Eph-mediated restriction of cerebrovascular arteriogenesis

By

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Stroke is a leading cause of morbidity and long-term neurological disability in the U.S. Ischemic stroke, which accounts for approximately 90% of all strokes, is the result of an occlusion in the arteriole cerebrovascular network. No effective treatment options exist to provide neuroprotection from occlusion, and limited success has been seen clinically when attempting to restore blood flow to vulnerable neural tissue regions. Enhancement of pial collateral remodeling (Arteriogenesis) has recently been shown to improve blood flow and mitigate neural tissue damage following stroke (1-3). Arteriogenesis is the remodeling of pre-existing arteriole vessel which are able to re-route blood to blood-deprived regions of tissue. Arteriogenesis requires endothelial cell (EC) and
smooth muscle cell proliferation, extracellular matrix degradation and recruitment of circulating bone marrow-derived cells (4-6). Unlike spouting angiogenesis, which requires weeks following occlusion to develop, arteriogenesis begins as early as 24-48hrs post-stroke (7, 8) and can expeditiously enhance blood flow to ischemic regions, making it an attractive target for therapeutic intervention. Our preliminary studies, in an EphA4 global knockout mouse model, indicated that EphA4 receptor tyrosine kinase severely limits pial arteriole collateral formation. The preliminary work also showed that activation of EC EphA4 receptor in vitro inhibited vascular formation. Additionally, ECs lining the collateral vessel have been shown to play a role in collateral remodeling (9). Taken together, the objective of this dissertation was to elucidate the cell autonomous role of the EphA4 receptor and given the central role of the EC in collateral remodeling, we postulated that EphA4 receptor on ECs limits pial collateral formations. Using a cell-specific loss-of-function approach, we tested the hypothesis that EC-specific EphA4 plays an important role in pial collateral development and remodeling after induced stroke. The results from this dissertation show that (1) EphA4 expression on ECs suppress the formation of pial collaterals during development and limits EC growth via suppression of p-Akt in vitro (2) EC-specific EphA4 ablation leads to increased collateral remodeling, enhanced blood flow recovery, tissue protection and improved neurological behavioral outcomes after stroke and (3) Mechanistically, EphA4 limits pial collateral remodeling via attenuation of the Tie2/Angiopoietin-2 signaling pathway. The work presented in this dissertation demonstrate that EphA4 can be targeted therapeutically to increase pial collateral remodeling to alleviate neurological deficits after ischemic stroke.

**General Audience Abstract**

Stroke is the fifth leading cause of death in the United States. Ischemic stroke is the most common type of stroke and occurs when blood flow to part of the brain is impeded. Lack of blood results in cell death and tissue damage in the brain. In an effort to restore blood flow, specialized blood vessels in the brain called collaterals remodel and become larger to allow re-routed blood to the blood-deprived region of the brain. The duration it takes to remodel these remarkable blood vessels and re-route blood varies in humans, and sometimes is not able to prevent adequate tissue damage. The current work explores novel therapeutic targets to accelerate collateral
remodeling in an effort to reduce tissue loss after stroke. We present studies which show that a protein called EphA4, found on endothelial cells restricts remodeling, and when inhibited in the brain can increase collateral remodeling and reduced adverse effects after ischemic stroke.
I dedicate this work to my family and loved ones. I am very grateful for all the support you have offered in my life. To my parents who have been a source of encouragement and inspiration to me throughout my life and for encouraging me to work hard and be diligent. To my siblings, for being role models in my life. To my fiancée, Adwoa, for her patience, care, love, and emotional support.
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Chapter 1

Introduction and Preliminary Work
Introduction

Arteriogenesis is an active remodeling response to arterial occlusion that leads to preservation of structure and function in ischemic tissues (10). Fluid shear stress activates the cascade of events which leads to arteriogenesis. Briefly, activated endothelium secret MCP-1 cytokine which leads to monocyte recruitment, infiltration and adhesion. This results in the production of cytokines, growth factors and proteases that mediate proliferation of endothelial cells (EC) and expansion of preexistent collateral arterioles, to re-route blood to the ischemic tissue (5, 6, 11). Arteriogenesis is not organ- or species-specific; cerebral, coronary or peripheral collateral vessels actively remodel and are principally the same in mice, rats, rabbits, or dogs (6, 12-14). This observation along with pre-clinical studies showing enhancement of this adaptive process following growth factor administration has raised hope for a new avenue of therapeutic intervention in vascular occlusive disease. However, knowledge of the mechanisms regulating this response in the brain is understudied and the development of safe and effective therapies targeting arteriogenesis remains a concern (15), indicating a need to improve our basic understanding of the coordinated process of arteriogenesis.

The present dissertation focused on the arteriogenic growth of the pial collaterals rather than on the sprouting capillary network. The arterial network carries ten times more blood volume compared to capillaries and therefore have a much greater capacity to restore blood flow after injury. In addition, induction of arteriogenesis in the ischemic brain may create a more hospitable microenvironment for neuronal plasticity leading to functional recovery (1). Arteriogenesis has been well documented to occur in cerebral arteriole collateral vessels, especially in the pial circulation, and is linked to neural tissue preservation and subsequent repair after stroke (2, 3, 16, 17). The pial collaterals link the anterior cerebral artery (ACA) and the middle cerebral artery (MCA) on both dorsal hemispheres. The density of cerebral pial collateral vessels varies widely amongst different strains of mice making each strain differentially vulnerable to ischemic stroke (18).

The Tie2 signaling pathway has recently emerged as a key player that regulates the density and diameter of arteriole collaterals during development and following injury (19-23). Steep pressure gradients across the damaged arteriole network increase fluid shear stress in the arteriole collateral walls (24), initiating the remodeling process (6). Given the emerging importance of collateral blood flow in the progression of neurovascular deficits and subsequent functional restoration, more emphasis should be placed on identifying
the molecular mechanism(s) underlying arteriogenesis. Our initial findings indicate that EphA4 receptor negatively regulates endothelial cell proliferation and migration via Tie2 signaling *in vitro*. Initial findings show significant enhancement of pial collateral diameter using EC-specific EphA4 KO mice, which corresponds with improvements in blood flow restoration and tissue preservation suggesting EphA4 may act as a negative regulator of arteriogenesis.

Eph receptor tyrosine kinases are known regulators of cell migration, proliferation and survival in the CNS (25-31). Both Eph receptors and their cognate ligands are membrane bound and require direct cell-cell contact to initiate signal transduction. Although several ephrin/Eph molecules play critical roles in other biological processes (32-37), the role of Eph signaling in *cerebral arteriogenesis* has not been identified. In addition to the known role of EphA4 as a negative regulator of stem cell proliferation and migration, it was recently described as a new member of the family of dependence receptors, which induces apoptosis in the absence of ephrinB3 ligand in neural stem cells (38). Given the importance of this signaling molecule, our studies utilized a cell-specific, transgenic approach to examine a *cell autonomous* role for EphA4 in arteriogenesis and tissue recovery after ischemic stroke.

**Significance.** The studies focus on an important, yet understudied area of brain research. Therapeutic stimulation of arteriogenesis is a novel approach for treatment of cerebral ischemia given this natural adaptive process can be expeditiously enhanced (39, 40). This is *unlike* sprouting angiogenesis, which develops slowly after occlusion. Despite the success of therapeutic arteriogenesis in a plethora of animal and small-scale human studies involving peripheral and heart disease (41-45), translating this promising approach to *occlusive stroke* has remained a challenge (3, 39, 40, 46). The few large scale clinical trials investigating arteriogenesis in stroke that have been conducted, show limited success (46), mainly because the mechanisms regulating endogenous cerebral arteriogenesis are still poorly understood. To that end, our studies addressed a novel role for EphA4 in limiting the active remodeling of the pial arteriole network thru regulation of the EC response. We examined the *cell autonomous* role of EphA4 using a transgenic loss-of-function approach. Recently, the ability to create cell-specific knockouts of EphA4 has become available (47) from which we have ablated EphA4 on ECs using the Tie2 promoter (EphA4^{f/f}/Tie2::Cre^+) to aid in our investigations. Knowledge gained from these studies will further our understanding of the cellular and molecular mechanisms regulating arteriogenesis following stroke.
Preliminary Work

Blocking EphA4 enhances proliferation, migration and tube formation in cultured endothelial cells (ECs). To investigate whether EphA4 regulates EC activities, we cultured primary brain-derived EC and assessed their ability to form vascular tubes in the presence and absence of EphA4 stimulation. After seeding on matrigel substrate, EC attach and degrade the extracellular matrix to form guidance pathways (ECM invasion) that facilitate cellular migration and tube formation (48). First, using immunofluorescence we showed that primary ECs express CD31 (Fig. 1A). Similar to our in vivo expression analysis, cultured ECs express both RNA transcripts for EphA4 and ephrinA5 (Fig. 1C). Next, we showed that stimulation of EphA4 on wild type (WT) ECs (isolated from WT transgenic mice) using pre-clustered ephrinA5 Fcs significantly disrupted tube formation at 8-72 hrs (Fig. 1F and 1H) compare to FC-control (Fig. 1D and 1H). Conversely, blocking EphA4 enhanced tube formation in WT ECs using soluble EphA4-Fc (Fig. 2E and 2H) or thru genetic knock down as seen with EphA4-null ECs (Fig. 1G and 1H). EphA4-null ECs are not affected by ephrinA5-Fc stimulation (Fig. 1H) suggesting that EphA4 forward signaling negatively regulates EC activities via ephrinA5 binding. EphA4-null ECs also demonstrated increased proliferation (Fig. 1J) as measured by BrdU incorporation, compare to WT cells (Fig. 1I-1K). Scratch assay also showed increased migration of EphA4-null ECs (Fig.1N, 1O) at 24 hr compared to WT (Fig. 1L,M). This loss-of-function, gain-of-function study supports a negative role for EphA4 in the proliferation, migration and tube formation (ECM invasion) of ECs which may impact arteriole collateral remodeling after injury.

Fig 1. (A-B) Brain-derived ECs express CD31 (A) and have RNA transcripts for EphA4 and ephrinA5 (C). (D-H) Vascular index (#tubes x tube length) during tube formation is enhanced in KO ECs or when EphA4 is blocked using soluble EphA4-Fc but disrupted when stimulated with ephrinA5-Fc compared to Fc-control. (I-K) KO ECs have increased BrdU incorporation and greater migration rate after scratch assay (N,O) compared to WT (L, M). I-O data generated by applicant.
EphA4 gene-targeted knockout mice have increased pial arteriole collaterals and reduced lesion volume after distal MCAO (dMCAO).

Our preliminary findings show that global EphA4−/− knockout mice have increased numbers of collaterals (Fig. 2B and 2C) compared to wild type and the infarct volume is reduced after pharmacologically inducing stroke in a distal branch of the middle cerebral artery (Fig. 2F and 2G). Initially, we used quantum dot-facilitated occlusion of the distal MCA branch to induce permanent focal cerebral ischemia in the presence and absence of EphA4. Twenty-four hours later we performed TTC staining and found a significant reduction in the infarct volume in EphA4−/− mice (Fig. 2F and 2G). This data indicate that deletion of EphA4 is neuroprotective. Given the restrictive role of EphA4 on arteriole formation (Fig. 2B) and on EC proliferation and migration (Fig. 1), we generated Tie2::Cre/EphA4flox/flox tissue-specific knockout (KO) mice to investigate the mechanism by which EphA4 regulates tissue injury after stroke. Specifically, we planned to investigate its limiting role during arteriogenesis and the impact on neural tissue loss and functional recovery using a much larger infarct model by permanent middle cerebral occlusion (pMCAO) of the main branch.

The overarching hypothesis of this dissertation was endothelial cell-specific EphA4 is a negative regulator of pial collateral formations and remodeling during vascular occlusion. To answer this hypothesis, we proposed the following specific aims:

**Aim 1: Examine the temporospatial expression of EphA4 and its effect on collateral formation.** Our preliminary findings showed that global EphA4 knockout increases collateral formations in mice. The endothelium is important to collateral development and work by Faber and colleagues have shown that
endothelial cell specific receptors are critical to the process (51). Thus our goal was to determine whether the effect seen in EphA4 global knockouts was endothelial cell specific. We hypothesized that endothelial cell specific EphA4 played a role in pial collateral development and the knocking it out on endothelial cells would recapitulate the global knockout phenotype. To test this hypothesis, we ablated EphA4 on Tie2-expressing ECs by crossing Tie2::Cre and floxed EphA4 mice to generate our experimental mice (EphA4\textsuperscript{flox/flox}; WT vs. EphA4\textsuperscript{flox/flox}/Tie2\textsuperscript{Cre+/-}; KO) mice. We used the Cre/lox system with a Tie2 promoter to ensure EphA4 was knocked out on endothelial cells. Effect of EphA4 on collateral development was assessed in postnatal day (P) 1, P7, P21 and in adult mice.

**Aim 2:** Examine the temporospatial expression of EphA4 and its ligand ephrinA5 on cerebral arteriole collaterals following pMCAO in the murine cortex. We hypothesized that EphA4 and/or its ligand were up-regulated on the arteriole cerebrovascular network following pMCAO. To test this hypothesis we analyzed the temporospatial changes in protein expression of EphA4, on pial collateral post stroke. We used the arteriole labeling technique, vessel painting (52) and immunofluorescence at 1, and 4 days post-sham or pMCAO to assess mural cell recruitment and EphA4 temporospatial expression.

**Aim 3:** Investigate whether EC-specific EphA4 negatively regulated arteriogenesis and neural functional recovery after pMCAO. Preliminary findings showed EphA4 is upregulated on pial collaterals following pMCAO and its ablation on ECs enhances their proliferation, migration and tube formation in vitro. We hypothesized that EC-specific up-regulation of EphA4 limits remodeling, neural tissue survival and behavioral outcome after pMCAO. To test this, we used our EphA4\textsuperscript{flox/flox} and EphA4\textsuperscript{flox/flox}/Tie2\textsuperscript{Cre+/-} mice. We quantified the number, size and proliferation of pial collaterals on the contralateral and ipsilateral hemispheres of stroke-injured mice at 1, 4, and 14 days using vessel painting. We also determined changes in cerebral blood flow, sensorimotor recovery as well as analyzed infarct volume and neuronal cell death using histology.

**Aim 4:** Elucidate the mechanism(s) by which EC-specific EphA4 negatively regulates arteriogenesis after pMCAO. KO endothelial cells exhibit increased proliferation and migration in vitro. The Tie2 signaling pathway is known to regulate endothelial cell proliferation and migration. To better address the role of EphA4 mechanistically during arteriogenesis we performed Tie2 inhibition experiments in vivo using soluble Tie2-Fc
infusion in our WT and KO mice. Furthermore, arteriogenesis as well as histological analysis of infarct volume and neuronal cell death was assessed in the presence and absence of the Tie2 receptor antagonist. These experiments provided a comprehensive examination of the molecular mechanism(s) suppressing stroke-induced collateral remodeling.

Overall, these studies were designed to define a novel mechanism that endothelial cell specific EphA4 regulates collateral development. The studies also delineated how EphA4 impairs arteriogenesis after ischemic stroke and evaluated its potential as an important new target for stroke therapy. Moreover, this dissertation yielded pertinent information regarding the cell autonomous role of EphA4 signaling during the coordinated process of arteriogenesis, using cell-specific transgenic mice. Successful completion of these studies allows us to pursue our long-term goal of enhancing arteriogenesis as a therapeutic strategy to improve neural preservation in stroke injured patients.
Chapter 2

Literature Review

Stroke therapy: Advances and viable therapeutic targets
Abstract

Stroke is the leading cause of mortality and morbidity worldwide. In the United States, it is the fifth leading cause of death but therapeutic interventions have remained staggeringly limited. Because of this, many stroke survivors are often burdened with exorbitant rehabilitation costs, limitations in their daily social activity, and significant residual disability. Thus identifying and treating stroke in the acute stage is critical to prevent forthcoming disability. This review highlights the clinical differences in stroke types and treatment regimens available for acute stroke. The review further discusses recent advances, as well as potential therapeutic targets for stroke treatment and recovery, with a focus on ischemic stroke. Additionally, we will focus on the collateral circulation and its potential as a therapeutic target for treating ischemic stroke.

What is stroke?

On average, the brain makes up 2% of the body’s weight. Interestingly, despite its relatively small size, it consumes about 20% of the body’s oxygen (53). Without oxygen, which is needed for oxidative metabolism of glucose, neuronal cells start to die within minutes. Oxygenated blood is carried via cerebral blood flow. Thus, a stroke occurs when the blood flow to part of the brain is impeded or severely reduced, depriving the brain tissue of oxygen and nutrients.

Stroke prevalence is increasing and statistics from the Center for Disease Control show that more than 795,000 people in the US have a stroke, with about 600,000 cases being first or new strokes. Stroke is the fifth leading cause of death in the United States, behind heart disease, cancer, chronic lower respiratory disease and accidents (cdc.gov). One out of every 20 deaths is a result of stroke – that is 140,000 deaths annually.

Stroke is a syndrome, and therefore patients with stroke present with several focal neurological deficits post-event. But clinical identification of the specific stroke type is key in acute treatment and recovery. Broadly, there are two types of stroke, either ischemic or hemorrhagic stroke. An ischemic stroke occurs due to vascular occlusion of a blood vessel that supply blood to the brain. Most often the occlusion occurs due to embolus or atherosclerosis. On the other hand, hemorrhagic stroke occurs when an aneurysm bursts or a weakened blood vessel leaks in the brain. The leaking blood seeps into or around the brain and creates swelling and pressure,
damaging cells and tissue in the brain. Thus, during stroke diagnoses, the source of blood flow reduction (occlusion or hemorrhage) and the location are further assessed using neuroimaging.

**Hemorrhagic stroke**

The two type of hemorrhagic strokes are intracerebral hemorrhage (ICH) and subarachnoid hemorrhage (SAH) based on the location of the hemorrhage in the brain. ICH which occurs when a ruptured vessel bleeds into the cerebrospinal fluid accounts for 10% to 15% of all strokes (54, 55). Adults with a hypertension condition are susceptible. ICH has a significantly higher mortality risks than ischemic stroke, with a mortality rate ranging from 35% to 52% within 30 days, which is more than five times the mortality for ischemic events (54, 55). Mortality risk gradually declines and by 3 months the ICH mortality risk is similar to ischemic stroke (56). The underlying mechanism of ICH is due to the effects of systemic blood pressure on small blood vessels that branch of major arteries, mostly the middle and posterior cerebral arteries. As a result of hypertension, these small blood vessels can develop intimal hyalinization and medial degeneration, which may result in focal necrosis and rupture. These vessel injuries can lead to microaneurysms, which are disposed to subsequent rupture, resulting in micro- or macro-hemorrhages (57).

Several neurovascular diseases occasionally progress into stroke (58). One such disease which may cause spontaneous ICH is cerebral amyloid angiopathy, and involves deposition of amyloid beta-peptide in small blood vessels of the basal ganglia, leptomeninges and cortex, and white matter (59). Subsequently, these vessels develop micro-aneurysms. These weakened sections of the blood vessel can subsequently rupture and cause microbleeds. Thus, in contrast to hypertensive ICH, cerebral amyloid angiopathy related ICH tend to occur in the cortex and subcortical white matter, specifically, the temporal and occipital lobes (60). Together, the symptoms and sometimes chronic debilitating neurological deficits presented by an ICH stroke patient coincide with the region of the brain affected. For example, a patient may complain of blurry vision, and this is normally due to ICH affecting the occipital lobes. Other symptomatic deficits of ICH include balance loss, weakness in the face, arm or leg, and loss of consciousness.

The second common type of hemorrhagic stroke is subarachnoid hemorrhage (SAH) and is less common than ICH. SAH accounts about 5% of all strokes (55, 61). Much like ICH, SAH-related deaths occur
early, with 61% of deaths happening within 2 days of the initial event (62). The typical cause of SAH is the rupture of a cerebral aneurysm (63), and accounts for more than 80% of all SAHs.

**Ischemic stroke**

Ischemic strokes occur due to occlusion a brain-supplying blood vessel. It is the predominant type of stroke, making up 87% of all stroke cases (64). The obstruction may be a result of embolism or thrombus. Thus, ischemic strokes can be further classified as either embolic or thrombotic.

Thrombotic events, which account for nearly 75% of ischemic strokes (65), are the result of a gradual pathophysiologic process associated with atherosclerotic disease. Plaque, which slowly accumulates in cerebral arteries, facilitates the formation of thrombi. The thrombi, in conjunction with the atherosclerotic plaque, can cause arterial narrowing and occlusion. This may lead to permanent focal neurological deficits. But in some events these deficits are transient. Transient ischemic attacks (TIAs) are thus thrombotic ischemic events that typically last only a few minutes and do not last longer than 24 hours. Although TIAs do not lead to permanent neurologic deficits, they are a critical warning sign that warrants emergency care and thorough diagnostic investigation. At least 10% of people who have experienced TIAs will develop thrombotic strokes. The distinction between TIAs and thrombotic ischemic stroke is the level of severity evaluated during diagnoses.

Embolic ischemic strokes are caused by emboli that interrupt cerebral blood flow. An embolus develops in the body, and travels through the blood to the brain. An embolus that reaches a cerebral blood vessel that is too narrow, lodges and then blocks blood flow to a part of the brain. The resulting sudden interruption in blood flow may result in stroke. Most often these emboli travel from the other parts of the body such as the heart and carotid artery and lodge in the MCA.

Although ischemic strokes may occur in any part of the brain, 80% of the cases are due to the occlusion of the middle cerebral artery (both embolic and thrombolic). The resulting impediment to blood flow leads to signs and symptoms of ischemia, including focal neurologic deficits. The severity of ischemic stroke ranges from clinically mild or transient, seen in TIA, to very severe, but the primary causes are identical – vessel occlusion.
**How is stroke diagnosed?**

Diagnosing the type of stroke that a patient presents with is critical in treatment options and prevention of further injury. Importantly, time is of the essence following stroke to prevent neuronal loss and further tissue damage. To put into context, for every minute spent without ischemic stroke resolution, 1.9 million neurons, 14 billion synapses and 7.5 miles of myelinated fibers are destroyed in the brain (66). Therefore, diagnosing and starting the appropriate treatment in time may be the difference between a full recovery from stroke and a patient having severe permanent neurological disabilities. Two important clinical tools used in the acute assessment are (1) the National Institute of Health Stroke Score (NIHSS) and (2) the use of imaging modalities such as computerized tomography.

The NIHSS is a 42-point standardized scale that is used as an indicator of the severity of neurologic dysfunction in a potential stroke patient and is mentioned for its interrater and intrarater reliability (67-69). The scale tests extraocular movements, level of consciousness, facial muscle function, visual fields, extremity strength, sensory function, language and speech, coordination, and hemi-inattention. NIHSS scores are interpreted as follow, 0 is no stroke, 1-4 is minor stroke, 5-15 is moderate stroke, 16-20 is moderate to severe stroke, and 21- 42 is considered as a severe and life threatening stroke. In addition to the NIHSS, patient manifestations such as headache, seizures, vomiting, coma and raised diastolic blood pressure increase prognosis of ICH compared to ischemic stroke, but only neuroimaging can provide a conclusive diagnosis (70). Altogether, the NIHSS is an initial clinical assessment that allows clinicians to estimate the type of stroke, as well as initialize therapies such as the administration of thrombolytic therapy.

After determining the severity of stroke symptoms, neuroimaging is used to differentiate between hemorrhagic and ischemic stroke. In the US, non-contrast computed tomography (CT) remains the principal imaging modality for the initial evaluation of patients with suspected stroke. It is able to detect acute ICH with great accuracy. Non-contrast CT is also used in the evaluation of acute intracranial hemorrhage as it produces good contrast between the high attenuating (bright) clot and the low attenuating (dark”) cerebrospinal fluid (71). Additionally, CT can be used to detect early ischemic changes, and most importantly will exclude confounding conditions such as ICH and lesions like a tumor. CT processing speed makes it critical for evaluation of suspected stroke patients. It is also important for determining continual use of thrombolytic therapy. Therefore,
thrombolytic therapy will be stopped in a patient who is determined to have hemorrhagic stroke since it may exacerbate the bleeding.

CT can diagnose about two-thirds of major stroke but falls short in diagnosing minor strokes (72-74). Additionally, superior imaging modalities are required to determine the area of vessel occlusion both pre and post-therapy. One of the commonly used modalities for these purposes is CT angiography (CTA) (75). CTA scans are more sensitive and requires intravenous injection of a contrast dye. Furthermore, 3D reorganizations of contrast-enhanced CTA reveal clear images of cerebral blood vessel occlusion (76). When a CTA reveals a vessel occlusion, the decision to administer the thrombolytic therapy can be made with more confidence, as administration of thrombolytic therapy will create increase bleeding in the case of a hemorrhagic stroke. The Moreover, because CTA can locate the specific segment of the vessel occluded with thrombi, it allows for more state-of-the-art therapies such as mechanical thrombectomy (77-80). In cases of hemorrhagic stroke, intracranial CT angiography can be used to determine the source of bleeding (81). Hence, CTA imaging during stroke prognosis is able to locate areas of vascular occlusion, as well as confirm intracranial hemorrhagic stroke.

Other neuroimaging modalities are used where CT or CTA are not adequate. Small-volume ischemic change, such as mini- or transient ischemic stroke, is just beyond the resolution of CT. Magnetic resonance imaging (MRI) has greater spatial resolution to detect brain ischemia in TIA or minor ischemic stroke (74). Although MRI has better sensitivity than CT and CTA in the mentioned ischemic stroke types, it is used in confirmatory assessments and when there is no time pressure to offer treatment.

A great portion of patients with major ischemic stroke arrive more than 4.5 hours after stroke onset and are therefore not eligible for thrombolytic therapy (82). The FDA has not approved rtPA use beyond 4.5 hours due to increased chances of secondary hemorrhaging and in some cases death. A critical imaging modality which expands the window of stroke treatment is perfusion CT (CTP). In CTP a contrast dye is administered to the stroke patient and sections of the brain are repeatedly imaged (83). Based on detected tissue blood flow after ischemic stroke, the technique recognizes potential areas of salvageable brain tissue around the ischemic core, termed the penumbra (83). If the penumbra is assessed as being salvageable in stroke patient, and within the time limits approved by the FDA, mechanical thrombectomy may be used to surgically remove thrombi and allow for reperfusion.
Using the precise imaging techniques are essential for the resolution and management of the acute stroke patient due to the time limitations for the few stroke therapies available. CT scan continues to be the initial choice for evaluating an acute stroke patient to rule out hemorrhagic strokes. This allows for continual administration of thrombolytic therapy. Additionally, CTAs allow the radiologist to detect the location of stenosis. If the CTA indicates an occlusion outside a three hour time from ischemic stroke onset, then endovascular thrombectomy may be a preferred treatment. Subsequently, CT perfusion informs the clinician of salvageable penumbra and thus expands the window of treatment to about 6-24hrs hours. Next, we consider the FDA approved therapies for stroke within these time windows.

**How is stroke treated?**

Unfortunately, as debilitating as stroke is and decades of research invested, there are very few modes of therapy available. While using the diagnostic tools outlined above are critical for indicating appropriate stroke treatment, time to treatment remains a critical consideration. From the onset of stroke, there are limited time windows for approved therapies of treatment by the FDA. This review discusses available stroke therapies approved by the FDA within appropriate time limits to prevent further injury to the patient. Although some hemorrhagic stroke therapies are discussed, the focus is to shed light on the limited therapies available for ischemic stroke

*Hemorrhagic stroke*

*Management therapy*

Immediate care is vital for a hemorrhagic stroke patient. After initial estimation of stroke and subsequent confirmation of the type of ICH stroke using neuroimaging, treatment focuses on management of other medical problems which may worsen stroke outcome. This commonly includes controlling the bleeding in the brain and decreasing the pressure caused by the bleeding. Drugs can be used to decrease the bleeding. But if you experience a hemorrhagic stroke while on blood thinners such as warfarin, you are at risk for excessive bleeding (84). Drugs to counteract the effect of the blood thinners are usually given during emergency treatment. For instance, intravenous vitamin K is administered to a patient who is on warfarin to gradually restore the clotting
ability of blood (85, 86). Other hemostatic therapies may treat platelet dysfunction, thrombocytopenia and heparin-related ICH.

Another preventative care performed during ICH is the reduction of blood pressure. Blood pressure is decreased to reach a systolic blood pressure below 160 mmHg and mean arterial pressure below 110 mmHg (87). Intravenous administration of hydralazine, labetalol, nicardipine, or nitrates is another route to regulate blood pressure (88). The principle element of reducing blood pressure is to reduce the hematoma.

*Surgical intervention*

Patients with SAH require emergency neurosurgery. The two popular options for treatment of a ruptured aneurysm are surgical clipping and endovascular coiling. In the latter, a catheter is passed through the groin up into the artery containing the aneurysm. Released platinum coils induce clotting of the aneurysm and by so doing prevent further bleeding. With surgical clipping, a small clip is placed across the neck of the aneurysm to occlude normal blood flow from flowing into the aneurysm. Treatment of a ruptured aneurysm reduces re-bleeding, and the benefit is related to the time when treatment is started (89, 90). The type of procedure depends on a range of characteristics including clinical status, medical comorbidities and age. Location of the aneurysm and size are also taken into consideration.

*Ischemic stroke*

*Intravenous thrombolytic therapy*

Recombinant tissue plasmin activator (rtPA) is the only FDA approved thrombolytic therapy for recanalization in acute ischemic stroke patients. It was first approved in 1996, after a robust study by the NINDS found that patients receiving rtPA within 3 h after the onset of clinical symptoms were significantly less disabled at 3 months post-stroke than those who received only a placebo (91). In 2008 another study conducted by the European Cooperative Acute Stroke Study (ECASS) indicated that the administration of the right dosage of alteplase, a rtPA, significantly improved clinical outcome in those patients who received this therapy between 3 and 4.5 h after onset of ischemic stroke symptoms (92). Although there are criteria difference between the US and Europe
for viable candidates, it is advised that patients receiving rtPA between 3 and 4.5 hours after onset should be (1) less than 80 years of age, (2) have no previous case of stroke, (3) should not be on an anti-vitamin K therapy, and (4) the patient should not have a pre-existing case of diabetic retinopathy. Following these guidelines prevents intracerebral hemorrhage (93). Thus, at the moment rtPA is approved by the FDA for treatment up 4.5 hours after onset of stroke. Unfortunately, in a study by Katzan and colleagues in 2004, they showed that only 15% of ischemic stroke patients make it to the hospital within 3 hours of onset, with only approximately 7% being eligible for rtPA (94). Since then initiatives by stroke organizations, such as FAST, have tried to educate the public about stroke symptoms. And although they have been somewhat successful, the qualifying candidates for rtPA have not increased much. Furthermore, rtPA is more effective for smaller vessel occlusions. Reperfusion of these vessels with rtPA reaches rates of between 40% and 80%, depending on the clot length (95, 96). As many as 75% of large vessel occlusions (LVO) remain occluded after tPA administration (95). Regrettably, LVO, which include occlusions of the carotid, basilar, and the middle cerebral artery are associated with the highest morbidity and mortality (97). The limitations of systemic thrombolysis for stroke, which include significant decrease in efficacy with prolonged ischemic time and risk of re-infarction and intracranial hemorrhage, encouraged development of other therapies such as endovascular mechanical thrombectomy.

**Mechanical thrombectomy**

A monumental hurdle in ischemic stroke therapy was treatment of patients, especially after 4.5 hours from the onset of symptoms. Moreover, a large number of patients are excluded as candidates for thrombolytic therapy due to risk of secondary hemorrhaging and in some cases death. Recently, clinical trials have shown the efficacy of mechanical devices for endovascular therapy. Patients with proximal intracranial occlusion are selected using CTA and therapy is carried out using these mechanical devices termed clot retrievers. Over the years, these clot retrievers have been improved to increase efficacy and safety.

The first trial using the Merci® retrieval system was published in 2005 and showed efficacy and safety of the first mechanical thrombectomy device (98). The first generation clot retriever achieved recanalization of large vessels in 48% percent of treated patients with 7% intracranial hemorrhage (98). Briefly, a cork screw structure is passed through a guide wire and catheter and deployed at the distal end of the occluding clot. Suction provided
by inflation of the catheter is used to pull the clot out of the vessel. Hence, the results established mechanical thrombectomy as the new opportunity for patients who were ineligible for thrombolytic therapy.

The second generation of mechanical thrombectomy, Penumbra®, uses a different mechanism in that the device is inserted just proximal of the clot instead of all the way through it. A small piston extends from the tip of the device to break up the clot into smaller pieces. At the same time a vacuum system is able suction the clot fragments. The Penumbra® System came out in 2008 and studies showed that it was able to obtain revascularization of large cerebral vessels in 81.6% of people (98, 99). Although more effective at revascularization, symptomatic hemorrhage was also greater at 11.2%. Also, a complication is re-infarction by clot fragments which are not vacuumed.

The third generation of these devices are called stent retrievers. Stent retrievers work similarly to the Merci® device. But in addition to the microcatheter being inserted through the clot, the device expands radially distal to, within, and proximal to the clot before the clot is mechanically pulled out. Thus, stent retrievers have the advantage of decreasing reinfarction by clot fragments and allows for retrieval the whole clot. Stent retrievers were the first to show both improved recanalization rates and improved outcomes since the Merci® trials (100, 101). One stent retriever, Solitaire®, had a recanalization rate of 61%. Even more impressive is the Trevo® stent retriever which had superior recanalization rates of 86% and better neurological outcomes at 90 days (100). Recently, the FDA approved Trevo® stent retriever to treat certain stroke patients up to 24 hours after symptom onset.

Time is essential in stroke treatment. And as much as the window for treatment has been extended over decades of research, there is a large patient population left with no treatment options for stroke. Thus, there is a compelling need to develop therapeutic approaches designed to decrease neurological deficits after ischemic stroke in the acute and hyper-acute phase of ischemia. A potential target for therapeutic intervention is the collateral vasculature of the brain, which already has underlying importance in all the therapies outlined. Collaterals are by-pass vessels which allow for retrograde reperfusion after occlusion of a blood vessel. The extent of collateral blood flow reperfusion has a major impact on the outcome of stroke, as it helps preserve the vulnerable penumbra (83). At hospital admittance, the severity of neurological deficits presented by patients depends on their collateral scores (102, 103). The more collaterals a patient has, revealed by CTA, the smaller the patients
infarct size. Additionally, several studies have demonstrated that the outcome after rtPA and thrombectomy therapy is highly dependent on patient collateral score (104-109). Patients with higher collateral scores have higher reperfusion rates after either rtPA or thrombectomy therapy. Finally of note, the risk for symptomatic bleeding after reperfusion therapy is significantly reduced in patients with more collaterals (110). Simply put, collateral score is a critical indicator of tissue protection of the penumbra and recovery. Hence, pharmacological acceleration of collateral remodeling may be a viable therapeutic options prior to and following endovascular therapies, to increase the window of treatment. Before deliberating possible pharmacological targets preclinically, we discuss the development of this unique vasculature.

**How does the pial collateral network develop?**

*General development of the cerebrovascular system*

During embryonic development, the brain forms from the ectoderm and is devoid of the capacity to generate endothelial cells. But the embryonic brain development depends on oxygen and nutrient delivery from blood vessels. Vascularization of the fetal brain begins when blood vessels from neighboring peri-neural vascular plexus (PNVP) ingresses the neural tube (111, 112). Prior to ingestion, the PNVP forms by a process called *vasculogenesis*, where angioblasts differentiate from the presomitic mesoderm to form blood vessels (113, 114). While blood vessels invade the CNS, the neural tube undergoes intricate developmental changes which gives rise to the medial and lateral surfaces of the neural tube. Vessels invade the neural tube on both sides of the floor plate, and then use cues delivered by the forming nervous system to migrate in specific directions. Recruitment of these vessels to the neural tube is by VEGF-A.

In the next step of vascularization, vessel sprouts from the PNVP grow into the neural tube pial surface to vascularize the hind- and forebrain. In the hindbrain, vessels grow radially towards the ventricular zone and turn laterally before reaching the ventricular surface (115-117). The laterally sprouting vessels then fuse with one another into a periventricular vessel network known as the subventricular vascular plexus (SVP). Sprouting of the PNVP is called *angiogenesis* and involves branching of the already existing blood vessels. Several signaling molecules are important for angiogenesis. VEGF and VEGF receptors activate intracellular signaling cascades in *endothelial cells* that drive their proliferation for vascular expansion. VEGF-A acts as a guidance cue for sprouting vessels by directing the filopodia that extend from the endothelial
‘tip cells’ to lead the vessel sprout into avascular areas. Subsequently, lateral sprouts emerge from radial vessels in deeper brain layers and then fuse into additional plexi. Vascularisation of the dorsal forebrain is not initiated by vessels originating from the PNVP, but via vascular spouting from the SVP in the ventral forebrain (118). Sprouts extend tangentially around the telencephalon to vascularize the dorsal regions.

During neural tube vascularisation, endothelial cells recruit mural cells called **pericytes**, which covers the blood vessel endothelium. The two cells do not make direct contact as they are separated by a basement membrane. Pericyte recruitment is critical and their loss leads to pediatric brain hemorrhage, due to endothelial cell apoptosis (119, 120). Subsequent specialization or interaction of the pericyte-ensheathed endothelial cell with **astrocytic end-feet** and their reciprocal signaling results in the formation of the **blood–brain barrier** (BBB). The BBB is beyond the scope of this review but is important for maintaining tissue homoeostasis by establishing selective permeability to fluid, molecules and cells between the blood stream and neural parenchyma.

The plexus however is inefficient to perfuse the growing brain. Primordia of arteries and veins appear when the endothelial cells from the primary plexus interact with un-differentiated mural cells. This begins the formation of an arterio-venous system capable of perfusing the developing brain. Recruitment of these perivascular mesenchymal cells, and development of blood vessel into a musculo-vascular phenotype is termed **arterialization** (121-124). Secreted angiopoietins by the perivascular cells bind to Tie2 receptors on the endothelial cells to stabilize the arterio-vascular vessel wall and promote vessel maturation. Later, the subcortical capillary plexus forms between the distal ends (‘tips’) of the arterial and venous vasculature, and is regulated by several molecular signals including the sonic hedgehog, VEGF-A, notch and eph/ephrin pathways (125-128). Of note is that ephrin-B2 is highly expressed in arteries while EphB4 is highly expressed in veins. The direct cell-to-cell contact of vein and artery endothelial cells via the eph/ephrin signaling leads to their fusion to form the capillary system.

**Development of the collateral network**

Collateral vessels form when the distal ends of arterioles join to form a continuous arteriole vessel. Collaterals are found in several tissue including skeletal muscles, heart, and brain. In humans, brain collaterals are found in the pia mater and form between the arterial branches in the brain. Very little is known about collateral development during embryogenesis as most of the information is based on animal models.
In mice, pial collaterogenesis is observed after the development of the cerebral artery trees between embryonic days 14 (E14) and 18 (E18) and undergo pruning to form a set number of collateral by from postnatal day (P)1 to P21 (129). Pial collateral formation is similar to capillary plexus formation. Initially, release of VEGF-A initiates the process of collateral formation or collaterogenesis (19), by sprouting and formation of microvessels larger than capillaries. Lucitti and colleagues further characterized this process by showing that endothelial tip cells extend filopodia toward areas of increased VEGF concentration by regulating notch/Dll4 signaling at the tip of the sprouting vessel (51). These extensions form a proto-collateral plexus followed by lumen formation and cross-connection of the opposing arterial trees. The continuous arteriole-to-arteriole in the brain are called pial leptominengial anastomoses or collaterals. Stabilization and maturation of newly formed collaterals occurs via PDGF-B expression and by differentiation of mesenchymal cells into pericytes and smooth muscle cells (SMC) (130, 131). In summary, VEGFR-2 and VEGF-A signaling initiates ligand Dll4 binding to Notch receptor. These events result in migration and anastomosis of endothelial tip cell structures of the most distal arterioles of the cerebral arterial tree. The collaterals undergo lumen formation and maturation when endothelial cells recruited mesenchymal cells are differentiated into pericytes and SMCs. Although the mechanism is not well known, we know that at the later stages of collateral development, post-birth, collaterals under pruning, where collaterals which are not being used are removed, thus reduces the collateral density. At this stages a person’s collateral number and density is set.

How do pial collateral remodel?

Importance of Collaterals

There are three main arterial trees in the brain, namely, the middle cerebral arteries (MCA), anterior cerebral arteries (ACA) and posterior cerebral arteries (PCA). By adulthood, these arterial vasculatures may anastomose to form the MCA – ACA, MCA – PCA, and ACA – PCA collateral vessels. Collateral arteries do not show a proximal-to-distal axis, with respect to the blood flow, as in large arteries. Instead, since the collateral connects two arterial branches with opposing flow direction, blood flow is bi-directional with along the vessel walls of the collateral vessel. In an event of vascular occlusion, as in ischemic stroke, collaterals undergo immense vascular
restructuring to allow for retrograde (uni-directional) blood reperfusion into the occluded arterial tree. For instance, when the MCA is occluded, the MCA – ACA collaterals restructure to allow retrograde reperfusion from the ACA into the MCA vasculature. The entire process by which these collaterals remodeling/restructure is called arteriogenesis.

Arteriogenesis is the outward growth of preexisting arterioles-to-arteriole collaterals into mature arteries capable of conducting large volumes of blood. An individual’s collateral extent, collateral number and ability to remodel, is imperative for tissue protection post-stroke (REF), as they help re-route blood to the blood-deprived regions of the brain. Collateral number vary among individuals, and may be regulated our different gene profiles. This is seen in different mouse strains where expression of higher VEGF-A expression correlates with increased number collateral formations. Clinically, stroke patients with a higher collateral number perform better after stroke as they tend to have better brain tissue protection (less neuronal cell death). Additionally, recanalization after administration of rtPA is higher in ischemic stroke patients with increased collaterals number. But even more essential than having increased collaterals is their ability to remodel and allow blood reperfusion and we tend to lose this ability as we age. Genetics of an individual, as well as age effects collateral remodeling. Studies in mice show that aging results in decrease of pial collateral diameter, as well as an increase in infarct volume after MCA occlusion (132, 133). This may explain why elderly stroke patients do not fare well, as preservation of the penumbra post-stroke may be less.

Pial collateral circulation is undoubtedly a critical determinant of ischemic stroke outcome and collateral therapeutics to enhance their response to large vessel occlusion has always been under investigation, since their discovery by Heubner in 1874. Since then several animal models, including mice, rats, guinea pigs, rabbits, and dogs, have been used to elucidate this remarkable process. Although, the signaling cascades which regulate arteriogenesis may not be always true compared human, these models have shed abundant information on the importance of collateral remodeling and cells which contribute to the process.

Cellular contribution during arteriogenesis

Arteriogenesis is a relatively rapid process compared to angiogenesis because it does not involve creation of new vessels. The remodeling of collaterals can be categorized into three main phases (134). In the first phase, endothelial cells are activated, leading to smooth muscle cell restriction and increase in vascular
permeability to recruited bone marrow-derived cells. With extracellular matrix breakdown, the vascular wall of the collateral becomes permeable and allows for migration of cell such as monocytes and other bone marrow cells. In the second phase the collateral artery matures via restructuring of smooth muscle cells into circular layers that establish cell-to-cell interactions with the other vascular cells. Later, pruning or termination of collateral arteries which are not being used for blood flow support marks the third and final phase. The less used collaterals are occluded with intimal proliferation over the course of several days.

Several cells are involved in the process of arteriogenesis. Next we discuss key arteriogenic cells which have been elucidated. These include endothelial, bone marrow-derived, and vascular smooth muscle cells.

**Endothelial cells**

*Endothelial cells (ECs)* lining the native collateral vessel are exposed to extremely low blood flow and shear stress under normal conditions (135). After vascular occlusion, the vasculature downstream of the occlusion experiences a drop in pressure. This distal drop in pressure creates a sharp pressure gradient that drives blood flow uni-directionally through collaterals to by-pass the occlusion. But due to the increased length and reduced radius of the collateral, the resistance to flow is much greater. The viscous force of increased blood flow is called **fluid shear stress** (FSS).

ECs of the collateral are important for the interaction with immune cells during the initial phase of arteriogenesis. First, mechanoreceptors on ECs detect FSS and cause them to be activated. Activated ECs express monocyte chemoattractant protein 1 (MCP-1) cytokine which recruits monocytes to the site of injury (136). Collateral remodeling is precipitously reduced in MCP-1-deficient mice due to aberrant monocyte recruitment (137). In addition, activated ECs express adhesion molecules such as vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) which promotes immune cell recruitment and attachment to the collateral endothelium (12, 138). Recruited monocytes initially secret cytokines which signal the recruitment of these immune cells from the bone marrow. Thus the ECs activation marks the initiation of collateral remodeling and the endothelium serves as a docking site for bone marrow cells critical for arteriogenesis.

**Bone marrow-derived cells**

In addition to endothelial activation, recruitment and accumulation of the immune cells is immensely important in the first phase of the collateral remodeling. Bone marrow-derived cells (BMDCs) were implicated in
arteriogenesis as early as 1976 when electron microscopy revealed monocytes in the endothelium of collaterals (139). In further studies of arteriogenesis, depletion of monocytes (by injection of 5-fluorouracil or bisphosphonate-containing liposomes) impaired blood flow recovery, while their inoculation increased arteriogenesis (11, 12, 140). Since monocytes were implicated and substantiated as an integral component in arteriogenesis, a variety of other BMDCs have also been shown or suggested to mediate arterial remodeling.

Another group of BMDCs which have been exhaustively researched is leukocytes, as there seems to be a complex interrelation between leukocyte recruitment and progression of the arteriogenesis. During the initiation phase, various adhesion molecules and chemokines, briefly discussed earlier, are upregulated in the ischemic niche. Neutrophils are one of the first leukocytes to respond to these signals within hours but also fade shortly after (141, 142). As a pro-inflammatory cell, neutrophils aid in clearing up dead cells but are also important in signaling other immune cells to the site of injury. To this, Soehnlein and colleagues demonstrated that neutrophil recruitment and secretion of their cytokines, aid in the recruitment of inflammatory monocytes (143). Neutrophils secret granulocyte colony-stimulating factor which can stimulate the recruitment of these non-resident monocytes.

Temporally, current research point to neutrophils as one of the first BMDCs to arrive in the ischemic niche and are able to stimulate the recruitment of monocytes and other lymphoid cells. Lymphoid cells such as natural killer cells and T cells have all been implicated in arteriogenesis (144). Research shows that both cells play roles tissue clearance and matrix degradation to allow for outward remodeling of collaterals, as well as in the recruitment of other inflammatory. Thus leukocytes are important post-initiation, where they are serve to clear dead tissue and extracellular matrix. Furthermore, stimulatory products that they secret signals the recruitment of other cells to milieu.

*Vascular smooth muscle cells*
Vascular smooth muscle cells (vSMC) are located in the media of the vascular wall. Their contractile ability leads to regulation of vessel volume and blood pressure. But getting to end of the first phase of arteriogenesis, vSMC switch their morphology from a contractile to a synthetic phenotype with the downregulation of contractile markers such as a-actin, calponin, and vinculin (145). This phenotypic change is partly due to extracellular matrix degradation by matrix metalloproteinase 2 (MMP-2) and MMP-9 (146) and is necessary for permeation of BMCs
during the second phase. vSMC proliferation and migration in the intimal and media layers ultimately leads to the outgrowth of collaterals during the second phase of arteriogenesis.

The termination phase, as mentioned, leads to stabilization of the remodeled vessel, and also pruning of collaterals which are not facilitating blood reperfusion. Complete occlusion of the vascular intima by vSMC proliferation is observed. This process is called pruning and results in a reduction in the number of small collateral vessels in favor of a large remodeled ones (139). Thus, smooth muscle cells are involved in the proliferation, maturation, and termination of the collateral remodeling post ischemia.

Current clinical methods of collateral remodeling and novel Pre-clinical indications

Animal models and clinical data of stroke patients demonstrate the importance of collaterals during acute ischemic stroke in determining stroke outcome and response to recanalization therapy. Moreover, the ability of the remodeled or remodeling collateral to rapidly supply blood to the penumbra after ischemic stroke preserves tissue and is key in allowing for thrombectomy therapy in some cases. Taking together, the benefits of therapy targeting collateral remodeling post-stroke are undisputable and will significantly improve stroke outcome. Although studies in animal models have shown promise, collateral therapy has shown little promise in the preclinical and clinical stages. We briefly review two of these therapies which have been clinically studied.

Nitric oxide

Statins have been shown to inhibit cholesterol synthesis. But their relevance as a stroke therapeutic is due to another mechanism of work; statins mediate nitric oxide (NO) synthase production of endothelial cells. Nitric oxide (NO) is closely involved both in normal physiological functions as well as in various disorders involving blood flow regulation, as it plays a role in blood vessel dilation. In addition to their role in physiological function in blood flow, NO has been shown to play role in collateral remodeling. In a study where NO knockout mice were compared to wild type mice, the results showed that EC NO was important for native collateral density maintenance, as well as collateral remodeling post-vascular occlusion. So how can NO be targeted as a therapeutic after stroke. The clinical study by Obviagele and colleagues showed that statin administration pre-stroke improves collateral circulation (147). In a recent study by Lee and colleagues showed that premorbid use of statin in atrial fibrillation-related stroke patients is associated with excellent collateral flow (148). It should be
noted that stroke related patients were administered NO-activated drugs pre-stroke. Although these clinical studies were not performed post stroke, they show clinical relevance of NO in augmenting collateral remodeling.

**Hypertension**

High blood pressure of hypertension is normally not a positive physiological state. But recently it has been associated with potential improvements in stroke. Studies in mice where mild hypertension was induced after ischemic stroke showed improved pial collateral flow (149). The mechanism of action relies on the fact that increased cerebral blood flow increases oxygen metabolism in the ischemic core and penumbra, leading to significant reduction in infarct volume. Clinically, several reports show the benefits of mild hypertension (induced with phenylephrine) after ischemic stroke (150, 151). These studies suggested that mild hypertensive therapy is feasible and relatively safe. But this therapy is not a suitable route for many ischemic patients. The American Heart Association does suggests that patients in the very acute period of stroke may be the only candidates for therapy (152).

**Conclusion**

Stroke is a debilitating disease of which the predominant type is ischemic stroke. Ischemic strokes are treated with either intravenous thrombolysis or endovascular therapy. But the time window for such treatments is small leaving many stroke patients untreated and with lifelong neurological deficits. Exhaustive reviewed literature in this review shows collaterals vessels are critical in recovery after stroke. Patients with a higher collateral extent have better recanalization rates and these remarkable vessel, are able to prolong blood flow into the ischemic penumbra, thus increasing the window of treatment a patient has for tissue-saving therapy such as endovascular thrombectomy. All this points to the fact that collaterals are undoubtedly at the core of ischemic stroke recovery and rigorous research is needed to elucidate new therapeutic targets for their augmentation post-stroke.

Clinical trials thus far have not been successful. But what is interesting is that most of collateral targeting therapies have an effect on the ECs of the collateral. Statins induce ECs to secret NO which effect collateral remodeling. Hypertension increases blood flow which in addition to subsequently increasing oxygen in the brain, may induce shear stress that can be detected by ECs for arteriogenesis initiation; the ECs lining the collateral vessel are first to detect drops in blood flow, through their mechanoreceptors. Therefore, therapies targeting the
collateral system maybe the most viable way of clinically augmenting collateral remodeling. Another reason why EC should be targeted is the fact that arteriogenesis depends on multiple cells and factors they release to coordinate the process. So for instance, targeting another cell such as SMC may not be adequate, since the process still requires immune cells such as neutrophils and monocytes to clear debris and extracellular matrices to allow for the growing collateral.

Chapter 3

*This chapter is published as a manuscript in the PLOS One Journal*

*Endothelial-specific EphA4 negatively regulates native pial collateral formation and re-perfusion following hindlimb ischemia*
Abstract

Leptomeningeal anastomoses play a critical role in regulating vascular re-perfusion following obstruction, however, the mechanisms regulating their development remains under investigation. Our current findings indicate that EphA4 receptor is a novel negative regulator of collaterogenesis. We demonstrate that EphA4 is highly expressed on pial arteriole collaterals at post-natal day (P) 1 and 7, then significantly reduced by P21. Endothelial cell (EC)-specific loss of EphA4, EphA4^f/f/Tie2::Cre (KO), resulted in an increase in the density but not diameter of pial collaterals compared to WT mice. ECs isolated from KO mice displayed a 3-fold increase in proliferation, enhanced migration, tube formation and elevated levels of phospho(p)-Akt compared to WT ECs. Attenuating p-Akt, using LY294002, reduced the proliferative and migration effects in the KO ECs. RNAseq analysis also revealed altered expression patterns for genes that regulate cell proliferation, vascular development, extracellular matrix and immune-mediate responses, namely MCP-1, MMP2 and angiopoietin-1. Lastly, we show that induction of hindlimb ischemia resulted in accelerated re-perfusion, collateral remodeling and reduced tissue necrosis in the absence of EC-specific EphA4 compared to WT mice. These findings demonstrate a novel role for EphA4 in the early development of the pial collateral network and suggests a role in regulating vascular remodeling after obstruction.
**Introduction**

Leptomeningeal anastomoses, first described in 1874 by Heubner (153), are arteriole-to-arteriole anastomoses in the pia mater that connect the anterior (ACA), middle (MCA) or posterior (PCA) cerebral artery branches on both dorsal hemispheres. This naturally occurring adaptation can help restore blood flow to vascular territories downstream of an occluded artery by providing retrograde perfusion. Leptomeningeal anastomoses constitute an important ‘by-pass’ system that provides an alternate route for oxygen, nutrients and potential therapeutic agents. Clinically, patients having greater numbers of pre-existing collaterals in skeletal muscle, heart and brain recover better following vascular occlusion (154-160). Similarly, collateral density varies widely amongst different strains of mice and dictates their outcome from vascular occlusion (18, 21, 135, 161). While an extensive, functional collateral network is inextricably linked to neuroprotection and is an undisputed target for therapeutic intervention, the mechanism(s) underlying native collateral formation remains poorly understood.

Collateral vessel formation arises through a series of orchestrated events. Recently it has been described that pial collaterals begin to form at embryonic day 15.5 (E15.5) in mice, after the MCA has extended across the cerebral cortex (162). This occurs after the formation of the primary vascular plexus where endothelial cells are mainly dividing, branching and migrating along astrocytes. Interestingly, astrocytes provide the main cues for endothelial cell guidance (163) during this time, similar to that in retinal vascular development (164-166). The establishment of the pial collateral network peaks at E18.5 to post-natal day 1, followed by significant post-natal pruning which is complete by P21 (162). The pial collateral density, which is set during embryonic development, is strain specific and these strain differences are maintained into adulthood (162). Furthermore, embryonic perturbation of collateral formation results in life-long changes in collateral density (51). These findings indicate that genetic factors largely dictate collateral establishment and that pre-natal exposures may influence their development. Although the genetic polymorphisms which create strain-specific variations in collateral numbers are unknown, vascular endothelial growth factor (VEGF), A disintegrin and metalloprotease Family Members (ADAM) 10 and 17 as well as chloride intracellular channel (Clic4) have been shown to regulate collateral density and diameter during development as well as remodeling after ischemia (19-21, 51).

Our current findings indicate that EphA4 receptor is a novel negative regulator of pial arteriole collateral formation in the brain. Eph receptors are widely known to control cell migration, proliferation, boundary formation
and repulsive/attractive cues (34, 167-170). Within the CNS, they regulate axonal guidance and fasciculation, neural crest migration, midline development, and synaptic plasticity (25-31). Eph receptors are the largest known family of transmembrane receptor tyrosine kinases which contain 14 members (25, 171) and are divided into two distinct subfamilies, EphA and EphB, both of which bind to membrane-anchored ephrin ligands and require cell-cell interaction. The EphA receptors bind to ephrin-A ligands that are anchored to the membrane by a glycosylphosphatidylinositol linkage, while the EphB receptors interact with three related transmembrane-spanning ephrin-B ligands (169). There is some promiscuity between subclasses, such as EphA4, which has been shown to bind with high affinity to both the A-class and B-class ephrins. This broad overlap of binding specificities within these subclasses suggests the possibility of compensatory activity and redundancy among family members. The involvement of Eph signaling in tumor and vascular development is well established for several family members (37). For example, ephrinB2 and EphB4 knockouts show defects in arterio-venous patterning (32) and the use of soluble recombinant ephrinB2 or EphB4 proteins can affect migration, adhesion and proliferation of cultured ECs and tumor angiogenesis (33-36). In addition, both ephrinB1 and ephrinA1 ligands have been shown to positively regulate angiogenesis (172, 173). Morphological differences in the vasculature of the spinal cord and hippocampus have also been recently described in EphA4- and ephrinA5-deficient mice, respectively (170, 174). Although ephrin/Eph interactions have been demonstrated during neural, cardiac, retinal and cancer development, few studies have addressed their role in cerebral arteriole development and whether this may influence tissue outcome after vascular obstruction.

The current study addresses the role of EphA4 in pial collateral formation using a cell-specific gene targeted approach. We find that endothelial cell (EC)-specific ablation of EphA4 significantly enhances the presence of leptomeningeal anastomoses which reflects an increase in the proliferation and migration of EphA4-null cultured ECs. We also show that p-AKT mediates these effects and is negatively regulated by EphA4 resulting in suppression of proliferation, migration and expression of key vascular proteins. Assessment of blood flow, collateral growth/remodeling and tissue necrosis following hindlimb ischemia also reveals substantial improvements in perfusion, collateral enlargement and appearance scoring in EC-specific EphA4 knockout mice. These findings indicate that EphA4 negatively regulates pial collateral formation in a cell-specific fashion and plays a key role in the peripheral vascular response to obstruction.
Results

Post-natal expression of EphA4 on pial arteriole collateral vessels

Previous studies have shown EphA4 to be expressed on platelet endothelial cell adhesion molecule-1 (PECAM/CD31)-positive cells in the cortex at E16, down regulated in adulthood and upregulated on astrocytes and ECs after CNS injury (170). To investigate the role of EphA4 in arteriole collateral formation we first assessed its expression on pial arterioles using vessel painting (VP), which selectively labels the adult arteriole vasculature, following transcardial Dioctadecyl-Tetramethylindocarbocyan (Dil) infusion (52). We modified this technique in order to visualize and assess post-natal (P1-P21) pial arteriole development. Post-natal pial collateral vessels are strongly labeled following VP (Fig 1A and 1B) whereas the associated smooth muscle cells, identified by smooth muscle actin (SMA), do not co-label with the Dil stain (Fig 1A, 1C and 1D; high mag confocal image). Using immunofluorescence labeling and confocal image analysis on whole mount cortical tissue from post-natal day 1, 7 and 21, we found EphA4 to be highly expressed on pial arteriole collaterals at P1 (Fig 1E-1G) and P7 (Fig 1H-1J) then reduced by P21 (Fig 1K-1M). Quantified expression analysis was performed at P1-P21 (n=3/group) for EphA4 using immunohistochemistry and densitometry which demonstrated a significant reduction in EphA4 immunoreactivity on VP-labeled pial collaterals (Fig 1N). This is the first demonstration of Eph/ephrin expression on the pial cerebral arteriole network.

Endothelial-specific ablation of EphA4 increases the presence of pial arteriole collateral vessels

Due to the widely expressed and complex nature of EphA4 in CNS development and injury-induced responses (170, 175, 176), we utilized a cell-specific approach to investigate its role in pial collateral development. We generated conditional knockout mice using the loxP/Cre system driven by the Tie2 promotor, which has been shown to be selectively expressed in endothelial cells (EC) (177). To demonstrate EC specificity, we bred Tie2::Cre mice with Rosa\textsuperscript{mTmG} double-reporter mice which expresses tdTomato (mT; Rosa\textsuperscript{mTmG}) prior to Cre-mediated excision (Fig 2A and 2B) and GFP (mG; Tie2::Cre/Rosa\textsuperscript{mTmG}) after excision (Fig 2C and 2D) (178). Coronal sections from Tie2::Cre/Rosa\textsuperscript{mTmG} mice were subjected to confocal microscopy in order to visualize exclusive GFP expression in Tie2::Cre-positive vessels in the pial surface (Fig 2F; white arrow), surrounded by tdTomato-positive support cells (Fig 2F; yellow arrow). Next, we generated EphA4 EC-specific,
Tie2::Cre/EphA4\textsuperscript{f/f} knockout mice (KO) and EphA4\textsuperscript{f/f} wild type mice (WT) (47), which carry the homozygous floxed alleles and Cre recombinase or floxed allele only, respectively (Fig 2G). Adult WT and KO mice were then analyzed for the presence of pial collaterals using vessel painting. In the absence of EC-specific EphA4, we find greater numbers of pial collaterals between the middle cerebral artery (MCA) and anterior cerebral artery (ACA) branches (Fig 2I compared to 2H). WT and KO ECs were then isolated and cultured from whole pup brain tissue of EphA4\textsuperscript{f/f} and Tie2::Cre/Rosa\textsuperscript{mTmG} mice using CD31 microbeads. Both WT and KO brain-derived ECs express CD31 (179) (Fig 2J and 2K, respectively) while mRNA transcripts for EphA4 are present in WT and absent in KO ECs (Fig 2L). These findings demonstrate that EC-specific ablation of EphA4 enhances the density of pial arterioles in the adult murine brain.

**Post-natal time course of pial arteriole collateral formation following EC-specific EphA4 ablation**

Recent studies have shown that pial collaterals are initially formed and peak during pre-natal development (E14-birth,) after which time there is a significant post-natal pruning process (P1-P21) followed by maturation of the collaterals (P21-adult) (162). To determine whether EphA4 limits collateral development by regulating these discrete phases, we modified the vessel painting technique to stain for post-natal pial collaterals at P1, P7 and P21. Analysis of the total number of collaterals show that KO mice (Fig 3A, 3G and 3H) display a significantly higher number of vessels at P1 (118.8 ± 14.6 vs. 72 ± 2.9), P7 (88 ± 5.1 vs. 56.4 ± 4.0), P21 (77.4 ± 3.9 vs. 44.0 ± 3.4) and adult (57.6 ± 1.4 vs. 42.6 ± 1.5) stages compared to WT mice (Fig 3A, 3E and 3F). No differences were seen in the tortuosity index (Fig 3B) or diameter (Fig 3C) of the collaterals during the post-natal and maturation stages. Smooth muscle cell coverage was also not affected on pial collateral vessels following EphA4 ablation at P1-P21 as observed using whole mount anti-SMA staining (data not shown). Interestingly, the extent of post-natal pruning is similar between WT and KO suggesting that pre-natal expression of endothelial cell (EC)-specific EphA4 plays a critical role in suppressing initial pial collateral formation.

**EphA4 limits EC proliferation, migration and tube formation via PI3K/Akt suppression**

Endothelial cell proliferation during this time has been shown to be critical in establishing the pial collateral network (19, 162). EphA4 may therefore suppress EC expansion to regulate pre-natal collateral formation. To
examine this possibility, we analyzed proliferation, migration and tube formation of cultured ECs derived from the post-natal day 1 brains of EphA4^{flox/flox}/Tie2::Cre (KO) and EphA4^{flox/flox} (WT) mice. Using bromodeoxyuridine (BrdU)-labeling, we found a significant increase in the percentage of BrdU-positive KO-ECs (30.95 ± 3.53; Fig 4B, 4B1, C) after 24 hrs of culture, compared to WT-ECs (4.9 ± 1.02%; Fig 4A, 4A1 and 4C). Next, we assessed migration using an *in vitro* wound closure or scratch assay (180). Under growth factor-free conditions, KO ECs display an increase rate of wound closure (0.8 ± 0.05 relative to post-scratch) (Fig 4D1 and 4F) compared to WT ECs (0.45 ± 0.04 relative to post-scratch) (Fig 4E1 and 4F) at 24 hrs post-scratch. Upon plating onto matrigel substrate, ECs attach and degrade the surrounding extracellular matrix to create guidance pathways that facilitate migration and tube formation (48). In the absence of EphA4, ECs display an increase in vascular index (number of tubes x tube length) during tube formation (Fig 4H and 4I) compared to WT ECs (Fig 4G and 4I) at 8 and 24 hrs after plating. KO ECs also displayed greater numbers of cells that appeared to sprout from the original formed tubes, which usually became apparent at 24 hours after plating (Fig 4 H1). These findings demonstrate that EphA4 plays a central role in suppressing the expansion and migration of brain-derived endothelial cells, which may influence the extent of collateral formation during critical periods of development.

Next we sought to identify key downstream mediators of EphA4 signaling in ECs. We isolated RNA from WT and KO ECs then assessed differences in global transcript levels using RNAseq analysis. Genome-wide differential expression analysis via RNA-sequencing showed significant changes in gene expression for EphA4-null ECs. Gene ontology analysis of significantly upregulated genes (≥2-fold expression change, p value ≤0.01) using DAVID found that knockout cells show increased expression of genes with products residing in the extracellular region as well as upregulation of genes involved in the immune response, cell proliferation and adhesion, and vascular development (Fig 5A). Overall ontological analysis of both upregulated and downregulated genes also found that knockout cells display significantly altered expression patterns for genes relevant to the extracellular matrix, immune response, cell proliferation, and vascular development (Fig 5B-5E). Based on the results of gene ontology analysis, differential expression of seven genes significant in vascular development was further assessed using qRT-PCR (Fig 5F). This assessment confirmed significant upregulation of Ang1, MCP1 and MMP2 downregulation of Ang2, as previously indicated by sequencing data.
We further assessed the role of the PI3K/Akt pathway in mediating EphA4 effects in cultured ECs. Protein lysates were collected from WT and KO ECs following 24-hour vehicle or PI3K inhibitor LY294002 treatment, then analyzed for phospo-Akt (p-Akt) by WesternBlot. P-Akt was significantly up-regulated in vehicle treated KO ECs (Fig 6A and 6B) compared to WT, which correlated with increased proliferation and migration. LY294002 significantly reduced p-Akt expression levels in KO ECs. Inhibition of p-AKT resulted in attenuation of proliferation (Fig 6C) and migration during scratch wound closure and a trend towards reduced tube formation (Fig 6D and 6E, respectively) in KO compared to WT ECs. These data indicate that EphA4 suppresses p-AKT which limits proliferation and migration of endothelial cells.

**EC ablation of EphA4 increases hindlimb collateral circulation and remodeling after femoral artery occlusion**

The extent of preexisting collaterals and the capacity of these vessels to remodel (enlarge) are major determinants of tissue injury following obstruction. The variation in collateral density within the hindlimb circulation as well as in vascular branching in the retina of different mouse strains has been shown to be consistent with that of the pial network (20, 135, 161, 181). Therefore, to address whether the density and/or remodeling of the collateral circulation in EC-specific EphA4 knockout mice correlates with tissue perfusion and ischemic outcome, we utilized the hindlimb ischemia model by performing femoral artery ligation (FAL) in adult WT and KO mice. We measured recovery of hindlimb plantar perfusion up to 7 days post-FAL using high-resolution laser Doppler imaging and performed necrosis scoring (number of necrotic toes). Plantar perfusion (index of leg perfusion) was reduced more immediately after FAL in WT mice compared to KO mice and recovered faster in KO at 3 and 7 days, although statistical significance was not reached until day 7 (Fig 7A and Fig 7B). Toe necrosis was also significantly reduced in KO compared to WT mice at 7 days post-FAL (Fig 7C) which correlates with greater re-perfusion. Serial sections of the injured and un-injured adductor muscles were stained for CD31 and the number and diameter analyzed. The adductor muscle from KO mice display greater numbers of arterioles both in the injured and un-injured tissue (Fig 7D) compared to WT mice. In addition, their size is significantly increased in the injured vs. un-injured limb (Fig 7I and 7M vs 7H and 7L, respectively) compared to WT (Fig 7G and 7K vs 7F and 7J, respectively) suggesting restoration of blood flow and reduced
tissue necrosis may result from greater collateral density and diameter following FAL. The capillary density in the adductor muscle was not significantly different between the two groups (data not shown). The density and diameter of pre-existing collaterals in the adductor thigh dictates acute plantar perfusion immediately following FAL (21, 135, 161) whereas outward remodeling of their lumen diameter (i.e., arteriogenesis) governs recovery of perfusion days to weeks post-occlusion (135, 182). Thus, our findings indicate that plantar perfusion and tissue recovery is negatively regulated by EC-specific EphA4.

Discussion

Leptomeningeal anastomoses represent a prominent structural feature in the pial surface of the brain. Although inactive under normal physiological conditions, shear stress following vascular occlusion induces collateral outward growth and remodeling which provides an alternate route for blood flow and preserves tissue function. This important adaptive response varies among individuals and is density dependent. Differences in the abundance of native pial collaterals also exists between different strains of mice, which is attributed to natural polymorphisms and is age-dependent (18, 21, 132, 133, 135, 181, 183). An expansive pre-existing collateral network is deemed favorable for patient outcome following vascular occlusion (105, 184), however, the key players involved in the development of this network remain unclear. Using a cell-specific transgenic approach, our current findings demonstrate that EphA4 ablation on endothelial cells increases the number of pial arteriole collaterals. This effect was prominent as early as post-natal day 1, indicating that EphA4 limits their pre-natal formation which peaks at embryonic day 18-P1(162). Post-natal pruning, however, was unaltered in the EphA4^flox/flox/Tie2::Cre (KO) compared to EphA4^flox/flox (WT) mice which further suggests that EphA4 negatively regulates an early embryonic event resulting in robust collateral formation upon EC-specific deletion. Smooth muscle cells (SMC) are also an integral part of collateral function (138, 185-188). Although we did not observe any difference in SMC coverage in the post-natal collateral, we cannot rule out any indirect effects of EC ablation of EphA4 on SMCs. Given that endothelial-to-smooth muscle cell junctions in collateral vessels have never been identified (5, 189), as they are separated by internal elastic lamina, we do not believe they play a major role during pre-natal collaterogenesis in the absence of EphA4.
Increased EC proliferation during primary vascular plexus formation has been attributed to an increase in collateral number (51). Our findings indicate that EphA4 significantly limits EC division by suppressing phosphorylation (p) of Akt. Attenuation of p-Akt using LY29004 in KO ECs reversed the proliferative effects. Likewise, EC migration was also restricted by EphA4 following scratch injury and during vascular tube induction. Preventing p-Akt attenuated the migration of KO ECs to WT levels during wound closure and tube formation. Upon plating onto matrigel substrate, ECs attach and degrade the surrounding extracellular matrix to create guidance pathways that facilitate migration and tube formation (48). Our data suggests that EphA4 restricts key players in this process, namely, PI3K/Akt, angipoeitin-1, MCP-1 and MMP2 (190, 191). Of note, the PI3K/Akt pathway has been shown to stimulate EC proliferation, migration and tube formation downstream of VEGFR (192-194) and angipoeitin signaling (191, 195-198), known mediators of collateral formation and remodeling (199, 200), respectively. Previous findings have demonstrated that Eph receptor signaling directly suppresses p-Akt upon ligand stimulation (201). EC-specific p-Akt may represent a major determinant in native collateral formation by balancing the signals between negative EphA4 and positive Flk-1 signaling (19, 21). While we show EphA4 does not directly regulate expression of VEGF or VEGFR1/R2, EphA4 may limit the known functions of VEGFR signaling indirectly by limiting downstream Akt phosphorylation to suppress proliferation, migration and vascular morphogenesis. Indeed, paracrine VEGF-stimulation of Flk-1-notch signaling on ECs was shown to positively regulate embryonic collaterogenesis (51). Adam 10 and Adam 17 were also implicated in this process. Our findings did not reveal a difference in expression of either Adam 10 or 17 suggesting EphA4 may regulate collateral formation though a novel pathway involving Akt. Interestingly, while angiopoietin-1 appears to play a critical role in regulating vessel branching during development (202, 203) as well as in stimulating remodeling (23, 204-206) and cytokine and chemokine induction, including MMPs, MCP-1, CXCL-5, -10, -9 and MMP-2, -9 (207-210), its role in collaterogenesis has not been established. Recently, using the Cre-lox system, angiopoietin-1’s role in regulating the vascular response to injury was confirmed (202). Given the embryonic lethality of germ-line deletion, floxed angiopoeitin-1 mice may be useful for future studies investigating its potential cell-specific role in pial collateral formation.

The density and diameter of pre-existing collaterals in adult tissue, as well as their ability to enlarge (remodel) following arterial occlusion dictates the severity of ischemic injury. While we have elucidated a novel
mechanism regulating collateral formation, it is unclear whether EphA4 is a key player in adult collateral remodeling post-obstruction (arteriogenesis). Using a well-established model of hindlimb ischemia, we find significant recovery of hindlimb blood flow within a week following artery ligation which correlated with reduced toe necrosis in EC-specific EphA4 knockout mice. Unlike sprouting angiogenesis, which is incapable of fully restoring the function of larger damaged vessels (211), arteriogenesis or outward growth of pre-existing collaterals (212-214) can result in an expeditious return of blood flow. They also carry ten times more blood volume compared to capillaries and therefore have a much greater capacity to prevent tissue loss. The quick return of plantar perfusion observed in the absence of EC-specific EphA4 after hindlimb ischemia suggests that arteriole remodeling may also be negatively regulated by EphA4. Currently it is unclear what major role EphA4 may regulate in this process, however, on-going studies are elucidating its role in several aspects of the remodeling process. Initiation of arteriogenesis occurs due to changes in fluid shear stress which activates the endothelium leading to recruitment and proliferation of vascular and peri-vascular cell types as well as reorganization of the extracellular matrix, all of which are coordinated through a temporal pattern of cytokine, chemokine, growth factor, and protease expression. The EC is the first to respond by sensing this change through mechano-transduction leading to transcription of downstream gene targets necessary for growth and remodeling (215-219). It is possible that EC-specific EphA4 could regulate some aspects of the mechano-transduction pathway leading to modulation of EC proliferation and immune cell recruitment. Future studies will address this important process.

The current investigation highlights a novel suppressive pathway involved in collateral formation during development, which focuses on the cell autonomous role of EphA4 using EC-specific knockout mice. EphA4 limits the proliferation and migration of ECs via p-Akt regulation, which may play a substantial role in pre-natal collateral formation. EphA4 also negatively regulates EC expression of angiopoietin-1, MCP-1 and MMP2 which may have a profound effect on collateral density during development and/or remodeling following ischemia. Future studies will evaluate the role of EphA4 on collateral remodeling in the adult brain following occlusion and the specific mechanism(s) driving the remodeling events using our EC-specific approach.
Materials and Methods

Animals

All mice were generated and housed in an AAALAC approved, virus/antigen-free facility with a 12 h light-dark cycle; food and water *ad libitum*. We employed a recently generated mouse strain carrying a LoxP-flanked (floxed) EphA4 gene (EphA4\textsubscript{floxed/flox}) (Jackson Labs, Epha4\textsubscript{tm1.1Bzh/J}) (47). EphA4\textsubscript{floxed/flox} mice were bred to transgenic mice expressing Cre recombinase under the direction of the tyrosine kinase Tek (Tie2) promoter/enhancer (Tie2::Cre\textsuperscript{gr+/}x) (Jackson Labs, B6.Cg-Tg(Tek-cre)12Flv/J), which is expressed in ECs during embryogenesis and adulthood (220). EphA4\textsubscript{floxed/flox}/Tie2::Cre male mice were bred to EphA4\textsubscript{floxed/flox} female mice to produce EphA4\textsubscript{floxed/flox} (WT) and EphA4\textsubscript{floxed/flox}/Tie2::Cre (KO) littermate pups. We confirmed that Cre activity was isolated to the vascular network by breeding the Tie2::Cre mice with double reporter labeled Rosa\textsuperscript{nTmG} mice (Jackson Lab) (178). DNA isolation from tail samples were performed using 25mM NaOH incubation at 98°C for 1 hour, 15°C for 20 minutes then neutralized using 40 mM Tris HCL (pH 5.5). Genotyping of DNA was performed using polymerase chain reaction analysis and the following primers (1 m): 5’-TGC TAA CAG GCA CTT AGA TCC C-3’ and 5’-TAA TTG TAA TCA GTG GGC GGG C-3’ to amplify floxed alleles; 5’- GCG GTC TGG CAG TAA AAA CTA TC-3’ and 5’- GTG AAA CAG CAT TGC TGT CAC TT-3’ to amplify Cre. The respective product sizes were 290 bp and 100 bp; DNA was amplified for 35 cycles (94°C for 1 minute, 51.7°C for 45s, 72°C for 1 minute) in a thermal cycler. Experiments comply with the ARRIVE guidelines for animal experimentation. Procedures related to animal use and care for this specific study was approved by the Virginia Tech Animal Use and Care Committee IACUC (protocols 15-063 and 14-044).

Hindlimb Ischemia

Unilateral femoral artery and vein excision was performed by an investigator blinded to the experimental groups of 2-4 month old female WT or KO mice (5-8 mice/group), as described above. Inhalation anesthesia was administered using 1.5-2% isoflurane. Several drops of Bupivacaine (0.5%) were given near the incision prior to surgery. The state of anesthesia (lack of response to firm toe pinch, no body or whisker movement), rectal temperature (read-out from rectal monitor), breathing and coloration was monitored throughout the entire procedure. Animals were kept on a heating pad (homeothermic blanket system; Harvard Apparatus) and rectal temperature was monitored and recorded throughout the surgery. Body temperature was maintained at 37°C.
Buprenorphine (0.1 mg/kg) was administer immediately following and for 2-days post-op. After dissection of the artery and vein from the nerve, ischemia was induced by twice electrocoagulation of the left femoral artery and vein, proximal to the superficial epigastric artery and vein and distal to sapheno-popliteal bifurcation. The artery was separated 1-2 mm between the cauterized ends at the end of the surgical procedure. Tissue outcome or appearance scoring was quantified and vascular perfusion was measured using non-invasive laser Doppler perfusion imaging prior to and immediately following surgery as well as at 3 and 7 days post-ischemia. No mice died during or following surgery nor were there any mice excluded from their respective groups. Animals were injected with Under 1.5-2% isoflurane and 37.0 ± 0.5ºC perfusion imaging was performed at each time point, then ROIs were drawn to anatomic landmarks around the ischemic and control limbs. The valid pixel (VP) and mean pixel (MP) of ROIs from both left (L) ischemic limb and right (R) control limb left were obtained using moorLDI Laser Doppler Imager software, the ratios of ischemic (LVP*LM) versus control (RVP*RM) limbs were used to plot the time-course. Appearance score was determined by counting the number of necrotic toes at 7 days-post ischemia score. At the completion of the study, mice were euthanized by cervical dislocation under ketamine cocktail (Ketamine 100mg/kg, Xylazine 10mg/kg).

Vessel Painting and pial collateral analysis

Vessel painting on post-natal murine pups was modified from previous studies (49). Briefly, mice were injected with heparin (2,000 units/kg), and sodium nitroprusside (SNP, 0.75 mg/kg) five minutes prior to euthanization using an overdose of carbon dioxide. When breathing stopped, the chest cavity was opened and then cardiac perfused using a Gilson MiniPuls3 peristaltic perfusion pump (Gilson Scientific, Bedfordshire, UK). Using a continuous infusion, 6-10 ml of 1x phosphate buffered saline (PBS) containing 20 units/ml heparin was perfused to flush blood from the cerebrovascular system, then 10 ml Dil (0.01 μg/ml, Invitrogen)– 4% sucrose–PBS-heparin mixture was perfused to label the vasculature (0.7 ml/min for P1, 1.0 ml/min P7 and 2ml/min for P21 and adult flow rate), and finally, 4% cold paraformaldehyde (PFA) was perfused to fix the tissue. All reagents were filter sterilized and debris free. After perfusion, brains were carefully removed from the skull and placed in PFA overnight. Fixed brains were imaged at multiple image planes at 4x magnification on an upright fluorescence microscope (BX-51, Olympus America), using mosaic tile imaging from StereoInvestigator software (MBF, Williston, VT). Scaled mosaic images were imported into ImageJ, then the total number of intra-
and inter-tree collaterals were identified between and within the MCA, ACA, and PCA artery branches and quantified using the counting tool in ImageJ on each mosaic image. Pial collateral diameters were also individually assessed on the scaled mosaic images using ImageJ by averaging three independent diameters along the collateral length.

Measurement of hindlimb arteriole diameter and capillary density

Adductor muscle was harvested 7 days after induction of ischemia and fixed in 4% PFA overnight, then embedded in OCT and serial cryosectioned at 30 μM. Four sections at 300 μM apart were collected per animal. Sections were then prepared and incubated with CD31 for the identification of endothelial cells and smooth muscle cells, respectively, to identify collateral arteries. Collateral artery diameter was measured using precalibrated microscope scale bars. Four sections were analyzed per animal by an investigator blinded to the groups and collateral diameters were quantified in 5 randomly selected low power (10×) fields per section; the mean value of these measurements was taken as a single data point for each animal. Capillary density was expressed as the ratio of CD31+ cells to myofibers. This measurement was determined in 5 randomly selected low power (200×) fields from each animal, and the average value taken and used as a single data point for each mouse.

Histology

For immunostaining, whole mount, perfused-fixed tissue sections were blocked in 2% cold water fish gelatin with 0.1% Triton for 3 hours and incubated in primary antibody overnight in block at 4°C (rabbit anti-EphA4: 1/200 Santa Cruz). Sections were washed 4 times with 1X PBS and incubated with anti-rabbit Alexa Fluor 488-conjugated secondary antibodies (Molecular Probes, Carlsbad, CA) for 1 h at RT. Whole mounts were counterstained with DAPI (1 μg/ml, Molecular probes, Carlsbad, CA) and mounted in Pro-Long anti-fade mounting solution (Molecular probes, Carlsbad, CA).

Isolation and culture of endothelial cells

Post-natal day 1-5-old EphA4flox/flox (WT) or EphA4flox/flox/Tie2::Cre (KO) pups were sacrificed by decapitation under anesthesia and whole brains were extracted and dissected using neural dissociation kit (Miltenyi Biotec, Auburn, CA). Four to five pups were used per group for each isolation. Single-cell suspension from fresh dissociated brain tissue was subjected to CD31+ magnetic beads and column separation, as per manufacturer
instructions (MACS; Miltenyi Biotec, Auburn, CA). Cells were seeded into one well of a 6-well plate (Corning) pre-coated with Fibronectin (10 µg/ml; Corning, Corning, NY) in complete endothelial cell media with growth factors (Cell Biologics, Chicago, IL; M1166). Media was changed every 2 days until confluent then passaged using 0.25% trypsin/EDTA (Hyclone, Logan UT) and plated one T75 flask pre-coated with fibronectin. Cells were expanded and frozen or used for experimentation. Complete media changes were performed every 3 days until cells were confluent and passaged. Experiments were conducted on cells <10 passages.

Proliferation, tube formation and migration assessment

ECs were plated in a 96-well plate containing EC complete media, pre-coated with 0.2% gelatin, at 20,000 cells/well. After 24 hour incubation, plates were removed and immediately assessed for proliferation by adding 10 µM bromodeoxyuridine (BrdU; Sigma Aldrich, St. Louis, MO). Following 1 h incubation with BrdU, cells were fixed with 10% buffered formalin and incubated in 2N HCl as previously described [24]. Cells were counterstained with DAPI (1 µg/ml) and analyzed under TRITC/DAPI filters on an inverted IX-71 Olympus epi-fluorescence microscope equipped with a digital XM-10 camera and Cell Sense software package (Olympus, Valley, PA). Four images per well were acquired and quantified as percentage of BrdU as previously described [29]. To assess tube formation, 15,000 cells were plated on a layer of approximately 60 µl solidified growth factor-reduced matrigel (Corning, Corning, NY) in EC base media without growth factors. Images were taken at 4x magnification using an IX-71 Olympus microscope. For migration assessment, we performed the scratch assay using 100,000 cells/well in a 24-well plate pre-coated with 0.2% gelatin, incubated overnight at 37°C; 5% CO₂ in complete media. After 24 hours, a 200 µl pipet tip was used to scrape the cell monolayer in a straight line creating a scratch. Wells were washed twice with warm 1x PBS, then incubated in EC base media and incubated 4-48 hours. Wound healing or migration into the scratch was assessed at 4, 24 and 48 hours post-scratch. Images were taken at 4x magnification using an IX-71 Olympus microscope. All quantifications were performed using the measurement tools from Cell Sense software (Olympus, Valley, PA). In experiments assessing the role of PI3K inhibition on proliferation and migration, 50 µM LY294002 (Sino Biological Inc, PA) was added to EC culture media and DMSO added as a vehicle control at the time of plating or following wound scratch.

qRT-PCR
Total RNA of endothelial cell cultures or fresh collateral zone tissue was isolated according to manufacturers instructions using the RNeasy Mini Kit for total RNA extraction (Qiagen, Valencia, CA). RNA quantification was carried out by measuring absorbance with spectrophotometer ND-1000 (NanoDrop). RNA was reverse transcribed into cDNA with Im-Prom II Reverse Transcription System (Promega, Madison, WI). RNA samples were treated with DNase I (ThermoFisher, Waltham, MA) before reverse transcription. Each DNase reaction (1 μg RNA, 1 μL 10X DNase I buffer, 1 μL DNase I, 0.5 uL RNase inhibitor, up to 10 μL with water) was incubated at 37 °C for 60 minutes before inactivation by addition of 1 μL 50 mM EDTA and incubation at 65 °C for 10 min. The DNase-treated RNA samples were incubated with oligo (dT) 15 primer at 70 °C for 5 min. (250 ng RNA, 1 μL oligo (dT) 15, up to 5 μL with water) and chilled on ice for 5 minutes to allow annealing before reverse transcription. To prepare the cDNA samples, 15 μL reverse transcription mix (3.7 μL water, 4 μL 5X ImProm-II buffer, 4.8 μL 25 mM MgCl₂, 1 μL 10 mM dNTPs, 0.5 μL RNase inhibitor, 1 μL ImProm-II reverse transcriptase) were added per 5 μL RNA sample and reverse transcription was performed using the following PCR scheme: 25 °C for 5 min.; 42 °C for 1 hour; 70 °C for 15 min.

Differential gene expression was then assessed using qRTPCR. Primer sequences used were: GapdhF, 5’AAT GTG TCC GTC GTG CAT CTG A 3’; GapdhR, 5’AGA TGC CTG CTT CAC CTT CTT CTT 3’; Adam10F, 5’-ACGCTGGGTCTTTTTGGTGTA-3’; Adam10R, 5’-AATTCTGCTCCTCTCCTGGG-3’; Adam17F, 5’-TCTTGTCTCTCAGACTACGACATCC-3’; Adam17R, 5’-CCACCACGACTCTCAAGTTTTTGTTG-3’; Ang1F, 5’-CGATAACCAGTCAGGGCAGT-3’; Ang1R, 5’-AGTAGGCTCGTGTTTCCTCC-3’; Ang2F, 5’-AGCAGATTGTTGCATCAGACC-3’; Ang2R, 5’-GTCCTTTTTCATGGACTGAG-3’; VegfF, 5’-GAAGTCCCATGAAGTGATCCAG-3’; VegfR, 5’-TCACCGCCCTTGCTGTCA-3’; Vegfr1F, 5’-TTGGGACAGACAGAAGTTCTGTT-3’; Vegfr1R, 5’-GACCTCGTAGTCACTGAGGTTTTG-3’; Vegfr2F, 5’-GAGACCTGACTGGCTTTTGG-3’; Vegfr2R, 5’-CCCGCATTCAGTGACCATACC-3’; Mcp1F, 5’-TCACCTGCTGCTACTCATTCAAC-3’; Mcp1R, 5’-TACAGCTTCTTTGGGACACCTGCT-3’. For qRTPCR analysis, 7 ng cDNA per reaction were amplified using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Expression changes were calculated using ΔΔCq values with reference to Gapdh internal control gene then calculated as relative expression compared to wild type samples.

RNAseq Analysis
RNA-seq libraries were constructed according to Illumina protocol and sequenced with the Illumina Hiseq 2000. Using TopHat (version 2.0.3), all the 101bp pair-end reads were mapped to the mouse reference genome (GRCm38/mm10) (221). Genome annotation files with GTF format for Known Genes were downloaded from UCSC. Reads per kilobase of transcript per million reads (RPKM) values were calculated for each gene using Cufflinks software (version 2.0.2) with default parameters (222) and normalized using quantile method. The files generated by the Cuffdiff program were then passed to the Cummberbund, an R package used to determine the significantly differentially expressed genes (P< 0.01) and to visualize the output. GO enrichment analyses were performed using DAVID functional annotation tools (223).

Western Blot analysis

Protein of EC cultures was extracted by lysing cells in RIPA buffer (1% NP-40, 1% sodium-deoxycholate, 0.1% SDS, 0.15 M NaCl, 2 mM EDTA, and 0.01M sodium phosphate) in the presence of complete protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail 2 (Sigma). Supernatant was collected by centrifuging at 13000 g for 30 min at 4 °C and the Lowry assay was used for determination of protein concentration (Pierce, Rockford, IL). Cell lysates (50 µg) were resolved on 8% SDS-PAGE gels and blotted onto PVDF membranes, blocked with 5% bovine serum albumin (BSA) in TBST buffer (20 mM Tris, 137 mM NaCl and 0.1% tween) then incubated in block overnight at 4 °C with primary antibody against Ang1 (Rabbit, 1:1000 Rockland, Limerick, PA), MCP-1 (rabbit 1:1000 Sant Cruz Biotech, Santa Cruz, CA), or β-actin (mouse, 1:5000 Cell Signaling, Danvers, MA). HRP-conjugated secondary antibodies (Jackson laboratory) were applied to the membrane and developed as previously described [24]. Blots were quantified by densitometry using acquisition into Adobe Photo Shop (Apple, Cupertino, CA, USA) and analyzing by the NIH Image software (National Institutes of Health, Bethesda, MD, USA). The level of protein expression was normalized according to β- actin control levels. Samples were run in quadruplicate.

Statistical analysis

Data was graphed using GraphPad Prism, version 4 (GraphPad Software, Inc., San Diego, CA). Student's two-tailed t test was used for comparison of two experimental groups. For three or more groups, multiple comparisons were done using one-way and two-way ANOVA where appropriate followed by Tukey or
Bonferroni test for multiple pairwise examinations. Changes were identified as significant if \( P \) was less than 0.05. Mean values were reported together with the standard error of mean (SEM).

**Acknowledgements**

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**Fig 1. Vessel Painting and co-labeling of post-natal murine pial vessels.** (A) Inverted VP image of a mouse brain showing selective labeling of the cerebral arteriole network using vessel painting. (B) Identification of pial collaterals using vessel painting (VP, red) and double labeling of with anti-SMA shows, VP does not label SMCs. (C) Confocal image showing cross section a vessel painted pial arteriole, immunostained for SMA, confirming that VP does not label SMCs. (D) Quantification of EphA4 on pial collaterals, represented as mean fluorescence intensity. (E – M) Double labeling of psot-natal day (P)1, P7 and P21 brains using VP (red) and anti-EphA4 (green). EphA4 is expressed on VP-labeled collaterals at P1, P7 and minimally at P21. *P<0.05; n=5 per group; represented as mean ± SEM.
Fig 2. Phenotype and genotype of littermates from EphA4f/f and Tie2::Cre breeding paradigm. (A - F) Reporter labeling of Tie2::Cre is specific to blood vessels. Panel F further confirms eGFP expression specifically to blood vessels. (G) Genotyping using PCR shows a flox primers of about 290bp and a bottom Cre band. Lane 1 and 2: EphA4f/f/Tie2::Cre (KO); Lane 3 and 4: EphA4f/f (WT). Vessel painting of surface pial arterioles of WT (H) show increased numbers in KO (I) mice. Panel J and K are WT and KO endothelial cells cultured from brain endothelial cell progenitor cells. ECs are labeled with CD31 (green) and DAPI (blue). (L) ECs from KO mice do not express EphA4 RNA transcripts.
Fig 3. EphA4 EC-specific ablation increases collateral formation in the post-natal murine brain. (A) KO mice have increased total collaterals in P1, P7, P21 and adult mice. (B) average tortuosity is increased in all KO groups except adult. (C) KO mice have more MCA – ACA collaterals but there is no significant difference in
average diameter of collaterals in the mice. (E – H) VP of P1 and adult WT and KO brains. *P<0.05; ***P<0.001; n=5-9 per group; represented as mean ± SEM.

**Fig 4. EphA4 EC-specific ablation increases EC proliferation, migration and tube formation.** Panel shows WT (A, A1) and KO (B, B1) BrdU analysis of ECs stained with DAPI (blue) and BrdU (red). (C) KO ECs have significantly increased BrdU incorporation compared to WT ECs. WT (D, D1) and KO (E, E1) EC scratch. (F) KO have greater migration rate 24 hours post scratch compared to WT EC. (G, G1) WT and (H, H1) KO ECs were seeded on growth factor-reduced matrigel to form tubes. (I) Vascular index (number of tubes x tube length) is enhanced in KO ECs compared to WT. ***P<0.001; n=16-20 wells/group; represented as mean ± SEM.
Fig 5. mRNA sequence profile of WT and KO ECs. (A) Genome-wide differential expression analysis via RNA-sequencing indicates KO ECs differing gene expressions that predominantly effect the extracellular matrix, immune response, wound healing, cell proliferation, cell adhesion and vascular development. (B - E) Overall ontological analysis of both upregulated and downregulated genes between WT and KO ECs. (F) Differential expression of seven genes significant in vascular development was further assessed using qRT-PCR which
confirms an increase in Ang-1, MCP-1, and MMP2 in KO RNA transcripts using PCR. *P<0.05; **P<0.01; represented as mean ± SEM.

Fig 6. PI3K inhibition attenuates endothelial cell functions following EphA4 ablation. (A) Western blot analysis indicates increased p-Akt expression in KO ECs compared to WT ECs. Expression is reduced in KO ECs after LY294002 treatment. (B) Densitometric analysis shows a three-fold increase of p-Akt in KO ECs. KO treated with LY294002 have identical p-Akt expression as WT vehicle control. (C – E) In addition treating KO ECs with LY294002 significantly reduced BrdU incorporation, migration and vascular index, respectively. ***P<0.001; n=16-20 wells/group; represented as mean ± SEM.
Fig 7. Plantar reperfusion and collateral remodeling post- hindlimb ischemia. (A) Laser Doppler images of blood flow pre and post ischemia in WT and KO mice. (B) Analysis shows significant increase in plantar blood flow perfusion at 7d post hindlimb ischemia or femoral artery ligation (FAL) in KO mice compared to WT. (C) KO mice also have a significant reduction in toe necrosis compared to WT mice at 7 days post-FAL. (D) The number of CD31-positive arterioles were are increased in serial sections of both un-injured and injured adductor muscles in the absence of EC-specific EphA4. (E) Average arteriole diameter in the injured aductor muscle is
increased in KO mice compared to WT and KO un-injured tissue at 7d post-FAL. (F-I) Representative high magnification (x20) images from un-injured and injured adductor muscles at 7d post-FAL using CD31 immunostaining and -fluorescence. Scale bar= 100µm. (J-M) Brightfield images of WT (J and K) and KO (L and M) adductor muscles at 7d post-FAL. *P<0.05; **P<0.01; n=5-8/group; represented as mean ± SEM. FA=Femoral artery.
Chapter 4

This chapter is published as a manuscript in the Journal of Neurological Methods

Temporal remodeling of pial collaterals and functional deficits in a murine model of ischemic stroke
Abstract

**Background:** Leptomeningeal anastomoses play a critical role in regulating reperfusion following cerebrovascular obstruction; however, methods to evaluate their temporospatial remodeling remains under investigation.

**New Method:** We combined arteriole-specific vessel painting with histological evaluation to assess the density and diameter of inter-collateral vessels between the middle cerebral artery and anterior cerebral artery (MCA-ACA) or posterior cerebral artery (MCA-PCA) in a murine model of permanent middle cerebral artery occlusion (pMCAO).

**Results:** While the overall density was not influenced by pMCAO, the size of MCA-ACA and MCA-PCA vessels had significantly increased 2 days post-pMCAO and peaked by 4 days compared to the un-injured hemisphere. Using a combination of vessel painting and immunofluorescence, we uniquely observed an induction of cellular division and a remodeling of the smooth muscle cells within the collateral niche following post-pMCAO on whole mount tissue sections. Vessel painting was also applied to pMCAO-injured Cx3cr1^GFP^ mice, in order to identify the spatial relationship between Cx3cr1-positive peripheral-derived monocyte/macrophages and the vessel painted collaterals. Our histological findings were supplemented with analysis of cerebral blood flow using laser Doppler imaging and behavioral changes following pMCAO.

**Comparison with existing methods:** Compared to polyurethane and latex methods for collateral labeling, this new method provides detailed cell-type specific analysis within the collateral niche at the microscopic level, which has previously been unavailable.

**Conclusions:** This simple and reproducible combination of techniques is the first to dissect the temporospatial remodeling of pial collateral arterioles. The method will advance investigations into the underlying mechanisms governing the intricate processes of arteriogenesis.
Introduction

Leptomeningeal anastomoses or pial collateral vessels are arteriole-to-arteriole connections joining the major branches of the cerebral arteries (middle MCA, anterior ACA and posterior PCA) in the pial surface of the brain. These specialized bypass vessels, which are normally inactive, are capable of re-routing blood flow around vascular obstruction (224-226). Although patients having greater collateral vessel density show improved recanalization and reduced infarct size following MCA occlusion, substantial variation in size, number and compensatory capacity of collaterals exist (227). The pial collateral network forms during prenatal development, although secondary changes related to various pathophysiological conditions may occur throughout life (228, 229). Vascular endothelial growth factor (VEGF), A Disintegrin and Metalloproteases (ADAM) 10 and 17, chloride intracellular channel (Clic4) and Eph receptor tyrosine kinase EphA4 have been shown to regulate murine collateral density and diameter during peri-natal development as well as remodeling after ischemia (19-21, 51, 230). Collateral vessel remodeling, also known as arteriogenesis, results in their enlargement or outward growth which enables restoration of retrograde re-perfusion into the territory of the occluded vessel (153, 231-236).

Arteriogenesis occurs in three phases: initiation, growth and maturation (134). Initiation of collateral remodeling occurs when collateral blood flow, which normally moves in both directions but in equilibrium, becomes disrupted leading to increased unidirectional flow within the collateral vessel. This steep pressure gradient causes fluid shear stress to activate the endothelium, invoking a cascade of events (224, 237, 238) that leads to the production of cytokines, growth factors and proteases which mediate the enlargement of the collateral vessels (6, 11, 182, 239). Enhancement of retrograde cerebral blood flow through remodeled collateral vessels into the territory of the occluded artery mitigates cellular damage and helps maintain tissue preservation (240-246). Thus, enhancing collateral growth and remodeling has become an attractive target for therapeutic intervention in patients suffering from an ischemic attack (247-249). However, the cellular and molecular cues regulating this response remains under investigation.

To investigate the dynamic changes occurring in the collateral network, in vivo imaging and histological analysis are often used. In vivo imaging techniques, such as optical coherence tomography, two-photon microscopy and laser speckle imaging have been used to observe collateral reperfusion and remodeling in
pathological states (239, 250-252). Such methods are particularly useful for non-invasive, longitudinal assessment of collateral function with high resolution. However, to improve our understanding of the cellular and molecular mechanism(s) underlying these processes we have developed a histological method combining vessel painting and immunocytochemistry using cortical whole mounts for quantitative and qualitative analysis of the structural changes within and around the wall of the collateral itself. This method will provide detailed cell-type specific analysis of the collateral niche at the microscopic level, which has previously been unavailable. Knowledge gained from such findings will expand our understanding of the individual cell response within and surrounding the collateral during active remodeling, including endothelial, smooth muscle and immune-derived cell morphology changes.

Few studies have evaluated the histological and morphological features of collateral remodeling. Coyle and colleagues developed the use of latex perfusion and casting of the cerebral-vasculature (253, 254). This technique, however, labels both the venal and arterial vascular system. The use of polyurethane to characterize the pial collaterals has also been described (255). While both techniques provide superior visualization of collaterals, they cannot be combined with immunofluorescence labeling. Our method provides detailed morphological and histological changes occurring in the pial collateral network, after surgically inducing stroke in the brain, and selectively labeling the arterial vasculature using a vessel painting technique. Using a combination of methods, our findings illustrate the stepwise changes in collateral growth following (230, 251) and quantify changes occurring in individual dorsal pial collateral vessels including cell proliferation, smooth muscle cell reorganization and recruitment of Cx3cr1GFP-expressing peripheral-derived immune cells (230, 251). We also describe the longitudinal relationship between these structural alterations and cerebral blood flow as well as behavioral deficiencies in the pMCAO model. These findings provide a standardized platform for evaluating the molecular and cellular mechanism(s) underlying collateral remodeling which may aid therapeutic interventions to enhance this adaptive response and improve acute neural functional outcome following stroke.

Materials and Methods

Animals.

All mice were generated and housed in an AAALAC approved, virus/antigen-free facility with a 12 h light-dark cycle; food and water ad libitum. CD1 mice were purchased from Charles Rivers, and bred until desired numbers
were generated for experimentation. All experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were conducted under the approval of the Virginia Tech Institutional Animal Care and Use Committee (IACUC; #15-063) and the Virginia Maryland Regional College of Veterinary Medicine. All efforts were made to minimize the number of animals used and their suffering.

*Surgical Procedure.*
Focal ischemic stroke was induced by pMCAO as previously referenced with slight modifications (50). Briefly, 8-12 week-old mice (25-35g) were injected with analgesic Buprenorphine-SR (0.15 mg/kg), and anesthesia induced with 2% isoflurane-30% oxygen mixture. Body temperature was monitored with a rectal probe and maintained at 37°C with a controlled heating pad (homeothermic blanket system; Harvard Apparatus). Subsequently, the skull was thinned, at the junction of zygomatic arch and squamosal bone, to expose the MCA. The main branch of the MCA, and two adjacent branches were ligated using a small vessel cauterizer. Sham controls received the same surgical procedures without ligation of the MCA. Following injury, the incision was closed using Vetbond tissue adhesive (3M, St. Paul, MN, USA) and animals were returned to their home cage on a heating pad for post-op monitoring.

*Vessel Painting.*
Vessel painting on adult CD1 mice was performed as previously described (230, 251). Briefly, mice were injected with heparin (2,000 units/kg), and sodium nitroprusside (SNP, 0.75 mg/kg) five minutes prior to euthanization, using an overdose of isoflurane. When breathing stopped, the chest cavity was opened and then cardiac perfused using a Gilson MiniPuls3 peristaltic perfusion pump (Gilson Scientific, Bedfordshire, UK). Using a continuous infusion, 10 ml of 1X phosphate buffered saline (1X PBS) containing 20 units/ml heparin was perfused to flush blood from the circulatory system, then 10 ml Dil (0.01 mg/ml, Invitrogen) diluted in 4% sucrose–PBS-heparin mixture was perfused using a flow rate of 2 ml/min followed by cold 4% paraformaldehyde (PFA) to fix the tissue. After perfusion, brains were carefully removed from the skull and placed in PFA overnight. Fixed brains were imaged at high resolution using multiple image planes at 4x magnification on an upright fluorescence microscope (BX-51, Olympus America), using mosaic tile imaging from StereoInvestigator software (MBF, Williston, VT).
Scaled mosaic images were imported into ImageJ (NIH), then the total numbers of intra- and inter-tree collaterals were identified between and within the MCA, ACA, and PCA artery branches and quantified using the counting tool in ImageJ on each mosaic image. Pial collateral diameters were also individually assessed on the scaled mosaic images using ImageJ by averaging three independent diameters along the collateral length. Diameters of individual collaterals were also confirmed using 20x magnification and the line measure tool on MBF StereoInvestigator software at three locations along the length of the collateral. Collateral length and span were also measured to determine collateral tortuosity. Length was measure with the straight line tool by drawing a straight line from the start to the end point of the collateral. Span was measured with the freehand line tool by tracing the collateral from the start to end point. Tortuosity index was calculated as a ratio of the length:span of each collateral.

**Immunostaining cortical whole mounts.**

For immunostaining, the surface cortical region of the brain was dissected and placed in 1X PBS overnight in preparation for whole mount staining. Whole mounts were subsequently blocked in 2% Fish gelatin (Sigma Aldrich, St. Louis, MO) with 0.1% Triton X-100 for 3 hours then incubated overnight in antibodies against smooth muscle actin (SMA) (1:1000; Abcam, Cambridge, UK; ab7817) in blocking buffer at 4°C. Whole mounts were washed 5 times with 1X PBS then incubated with anti-mouse Alexa Fluor 488 conjugated secondary antibody (Molecular Probes, Carlsbad, CA) overnight at 4°C. After washing 5x with 1X PBS, whole mounts were counterstained with DAPI (ThermoFisher Scientific, Waltham, MA) for 5 minutes, washed 3x with 1X PBS and embedded in mounting media (SouthernBiotech, Birmingham, AL) in a 35mm glass dish, cover slipped, then imaged on an inverted Zeiss 880 confocal microscope (Carl-Zeiss, Oberkochen, Germany). For PCNA staining, whole mounts were incubated in HCL for 45 minutes at 37°C and then neutralized with sodium borate as previously described (256). This method is used to unmask the PCNA antigen. Tissue was blocked then incubated in rabbit anti-PCNA antibody (1:1000; Cell signaling, Danvers, MA) overnight, and then incubated with anti-rabbit 488 (Invitrogen, Carlsbad, CA) secondary antibody. The contralateral hemisphere was used as a negative control.
Behavioral Testing.

Rotarod. We used the Rotarod (Rotamex, Columbus Inst) to assess motor function as previously reported (257, 258). Briefly, animals were pre-trained for 4 consecutive days prior to pMCAO or sham injury. The starting velocity was set at 10 rpm and accelerated to 0.1 rpm/sec. A baseline was collected on the fourth day, then again at 3, 7, 14, 28, and 35 days post-pMCAO or sham injury for each mouse. Data is graphed as the mean of the individual scores relative to baseline. Grip test. A rodent grip strength meter (DFIS 10, Columbus Instruments, Columbus, OH) was used to measure strength in the forelimbs. Mice were first allowed to grasp the apparatus bar, then pulled perpendicular to the bar. Grip strength was recorded in Newtons (N) at baseline (one day prior to injury) then at 3, 7, 14, 28, and 35 days post-pMCAO or sham injury. Data is represented as relative to baseline. Inverted Screen or grip Test. We used the Kondziela inverted screen test to assess muscle strength after injury (259). The inverted screen contained a 43 cm$^2$ wire mesh consisting of 12 mm squares of 1 mm diameter wire. Mice were pre-trained, for 4 consecutive days, to grip the screen as it is inverted and a baseline time to fall was recorded on the last day of training then again at 3, 7, 14, 28, and 35 days post-pMCAO or sham injury. Beam walk. The beam walk test was used to assess fine motor coordination and balance. The beam was 30 cm in height, 6 mm in diameter and 80 cm long. Mice were pre-trained and the time to traverse the beam was recorded on the last day of training and at 3-35 post-injury. Neurological severity scoring (NSS). The NSS was used to assess sensorimotor deficits pre-injury and at 3-35 days post-injury. NSS is a composite of motor, sensory (visual, tactile and proprioceptive), reflex, and balance tests. Function was graded on a scale of 0 to 14 (normal = 0; maximal deficit = 14), where 1 point is awarded for the inability to perform the task or for the absence of the tested activity as previously described (260). Novel object recognition (NOR). Cognitive and spatial deficits were tested using the novel object recognition task. Briefly, mice were introduced to two identical objects on day one and then one of the objects was replaced with a new object the following day (test day) in a 40 cm$^3$ arena. Time of exploration of the old and new object was recorded over 5 minutes. Preference of object was calculated as a ratio exploration time of the specific object to the total time of exploration of both objects. NOR was also administered at 3-35 days post-injury.

Cerebral blood flow
Cerebral blood flow (CBF) was assessed at pre-, 5 minutes and 1-4 day post-injury using the Moor LDI2-HIR Laser Doppler flowmeter with Moor Software Version 5.3 (Moor Instruments, Wilmington, DE). Briefly, mice were anesthetized with 1.25% isoflurane-O2, the skin opened to expose the scalp transversely, and head placed in a stereotactic device. Rectal temperature was maintained at 37.0 ± 0.5°C and cerebral blood flow, in perfusion units (PFU) was scanned using a 2.5cm x 2.5cm scanning area. Tissue perfusion was quantified with a region of interest (ROI) define in the same left hemisphere of injury and represented a ratio of post-perfusion units (PFU) relative to the pre-injury scan of each mouse.

**Statistical analysis.** Data was graphed using GraphPad Prism, version 4 (GraphPad Software, Inc., San Diego, CA). Student’s two-tailed t test was used for comparison of two experimental groups. Multiple comparisons were done using one-way or two-way ANOVA where appropriate followed by post hoc Bonferroni test. Changes were identified as significant at P value < 0.05. Mean values were reported together with the standard error of mean (SEM).

**Results**

**Murine permanent middle cerebral artery occlusion (pMCAO) model and cerebral blood flow**

Collateral density and remodeling plays a critical role in restoring cerebral blood flow after arterial occlusion (254). First we evaluated the time course of reperfusion to the ipsilateral cortex following pMCAO, by assessing cerebral blood flow (CBF) using high resolution Laser Doppler imaging (moorLDI2-HIR). Laser Doppler images revealed distinct difference in ipsilateral CBF between sham operated and pMCAO mice immediately after injury (Fig. 1A vs 1B; respectively). Statistical analysis correlated with reperfusion images. CBF was represented by quantifying the perfusion units (PFU) relative to pre-injury scan values (Fig. 1C). Immediately after pMCAO, CBF is significantly reduced (0.42 ± 0.03 PFU) in the ipsilateral hemisphere compared to pre-injury levels (0.99 ± 0.02 PFU). However, perfusion values are increased at day 1 (0.65 ± 0.01 PFU), day 2 (0.73 ± 0.03), day 3 (0.75 ± 0.03 PFU) and day 4 (0.77 ± 0.03 PFU) compared to 5min post-pMCAO. A significant increase (16%) in CBF was observed between 1d and 4d post-pMCAO. These findings demonstrate a 57% reduction in CBF following pMCAO in CD1 mice, which is restored to 65% of the pre-injury levels by 1 day and 80% by 4 days post-pMCAO.
TTC staining of coronal brain slices confirms a focal area of cortical tissue loss in the left hemisphere (Fig. 1E) compared to sham-injured control (Fig. 1D) as a result of these changes in CBF.

**Collateral Remodeling after pMCAO**

The MCA is one of the major arteries that provides blood supply to the brain and connects to the anterior and the posterior cerebral arteries via the MCA-ACA and MCA-PCA collateral networks, respectively. Following pMCAO, the outward growth and remodeling of pial collateral vessels provides an alternative route for retrograde reperfusion into the MCA territory. To correlate changes in CBF after pMCAO with collateral density and remodeling, we used vessel painting to label the adult arterial network, as previously demonstrated (230), and assessed the pial collateral remodeling at 1, 2, 4, 7, and 65 days post-pMCAO on the ipsilateral and contralateral hemispheres. Collateral diameter and tortuosity was assessed as parameters of collateral remodeling at each time point. The overall collateral diameter was significantly increased in the ipsilateral cortex at 2d (34 ± 2 µm vs 20 ± 1 µm), 4d (39 ± 3 vs 20 ± 1 µm), 7d (38 ± 1 µm vs 20 ± 1 µm) and 65d (40 ± 2 µm vs 20 ± 1 µm) post-pMCAO compared to contralateral. No significant change was observed at 1 day (24 ± 2 µm vs 19 ± 1 µm) (Fig 3A). Similarly, we found a significant increase in collateral size between both MCA-ACA (Fig. 3B) and MCA-PCA (Fig. 3C) indicating both MCA-ACA and MCA-PCA collaterals enlarge in response to pMCAO. We then analyzed the percentage of pial collaterals at 10-20 µm, 21-30 µm, 31-40 µm, <40 µm in the contralateral and ipsilateral hemisphere at 1-65d post-pMCAO (Fig. 2D). Approximately ninety-percent of uninjured collaterals were 10-30µm in size, which reduced to ~70% at 1d, ~40% at 2d, and ~30% at 4, 7 and 65d in the ipsilateral cortex after pMCAO. A concomitant increase in the percentage of >40 µm collaterals at 1d (8.40 ± 0.07%), 2d (27.59 ± 5.19%), 4d (56.30 ± 5.23%), 7d (56.09 ± 5.44%) and 65d (45.73 ± 9.83%). No significant differences were seen in inter-collateral counts (MCA-ACA and MCA-PCA) or total collateral counts (inter- and intra-collaterals) between ipsilateral and contralateral sides (Fig. 2E and 2F; respectively). We observed an increased trend in the average tortuosity index (TI) between 1d (1.13 ± 0.01 TI) and 4d (1.22 ± 0.02 TI) in the ipsilateral compared to contralateral collaterals, although its difference was non-significant. These data indicate that a majority of the collateral growth in size occurs between 1-4 days in CD1 mice, an effect maintained up to 65 days post-pMCAO.
Histological evaluation of collateral remodeling after pMCAO

The acute temporal remodeling of pial collaterals in CD1 mice can be characterized after pMCAO using vessel painting (230) and standard histological evaluation of cortical whole mounts. We determined that vessel painting can be used in combination with antibodies against smooth muscle actin (SMA) to qualitatively assess the dynamic changes in vessel shape and smooth muscle cell (SMC) location as well as quantify cellular processes, such as proliferation, during collateral remodeling. Following vessel painting (VP) with the lipophilic and fluorescent Dil at 1, 2 and 4 days post-injury, perfused whole mount tissues were stained with anti-SMA and imaged using confocal microscopy to visualize smooth muscle cell reorganization during arteriogenesis, a key step in collateral remodeling (12, 134). Visible changes in the size of pial collaterals are seen following pMCAO and 4 days post-pMCAO compared to sham injury (Fig. 3A-3D). The number of collaterals undergoing remodeling is also increased during this time (Fig. 3B-3D; white arrows). Whole mounts stained with anti-SMA and imaged using confocal microscopy show reduced staining and significant re-organization of smooth muscle cells in collateral vessels at 4d post-pMCAO (Fig. 3C-3E and 3C1-3E1) compared to 7d day post-pMCAO (Fig. 3F-H) where increased SMA coverage is seen and coverage of the vessel is underway. During this time it is known that SMCs exit the vessel wall, expand and reengage once the vessel has grown to full capacity. These findings demonstrate a useful histological tool for evaluating SMC changes on vessel painted pial collaterals as a means to delineate dynamic temporospatial structural features of remodeling vessels during arteriogenesis (135).

Collateral remodeling in Cx3cr1GFP mice after pMCAO

The contribution of peripheral-derived immune cells during collateral remodeling has recently been demonstrated (8, 261). For example, pharmacological monocyte depletion and athymic nude mice that lack T cells display impaired arteriogenesis (11, 262). CD4- and CD8-deficient mice also show reduced collateral circulation and are thought to be mediators of collateral recruitment (263, 264). To test whether we could visualize with high resolution the recruitment of immune-derived cells to the peri-vascular region of remodeling collaterals on vessel painted whole mounts, we used genetic Cx3cr1GFP knock-in reporter mice. Cx3 chemokine receptor 1 (Cx3cr1) is expressed by monocytes, macrophages, subsets of NK and dendritic cells and brain microglia (265). At 4 days post-pMCAO, using confocal image analysis, we observed a significant recruitment of GFP-positive cells on and
around the remodeling collateral vessels in the ipsilateral hemisphere (Fig. 4A1-A3) compared to contralateral (Fig. 4B-D). Higher magnification of a collateral vessel undergoing remodeling (identified as vessels at or greater than 30µm in the ipsilateral hemisphere) clearly shows the peri-vascular nature of the immune-derived GFP-positive cells (Fig. 4E-4G; 4E ortho view showing non-overlap of GFP and the vessel painted arteriole, compared to the underlying ramified microglia in the brain parenchyma (Fig. 4A) of the whole mounts. To our knowledge, this is the first high resolution demonstration of Cx3cr1<sup>GFP</sup>–positive cell recruitment to the pial collaterals undergoing remodeling following ischemic stroke. Because these cells likely represent peripheral-derived immune cells and not brain parenchymal microglia. Additional co-labeling using this technique could also be employed to specifically identify and quantify the Cx3cr1<sup>GFP</sup> expressing cells during the remodeling phases.

**Cell proliferation in the collateral niche**

Endothelial and smooth muscle cell proliferation is a key step in the arteriogenic process. Several biological markers such as BrdU (5-Bromo-2'-deoxyuridine) and EdU (5-ethynyl-2'-deoxyuridine) can be used to identify proliferating cells during remodeling, but they can be expensive and only label a snapshot of cells in the s-phase of the cell cycle. In order to identify all cells undergoing cellular proliferation we used antibodies against proliferating cell nuclear antigen (PCNA), which is expressed in the nucleus of dividing cells and is a cofactor of DNA polymerase delta (266, 267). In addition to the recruitment of peripheral-derived immune cells to the arteriogenic region, numerous PCNA-positive cells are seen on and around growing collaterals in the ipsilateral hemisphere (Fig. 5B) compared to contralateral (Fig. 5A) following pMCAO. High magnification confocal imaging demonstrates numerous PCNA-positive cells within the VP-stained vessel wall (Fig. 5C-5E). These findings highlight the unique cellular changes that occur during active remodeling using vessel painting and histological examination in a murine model of ischemic stroke.

**Comprehensive behavioral assessment after pMCAO**

In order to determine whether the time course of behavioral deficits correlates with that of collateral remodeling we administered a comprehensive battery of behavioral tasks after ischemic stroke. Sensorimotor deficits have not been previously described for this model. We assessed sensory and balance coherence by using a 14-point
neurological severity scoring system, which tests motor, sensory and reflex activity. Injured mice had consistent
deficits in head axis placement, balancing, proprioception in forelimbs and occasionally in their hind limbs, as
well as corneal and startle reflexes. Neurological severity scores were significantly increased at each time point
compared to sham injury (Fig. 6A). Motor activity was also determined by several tasks. Rotarod performance
showed that mice develop severe motor deficits after pMCAO (Fig. 6B). Further motor functional assessment
using the beam walk test (Fig. 6D) and grip test (Fig. 6F) confirmed the motor deficits in the pMCAO model.
Moreover, we tested murine strength after injury using the inverted screen and grip strength tests. Although the
inverted screen test did not yield consistent significant differences in performances between sham and pMCAO-
injured mice (Fig. 6E), the grip meter test for forelimb strength displayed consistent deficits (Fig. 6F) at 3, 7, 14
and 28 days. Finally, the use of the novel object recognition test, a simple assay of memory and novelty
preference that relies on a rodent’s innate exploratory behavior (268), was evaluated following pMCAO or sham
injury. As early as 3 days post-injury, pMCAO mice had deficits in their memory function which persisted up to
35 days compared to sham (Fig. 6C). These data show that focal cerebral ischemia induces long-lasting
sensorimotor, as well as learning and memory deficits following pMCAO. Since no further growth of collateral
vessels occurs after 4 days post-pMCAO, the sustained behavioral impairments from 3-35 days post-pMCAO
may be affected by early collateral remodeling.

Discussion

Targeting the adaptive response of the collateral circulation is an emerging strategy for effective therapy
against ischemic stroke, where several pre-clinical studies show enhancement of arteriogenesis greatly affects
the severity of neural injury (107, 269). However, the mechanisms underlying collateral remodeling remain under
investigation. The current study aimed to catalog changes in the murine pial collateral network in a pMCAO
stroke model (270) using a novel method that combines histological detection for cell-type specific changes on
vessel painted collaterals. Analysis of collateral remodeling was complemented with histological analyses to
show the versatility of the technique. Finally, CBF restoration and behavioral outcomes that occur following
pMCAO were assessed to develop a platform for quantifying functional recovery in parallel with collateral
analysis, which have not previously been demonstrated for this model.
Collateral circulation and injury-induced remodeling have been assessed by standard angiographic techniques, including digital subtraction angiography, computed tomography and magnetic resonance (MR) angiography, as well as a growing array of advanced MR techniques including arterial spin labeling and dynamic MR angiography (271), as well as other in vivo methods. Recently, studies have utilized latex, polyurethane-filled arteriograms, or genetically modified mice, ephrinB3lacZ, to label and analyze collateral remodeling (135, 228, 253, 254). The current study evaluated the ease of use and the histological flexibility of vessel painting to identify and evaluate the remodeling of pial collaterals after pMCAO. When whole mount cortical tissue preps following vessel painting are assessed using confocal image analysis, detailed information at the cellular level can be provided at high resolution. This includes temporospatial determination of cell-to-cell interactions (SMC, immune cells and vessel painted ECs), cell dynamics (proliferation and survival) and regional distribution of the remodeling process along the length of the collateral vessel. Although not emphasized in the results, we found ‘hot spot’ areas of remodeling on the growing collaterals. The significance of these findings, underlying mechanism(s) and evaluation across different species such as mouse, rat and swine are currently underway. Moreover, this technique has been successfully used in other models of brain injury in our laboratory, including controlled cortical impact and repeated mild traumatic brain injury. The current study shows that the active remodeling of pial collaterals occurs predominantly between day 1 and 4 post-pMCAO. Similar results of collateral remodeling have been reported elsewhere using different murine strains of mice (255). Both MCA-ACA and MCA-PCA collaterals increased in size at a similar rate in the ipsilateral hemispheres compared to the un-injured contralateral hemisphere. Furthermore, the peak of collateral enlargement correlates with the greatest improvement in CBF. These findings suggest retrograde reperfusion may occur via the ACA as well as PCA following MCA occlusion.

Few studies have analyzed the temporal dynamic changes in SMCs on pial collaterals during arteriogenesis. Confocal microscopy revealed distinct temporal changes in expression of SMA during arteriogenesis. As early as day 4 post-pMCAO, ipsilateral collaterals undergoing remodeling showed reduced expression of SMA compared to contralateral collaterals. Interestingly, we observed numerous expanding or bulging sites on collaterals undergoing remodeling, which coincided with a reduction in SMA expression and a change in location and organization of SMCs. The inverse correlation between the increasing size and SMA
expression suggests SMC reorganization is necessary for the outward growth and remodeling. These temporal expressional changes have been suggested to be required for the infiltration of peripheral-derived immune cells during luminal expansion of the collateral vessel (12, 145). Owing to an increase in uni-directional flow through the collateral vessel, shear stress activates mechanoreceptors on endothelial cells (ECs) to initiate the production of key signals that influence the surrounding cells, resulting in the adaptive outward growth response in including EC and SMC division (6, 182, 225, 237). Indeed, the PCNA-positive cells located within and surrounding the collateral wall in the ipsilateral hemisphere after pMCAO likely represents actively dividing SMCs and ECs, as well as infiltrating monocytes/macrophages. The versatility of vessel painting can allow for further assessment of the location and identity of PCNA-positive cells.

Finally, a battery of sensorimotor behavioral tests were performed to assess reliable modes of analyzing neurological deficits after stroke. A longitudinal behavioral assessment was administered to experimental mice to gauge neurological deficits in our model. The hippocampus plays an important role in the spatial memory in animals (272-274). Although the MCA does not directly supply blood to the hippocampus, it has been postulated that cognitive deficits may be due to hippocampal denervation from the infarcted cortex, thalamic atrophy, or denervated basal nucleus cholinergic fibers to the cortex, as well as neocortical sites within the MCA region (275, 276). Numerous behavioral analyses have been used to assess cognitive deficits including the Morris water maze (MWM) and the novel object recognition (NOR) tests. However, investigation of deficits in spatial memory using the MWM may be confounded by coexisting sensory and motor impairments (277). Thus, learning and memory were evaluated using NOR because it is sensitive to hippocampal function (278). Ischemic-injured mice showed NOR deficit up to 65 days post. In addition, a comprehensive neurological severity test was used to assess sensorimotor function. The NSS constitutes motor, sensory, and reflex activities. Our studies showed significant and clear deficits in pMCAO-injured mice compared to shams. Finally, because the injury also involves the primary motor cortex, we performed the Rotarod, beam balance, screen inversion and grip strength tests in order to gauge motor and muscular strength deficits. These studies have been performed in a rat model of MCAO and were modified for mice (260). These current findings provide a standardized platform for evaluating the molecular mechanism(s) controlling collateral remodeling in order to develop novel strategies aimed at enhancing this adaptive response and improving neural functional outcome following stroke.
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Fig. 1. Cerebral Blood Flow (CBF) following pMCAO. Transverse Laser Doppler image analysis of CBF at 1-4 day pre- and post-sham (A) and –pMCAO (B) in adult CD1 mice. Arrow indicates region of reduced CBF and area of reperfusion in the ipsilateral hemisphere after pMCAO. (C) Quantitative analysis shows a significant decrease in ipsilateral CBF at 5 min post-pMCAO relative to pre-injury. Partial restoration of ipsilateral CBF occurs at 1-4 days compared to 5 min post-pMCAO. There was also a significant restoration of CBF between 1d and 4d post-pMCAO. No differences in CBF were observed in sham-injury mice. (D) Triphenyl tetrazolium chloride (TTC)-stained mouse coronal brain slices after 1 day sham injury compared to pMCAO (C). Area of infarct appears white compared to healthy red-stained tissue. Hash marks = 1 mm in D and E.***P<0.001 compared to sham; #P<0.05 compared to 1d pMCAO; ###P<0.001 compared to 5 min post-pMCAO (n= 5-7 mice per group). Data represented as mean ± SEM.
Fig. 2. Analysis of pial collateral remodeling after pMCAO. (A) Average collateral diameter (µm) in the contralateral and ipsilateral hemispheres at 1, 2, 4, 7 and 65 days post-pMCAO. Ipsilateral collateral diameter is significantly increased following pMCAO compared to contralateral at all time points except at 1 day (***P<0.001). Additionally, there was a significant increase in ipsilateral collateral diameter at 2, 4, 7 and 65 days compared to 1 day post-pMCAO (##P<0.01; ###P<0.001). These findings were similar when analyzing MCA-ACA (B) and MCA-PCA (C) collaterals. (D) Percentage of collaterals measuring 10-20 µm, 21-30 µm, 31-40 µm, >40 µm on the contra- and ipsilateral hemispheres. The largest change was observed in vessels >40 µm at 2-65 days post-pMCAO. Less than 1% of contralateral collaterals measured >40 µm, however, a significant increase was seen in ipsilateral vessels at 2 days (~30%) and 4, 7 and 65 days (~60%) compared to 1 day (~10%). (E) The numbers...
of inter-collaterals (MCA-ACA and MCA-PCA) and (F) total number of collaterals was unchanged following pMCAO. (G) A trend towards increased tortuosity of ipsilateral collaterals was also seen at 2-65 days post-pMCAO compared to contralateral. *p<0.05; **p<0.01; ***p<0.001 compared to contralateral at each individual time point; #p<0.05; ##p<0.01; ###p<0.001 compared to 1 day ipsilateral post-pMCAO (n = 5-9 mice/group). Data represented as mean ± SEM.
Fig. 3. **Histological evaluation of pial collateral remodeling.** (A) Identification of pial collaterals using vessel painting (VP, red). Arrows indicate MCA-ACA collaterals at 1 day post-sham in the ipsilateral hemisphere compared to 1d (B), 2d (C) and 4d (D) post-pMCAO. (E-H) Confocal image analysis of VP co-labeled with anti-
SMA showing dynamic changes in SMA expression at 1 day (E) and 4 day (F) post-pMCAO. (G) Representative high magnification images of a stable collateral vessel in the contralateral region compared to a remodeling collateral in the ipsilateral hemisphere (H) at 4 days post-pMCAO. Scale bar=200 µm in E and F; Scale bar= 20 µm in E1-E3, F1-F3 and G-H.

Fig. 4. Analysis of collateral remodeling in vessel painted Cx3Cr1GFP mice after pMCAO. (A) Representative confocal microscopy showing Cx3Cr1GFP-expressing immune-derived cells (green) co-labeled with vessel painting (VP; red) and 4',6-diamidino-2-phenylindole (DAPI; blue) (A1-A3) in the ipsilateral hemisphere of whole mount cortical tissue at 4 days post-pMCAO. Numerous GFP-positive cells were attached to and surrounding the collaterals undergoing remodeling. (B-D) Few to no GFP-positive cells are present on
contralateral collaterals. GFP-positive cells are also observed in the underlying tissue, likely parenchymal microglia. (E-G) High magnification confocal image demonstrating extensive recruitment of GFP-positive cells to the remodeling collateral vessels in the ipsilateral hemisphere. Scale bar= 200µm in A; 50µm in A1-A3, E-G; 20µm in B-D.

Fig. 5. Analysis of cellular proliferation in the pial collateral niche after pMCAO. (A and B) PCNA labeling of proliferating cells in collateral zone of whole mount cortical tissue at 4 days post-pMCAO. Extensive PCNA labeling is seen in the underlying injured cortex, remodeling collaterals and large veins. PCNA expression (green) is present around remodeling collateral vessels in the ipsilateral side (B1-B2) but not on vessels in the contralateral hemisphere (A1-A3). Scale bar = 200µm in A and B; 20µm in A1-A3 and B1-B3. (C-E) Representative high magnification images of a growing ipsilateral collateral at 4d post-pMCAO showing PCNA expression in the vessel painted wall of the collateral. Scale bar = 20µm.
Fig. 6. Comprehensive analysis of behavioral deficits after pMCAO. Comprehensive sensorimotor and cognitive behavioral tasks were quantitatively assessed at 3-35 days post-sham and –pMCAO injury. (A) Quantitative assessment of Neurological Severity Scoring (NSS) post-injury showed sham-injured animals have significantly lower severity score compared to pMCAO mice. (B) Rotarod assessment demonstrated focal ischemia causes significant motor impairment that is sustained from 3 to 35 days after injury. (C) Novel object recognition was also significantly reduced in pMCAO mice at all the time points tested post-injury. (D) Completion
time on the beam walk was also significantly increased following pMCAO, indicating mice required more time to traverse a narrow beam owing to functional impairments. (E) Inverted screen assessment shows no consistent performance difference in sham or pMCAO-injured mice. (F) Assessment of relative strength in injured mice using grip assessment. Analyses indicate relative deficits in strength at each time point and significant difference at 3d, 14d and 28d post-pMCAO compared to sham injury. *P<0.05; **P<0.01 and ***P<0.001 compared to sham controls.
Addendum

Results

EphA4 expression is decreased on remodeling collaterals

To assess EphA4 expression on remodeling collaterals, 1 day stroke-d mice were vessel painted and immuno-labeled with anti-EphA4, as well as smooth muscle actin (SMA). Our results show that in the ipsilateral hemisphere, remodeling collaterals express less EphA4 compared to non-remodeling collateral in the contralateral hemisphere (Fig 1).

Fig 1. EphA4 expression during arteriogenesis. Pial collaterals were labeled by vessel painting (VP) and co-labeled for SMA and EphA4, 1 day post stroke shows reduced EphA4 expression on remodeling collaterals compared to contralateral hemisphere collaterals.
Chapter 5

EphA4 constrains pial collateral remodeling by suppressing endothelial cell-specific pAkt/Tie2 receptor signaling following ischemic stroke
Abstract

Leptomeningeal anastomoses or pial collateral vessels play a critical role in cerebral blood flow (CBF) restoration following occlusion. While the active remodeling response is known to be driven by activation of the endothelium, the molecular mechanism(s) involved remain under investigation. Our previous findings suggest endothelial cell-specific EphA4 receptor tyrosine kinase is a negative regulator of collateral remodeling. Here, we demonstrate using EphA4<sup>fl/fl</sup>/Tie2-Cre conditional knockout mice, that ablation of EC-specific EphA4 significantly enhances pial collateral remodeling and functional recovery following permanent middle cerebral artery occlusion (pMCAO). This correlated with a significant improvement in CBF and reduced infarct volume compared to EphA4<sup>fl/fl</sup> wild type mice. Interestingly, EphA4<sup>fl/fl</sup>/Tie2-Cre mice showed increased levels of phosphorylated (p)-Akt and angiopoietin-2 protein expression at 24hrs post-pMCAO. We further elucidated the cross-talk between EphA4 and angiopoietin-2/Tie2 pathways using soluble Tie2 receptor (sTie2-Fc) following pMCAO. Inhibition of Tie2 signaling ameliorated pial collateral vessel remodeling, neuroprotection and p-Akt activation in EphA4<sup>fl/fl</sup>/Tie2-Cre mice which coincided with an attenuation endothelial cell proliferation in vitro. Lastly, we demonstrate that blocking EphA4, using KYL peptide inhibitor delivered via mini-osmotic pumps, can enhance pial collateral remodeling after pMCAO. Taken together, these findings suggest that EphA4 negatively regulates arteriogenesis by suppressing p-Akt/Tie2 signaling. Therapeutic targeting of EphA4 or Tie2 represents an attractive new strategy for improving collateral function, neural tissue health and functional recovery following stroke.

Introduction

Ischemic stroke occurs following vascular occlusion resulting in significant cerebral blood flow loss to brain regions predominately served by the middle cerebral artery, leading to cell death and neural tissue dysfunction. However, restoration of blood flow can occur through natural adaptations to the vascular network called, leptomeningeal anastomoses or pial collateral vessels, which provide retrograde reperfusion to vulnerable tissue regions. This phenomenon is essential for preventing or reducing the consequences of an ischemic attack, however, the molecular mechanism(s) regulating pial collateral vessel function and remodeling, as well as the patient-specific collateral response remains understudied. Currently, the gold standard for stroke therapy is the
use of tissue plasminogen activator to dissolve the occluding thrombus. Unfortunately, this therapy is not effective in large vessel occlusions (LVO) or when treatment is started 4.5 hours post-ischemic attack(92, 95). Recently, the use of endovascular thrombectomy such as stent retrievers has made it possible to treat patients beyond the 4.5 hour window and provide a therapeutic option for patients with LVOs(77, 79, 80, 279). The advent of endovascular thrombectomy has also been reported to have extended the treatment window up to 24 hours(100). This is thought to be possible in certain patients, due to the perfusion and tissue preservation intervention of pial collateral vessels.

The extent of collateral-mediated reperfusion has a major impact on preserving the penumbra and often dictates stroke outcome(83, 269, 280-282). The neurological severity and tissue infarct has been directed correlated with patient collateral scoring(102, 103). Additionally, several studies have demonstrated that the outcome after thrombolysis and thrombectomy therapy is highly dependent on patient collateral score (104-109). Patients with higher collateral scores also have higher reperfusion rates after rtPA therapy and a lower risk for symptomatic bleeding after reperfusion therapy(110). Pharmacological targeting of the pial collateral network therefore represents a viable therapeutic option to improve outcomes following embolic stroke by sustaining penumbral blood flow and preserving neural tissue health. The mechanism(s) regulating collateral remodeling, however, remain under investigation.

There are three main pial arterial vasculatures in the surface of the brain, namely, the middle cerebral arteries (MCA), anterior cerebral arteries (ACA) and posterior cerebral arteries (PCA), all of which branch off the Circle of Willis. Their distal arteriole ends anastomose to form the MCA-ACA and MCA-PCA collateral vessels and are important after vascular occlusion(283). Collateral arterioles do not show a proximal-to-distal axis with respect to the blood flow, as in large arteries, rather they connect two arterial branches with opposing flow. Therefore, collateral blood flow is bi-directional along the collateral vessel wall(224). Following an obstruction, collateral vessels undergo immense vascular restructuring and remodeling (enlargement), also called arteriogenesis, to allow for uni-directional retrograde blood reperfusion into the area of an occluded arterial branch(224, 239). Although the benefits of targeting the collateral system are evident, further research is needed to elucidate the mechanism(s) underlying this remarkable tissue saving adaptation. It is known that collateral remodeling is initiated when mechanoreceptors on endothelial cells detect fluid shear stress after a vascular
occlusion(284-286). Thus the endothelial cells lining the collateral vessels are crucial to the remodeling process. Recently, we implicated the Eph receptor tyrosine kinase family in collateral formation and remodeling(230). We demonstrated that endothelial cell knockout of EphA4 receptor increased collateral remodeling, reduced hindlimb ischemia and improved blood flow following femoral artery ligation. The Eph/ephrin family plays an integral role in embryonic neural development (REF). Here we discovered a novel role for EphA4 in the negative regulation of the pial collateral arteriogenic response by suppressing Tie2/pAkt signaling in a murine model of pMCAO. These findings highlight an attractive new target for improving collateral function in patients with cerebral vascular obstruction.

**Materials and Methods**

**Animals**

All rodents were bred and housed in an AAALAC accredited, virus/antigen-free facility with a 12 h light-dark cycle; food and water were provided *ad libitum*. EphA4f/f and Tie2-Cre mice (#012916 and #008863, respectively, Jackson Laboratory, Bar Harbor, ME) were backcrossed on the CD1 background and bred until the desired experimental animals were generated. All procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals, the Virginia Tech Institutional Animal Care and Use Committee (IACUC; #15-063; #18-088) and the Virginia Maryland Regional College of Veterinary Medicine. All mice were coded for double blinded experimentation.

**Surgical Procedures and treatments.**

Ischemic stroke was induced by pMCAO as previously described (287). Briefly, 8-12 week-old mice were injected with analgesic Buprenorphine-SR (0.15 mg/kg, ZooPharm,Laramie, WY) followed by induction of anesthesia using 2% isoflurane-30% oxygen. Mouse body temperature was monitored and regulated with a rectal probe and maintained at 37±0.5 °C with a heating pad (homeothermic blanket system; Harvard Apparatus). An incision was made and skull thinned to expose and cauterize the main and two distal branches of the left MCA. Sham controls received identical procedures without ligation. The incision was closed using Vetbond (3M, St. Paul, MN, USA) and mice were returned to their home cage on a heating pad until recovery. For Tie2-Fc study,
immediately following pMCAO, mice were administered either 5 mg/kg of soluble Tie2-Fc or 2.5 mg/kg soluble human Fc-control via tail vein injection then euthanized at 24hrs. EphA4 blocking peptide, **KYLPYWPVLSSL** was administered subcutaneously via mini-osmotic pumps (Alzet, Inc) at 10mg/ml/day or vehicle immediately following pMCAO. Mice were euthanized by vessel painting at 4 days post-pMCAO.

**Vessel Painting and collateral quantification**

Vessel painting was performed as previously described (230, 251). Briefly, mice were initially administered heparin (2,000 units/kg), and sodium nitroprusside (SNP, 0.75 mg/kg) five minutes before sacrificing mice. Next, the chest cavity was opened and then cardiac perfused using a Gilson MiniPuls3 peristaltic perfusion pump (Gilson Scientific, Bedfordshire, UK). 10 ml of 1X phosphate buffered saline (1X PBS) containing 20 units/ml heparin was continuously perfused to flush blood from the vascular system, then 10 ml DiI (0.01 mg/ml, Invitrogen) 4% sucrose-PBS-heparin mixture was perfused to label the arteriole vasculature using a flow rate of 2 ml/min followed by 50 ml of 4% cold paraformaldehyde (PFA). Fixed brains were imaged on a fluorescence microscope (BX-51, Olympus America), using mosaic tile imaging from StereoInvestigator software (MBF, Williston, VT). Scaled mosaic images were imported into ImageJ (NIH) for quantification of the number and diameter of inter-collaterals between the MCA, ACA, and PCA branches as described (287).

**Infarct volume**

Fresh frozen brains were embedded in OCT, snapped frozen and serial cryo-sectioned. Infarct volume (mm³) was assessed by using the Cavelieri Estimator from non-biased StereoInvestigator software (MicroBrightField, Williston, VT, USA) and an upright Olympus BX51TRF motorized microscope (Olympus America, Center Valley, PA, USA) as previously described (288). Serial coronal sections were then stained with 0.2% cresyl violet solution (Electron microscopy science, Hatfield, PA) and the infarcted area was identified by loss of Nissl staining and pyknotic neurons. Volume analysis was performed by estimating the area of tissue loss in the ipsilateral cortical hemisphere using six, 30µm serial coronal sections. A 100 µm spaced grid was placed over the ipsilateral hemisphere in the Cavalieri probe and infarcted area scored.
**Immunohistochemistry of cortical whole mounts**

After perfusion, the surface of the brain cortex was dissected and placed in 1X PBS overnight for whole mount staining as previously described(287). Briefly, whole mounts were blocked in 2% Fish gelatin (Sigma Aldrich, St. Louis, MO) with 0.1% Triton X-100 and incubated overnight in mouse anti-smooth muscle actin (SMA) (1:1000; Abcam, Cambridge, UK; ab7817) at 4°C. Whole mounts were washed then incubated with anti-mouse Alexa Fluor 488 conjugated secondary antibody (ThermoFisher, Waltham, MA) then counterstained with DAPI (ThermoFisher Scientific, Waltham, MA) then imaged on Zeiss 880 confocal microscope (Carl-Zeiss, Oberkochen, Germany). For PCNA staining, whole mounts were incubated in 2N HCl with 0.1% Triton X-100 for 1 hour at 37°C and neutralized with sodium borate as previously described(256). For imaging, the uninjured contralateral hemisphere was used as control.

**Western Blot analysis.**

Ipsilateral cortical tissue was homogenized in RIPA buffer (Tris-base 50 mM, NaCl 150 mM, EDTA 1 mM, NP-40 1%, Sodium deoxycholate 0.25%, NaF 20 mM, 1 mM Na3VO4 1 mM, β-glycerophosphate 10 mM, Azide 0.02%) with the Roche Proteinase Inhibitor Cocktail (Catalog # 25178600, Indianapolis, IN) and Thermo Fisher Scientific Pierce™ Phosphatase Inhibitors (Catalog # 88667, Waltham, MA). Protein was quantified by Lowry method (DC Protein Assay Kit, catalog # 500-0116, Bio-Rad, Hercules, CA) then 100µg protein was reduced in 4X sample buffer with β-mercaptoethanol then separated by 10% SDS-PAGE and blotted on to Bio-Rad Laboratories Immobilon™ PVDF membrane (Catalog # 162-0177, Hercules, CA). Membranes were blocked in TBS/0.1% Tween20 (TBST)/5% bovine serum albumin (BSA) then incubated in the following primary antibodies, phospho-Akt, Cat # 4051, Cell Signaling Technology, Danvers, MA; pan Akt, Cat # 4691, Cell Signaling Technology, Danvers, MA; Angiopoietin 1, Cat # AF923, R&D Systems, Minneapolis, MN, and Angiopoietin 2; ab155106, Abcam, Cambridge, MA) in blocking solution overnight, washed 4x with TBST, and incubated with secondary antibodies (anti-rabbit IgG Dylight™ conjugate 680 or anti-mouse IgG Dylight™ conjugate 800; Cell Signaling Technology, Danvers, MA) then imaged using LI-COR Odyssey Imaging Systems (LI-COR, Inc), and band intensities were quantified by using LI-COR’s Image Studio software.
Behavioral Testing.

Rotarod. The Rotarod test (Rotamex, Columbus Inst) to assess motor function as previously reported (257, 258). Briefly, animals were pre-trained for 4 consecutive days prior to pMCAO or sham injury at 10 rpm and an acceleration of 0.1 rpm per second. A baseline was collected on the fourth day, then again at 3 days, 7 days, and 14 days post-injury, for each mouse. Data is graphed as the mean of the individual scores relative to baseline.

Neurological severity scoring (NSS). The NSS was used to assess sensorimotor deficits pre-injury and at 3-14 days post-injury. NSS is a composite of motor, sensory (visual, tactile and proprioceptive), reflex, and balance tests. Function was graded on a scale of 0 to 14 (normal = 0; maximal deficit = 14), where 1 point is awarded for the inability to perform the task or for the absence of the tested activity as previously described (260).

Novel object recognition (NOR). Cognitive and spatial deficits were tested using the novel object recognition task. Briefly, mice were introduced to two identical objects on day one and then one of the objects was replaced with a new object the following day (test day) in a 40 cm³ arena. Time of exploration of the old and new object was recorded over 5 minutes. Preference of object was calculated as a ratio exploration time of the specific object to the total time of exploration of both objects. NOR was also administered at 3-14 days post-injury.

Cerebral blood flow

Cerebral blood flow (CBF) was measured at pre-, 5 minutes and 1-4 day post-injury using the Moor LDI2-HIR Laser Doppler and Moor Software Version 5.3 (Moor Instruments, Wilmington, DE) as previously described(287). Briefly, mice were anesthetized with 2% isoflurane-30% O₂, then secured in a stereotactic device. Rectal temperature was maintained at 37.0 ± 0.5°C and cerebral blood flow, in perfusion units (PFU) was scanned using a 2.5cm x 2.5cm scanning area. Tissue perfusion was quantified with a region of interest (ROI) define in the same left hemisphere of injury and represented as a ratio of post-perfusion units (PFU) relative to the pre-injury scan.

Statistical analysis. Data was graphed using GraphPad Prism, version 7 (GraphPad Software, Inc., San Diego, CA). Student’s two-tailed t test was used for comparison of two experimental groups. Multiple comparisons were done using one-way or two-way ANOVA and repeated measures where appropriate followed by a post hoc Sidak test. Changes were identified as significant at P value < 0.05. Mean values were reported
together with the standard error of mean (SEM). All quantifications were performed by an experimenter blinded to the conditions.

Results

Genetic deletion of EC-specific EphA4 improves CBF, neural tissue and behavioral recovery following pMCAO

We recently established EphA4 as a novel negative regulator endothelial cell (EC) growth, p-Akt signaling and hindlimb collateral remodeling after FAL(230). Here we determine whether EphA4 plays a role in pial collateral remodeling and functional recovery following permanent middle cerebral artery occlusion (pMCAO). Using EphA4floxed/Tie2-Cre conditional knockout (KO) mice we evaluated changes in cerebral blood flow (CBF) following acute ischemic stroke and subsequent outcomes compared to EphA4floxed wild type (WT) mice. CBF was measured by Laser Doppler prior to and at 5 minutes (m), 1-4 days (d) post-pMCAO in the ipsilateral hemisphere and the perfusion units quantified and represented relative to baseline pre-injury CBF (Fig. 1A-1B). No significant difference in CBF was observed at 5m post-pMCAO between WT and KO mice (relative PFUs: 0.532 ± 0.026 vs 0.502 ± 0.034). However, we observed a significantly increase in CBF at 1d (0.87 ± 0.05 vs 0.69 ± 0.05), 2d (0.94 ± 0.06 vs 0.73 ± 0.05), 3d (0.95 ± 0.05 vs 0.79 ± 0.06) and 4d (0.97 ± 0.04 vs 0.82 ± 0.04) in KO compared to WT mice. These findings correlated with reduced infarct volume in KO mice (15.57 ± 3.26 mm³) compared to WT mice (26.77 ± 3.13 mm³) at 4d post-pMCAO (Fig. 1C-1E). Likewise, we found KO mice showed improvements in behavioral recovery. Rotarod assessment demonstrated a significant increase in motor function in KO compared to WT mice at 7d and increased trend at 3d and 14d post-pMCAO (Fig. 1F). While increased neurological severity scoring was observed following p-MCAO, no significant difference was found between WT and KO mice (Fig. 1G). However, assessment using NOR showed p-MCAO reduced the novel object preference index in WT but not KO mice at 3d (62.75 ± 1.08 vs 45.58 ± 2.61), 7d (63.81 ± 2.57 vs 50.01 ± 1.68) and 14 day (69.42 ± 3.15 vs 50.16 ± 0.87) (Fig. 1H). These findings demonstrate EC-specific EphA4 is novel mediator of functional deficits and neural tissue damage following pMCAO.

Genetic deletion of EC-specific EphA4 enhances pial collateral remodeling following pMCAO
To evaluate whether improvements in CBF and behavioral recovery coincided with changes in pial collateral remodeling, we performed vessel painting at 1d and 4d post-pMCAO on EphA4<sup>f/f</sup> and EphA4<sup>f/f</sup>/Tie2-Cre mice. While we observed an increase in ipsilateral compared to contralateral pial collateral diameter in WT (Fig. 2A and 2B) and KO (Fig. 2C and 2D) mice, EC-specific EphA4 ablation significantly enhanced remodeling of MCA-ACA collaterals at 1d (KO 41.08 ± 2.16µm vs WT 29.59 ± 1.79µm) and 4d (KO 53.29 ± 2.39 µm vs WT 39.03 ± 1.84µm) post-pMCAO (Fig. 2E). Likewise, MCA-PCA showed increased collateral diameter in KO compared to WT at 1d (37.52 ± 1.88 vs 26.90 ± 1.20 µm, respectively) and 4d (50.43 ± 2.83 µm vs 37.72 ± 2.15µm, respectively) (Fig. 2F). Total inter-collaterals combined further show KO mice have increased collateral diameter compared to WT at 1d (39.30 ± 1.45µm vs 28.31 ± 1.11µm, respectively) and 4d (51.75 ± 1.85µm vs 38.56 ± 1.36µm, respectively) (Fig. 2G). No significant difference was observed in contralateral collaterals between groups or in total number of inter-collaterals (Fig. 2H) post-pMCAO. Moreover, breakdown of collateral size shows the largest increase in collateral diameter occurred in the ipsilateral KO mice where 85% of collaterals were greater than 31µm at 1d and 91% at 4d post-pMCAO compared to only 10-15% on the contralateral side. Conversely, 40% of the WT ipsilateral collaterals were greater than 31µm at 1d and 75% at 4d. Additionally, a greater number of KO collaterals were larger than 50µm, 52% compared to 18% in WT mice (Fig. 2I and 2J). These findings demonstrate EphA4 restricts collateral remodeling and the loss of EC-specific EphA4 can accelerate arteriogenesis as early as 24hrs post-pMCAO.

**Evidence of early cellular remodeling in EphA4<sup>f/f</sup>/Tie2-Cre vessel painted collaterals**

We recently demonstrated that EphA4 suppresses EC proliferation in vitro<sup>28</sup> and the pial collateral niche undergoes active cellular remodeling, including proliferation and smooth muscle cell reorganization, following pMCAO using vessel painting and immunohistochemistry<sup>35</sup>. To address whether the loss of EC-specific EphA4 improved early remodeling of pial vessels by enhancing EC growth properties, we performed immunolabeling for cell division marker, PCNA on wild type and KO cortical tissue whole mounts at 1d post-pMCAO and vessel painting. Confocal image analysis shows KO mice (Fig. 3A-3C) displayed increased cell division in the pial collateral wall compared to WT (Fig. 3D, 3E). These findings correlate with increased collateral diameter and suggest EphA4 limits early remodeling, in part, by suppressing EC proliferation in the collateral niche.
**Endothelial cell loss of EphA4 increases p-AKT and Angiopoietin-2 cortical expression after pMCAO**

Previously we found increased p-Akt and angiopoietin expression in EphA4-null endothelial cells which coincided with increased proliferation compared to WT cells in vitro(230). To test whether the loss of EC-specific EphA4 could influence these pathways after stroke, we assessed the protein lysates derived from the ipsilateral cortex at 1d and 4d post-pMCAO in EphA4<sup>f/f</sup> and EphA4<sup>f/f</sup>/Tie2-Cre mice. Using western blot analysis, we observed a significant decrease in the expression of p-Akt in WT pMCAO-injured cortices compared to WT sham (0.06 ± 0.03 vs 0.27 ± 0.05, relative to total Akt, respectively), however the level of p-Akt was maintained in the KO injured cortex compared to sham at 1d (0.23 ± 0.02 vs 0.38 ± 0.14, relative to total Akt, respectively) (Fig. 4A and 4B). No significant change was seen at 4d post-pMCAO (Fig. 4C and 4D). We also evaluated the expression of angiopoietin-1 and -2 at 1d post-pMCAO and found a significant reduction in angiopoietin-2 in the WT injured cortex compared to sham (0.61 ± 0.10 vs 1.35 ± 0.13, respectively) that was not observed in KO mice (1.19 ± 0.17 vs 1.32 ± 0.10, respectively) (Fig. 4E and 4F). Angiopoietin-1 showed no significant difference between samples (Supplementary Fig 1). These data reveal EphA4 as a novel early suppressor of angiopoietin/Tie2 signaling which could impact collateral function following pMCAO.

**Blocking Tie2 receptor prevents collateral remodeling, p-Akt expression and neuroprotection in EphA4<sup>f/f</sup>/Tie2-Cre mice**

To investigate whether the genetic deletion of EC-specific EphA4 enhances collateral remodeling, p-Akt expression and neuroprotection via Tie2 receptor signaling, we administered 5mg/kg/day soluble Tie2-Fc or human Fc-control via tail vein injection immediately after pMCAO in EphA4<sup>f/f</sup> and EphA4<sup>f/f</sup>/Tie2-Cre mice. We observed a significant attenuation of ipsilateral collateral remodeling in KO mice receiving soluble Tie2-Fc (31.84 ± 1.06µm) (Fig. 5B and 5C) compared to Fc-control (41.00 ± 4.80µm) (Fig. 5A and 5C) at 1d post-pMCAO. No effect on collateral diameter was found in the ipsilateral hemisphere of WT mice (Fig. 5C). Similar changes were seen when comparing MCA-ACA (Fig. 5D) and MCA-PCA (Fig. 5E). These findings coincided with increased infarct volume in KO mice receiving Tie2-Fc (24.93 ± 3.60mm<sup>3</sup>) compared Fc-control (12.16 ± 3.52mm<sup>3</sup>) (Fig. 5F and 5G). Furthermore, blocking Tie2 receptor signaling suppressed p-Akt expression in the cortex of KO mice.
treated with soluble Tie2-Fc (0.05 ± 0.03, relative to total Akt) compared to Fc-control (0.17 ± 0.02, relative to total Akt) (Fig. 5H and 5I). These data demonstrate endothelial-derived EphA4 prevents collateral remodeling and tissue recovery by restricting Tie2/p-Akt signaling following pMCAO.

Additional cellular analysis was performed on WT and KO brain-derived endothelial cells in vitro, as demonstrated by our lab(230), we found KO ECs display increased bromodeoxyuridine (BrdU) incorporation compared to WT (Fig. 6), which was significantly ameliorated after 24hrs in the presence of 20µg/ml soluble Tie2-Fc (Fig. 6D and 6E) compared to Fc-control (Fig. 6C and 6E). No changes were observed in WT ECs exposed to Tie2-Fc compared to Fc-control (Fig. 6A, 6B and 6E). Lastly, we observed a dose-dependent response following treatment of KO ECs with 2, 10 and 20µg/ml soluble Tie2-Fc (Fig. 6F-6I). This suggests EphA4 suppresses EC growth by limiting Tie2 receptor signaling in vitro.

**Pharmacological inhibition of EphA4 increases collateral remodeling after pMCAO**

Lastly, we evaluated whether pharmacological inhibition of EphA4 could recapitulate the enhanced collateral remodeling seen in EphA4f/f/Tie2-Cre mice. To test this, we utilized mini-osmotic pump infusion of a recently described peptide inhibitor of EphA4, KYLPYWPVLSSL (KYL)(289, 290). At 4d post-pMCAO, we observed a significant increase in the ipsilateral pial collateral diameter in KYL-treated mice (Fig. 7E) compared to vehicle control (Fig. 7D). This effect was observed in the MCA-ACA (50.61 ± 1.55µm KYL vs 42.35 ± 2.67µm vehicle), MCA-PCA (49.15 ± 2.99µm KYL vs 35.90 ± 3.22µm vehicle) and combined (49.88 ± 1.64µm KYL vs 39.13 ± 2.19µm vehicle) arteriole branches (Fig. 7A-7C). A breakdown of collateral size showed a significant increase in the percentage of collaterals greater than 50µm compared to vehicle (47% vs 21%, respectively) (Fig. 7F). These findings are similar to the effects seen in the EphA4f/f/Tie2-Cre mice and suggests EphA4 may be a key pharmacological target therapeutic enhancement of collateral remodeling after stroke.

**Discussion**

Since their discovery by Heubner in 1874, leptomeningeal anastomoses have been implicated as critical determinants of injury severity, however the cellular and molecular underpinnings of the arteriogenic response remain under investigation following ischemic stroke. Findings from the current study describe a novel
mechanism that restricts outward growth and remodeling. The presence of EphA4 on the endothelium acts a negative cue within the collateral niche to restrict Tie2 receptor activation and function via the p-Akt pathway. Loss of EC-specific EphA4 increased p-Akt signaling, collateral remodeling, functional recovery and tissue protection which was ameliorated in the presence of soluble Tie2 inhibitor. Our results are the first to implicate the angiopoietin/Tie2 axis as a critical regulator of pial collateral remodeling following stroke. Finally, we show that inhibition of EphA4 using the KYL peptide inhibitor mimicked the enhanced collateral response we observed in EphA4\textsuperscript{f/f}/Tie2-Cre mice. Overall, these findings highlight EphA4 and the angiopoietin/Tie2 axis as an important target for collateral therapeutics.

The extent of collateralization and function dictates retrograde reperfusion and preservation of blood flow to vulnerable regions of the brain affected by vessel obstruction. Several animal models, including mice, rats, guinea pigs, rabbits and dogs have been used to elucidate changes in this remarkable process\cite{6, 12-14, 255}. Fluid shear stress activates a cascade of events which leads to collateral remodeling\cite{284-286}. Central to this process is the endothelial cell response \cite{9}. The endothelium release cytokines, etc which lead to the recruitment of immune cells, induction of endothelial cell proliferation and smooth muscle cell reorganization within the collateral niche\cite{5, 6, 11}. Our findings further demonstrate this in the brain using our newly developed vessel painting histological method which provides detailed cellular structural analysis of the collateral niche\cite{287}. Following pMCAO, we find substantial changes including cell division in the vessel wall and smooth muscle cell reorganization as early as 24hrs, which is augmented in the EphA4\textsuperscript{f/f}/Tie2-Cre mice. This suggests EphA4 on the endothelium or 'first responder' is an early negative regulator of cellular changes involved in mediating the arteriogenic response.

Eph receptor tyrosine kinases are widely known to control cell migration, proliferation and survival in the CNS \cite{25, 26, 28, 29}. Both Eph receptors and their cognate ligands, ephrins, are membrane bound and require direct cell-cell contact to initiate signal transduction. Although ephrin/Eph molecules play critical roles in numerous biological processes\cite{35, 291, 292}, the role of Eph/Ephrin signaling in cerebral arteriogenesis has not been investigated. Previous studies have demonstrated ephrinB2 expression, an arterial-specific marker involved in arteriovenous specification\cite{291, 293, 294}, is induced following cyclic stretch and limits the migration of smooth muscle cells and transmigration of monocytes \textit{in vitro}\cite{138}. EphA4 displays remarkable ligand binding
promiscuity and its binding complex with ephrinB2 has been described(295). While we cannot rule out associations with other A- or B-class ligands, it is plausible that EphA4-ephrinB2 interaction are induced on collateral arterioles following MCA occlusion and that this region-specific partnership acts to prevent outward growth and remodeling. EphrinB2 is located at the luminal and junctional endothelial cell surface where it associates with CD31(296). Korff T, et. al., demonstrated that quiescent smooth muscle-contacting ECs show uniform luminal expression of ephrinB2, which translocates to inter-endothelial cell junctions in a context-dependent manner. If this could occur under shear conditions within the collateral vessel wall, then association with and activation of EC-specific EphA4 forward receptor signaling may result in Tie2/p-AKT pathway suppression during remodeling. Given that ephrinB2 reverse signaling does not mediate EC activities(297) and our in vitro findings show clustered EphA4-Fc treatment does not attenuate enhanced EC proliferation in EphA4-null ECs (data not shown), we conclude that EphA4 forward signaling mediates collateral growth restriction. Although further studies evaluating ephrinB2−/− or ephrinB2LacZ mice could provide additional insight.

Our findings are the first to implicate EphA4-Tie2 receptor crosstalk in pial collateral remodeling. Angiopoitins (angpt), ligands of the Tie2 receptor, are known regulators of arteriogenesis which have been shown to affect blood flow recovery in ischemia(204, 298, 299). Although previous studies demonstrate these effects in non-CNS ischemic conditions, no studies have been conducted to assess the functional relevance of Tie2 signaling in the cerebrovascular collateral network after stroke. Our studies revealed a significant reduction in angpt-2 and no change in angiopoietin-1 protein expression in the ipsilateral cortex of WT mice at 1d post-pMCAO. Angpt-2 levels were maintained in KO mice alongside increased collateral growth suggesting heightened Tie2 activation in the absence of EC-specific EphA4 can accelerate collateral remodeling at this early time point. Indeed, blockade of the Tie2 receptor restored the size of KO pial collaterals to WT levels while having no effect in WT mice at 1d post-pMCAO. Although we did not evaluate angpt-2 after 4 days, we postulate the levels in WT mice would return to normal, similar to the p-Akt expression, concomitant with an increase in collateral size that matched our KO mice at 1d post-pMCAO. These findings reveal pMCAO induces an acute downregulation of angpt-2 expression that is mediated by EphA4 signaling and prevents early induction of pial collateral remodeling. Given the importance of collateral function in maintaining blood flow to the vulnerable
penumbra region prior to thrombectomy(300), strategies that target the EphA4/angpt-2 discord to accelerate this process within the first 24hrs may help alleviate neural tissue damage and dysfunction.

The current study expands our knowledge of the wide net that Eph receptors cast on cellular function and provides key mechanistic insight into the growth constraints that limit the unique adaptive response of cerebral collateral vessels. To date, it remains unclear why some patients display high collateral function while others do not. Understanding the mechanism(s) that regulate and importantly restrict this process will be crucial for devising approaches to predict collateral function while improving collateral health and neurological outcome in patients with ischemic stroke.
Fig 1. Cerebral blood flow (CBF) and infarct volume post-pMCAO. (A) Laser Doppler images pre- and post-pMCAO. Panel shows a representative image from EphA4^{fl/fl} wild type (WT) and EphA4^{fl/fl}/Tie2::Cre knockout (KO) mice pre and post pMCAO. (B) Quantified analysis shows increased CBF in KO compared to WT mice at 1-4 day post-stroke. (C) Representative NISSL images of WT mice at 1 day post-pMCAO at same bregma levels. (D) Representative NISSL images of KO mice at 1 day post-pMCAO. (E) Quantified infarct volume shows a significant reduction in infarct volume in KO mice compared to WT mice. (F) Rotarod assessment of WT and KO mice. Data was analyzed as two-way ANOVA with Tukey post test. KO mice performed significantly better than WT mice 7 days after stroke. (G) Neurological severity score of experimental mice, analyzed as two-way ANOVA with Bonferroni post test. There is no performance difference between WT and KO mice. But there was significance difference when shams were compared to pMCAO in both WT and KO mice. (H) Novel object recognition test analyzed as two-way ANOVA with Bonferroni post test. Stroke KO mice performed better at all
time points when compared to WT stroke mice. Furthermore, WT pMCAO mice performed significantly worse compared to their sham counterparts. *P<0.05; *P<0.01, ***P<0.001; ****P<0.0001 compared to corresponding WT mice.

Fig 2. Collateral remodeling post-pMCAO. After pMCAO, MCA-ACA and MCA-PCA collaterals remodel to allow for retrograde reperfusion. (A – B) show a vessel painted WT brain 1 day after pMCAO. Arrows point to
remodeling pial collaterals in the ipsilateral hemisphere. (C – D) KO vessel painted brain 1 day post pMCAO. Arrows indicate remodeling collaterals in the ipsilateral hemisphere. (E) MCA – ACA collaterals diameter analyses at both 1- and 4-days post pMCAO. At 1 day and 4 day post pMCAO, KO mice have significantly larger collateral diameters compared to WT mice (*). Ipsilateral collaterals are larger in diameter compared to contralateral collaterals at both time points (#). Additionally, 4-day ipsilateral collaterals are significantly larger than 1 day post ($). (F) MCA – PCA collateral analyses. KO collaterals are larger at 1 day and 4 days post stroke in ipsilateral hemisphere (*). Additionally, at both time points, ipsilateral collateral are larger than contralateral collaterals in both WT and KO mice (#). Analyses also shows that MCA – PCA collaterals are significantly larger at 4 days compared to 1 day post pMCAO ($). (G) Average inter-collateral analyses. At 1 day and 4 day post pMCAO, KO mice have significantly larger collateral diameters compared to WT mice (*). All ipsilateral collateral are larger in diameter compared to contralateral collaterals (#). Additionally, 4-day ipsilateral collaterals are significantly larger than 1 day post ($). (H) Total inter-collateral counts show no significant difference between time points, and also when ipsilateral and contralateral hemispheres are compared. Breakdown of collateral sizes 1 day post (I) and 4 days post pMCAO (J). One way ANOVA with Bonferroni post test was used to analyze all data. *P<0.05; *P<0.01, ***P<0.001; ****P<0.0001.
**Fig 3. Cell proliferation during collateral remodeling.** Vessel painted brains were immuno-labeled with PCNA to assess cell division on remodeling collaterals. (A) Vessel painted ipsilateral KO brain co-labeled with PCNA. (B) PCNA expression on remodeling collaterals in ipsilateral hemisphere of KO mouse and (C) is the KO vessel painted pial collateral. (D) shows PCNA expression on a WT remodeling collateral. (E) shows the vessel painted WT.

**Fig 4. Protein expression post pMCAO.** (A) Western blot analysis shows 1 day post pMCAO. (B) Densitometric analysis shows a significant increase of p-Akt in KO mice protein lysates compared to WT mice. (C) Western blot analysis from ipsilateral hemisphere lysates 4 days post pMCAO. (D) There was no significant difference in p-Akt expression at this time point. (E) Angiopoietin-2 (Ang-2) western blot analysis. (F) 1 day post stroke, there is significantly elevated Ang-2 protein in KO mice lysates compared to WT mice. One way ANOVA with Bonferroni post test was used to analyze all data. *P<0.05; *P<0.01, ***P<0.001; ****P<0.0001.
**Fig 5. Tie2 inhibition.** KO mice were treated with soluble Tie2Fc or Fc control immediately following pMCAO. (A - B) Ipsilateral hemisphere of vessel painted pMCAO KO mice after treatment with Fc or TieFc. (C) Quantitative analysis shows that 1 day post stroke, KO mice treated with Tie2Fc have significantly reduced average collateral diameters compared to Fc treated. Also, WT mice did not show a significant difference in collateral diameter when treated with Tie2Fc after pMCAO. (D) MCA – ACA collateral diameters after Tie2Fc treatment. (E) MCA – PCA collateral diameters. (F) Representatively Nissl stains brains of both Fc and Tie2Fc
brains after 1 day pMCAO, demarcating the area of infarct. (G) Statistical analysis shows that Tie2Fc treated KO mice have a significantly larger infarct volume compared to Fc treated mice. (H) Western blot run from ipsilateral brain lysates of both Fc and Tie2Fc treated KO mice after pMCAO. (I) Analysis shows that Tie2Fc treated KO mice have significantly reduced p-Akt expression compared to Fc treated mice.

**Fig 6. Soluble Tie2Fc inhibits proliferation of brain endothelial cells.** Seeded WT and KO brain endothelial cells (EC) were treated with either soluble Tie2Fc or Fc control antibody. (A - D) Images are a representation of WT and KO BrdU assay 24-hours after treatment. (E) Analysis shows that KO ECs treated with Tie2Fc significantly decrease in BrdU positive cells, while there is no significant difference when WT cells are treated with Tie2Fc. (F - H) shows dose dependent effect of soluble Tie2Fc on KO EC BrdU positive cells. (I) Analysis shows that there is a significant reduction in BrdU positive cells with 20 µg/mL of Tie2Fc.
Fig 7. Pharmacological EphA4 inhibition. Osmotic pumps were filled with KYL peptide. WT mice were stroke and pumps were immediately inserted at the neck region of mice to continuously administered KYL to brain surface. Brains were analyzed 4 days post pMCAO (A) Statistical analysis of MCA-ACA collaterals. Ipsilateral collaterals of WT mice treated with KYL significantly increased in diameter compared to saline treated (*). Both saline and KYL ipsilateral collaterals are significantly larger than contralateral collaterals (#). (B) Analysis of MCA – PCA collaterals. Ipsilateral collaterals of animals treated with KYL are significantly larger compared to saline ipsilateral collaterals (*). Ipsilateral collateral diameters are also larger compared to the same brains contralateral collaterals (#). (C) Graph depicts the average inter-collateral diameter post stroke. There is a significantly increase in ipsilateral collateral diameter of KYL treated mice compared to saline treated (*). (D) WT Ipsilateral hemisphere, vessel painted brain 4 days post stroke with saline treatment. (E) WT Ipsilateral hemisphere, vessel painted brain 4 days post stroke with KYL peptide treatment. (F) Breakdown of collateral sizes after pMCAO. In the ipsilateral hemisphere, there is a significant increase in collaterals with diameters
greater than 51 µm, and precipitous drop in collaterals between 21 – 30 µm. One way ANOVA with Bonferroni post test was used to analyze all data. *P<0.05; *P<0.01, ****P<0.0001.
Chapter 6

Summary and Conclusion
Summary and future direction

Pial collaterals and their ability to remodel is critical post stroke as it determines severity of stroke and neurological outcome. This is because blood is rerouted to the blood-deprived region of the brain through the remodeling collaterals, and by so doing preserves the penumbra and prevents further neuronal cell death. The endothelial cells lining the collateral vessel are central to and initiate collateral remodeling. Our preliminary data implicated EphA4 in collateral development and remodeling. Thus, in this dissertation, the objective was to elucidate the endothelial cell autonomous role of EphA4 on collateral development, and collateral remodeling after stroke. In chapter 3 we show that endothelial cell-specific EphA4 is a negative regulator of collateral formations during development. Specifically, ablation of EphA4 on collaterals resulted in increased pial collateral formation and EphA4 expression on collaterals gradually decreased through development. Investigation of cultured brain endothelial cells showed that knocking out EphA4 also increased proliferation and cell migration via phospho-Akt. With EphA4 affecting collateral development, we initially assessed its effect on collateral remodeling after hind limb ischemia. The EphA4 knockout mice had increased blood reperfusion after ischemia leading us to postulate that endothelial cell specific EphA4 plays a role in arteriogenesis or collateral remodeling. The goal thereof was to assess EphA4 mediated pial collateral remodeling after stroke.

Our preliminary data in the introduction (chapter 1) showed that global EphA4 knockout mice had better neuroprotection after induced stroke in mice. Thus, to determine the endothelial cell autonomous role of EphA4 on pial collaterals we first assessed their remodeling in CD1 mice (reported in Chapter 4). Faber and colleagues had shown that different mouse strains had differing collateral numbers, with C57BL/6 mice having the most and BALB/c’s having the least. Our assessment confirmed that CD1 mice, an outbred strain, had moderate collateral numbers. Furthermore, our work was the first to show that CD1 collateral remodeling peaked between 4 – 7 days post injury. Lastly, we assessed EphA4 expression on remodeling collaterals after stroke. We noticed that larger remodeling collaterals has less EphA4 expression. Of note, we adopted the permanent middle cerebral ischemia model (pMCAO) in this work, as well as this dissertation, because it induced a large enough injury to be able to clearly show the effect of collateral remodeling post injury.

With the discoveries in chapters 3 and 4, we elucidated the cell autonomous role of endothelial cell EphA4 on collateral remodeling. The rationale was that if larger remodeling collaterals expressed less EphA4, then
perhaps EphA4 is a negative regulator of arteriogenesis. Our studies using our transgenic mice showed that (1) EphA4 restricted collateral remodeling post pMCAO as KO mice had increased collateral diameters compared to WT mice (2) EphA4 knockout mice had better tissue protection after injury as well as better neurological behavioral recovery and (3) protein assessment showed increased angiopoietin 2 levels in the ipsilateral hemisphere in KO mice.

To determine whether there is a cross-talk between the Tie2/Ang-2 axis and EphA4, in mediating collateral remodeling, we first isolated brain endothelial cells and treated them with a Tie2 receptor inhibitor. Our data showed that blocking Tie2 decreased the proliferation capacity in KO endothelial cells. This led us to postulate that Tie2 inhibition in vivo would decrease collateral remodeling in KO mice. In vivo studies confirmed our hypothesis. KO mice treated with Tie2 inhibitor has significantly reduced collateral diameters similar to WT mice. In one of our final studies, we were able to recapitulate the collateral remodeling capacity of KO mice in WT mice by administering KYL peptide, an EphA4 antagonist. So, in conclusion, endothelial cell specific EphA4 restricts pial collateral remodeling by mediating Tie2/Ang-2 cellular pathway activation.

The discovery that EphA4 mediates arteriogenesis through the Tie2/Ang-2 pathway is intriguing and one that could be further investigated. For one, Ang-2 was recently shown to play a role in smooth muscle cell recruitment. Also the Ang-2 pathway cross-talks with the NF-κB pathway in regulating immune cell recruitment and adhesion. Both of these cell groups are critical in collateral remodeling. Thus, further research would shed more light on how endothelial cell specific EphA4 regulates collateral remodeling and even inflammation post pMCAO.
Figure 1 Summary of findings. EphA4 receptor ablation on endothelial cells leads to increased pial collateral formations during development. Furthermore, after stroke more collateral remodeling is accelerated in transgenic mice leading to increased blood reperfusion, reduced neuronal cell death and better behavioral recovery.

Conclusion

Up until 2005, the only approved route for treating ischemic stroke was the use of the clot bluster called tissue plasminogen activator (tPA). Although tPA is used for treating ischemic stroke to thrombolytically break up a blood clot when administered, it is ineffective when the therapy is initiated outside of 4.5 hours after onset of stroke. Furthermore, the drug is not effective in patients with large vessel occlusions, which make up 75 percent of ischemic strokes (95). Recently, the advent of thrombectomy using mechanical endovascular devices have widened the window of ischemic stroke treatment up to 24 hours.

However, the extended time to clot removal and subsequent blood flow restoration is most suitable for patients with good collateral function, which maintains or preserves the penumbra tissue. The research presented in this dissertation shows that pial collateral remodeling is malleable and can be greatly enhanced through genetic or pharmacological inhibition of EphA4 to reduce tissue loss and neurological deficits after
ischemic stroke. It would be ideal to next identify whether human patients that present with excellent or poor collateral scores on angiography also correlate with changes in EphA4 expression and if this can be used as a biomarker for collateral status to predict outcome and guide therapy.

Also, future animal studies should be completed to identify whether EphA4 can enhance collateral outgrowth within hours of occlusion. It would be interesting to determine if the outgrowth is by active cellular remodeling, as shown in this dissertation, or by acute hemodynamic cues which modulate smooth muscle cells to help expand the collateral vessel wall. Activation of another Eph receptor, EphB4, on endothelial cells regulate eNOS activity (301, 302), which dilate blood vessels via smooth muscle cell modulation. It is possible that EphA4 is able to elicit a similar response in endothelial cells or interact with EphB4 to induce eNOS-stimulated collateral dilation. These studies will further validate the used of EphA4 targeting drugs to enhance pial collateral remodeling after stroke.

Clinically, implementation of such a drug during and after the acute phase of stroke treatment would accelerate collateral remodeling, re-perfuse the ailing penumbra and thus preserve this region for several hours after stroke. This will increase the number of patient candidates for endovascular thrombectomy, as the main criterion for a candidate is whether he or she has a viable penumbra. Moreover, tPA therapy is more effective when patients have better collateral blood flow. A combination therapy of tPA and KYL, for instance, would enhance collateral blood flow and further improve the effectiveness of tPA. The pial collateral system is undeniably crucial to improvements after ischemic stroke. Therapy to enhance its remodeling in ischemic stroke patients can lead to significantly better outcomes, but ultimately will preserve the penumbra and increase the window of treatment beyond 24 hours.
Dissertation References


111. Feeeney JF, Jr., and Watterson RL. The development of the vascular pattern within the walls of the central nervous system of the chick embryo. J Morphol. 1946;78(231-303).


Appendices
Appendix I
Academic and Professional Honors

1. Excellence for PhD poster, Central Virginia Chapter of the Society for Neuroscience, Richmond, VA, March 2018.
   Awarded to best graduate student for their research and presentation


3. International Center for Professional Development scholarship, 2017
   Scientist mentoring and development program (SMDP)
   Received direct mentoring from leaders of leading biotech companies including: Johnson and Johnson, Janssen, Merck, Amgen, Baxter, Immucor, Medtronic, Braun and Fenton

4. The Ruth L. Kirschstein Predoctoral Individual National Research Service Award (F31), 2017
   NIH award given to exceptional pre-doctoral students with potential to develop into productive, independent research scientists

5. VMRCVM Graduate Fellowship, 2017
   Scholarship awarded to an outstanding graduate student in the Biomedical and Veterinary Science Department

6. Outstanding PhD Poster, 28th Biomedical and Veterinary Science Symposium, Virginia-Maryland Regional College of Veterinary Science, Virginia Tech, 2017
   Best research presentation by a PhD student at the annual research symposium

7. Best Initiative for Maximizing Student Development (IMSD) Graduate Presentation, Mid-Atlantic PREP/IMSD Research Symposium, 2016
   Scientific research symposium meeting of mainly ACC conference schools
   Best presentation and speaking by a graduate student

8. Graduate School Association Silver Poster Presentation Award, Virginia Tech, 2015
   Interdisciplinary research symposium for all graduate students at Virginia Tech

   Scholarship to attend NAVBO’s Vasculata workshop
   Learnt skills and techniques important in vascular research

10. The Initiative for Maximizing Student Development (IMSD) Program Award, Virginia Tech, 2014
    NIH funded fellowship for developing minority research scientist

11. The Regenerative Medicine Interdisciplinary Graduate Education Program Award, Virginia Tech, 2013
    Scholarship awarded to promising graduates students in interdisciplinary and translational science

    Best research presentation by a Master’s student at the annual research symposium
13. Graduate Research and Development Program Award, Virginia Tech, Graduate Students Association, 2012
   Graduate student grant award for innovative public health scientific research

14. Outstanding PhD Poster, 23rd Biomedical and Veterinary Science Symposium, Virginia-Maryland Regional College of Veterinary Science, Virginia Tech, 2011
   Best research presentation by a PhD student at the annual research symposium


Appendix III
Selected Presentations


