knock down CRIM1 by 60-70% at the protein (Figure 2.14 A) and transcript (Figure 2.17 D) levels. Measured by MTT assay, growth of CRIM1-knockdown HUVECs cultured in complete medium was greatly suppressed (Figure 2.14 A), suggesting a functional requirement. Localization of VE-CADHERIN and β-CATENIN as well as VEGFR2 was not changed in these knockdown cells (Figure 2.15A-F, J-O).

To test directly whether CRIM1 was involved in VEGFA-VEGFR2 signaling, we stimulated HUVECs with recombinant VEGFA and assessed the VEGFR2 phosphorylation level using ELISA (Figure 2.14 B). With exogenous VEGFA at 80 ng/ml,
Figure 2.14 Crim1 promotes VEGFA-VEGFR2 autocrine signaling in endothelial cells. (A) Growth curve of HUVECs infected by lenti-viral particle expressing scramble shRNA and shRNAs specific for CRIM1. 5,000 cells were plated into each well of fibronectin coated 96-well plates at time zero. Efficiency of knocking-down CRIM1 protein with shRNAs was shown by immunoblot (inset in A). (B) VEGFR2 phosphorylation in response to VEGFA stimulation. Control and CRIM1 deficient cells were starved for 24 hours and stimulated with 80ng/ml recombinant hVEGFA for 5 minutes. Cell lysates were collected and applied to phospho-VEGFR2 and VEGFR2 ELISA kit and ratio of phospho-VEGFR2 and total VEGFR2 readouts calculated. Measurements were normalized to that of control HUVECs stimulated with rhVEGF. (C) VEGFR2 phosphorylation without exogenous VEGFA. Control and CRIM1 knockdown HUVECs were cultured in serum-free and VEGFA depleted culture medium with or without 0.1 mg/ml Na$_3$VO$_4$ (to inhibit phosphatase activity) for 24 hours. Cell lysates were collected and applied to phospho-VEGFR2 and VEGFR2 ELISA kit and ratio of the two calculated. All readouts were normalized to that of control HUVECs. Error bars represent s.e.m. of three independent experiments. (D) Immunoblot of whole cell lysates of control and CRIM1 deficient HUVECs cultured in VEGFA depleted medium with or without addition of 0.1 mg/ml Na$_3$VO$_4$. Error bars represent s.e.m of three experiments.

Figure 2.15 Adhesion junctions and VEGFR2 level and distribution were not changed when CRIM1 and VEGFA were knocked down in cultured endothelial cells. (A-I) VE-CADHERIN and β-CATENIN labeling with Hoechst staining in control HUVECs or HUVECs expressing shRNAs targeting CRIM1 and VEGFA. (J-R) VEGFR2 and GOLGA2 labeling with Hoechst staining in control HUVECs or HUVECs with CRIM1 and VEGFA knockdown. GOLGA2 antibody was used to label Golgi apparatus.
CRIM1 knockdown HUVECs showed no change in the ratio of phosphorylated to total VEGFR2 (Figure 2.14 B). When we tested different concentrations of exogenous VEGFA from 2 to 20 ng/ml, CRIM1 knockdown also had no consequence for VEGFR2 phosphorylation (Figure 2.16 B). Interestingly however, when we cultured HUVECs in VEGFA-deficient medium for 24 hours and added Na$_3$VO$_4$ to inhibit phosphatase activity, we recorded a significant reduction of VEGFR2 phosphorylation in the CRIM1 knockdown cells. This response was quantified by ELISA (Figure 2.14 C) but was also observed using immunoblot (Figure 2.14 D). These data suggested that CRIM1 modulates the autocrine signaling response to VEGFA.

**Figure 2.16 Crim1 was required for cultured endothelial cell survival.**

(A) Growth curve of control and CRIM1 knockdown HUVECs cultured in serum-free, VEGFA depleted medium. 20,000 cells were plated into each well of fibronectin coated 96-well plates at time zero. (B) VEGFR2 phosphorylation in response to different concentration of exogenous VEGFA stimulation. HUVECs infected with scramble shRNA or shRNA targeting CRIM1 were starved for 24 hours and stimulated with different concentration of recombinant VEGFA for 5 minutes. Cell lysates were applied to phospho-VEGFR2 and total VEGFR2 ELISAs and the ratio of the two readouts calculated. In one set of experiments, conditional medium with 20 ng/ml VEGFA added was incubated with 10 μg/ml Avastin for 2 hours before applied to the cells. In another set of experiments, cells was incubated with 0.5 μM SU5416 for 1 hour before stimulated with conditional medium containing 20 ng/ml VEGFA with same concentration of SU5416. Error bars represent s.e.m of three experiments. *: p<0.05; ***: p<0.005.
To examine this possibility further, we assessed the relative impact and the combined impact of VEGFA and CRIM1 knockdown on the growth of HUVECs and on VEGFR2 signaling. In complete medium with a low supplemental level of VEGFA (about 2 ng/ml) VEGFA and CRIM1 knockdown HUVECs both exhibited slower growth kinetics (Figure 2.17 A) as shown previously (Figure 2.14 A). However, in addition, CRIM1/VEGFA double knockdown cells showed a more severe and statistically distinct effect that either single knockdown (Figure 2.17 A-B, green trace). In similar experiments assessing cell number after 48 hours, we found that the effects of single and combined knockdowns were not compensated by additional exogenous recombinant VEGFA (Figure 2.17 B). When we performed growth kinetics for CRIM1 knockdown HUVECs in serum free, VEGF-deficient medium, we found that cell number actually reduced (Figure 2.16 A), indicating that some cells die, as might be expected with limited VEGFA and limited alternative survival stimuli. A comparison of the separate or combined effects of VEGFA and CRIM1 knockdown showed that under these conditions as well, the combination produced the most severe effect (Figure 2.17 C). An assessment by QPCR of the expression levels of BCL-2, a known anti-apoptosis molecule, and BAX, a pro-apoptosis molecule showed that the former showed reduced expression while the latter showed an increase with VEGFA, CRIM1 and combined VEGFA/CRIM1 knockdown (Figure 2.17 D). This establishes that VEGFA and CRIM1 affect the same target genes in a survival pathway that functions within VECs.

The differential effects of an anti-VEGFA antibody (Avastin) and the VEGFR2 kinase activity inhibitor has been used to show that there is an exclusively autocrine signaling pathway in VECs (Lee et al., 2007). Using VEGFR2 phosphorylation in the
Figure 2.17 Relative impacts and combined impact of VEGFA and CRIM1 knockdown on VEGFR2 signaling. (Figure legend see next page)
presence of orthovanadate in minimal medium as a read-out for autocrine signaling activity, we assessed the relative consequences of either VEGFA or CRIM1 knockdown and determined whether Avastin or SU5416 could modulate signaling. As already shown, VEGFA or CRIM1 knockdown had very similar consequences for VEGFR2 phosphorylation (Figure 2.17 D). However, in addition we found that Avastin had a very limited ability to suppress VEGFR2 phosphorylation while SU5416 abrogated this completely. Quantitatively, Avastin suppressed VEGFR2 phosphorylation by about 25%. This means that 75% of the signaling activity can be attributed to an Avastin-resistant, autocrine signaling pathway. Since CRIM1 knockdown can suppress VEGFR2 phosphorylation by significantly more than this, there is a strong argument that, like VEGFA knockdown, CRIM1 knockdown also suppresses autocrine signaling by endogenous VEGFA. This notion is reinforced by the observation that when CRIM1 and

Figure 2.17 Relative impacts and combined impact of VEGFA and CRIM1 knockdown on VEGFR2 signaling. (A) Growth curve of HUVECs expressing scramble shRNA and shRNAs targeting CRIM1, VEGFA or combined. Colored asterisks represent statistical significance between control and respective experimental groups. (B) Fold change of cell number after 48 hours compare to time zero of cells incubated in complete medium (EGM-2) or complete medium with addition of 10 ng/ml rhVEGFA. For (A and B), 5,000 cells were plated into each well of 96-well plates at time zero. (C) Fold change of cell number after 48 hours incubation in serum-free EGM-2 without addition of supplemented VEGFA. 20,000 cells were plated in each well at time zero. (D) Gene expression measured by quantitative RT-PCR. Cells were cultured in fresh complete medium for 24 hours. Quantity of mRNA expression of each was normalized to the respective β-Actin expression level. All error bars represent s.e.m of three experiments. (E) VEGFR2 phosphorylation without exogenous VEGFA. Cells were incubated in serum-free EGM-2 without addition of supplemented VEGFA and with 0.1 mg/ml Na$_3$VO$_4$. In one set of experiment, the medium was pre-incubated with 10 μg/ml Avastin for 2 hours and then applied to the cells. In another set of experiments, medium contained 0.5 μM SU5416. (F) VEGFR2 phosphorylation without exogenous VEGFA. HUVECs infected by lenti-viral particle expressing scramble shRNA and shRNAs targeting CRIM1, VEGFA or combine of the two were incubated in serum-free, VEGFA depleted medium containing 0.1 mg/ml Na$_3$VO$_4$ for 24 hours before cell lysates were applied to phospho-VEGFR2 and VEGFR2 ELISA and the ratio of the two readouts calculated. All error bars represent s.e.m. *: p<0.05; **: p<0.01; ***: p<0.005; ****: p<0.001; NS: not significant.
VEGFA knockdown are combined under the same conditions, there is a greater effect than either single knockdown on VEGFR2 phosphorylation (Figure 2.17 E). Combined, these data suggest the CRIM1 functions to regulate the activity of VEGFA within the autocrine pathway known to be active within VECs.
SECTION 2.4 Discussion

We have investigated the function of Crim1 in microvascular development in vivo. Using conditional deletion of a \textit{Crim1}^{\text{flox}} allele, we identified a critical function for Crim1 in the vascular endothelial cells (VECs) of the developing retinal vasculature. We also provide evidence that in VECs, Crim1 regulates autocrine signaling by Vegfa.

Section 2.4.1 Crim1 stabilizes nascent vessel connections

Angiogenic sprouting forms a draft vessel network that subsequently undergoes remodeling to become functional (Potente et al., 2011). Endothelial cell death (Lobov et al., 2005) and retraction of endothelial cells (Chen et al., 2012) can both contribute to vessel regression. In the mouse retina, some segment regression is likely triggered by the lack of oxygen demand close to large vessels (Sun et al., 2005) but in addition, there is sporadic segment regression throughout the forming network. Vessel “ghosts” that indicate a regressing segment can be found even at the advancing front of an angiogenic network and indicate that remodeling is not completely restricted spatially. Disruption of certain molecular pathways, including the Notch pathway (Phng et al., 2009) will cause excessive vessel regression. The current analysis suggests that the trans-membrane protein Crim1 has a role in stabilizing newly formed vessel segments; when conditionally mutated in VECs, the consequence is many more vessel ghosts and overall compromise of the forming vascular network.

Section 2.4.2 Does Crim1 regulate cadherin adhesion in the retinal vasculature?
There is evidence that Crim1 can regulate cell-cell adhesion. Crim1 is expressed in adherent cells, especially in those making new cell-cell contacts (Glienke et al., 2002). Crim1 can also form complexes with β-catenin and N-Cadherin, and consistent with this, in *Xenopus*, Crim1 stabilizes adhesion junctions and maintains the integrity of neuroepithelium during morphogenesis (Ponferrada et al., 2012). These data suggest that some functions of Crim1 could result from the formation of complexes with VE-cadherin, a cadherin family member known to be critical for vascular development (Dejana et al., 2009; Strilic et al., 2009). Several features of the Crim1 VEC-specific conditional knockouts also suggested a consequence for adhesion. These included, (1) an increase in non-perfusible, VE-cadherin negative vessel segments, (2) an increase in the bifurcation of tip cells that mimics the behavior of the VE-CADHERIN knockdown tip cells (Montero-Balaguer et al., 2009), and (3) VECs separated from extending vessels at the angiogenic front, a feature also observed in VE-cadherin endothelial conditional mutants (Gaengel et al., 2012) suggesting compromise of stable junctions.

However, there is also evidence countering the suggestion that Crim1 regulates VE-cadherin function. Firstly, when VE-cadherin is depleted from endothelial cells, vessels show excessive branching and over-growth (Gaengel et al., 2012). This contrasted with Crim1 VEC conditional mutants where VE-Cadherin discontinuous vessel connections are thin and usually lacking a lining of endothelial cell nuclei (Figure 2.7 E-H). Secondly, though we could identify α-catenin and β-catenin in immunoprecipitates of VE-Cadherin from cultured confluent endothelial cells, we were not able to identify Crim1, even if we used an epitope-tagged Crim1 to increase detection efficiency (data not shown). These finding suggest that the EC-EC contact
and vessel lumen defects in Crim1 conditional mutants might be an indirect consequence of *Crim1* loss-of-function.

**Section 2.4.3 Crim1 regulates autocrine Vegfa signaling**

Previous studies on Crim1 in various organisms have suggested it may regulate the bioavailability of growth factors and morphogens. In vitro studies demonstrate that Crim1 regulates the rate of processing and delivery of Bmp4 and Bmp7 to the cell surface (Wilkinson et al., 2003), probably in the Golgi compartment. Another study provides evidence that Crim1 can physically bind signaling ligands such as Vegfa, Plgf or Pdgf before their secretion (Wilkinson et al., 2007). In the kidney, Crim1 sequesters Vegfa at the surface of podocytes so that loss of Crim1 causes excessive Vegfa-Vegfr2 signaling and overgrowth of the glomerular vasculature (Wilkinson et al., 2007). There are also invertebrate homologues of Crim1. The *C. elegans* homologue of Crim1 (crm-1) controls body size through regulation of BMP signaling (Fung et al., 2007). *Drosophila Crimpy*, which has sequence homology to Crim1, inhibits Glass bottom boat (a BMP family ligand) in motor neurons during neuromuscular junction development (James and Broihier). However, *in ovo* knock-down of CRIM1 in both *Xenopus* and *Zebrafish* and germ line loss-of-function of Crim1 in the mouse do not produce defects in the early patterning of the body axis (Chiu et al., 2012; Kinna et al., 2006; Ponferrada et al., 2012) as would be expected if Crim1 was a critical universal regulator of BMP signaling.

Our current findings are consistent with previous analysis of the phenotype after germ line deletion of a Crim1 conditional allele (Chiu et al., 2012). When a germ line
loss-of-function homozygous mutant was produced by recombining our loxP-flanked Crim1 allele with Elia-cre, we found a constellation of changes (including hemorrhage, edema and syndactyly) that was very similar to the phenotype of the previously characterized insertional (Pennisi et al., 2007) and germ line conditional (Chiu et al., 2012) mutants. Though Crim1 mutant mice show hemorrhage, and Crim1 has been implicated in binding Vegfa (Wilkinson et al., 2007), germ line Crim1 mutant mice do not show a major failure in early vascular patterning as would be anticipated if Crim1 was a critical regulator of Vegfa signaling (Carmeliet et al., 1996; Ferrara et al., 1996). Combined, these findings suggest that Crim1 regulates signaling ligands in a temporally and spatially restricted manner.

It has been shown that VECs can produce Vegfa and that this autocrine source of Vegfa is indispensible for blood vessel homeostasis (Lee et al., 2007). The current model suggests that autocrine Vegfa is critical for stimulating VEC survival in response to stress signals such as hypoxia, irradiation and reactive oxygen species, factors that would be an ongoing challenge for mature vessels. The current analysis, where we have deleted Vegfa in VECs during development, also indicates that autocrine Vegfa is important at developmental stages.

One consequence of VEC-specific Crim1 loss-of-function is a deficient angiogenesis in the retina. Prompted by data showing that Crim1 can regulate Vegfa (Wilkinson et al., 2007) and by analysis showing that Vegfa is the major stimulus for angiogenesis in the retina (Carmeliet and Jain, 2011a), we investigated the possibility that the Crim1 phenotype resulted from a Vegfa signaling deficiency. We showed that in the developing retinal vasculature, Vegfa or Crim1 conditional deletion in VECs gave
very similar phenotypes characterized by increased number of vessel ghosts, discontinuous VE-cadherin labeling and increase in the number of segments that showed the absence of a vessel lumen. In addition, we showed that conditional compound heterozygotes had a phenotype more severe than each single heterozygous conditional mutant and one that was indistinguishable from the homozygous conditional phenotype. The severe phenotype of the double heterozygotes is surprising, and this additive effect is consistent with the hypothesis that this is a synthetic phenotype resulting from the function of Crim1 and Vegfa in the same pathway.

Culture experiments showed quite directly that CRIM1 regulates autocrine VEGFA signaling. First we showed, as expected, that proliferation of HUVECs in culture is enhanced by VEGFA supplementation. In addition, we showed that HUVEC expansion was almost absent if we used shRNA targeting of CRIM1 even with an exogenous source of VEGFA. Starvation-stimulation experiments with exogenous VEGFA in CRIM1 knockdown HUVECs were not, however, accompanied by any change in the level of VEGFR2 phosphorylation. This suggested that CRIM1 was required for proliferation and survival in HUVECs but not because it regulated the activity of exogenous VEGFA. By contrast, when HUVECs were denied exogenous VEGFA and asked to rely on autocrine signaling, the outcome for VEGFR2 activation was different. In the absence of exogenous VEGFA, control HUVECs in serum-free medium do not proliferate but survived for 2 days or more. CRIM1 knockdown resulted in rapidly reducing cell numbers over this time-course. Accompanying this elevated level of cell death was a reduced proportion of VEGFR2 in its active, phosphorylated state. Evidence that the documented VEGFR2 phosphorylation was a consequence of
autocrine signaling came from a comparison of the effects of an anti-VEGFA antibody, which had a very limited effect and the effects of a small molecule VEGFR2 inhibitor, which completely abrogated phosphorylation. Combined, these data support a model in which CRIM1 regulates VEGFA autocrine activity in the previously defined “private loop” (Lee et al., 2007) and is consistent with the observation that Crim1 can form physical complexes with Vegfa (Wilkinson et al., 2007).
SECTION 2.5 Materials and Methods

Section 2.5.1 Animals

*Pdgfb*-iCreER (Claxton et al., 2008), *Ella-Cre* (JAX® 003724), *Tg(CAG-Bgeo/GFP)* (Z/EG, JAX® 003920) and *Vegfa*\(^{\text{loxP}}\) line (Gerber et al., 1999) were described before. To induce endothelial cell gene deletion in *Pdgfb*-iCreER mouse pups, peanut oil-dissolved tamoxifen (Sigma) was injected intraperitoneally daily at 20 \(\mu\)g/g body weight. Tamoxifen-injected *Pdgfb*-iCreER negative littermates were used as controls. Birth was defined as postnatal day 1 (P1).

Section 2.5.2 Generation of the Crim1 conditional allele

We generated a conditional loss-of-function allele, *Crim1\(^{\text{flo}x}\)*, using conventional gene targeting. With the very high GC-content surrounding exon 1 (5’-UTR and start codon), we targeted exons 3 and 4 (Figure 2.2 A). This design left open the possibility that exons 1 and 2 might produce a functional truncated protein. Incorporation of sequences from the C-terminus of ornithine decarboxylase (cODC) as a signal for rapid degradation (Matsuzawa et al., 2005) was designed to eliminate any expressed CRIM1 N-terminal sequence. The allele design incorporated lox\(P\) sites into an artificial exon that is initially in reverse orientation. Through two steps of lox\(P\)-mediated recombination, mediated by a pair of wild-type and a pair of variant lox\(P\) sequences that are incompatible, the ODC degradation signal is spliced into the mRNA, simultaneously deleting exons 3 and 4. This allele design strategy has used previously (Schnutgen et al., 2003).
Section 2.5.3 Whole-mount immunofluorescence of retinas

Antibody and isolectin labeling of retinas was performed as previously described (Stefater et al., 2011) with the following: Alexa 488-Isolectin-IB4 (Molecular Probes), Crim1 (home-made anti-serum), collagen IV (abcam), BrdU (Dako), VE-Cadherin (BD and Santa Cruz), CD31 (BD), active caspase-3 (R&D), podocalyxin (R&D), PDGFRα (R&D), desmin (abcam), Smooth muscle actin (Sigma).

Section 2.5.4 Cell culture

Pooled Human umbilical vein endothelial cells (HUVECs) (Lonza, CC-2519) were cultured on 0.1% gelatin coated culture dishes in EGM-2 BulletKit medium (Lonza CC-3162). Cells before passage 5 were used in all experiments. Lenti-viral particles containing shRNA targeting CRIM1 mRNA were acquired from TRC Mission® Library (Invitrogen). Cells reaching 90% density were incubated with diluted lenti-viral particles together with 8 μg/ml polybrene for 16 hours and then treated with 2 μg/ml puromycin for 2 days to enrich infected cell population. 24 hours after puromycin removal cells were applied to subsequent experiments. We tested 4 different shRNA targeting CRIM1 and chose 2 shRNAs that worked most effectively: TRCN0000063860 (shCRIM1(60)) and TRCN0000063862 (shCRIM1(62)). Similarly, VEGFA was knocked down using TRCN0000003343, whose effectiveness has been validated before (Segarra et al., 2012).

Section 2.5.5 Growth curve of HUVECs

5,000 HUVECs expressing scramble shRNA or shRNA targeting CRIM1 and VEGFA
were plated in each well of 96-well plates coated with 10 μg/cm² human fibronectin (Sigma F2006) and cultured in EGM-2 medium. To monitor cell survival in VEGFA deficient medium, 20,000 cells per well (confluent) were plated each well and cultured in EGM-2 without addition of serum and supplemented VEGFA.

**Section 2.5.6 VEGFR2 activation in cultured HUVECs**

To detect the response to exogenous VEGFA, HUVECs expressing shRNAs were starved in EBM-2 (Lonza CC-2156) containing only antibiotics and heparin for 24 hours and stimulated with recombinant human VEGFA protein (R&D 293-VE) with 2 mg/ml Na₃VO₄ for 5 minutes. To detect VEGFR2 activation by autocrine VEGFA, HUVECs expressing shRNAs were cultured in the same medium for 24 hours with or without 0.1 mg/ml Na₃VO₄. For control experiments, cells were cultured in medium either pre-incubated with 10 μg/ml Avastin (Genentech) or with 0.6 μM SU5416 (Sigma) added. Cell lysates were collected in RIPA buffer containing 2 mg/ml Na₃VO₄ and phosphorylated VEGFR2 level detected either by ELISA (Cell signaling Kit 7335 and 7340) or immunoblot (phospho-VEGFR2 (Y1175, cell signaling 2478), VEGFR-2 (cell signaling), CRIM1 (Sigma WH0051232M1), α-tubulin (Abcam)).

**Section 2.5.7 Quantitative RT-PCR**

RNA was extracted using RNeasy Micro Kit (Qiagen) and cDNA was prepared using Superscript III reagent (Invitrogen). Quantitative PCR reaction was performed with iQ SYBR Green Supermix (BioRad). Signals were detected using BioRad CFX1000 system and quantities of each gene expression were calculated using the standard
curve method.

Section 2.5.8 Fluorescent activated cell sorting (FACS)

Freshly isolated mouse retinas were incubated in DMEM (Gibco) containing 1 mg/ml Collagenase A (Roche) and 3 U/ml DnaseI on a shaker at 37˚C for 30 minutes with gentle pipetting every 10 minutes. Cells were passed through cell strainers (BD) and spun down at 500g for 5 minutes. After washes in PBS containing 2 mM EDTA and reconstituted in the same buffer, cells were labeled with PE-CD31 and APC-CD45 antibodies (BD Pharmingen) at 4˚C for 20 minutes. After washes, cells were reconstituted in PBS containing 2 mM EDTA and passed through cell strainer again before FACS analysis. PE (CD31) positive and APC (CD45) negative populations were sorted and collected as vascular endothelial cells.

Section 2.5.9 Statistical Analysis

Statistical analysis was performed using Student’s t test.
CHAPTER THREE

Conclusion
Research significance and future directions

In data presented in Chapter two, we demonstrate that Crim1 maintains retinal vascular stability during development by regulating endothelial cell Vegfa autocrine signaling. The strength of the study is that we used mouse genetics, taking advantage of loxP-Cre system, to elucidate endothelial cell specific functions of Crim1 and Vegfa in angiogenesis in vivo. Previous studies about role of Crim1 in vascular biology were either restricted in vitro, or lacking cell-specific functional analysis. The current study showing that Crim1 is required in developmental angiogenesis, at least in the retinal vasculature, is quite significant in that:

1) It underscores the close coupling between microvasculature growth and remodeling. As has been shown in other studies, vessel pruning not only happens in completely formed plexus, but also happens in very newly formed plexus. When endothelial cells at the frontier of sprouting turn on pro-angiogenic and pro-proliferation molecular machinery, vessel segments closely training the angiogenic front switch to a pro-survival molecular kit, and vessel pruning is inseperable from branching morphogenesis.

2) It enlarges our knowledge of Crim1 protein. In addition to previous notions of its involvement in regulating growth factor secretion and/or cell-cell adhesion, it also regulates the autocrine Vegfa-Vegfr2 signaling within endothelial cells, probably in intracellular vesicle compartment. As discussed in Chapter Two, these different functions may be linked together, which requires further investigation.

3) It deepens our understanding of the Vegfa-Vegfr2 autocrine signaling. The Vegfa-Vegfr2 autocrine signaling has been demonstrated within endothelial cells and in
other cell types, specifically tumor cells. In endothelial cells, current data supports the model that the signaling happens even before the ligand being secreted out the cell. Deletion of Vegfa ligand of course abrogates the signaling. Very few other mutants have been identified in regulating the pathway; this includes Semaphorin6A which regulates Vegfr2 expression directly, thus deletion of Semaphorin6A in endothelial cells caused defected signaling exerted by both exogenous and endogenous Vegfa source (Segarra et al., 2012). Crim1 seems to be the first molecule identified to be affecting only the autocrine signaling. This indicates segregation of Vegfr2 in different pathways.

The active role of Vegfa in expanding microvasculature has important implications. As has been discussed in Chapter One, no matter how many new pathways were found in the past several years, the central role of Vegfa signaling in driving developmental and pathological angiogenesis is unshakeable and thus Vegfa and its receptors are the most important targets in proangiogenic or antiangiogenic therapies. It is interesting to consider taking into account the balancing of autocrine and paracrine Vegfa signaling in these therapies.

Future studies regarding Crim1 protein and Vegfa autocrine signaling in vascular development and homeostasis could consider these directions:

1) Role of Crim1 beyond retinal angiogenesis needs to be elucidated. This includes whether the vascular defects and perinatal lethality in Crim1 germline knockout embryos are directly related to an endothelial function, and whether microvasculature in other organs that form through angiogenesis (discussed in Chapter One) is also affected by endothelial Crim1 loss-of-function. Also, we have showed that Crim1 is also
expressed in mural cells. Preliminary data from our lab showed that Crim1 in pericytes negatively regulates retinal angiogenesis (data not shown), and the precise mechanism remains unclear.

2) The precise mechanism of how Crim1 regulates Vegfa-Vegfr2 autocrine signaling requires further investigation. We hypothesized in Chapter Two that this function may be related to the ability of Crim1 to bind the ligand directly through extracellular domain, whether this means more availability of ligand in intracellular vesicles or higher accessibility of endogenous Vegfa to internalized Vegfr2, remains unknown. Given that Crim1 can form complex with Cadherin-catenin complexes in other context, which suggests the possible binding capacity of the cytoplasmic domain to other cytoplasmic proteins, we could not rule out the possibility that Crim1 directly or indirectly regulates the Vegfr2 receptor and other molecules in the signaling cascade as well.

3) Vegfa endothelial autocrine signaling in angiogenesis in other contexts is important. Our data is to some extent in contrary to previous study (Lee et al., 2007) in which researchers showed that the primary vasculature patterning in Vegfa conditional knockout mice seemed not affected and mice did not start to die until juveniles. Although we can argue that decreased ratio of knockout mice at birth could be attributed to prenatal death of mice with severe vascular defects, which were not examined in that study, another postulation is that Vegfa autocrine promoting angiogenesis is context-dependent and requires validation.

4) The convergence of autocrine and paracrine Vegfa signaling remains to be expounded. Our data also suggest the autocrine pathway possibly regulates expression
of pro- and anti-survival genes. Molecular machinery transduces the signaling remains unclear. Current data suggests that Vegfr2 is phosphorylated in the same way upon activation by endogenous and exogenous source of Vegfa (Lee et al., 2007). It is very likely they share similar molecular cascade in the cytoplasm. If this is the case, how the signaling divergent and convergent within the cytoplasm might be related to the balancing of the signals and behaviors of the cells. Recently, researchers found that the delayed trafficking of endocytosed Vegfr2 after ligand binding from Rab5+ to EEA+ endosomes resulted in dephosphorylation of the receptor and thus the signaling activity. It would be interesting to investigate the mechanisms in initiating the internalization of the Vegfr2 to render the signal from endogenous Vegfa and whether similar trafficking systems are used to regulate the signaling activity.


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