MAJOR COLLATERAL VESSELS DEVELOP FROM PRE-EXISTING SMALL ARTERIES THROUGH RAC2/NOX2 INDEPENDENT MECHANISMS

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ABSTRACT

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Major Collateral Vessels Develop from Pre-existing Small Arteries

Through Rac2/Nox2 Independent Mechanisms.

There is no consensus on which vascular segment or what size of vessels is most important in the process of collateral growth, the degree to which these vessels can enlarge, or the mechanisms that mediate collateral vessel expansion and its impairment. Chapter I identifies the major collateral vessels that develop in response to femoral arterial occlusion in the pig, rat, and mouse hindlimbs for comparison to humans. Pre-existent small named arteries enlarged ~2-3-fold to become the major collateral vessels in each species, these major collaterals displayed characteristics similar to large arteries experiencing flow-mediated outward remodeling, and important differences in vascular wall thickness were observed between rodents and pigs. Chapter II utilized *Rac2-/-* and *Nox2-/-* mice to investigate the hypothesis that Nox2-NAD(P)H oxidase is required for major collateral growth subsequent to femoral arterial occlusion. Previous studies suggest bone marrow cell (BMC)-derived reactive oxygen species (ROS) produced by the Nox2 subunit of NAD(P)H oxidase plays an important role in neovascularization and recovery of hindlimb perfusion subsequent to femoral arterial occlusion; but did not investigate collateral growth. The hematopoietic cell restricted protein Rac2 has been shown to bind to and activate Nox2-NAD(P)H oxidase and *Rac2-/-* and *Nox2-/-* leukocytes display impaired ROS related functions. The data demonstrated that Rac2 and Nox2 are not essential for major collateral growth, but both are important for the

recovery of hindlimb perfusion and preservation of distal tissue morphology. Chapter III investigated BMC and antioxidant therapy in the age-related impairment of collateral growth. Aging, like all cardiovascular disease risk factors is associated with elevated ROS and impaired collateral growth. Studies also suggest BMCs promote collateral growth by secreting paracrine factors but elevated ROS may affect the efficacy of BMCs. The data revealed that neither BMC injection nor antioxidant therapy via apocynin enhanced the process of major collateral artery growth in aged mice.

Joseph L. Unthank, PhD, Chair

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LIST OF ABBREVIATIONS

BM-MNCs = Bone marrow mononuclear cells

CLI = Critical Limb Ischemia

GA = Gluteal Artery

IEL = Internal Elastic Lamina

LDPI = Laser Doppler perfusion imaging

NAD(P)H = Nicotinamide Adenine Dinucleotide Phosphate

PAD = Peripheral Artery Disease

PFA = Profunda Femoral Artery

ROS = Reactive Oxygen Species

SFA = Superficial Femoral Artery

WT = Wild Type

INTRODUCTION

While great strides in the fight against cardiovascular disease (CVD) have been made in recent years, as indicated by the ~25% decline in CVD associated death rate from 1994-2004, Americans are still dying from CVD at a rate of 1 death every 37 seconds (~2400 per day) (110). Peripheral arterial disease (PAD) - a component of CVD resulting from arterial occlusion in the extremities - is known to affect ~8 million Americans (110) and is associated with significant morbidity and mortality (110). One of the endogenous compensation mechanisms for arterial occlusion is the enlargement of blood vessels which bypass the site of occlusion, a process known as collateral growth. However, effective pharmacotherapy specifically designed to increase limb perfusion in PAD patients is not currently available (159). Molecular therapies utilizing growth factors designed to increase hindlimb perfusion via promoting generalized vascular growth showed considerable promise in pre-clinical animal models; however, results from molecular therapy-based clinical trials have been mixed at best (153). In addition, subsequent studies suggest many of these growth factor therapies were more readily able to modulate capillary vascular growth rather than collateral vascular growth. While there are numerous potential explanations for the mediocre clinical results observed, two possibilities include: 1) that the pre-clinical models being used may not be an optimal representation of the human condition and 2) that previous studies have suggested capillaries are not the major determinant of hindlimb perfusion after arterial occlusion (46, 62, 76, 112, 121, 150, 159, 163). Unfortunately, it appears that clinical results with cell-based therapies will be similar to those seen with the molecular therapies (74). Thus, the fundamental question remains (which will serve as the major theme for this

introduction): do the current pre-clinical rodent models adequately reflect the clinical, human situation? To adequately answer this question, this introductory chapter will: 1) review PAD, 2) discuss the endogenous mechanisms used by the body to combat arterial occlusion, 3) critique some of the current animal models and analytical methods used to study PAD, 4) suggest whether compensation via capillary angiogenesis or collateral growth is more important, 5) consider the shift in emphasis from the study of collateral growth to angiogenesis and arteriogenesis research, 6) discuss the confusion brought on by the definition of arteriogenesis, and finally, 7) propose what is now needed to advance the field of collateral growth research.

Review of Peripheral Arterial Disease (PAD) and its Therapeutic Interventions

As reviewed in Greenfield's vascular surgery textbook (by Mulholland, et al. 2006) (88), chronic obliterative atherosclerosis of the infrainguinal vessels is the most prevalent manifestation of arterial occlusive disease encountered by the vascular surgeon and the most common lesion seen below the inguinal ligament is that of a short-segment total occlusion of the superficial femoral artery. The patient populations most at risk for significant morbidity and mortality associated with PAD are aged patients, those with diabetes, and those who smoke. PAD prevalence increases dramatically with age and affects 12-20% of Americans age 65 and older (110). The risk of lower extremity PAD increases 2-4X in the presence of diabetes mellitus and 12-20% of patients with lower extremity PAD have diabetes (64). The risk of developing lower extremity PAD increases 2-6X with smoking and >80% of patients with lower extremity PAD are current or former smokers (64). As the occlusive process progresses, alterations in the hemodynamic forces within the vasculature lead to the enlargement of a network of

bypass (or collateral) channels around the occluded segments (102). The blood flow provided by this collateral network is usually sufficient to meet the metabolic needs of the lower extremities while at rest with slowly developing occlusive lesions; however, this network of bypass vessels cannot increase blood flow enough to meet the metabolic demands of exercising leg musculature (102). The characteristic exercise-induced, cramping pain in the lower extremity muscles caused by chronic atherosclerotic occlusive disease is known as intermittent claudication (102). When collateral pathways cannot meet even the basal metabolic needs of non-exercising tissue and occlusive disease in both the aortoiliac and infrainguinal segments is present, the outcome is pain at rest, tissue loss (gangrene, non-healing ulceration), and threatened limb viability – a condition known as critical limb ischemia (102).

Two non-invasive methods of clinically assessing the level of disease present in the ischemic limb are by calculation of the ankle-brachial index and via segmental blood pressure measurements. The ankle-brachial index (ABI) is a good indicator of the degree of ischemia present (102) and is defined as the ratio of the ankle systolic pressure to the brachial systolic pressure. Normal ABIs are generally ≥ 1, while patients with claudication usually have ABIs ranging from 0.5-0.9 and patients with rest pain and tissue loss have ABIs of <0.5 (102). When the arterial stenosis is not severe enough to cause a pressure drop while the limb is at rest, patients with claudication can occasionally have normal or nearly normal ABI. However, higher rates of flow due to exercise-induced vasodilatation in order to meet the increased metabolic demands of exercising muscle tissue will produce a hemodynamically observable effect (102). In these patients, ABIs are measured before and after a treadmill exercise test and the sign of

hemodynamically significant occlusive disease is a drop of 15% or more in the ABI following exercise (102). Determination of the arterial segment involved with occlusive disease can be done non-invasively via segmental blood pressure measurements. This method utilizes four blood pressure cuffs placed on the upper thigh, lower thigh, calf, and ankle. Significant disease within the intervening arterial segment is indicated by a pressure drop of >20 mmHg between consecutive levels of measurement and an upper thigh pressure that is below normal signifies occlusive disease in the aortoiliac or common femoral segments (102). Intra-arterial vasodilator injection can be used to mimic exercise hyperemic hemodynamics during the measurement of segmental pressures when the arterial stenosis is not severe enough to cause a pressure drop while the limb is at rest (102).

Therapeutic intervention for PAD can range from medical stabilization of the symptoms through minimally or maximally invasive surgical procedures to limb amputation. The selection of the most appropriate therapy is patient specific; depending on patient risk, surgeon experience, and the pattern of the occlusive process (102). Due to the relatively stable natural history of claudication, endovascular or surgical intervention may or may not be needed; however, patients with critical limb ischemia face inevitable limb amputation unless surgical correction is performed (88). In percutaneous transluminal angioplasty (PTA), a balloon catheter is inserted into the arterial system, guided to the point of atherosclerotic occlusion/stenosis, and then inflated in order to re-establish the lumen of the vessel. PTA is best suited to the treatment of short, focal, non-occlusive lesions (102). Placement of an intravascular stent to assist in maintaining long-term patency often supplements balloon angioplasty (41). Iliac artery

angioplasty has a 3-year patency rate of 70-90% and a 5-year rate of 50% (102); however, the results of PTA in the femoropopliteal system have been mixed: 43-58% patency at 1 year, 41-46% at 2 years, 38-41% at 3 years, and 26-38% at 5 years (41). Endarterectomy is limited to only good-risk patients with focal occlusive disease at the aortic bifurcation and common iliac arteries, is less well suited for disease extending in the external iliac arteries, and is to be avoided when aneurysmal degeneration complicates the primary atheroocclusive aortoiliac process (102). Patients with diffuse atherosclerotic disease, long (>10 cm) and complex stenoses or occlusions benefit more from bypass/revascularization than from PTA (102). Indeed, when medical therapy or percutaneous treatment has proven inadequate or is technically inadvisable, open surgical revascularization remains the gold standard for patients with disabling claudication, ischemic rest pain, and ischemic ulceration or gangrene (88). The 5- and 10-year graft patency rates in aortofemoral bypass are excellent (at ~85% and 75%, respectively) and perioperative morbidity and mortality are reported to be below 10% and 5%, respectively (102). 5-year results of saphenous vein grafting to combat infrainguinal atherosclerotic diseases (using modern techniques) have also been excellent, with patency rates of 75-80% and limb salvage rates as high as 90% (88). However, despite this relative success, there are still patients who are not suitable candidates for revascularization, such as those with diffuse femoropopliteal and tibioperoneal disease, since long-distance reconstruction does not necessarily provide symptomatic relief and graft failure may result in distal arterial thrombosis and a jeopardized limb (88). In addition, CLI is associated with a 20% 1yr mortality rate, nearly half of patients will require revascularization for limb salvage, and (of those with un-reconstructable disease) ~40% will require major limb

amputation within 6 months (64). Therefore, novel therapies which could enhance endogenous mechanisms of increasing perfusion of the distal tissues would have tremendous potential for improving the morbidity and mortality caused by arterial occlusion within PAD.

What are the Endogenous Mechanisms of Compensation for Arterial Occlusion?

As previously mentioned, collateral growth is one endogenous mechanism the body utilizes to maintain resting tissue perfusion in the presence of arterial occlusion. Collateral growth is characterized by the enlargement of pre-existing bypass vessels which allow blood to circumvent the site of occlusion and continue to perfuse the distal tissues.

Several early studies provide anatomical and functional evidence of pre-existing collaterals. In 1911, Matas (84) recognized the importance of the collateral circulation stating "The surgery of the vascular system bristles with problems which still await solution, but none appear to me more important or fundamental than the study of the collateral circulation in its behavior to occluded arteries" and then designed a method of testing the efficiency of the collateral circulation in humans which he utilized to determine which patients were amenable to surgical aneurisms repair. Eckstein (1941) (42) measured distal pressure and flow recovery after femoral, carotid, and coronary occlusion in the dog and demonstrated the presence of pre-existent vessels by observing an ~50% recovery of distal pressure within the first 90 min after occlusion; far too little a time for new vessels to form. In a review on arterial injury in World War II, DeBakey and Simeone (1946) (37) state that "after interruption of a major artery of a limb, the circulation may be so seriously impaired as to necessitate amputation of the limb; or it

may be entirely adequate, so that a few weeks after the injury, there may be no detectable abnormality", thus suggesting the presence of pre-existent collaterals. DeBakey and Simeone (37) go on to repeatedly discuss the importance of maintaining the collateral circulation after arterial injury. Shepherd (1950) (125) measured calf blood flow upon mechanical compression of the femoral artery in humans and observed that calf blood flow initially dropped to ~1/6th of the resting pre-occlusion value and then recovered to near the resting level within an average of 2 min, again indicating the presence of pre-existing collaterals. Longland (1953) (81) identified pre-existing collaterals in the hindlimbs of rabbits and serially observed their enlargement over the course of a year. Rosenthal and Guiton (1968) (111) used conductance to estimate the dilation of pre-existent collaterals within the first hour after femoral artery occlusion in the dog and observed a ~277% rise in conductance in the first 70 seconds following occlusion, demonstrating the presence of pre-existing collaterals. Gorey et al. (1979) (51) reported that in 14 cases of iliac artery ligation to control hemorrhage during renal transplantation none of the patients lost their limbs, none of the patients required immediate iliac bypass surgery, and only 2 eventually required reconstruction due to symptoms of claudication, demonstrating the presence of pre-existent collateral vessels. Gorey et al. (51) went on to list both the parietal and the visceral pre-existent named arteries which could provide potential collateral circulation around an occluded iliac artery.

The evidence put forth by these and other foundational studies, in combination with decades of clinical observation, were compelling enough that pre-existent collaterals are now standard textbook knowledge (60, 92, 122, 134) for all medical students and have been since the publication of Gray's Anatomy, 20th edition, in 1918 (78). Some

examples of collateral circulation readily identified in medical textbooks include: 1) the circle of Willis; 2) the intercostal and lumbar arteries to the circumflex iliac and iliolumbar arteries, the superior to inferior epigastric arteries, and the superior and inferior mesenteric arteries to the rectal and internal pudendal arteries, all of which are important collateral arterial pathways around the aortic bifurcation and common iliac segments; 3) the hypogastric-to-circumflex femoral channels which provide collateral pathways around occlusive lesions of the external iliac arteries; 4) collateral perfusion from the profunda femoral artery around a heavily diseased or occluded superficial femoral artery which frequently reconstitutes the distal superficial femoral artery or popliteal artery with enough well perfused arterial blood to ensure sufficient resting tissue perfusion (88); 5) the rich network of geniculate collaterals in the knee which can compensate for a diseased popliteal arterial segment to a sufficient degree to prevent rest pain or overt tissue loss (88, 122); and 6) collaterals between radial and ulnar arteries as well as between digital arteries in upper lower extremities (60). The exact mechanism of collateral growth is still under investigation, but most investigators believe the initiating stimulus is the increased shear stress experienced by the endothelial cells of the collateral due to the increased flow passing through the bypass vessels upon parent artery occlusion (12, 45, 96, 131, 148, 154, 158).

Another endogenous mechanism utilized by the body in an attempt to maintain perfusion is increasing the number of capillaries within an ischemic region, otherwise known as angiogenesis. As reviewed by Simons (131), angiogenesis is stimulated by tissue hypoxia and proceeds via sprouting of new capillaries from post-capillary venules. Tissue hypoxia stimulates the expression of hypoxia-inducible factor (HIF)-1α which

then activates the transcription of numerous genes, including vascular endothelial growth factor (VEGF) (131). VEGF then mediates increased vascular permeability and endothelial cell proliferation and migration (158). The endothelial cells migrate into the extracellular matrix, form tubes, and fuse to existing vessels (158). Angiogenesis is known to play a role in both physiologic and pathophysiologic processes (such as cardiovascular disease, among others) (158) and can improve oxygen delivery to individual cells (80).

A third purported endogenous process of compensating for arterial occlusion is vasculogenesis, or the growth of entirely new vessels (larger than capillaries) driven by circulating endothelial or vascular progenitor cells (131). The benefit from vasculogenesis has been reported as a wide spectrum from none at all to significant (131). Two recent reviews suggest that it is unclear whether vasculogenesis actually occurs and whether vessels formed by this process are physiologically relevant (55, 57).

Critique of the Animal Models and Analytical Methods Utilized in Recent Studies

Major limitations of the animal models currently in use may have contributed to the failure of small animal models to predict clinical outcomes in the field of therapeutic angio-/arterio-genesis and collateral growth. First, as reviewed by Waters (159), surgical ligation alone often does not result in a significant/sustainable reduction in resting blood flow in some animal models (14, 22, 61, 70, 123). Waters also points out that the timing of perfusion assessment in animal models is critical, as these models tend to be stable after 1 week and, thus, it is more difficult to detect efficacy of therapy (159). On the other hand, simple ligation can result in impaired blood flow capacity under stress conditions, such as exercise, and ligation plus excision of the femoral artery can result in

reduced blood flow at rest, especially if in a diseased background (159, 161). Unfortunately, the more extensive the surgical ligation strategy, the more potential pre-existing collateral pathways are eliminated and are thus unable to provide compensation. Thus, preclinical models which demonstrate sustained hemodynamic impairment at rest would roughly mimic CLI while those which demonstrate hemodynamic impairment only upon exercise would approximate claudication (159).

Second, despite these rough approximations, surgically induced hindlimb ischemia in young healthy animals is not equivalent to human PAD. Animal models primarily employ sudden ligations that usually involve only one segment and occur in a healthy surrounding vasculature. In contrast, the most serious syndromes in human PAD usually result from gradual obstruction at multiple vascular sites and the surrounding vasculature contains atherosclerotic disease as well as other co-morbidities such as age, diabetes, hypertension, and hypercholesterolemia. Therefore, pre-clinical models utilizing a single ligation to study collateral growth in the context of infrainguinal vessel occlusion should place their ligation on the short segment of the superficial femoral artery as that is known to be the most common site of occlusion in humans (88). However, a more accurate pre-clinical model would be one with an initially gradual occlusion that would eventually experience a sudden obstruction. In addition, pre-clinical models should utilize animals with the cardiovascular risk factor co-morbidities of age, diabetes, and/or smoking to most accurately mimic the human condition. As models with this degree of complexity are costly and difficult to obtain, the work performed in the remainder of this thesis is done with either a single ligation to mimic

moderate disease or a double ligation and excision of the femoral artery to mimic more advanced PAD and the co-morbidity of age was also investigated.

Third, largely due to the failures of molecular therapies derived from small animal studies to provide clinical benefit, some investigators have suggested small animals are irrelevant to the human situation. These investigators suggest that because of the size difference, mechanisms and/or characteristics of collateral growth, the process may be different in mice versus humans (this will be addressed at greater length in Chapter I). It should be noted, however, that a majority of small animal studies were intended to investigate or enhance angiogenesis rather than collateral growth and that the molecular therapies derived from these studies were primarily developed in young healthy animals experiencing a sudden focal occlusion of their vasculature; which, as previously mentioned, is a very different situation from the aged patient with diffuse atherosclerotic disease presenting with multiple co-morbidities and experiencing gradual occlusions of their vasculature. The use of mice does still have advantages, as outlined by Casal and Haskins (20): mice have been invaluable for the study of gene transfer methods and gene therapy of inherited diseases, they are relatively inexpensive, have a short generation time and large litters, transgenic techniques allow the development of almost any monogenetic disease model, and the various mouse strains are highly inbred which provides uniform conditions in which experiments can be easily reproduced and statistical significance achieved. However, the problem of compensation by secondary mechanisms arises when using transgenic animals in which some portion of the primary mechanism under investigation has been genetically deleted. Therefore, the failure of current molecular therapies which showed promise in pre-clinical animal models to translate to the clinical

situation may largely be due to these limitations in the animal models utilized in those studies.

In addition to limitations in the animal models utilized to study vascular compensation, limitations in the analytical methods used to assess vascular compensation are also prevalent. For instance, few studies have specifically identified the pre-existing vessels which form the major or most functionally important collateral pathways and investigated their mechanism of enlargement. Previous studies relied primarily on the following 5 methods to assess collateral development: 1) counting the number of angiographically visible collaterals, 2) obtaining collateral vessel density or angioscore (also essentially counting), 3) using micro-CT to obtain vascular volume of entire hindlimb, 4) using microspheres to calculate a collateral dependant flow, or 5) using laser Doppler perfusion imaging without ever directly assessing collateral growth. Fuchs et al. (50) noted that collateral vessel density in angiograms does not correlate with collateral flow and angiographic scoring does not reflect tissue perfusion as assessed by microsphere techniques. In addition, counting collaterals or obtaining entire hindlimb volume will result in the inclusion of regressing collaterals. Regressing collaterals have been observed by multiple investigators (12, 63, 81, 95, 122) and are probably not a significant component of hindlimb perfusion, since the reason these vessels are regressing is due to reduced blood flow. In addition, regressing collaterals may be undergoing an entirely different process than enlarging collaterals which would confound the results of studies aimed at investigating the mechanisms of collateral enlargement. Using microspheres that are too small may cause misinterpretation of an animal's ability to grow collaterals; 15 μm microspheres utilized by many studies are good for overall

perfusion but not for collateral growth as they are too small, readily passing well beyond collaterals ranging 75-125 μm in diameter (in mice). The most commonly used laser Doppler perfusion imagers cannot penetrate the tissue deeply enough to directly assess collateral flow, and thus, can only measure what is thought to be collateral-dependant flow at sites distant to the location of the actual collateral vessels. Clinically, assessment of limb ischemia is performed non-invasively via segmental pressure measurements or obtaining the patient's ankle-brachial index (ABI); however, these methods are seldom utilized in pre-clinical models of limb ischemia. To mimic these non-invasive clinical assessments of ischemia, larger animal models would probably be necessary (such as sheep, pigs, dogs, cats, and possibly rabbits) for the segmental pressure techniques while animals as small as rats would probably be amenable to ABI measurement. If experimental design allows invasive techniques to be performed, then either ABI or segmental pressure measurement can be performed in animals as small as mice (via catheterization of vessels proximal and distal to the occlusion); however, the measurement of distal pressures becomes increasingly difficult with decreasing animal size due to decreasing vessel size. Therefore, the limitations of the current analytical techniques which do not focus on the enlarging, functionally important collaterals and allow for the inclusion of regressing vessels may have played a role in the inability of promising pre-clinical therapies to be translated into clinical outcomes. In addition, incorporating analytical techniques into pre-clinical studies that more closely mimic the analytical techniques of the clinic may allow for a more direct comparison of therapeutic pre-clinical and clinical outcomes.

Collateral Growth is Probably More Important in Restoring Hindlimb Perfusion after Arterial Occlusion than Capillary Angiogenesis

Even though capillary density is the most commonly assessed histological measure of perfusion in pre-clinical models, a context for the interpretation of these findings is unclear (159). As reviewed by Waters et al. (159), while capillary density is directly correlated with oxidative capacity and muscle endurance in the absence of disease, increased capillary density was correlated with the degree of skeletal muscle hypoxia (and not increased perfusion) in the presence of PAD. In addition, the common phenomenon of muscle atrophy in hindlimb ischemia models complicates the measurement of capillary density (in capillaries per mm^2) as this value is influenced by changes in muscle fiber size (159). In his review, Wahlberg identifies a lack of reports in the literature that demonstrate active angiogenesis occurring in the chronically ischemic limb of patients (158). Simons review suggests that increased capillary number can increase blood flow 2-3X but that collateral growth can increase blood flow 20-30X (131). Scholz et al. demonstrated that the largest increase in capillary density occurred in the strain of mice that showed the poorest recovery of hindlimb perfusion (Balb/C) while capillary density was unchanged in the mice that experienced the greatest recovery of hindlimb perfusion (C57BL/6) (121). Hershey et al. showed that increased capillary density occurred at the same time as reduced resting blood flow while the growth of collateral vessels coincided with a large functional improvement in the reserve blood flow capacity of the limb (62). Thus, increases in capillary density and improved hindlimb perfusion were not linked (62). Emanueli et al. showed in the ischemic rat hindlimb that hemodynamic improvement can occur in an absence of changes in

capillarity (47). Sanne and Sivertsson measured hindlimb resistance and observed that the resistance of the "local" vascular bed (distal to the collaterals, which would include the capillaries) did not change over the course of 5 weeks after femoral arterial occlusion while the collateral vessel resistance was reduced (112). Unthank et al. (150) and Lash et al. (76) demonstrated that: 1) distal tissue perfusion is determined by the vascular resistance of the bypass collaterals and the distal microvasculature, 2) the collateral vessels account for the majority of vascular resistance distal to occlusion, and 3) the greatest decrease in vascular resistance occurs in the collaterals (not the distal microvasculature) as compensation to occlusion occurs and hindlimb perfusion is increased after femoral artery ligation in the rat. Terjung's group corroborated the findings of Unthank and Lash, demonstrating that collateral resistance but not distal tissue resistance is the major site of resistance determining downstream blood flow after femoral artery in the rat (75-85% total limb resistance) (163). Lastly, not only does the microvasculature comprise a small amount of hindlimb resistance after arterial occlusion, but Bohlen et al. (7) and Fronek and Zweifach (49) have also previously shown that capillaries themselves normally comprise only a small component of skeletal muscle microvascular resistance in the absence of arterial occlusion. Indeed standard physiologic textbooks state: 1) vascular resistance in the non-diseased limb is primarily controlled by the pre-capillary arterioles because of their ability to regulate vascular tone due to the presence of smooth muscle within their wall and 2) that the total resistance of the capillaries is much less than that of the arterioles due to the capillaries tremendous cross-sectional area (127). Thus, these data suggest not only that angiogenesis may not be of primary importance in perfusion of the chronically ischemic limb in patients but

also that collateral growth seems to be more important in restoring perfusion in animal models of hindlimb ischemia than angiogenesis.

Additional evidence for collateral growth being more beneficial at restoring hindlimb perfusion comes from Poiseuille's law. Poiseuille's law dictates that vascular resistance is inversely proportional to the fourth power of radius. Thus, small changes in collateral diameter can be responsible for extremely large changes in collateral conductance (147). Poiseuille's Law estimates the volume flow (Q) of a fluid through a tube of length (L) and radius (r) as follows:

$$Q = \pi r^4 \Delta P / 8L\eta$$

where ΔP is the change in pressure from the beginning to the end of the tube and η is the viscosity of the fluid, assuming Newtonian fluid dynamics. Hemodynamic resistance is defined as the energy drop along the vessel divided by the volume flow and, as long as gravitational potential energy and kinetic energy are considered to be small, the ΔP can represent the energy loss along the vessel. Therefore, Poiseuille's equation can be re-written to calculate vascular resistance:

$$R = 8L\eta / \pi r^4$$

Poiseuille's law has been invoked to suggest the importance of collateral growth over capillary angiogenesis in several ways. Rockstroh and Brown (109) used the Poiseuille equation to calculate the theoretical flow capacity of various caliber tubes: a 1 mm tube will have a flow capacity 7.7 times that of a 0.6 mm tube, and 39 times that of a 0.4 mm tube. They then used a cineangiographic approach to directly quantify the lumen caliber and flow capacity of dominant collaterals by measuring the transit time of the dye through the collateral as well as the collateral length and diameter (109). This direct

method demonstrated the flow in the 1 mm diameter collateral was 6.5 times that through the 0.6 mm collateral and 32 times that through the 0.4 mm collateral, in agreement with what was calculated from Poiseuille's equation (109). Based on both this direct and theoretical evidence, Rockstroh and Brown then concluded the 2-3 largest collaterals in the angiographically visible spectrum carried the majority of flow capacity of a collateral network (109). Similarly, De Lussanet et al. utilized magnetic resonance angiography (MRA) and Poiseuille's law to determine that almost 100% of total blood flow in the rabbit hindlimb after femoral artery occlusion is supplied by collaterals visible in both MRA and X-ray angiography (XRA; i.e., collaterals ≥0.3 mm), while collaterals visible only in XRA (i.e., collaterals 0.1-0.3 mm in diameter) contributed little to blood flow (36). Scholz et al. (121) reported that even though a 1.7X increase in capillary density was observed in the Balb/C mice, this did not adequately restore perfusion and would only theoretically restore perfusion ~1.7X. Conversely, the C57BL/6 mice they studied experienced extensive collateral enlargement, minimal increases in capillary density, and restored perfusion ~4X (121). Upon femoral artery occlusion in our mice, we have observed 50-60 μm pre-existent collateral vessels enlarging ~2.5 fold to compensate for the occlusion of the ~350 μm femoral artery, providing ~80% of the hindlimb perfusion that was present before the occlusion. If compensation to occlusion occurs through a mechanism in which the pre-existent vessels only increase in number but do not enlarge, as is the case with angiogenesis, Poiseuille's equation states it would take 2401 of the 50-60 μm pre-existent collateral pathways or 24 million of the ~5 μm (4) capillaries to completely compensate for the occluded femoral artery. However, it would take only ~61 of the enlarged collaterals (~125 μm in diameter) to compensate for femoral arterial

occlusion. This is one reason Schaper suggested an impossible number of capillaries would be needed to compensate for an occluded parent artery, because the tissue volume created by such an increase in capillarity would in essence replace the organ to be perfused with blood (115). Indeed, Scholz et al. observed a permanent flow deficit in Balb/C and sv129 mice even though the combined diameter and tissue mass of the six collateral arteries identified bypassing the site of femoral occlusion exceeded that of the original femoral artery (121). This was attributed to increased length of the collaterals versus the length of the occluded segment of the femoral artery (a >2X increase in length), increased collateral vessel tortuosity, and the energy losses predicted by Poiseuille's law when one larger vessel is replaced by several smaller ones (121).

In their review, Heil and Schaper (56) also suggest that the arrangement of having multiple collaterals replace a single occluded parent artery is not efficient according to Poiseuille's law because the energy losses created by the additional resistances of the contributing vessels are additive. These authors go on to suggest that this is the reason only a few collaterals which experience the greatest degree of enlargement eventually remain while the rest of the many collaterals that initially enlarged undergo regression (56). Thus, having a network of multiple vessels compensate for the occlusion of one main artery, as would occur with angiogenesis, is energetically unfavorable. Heil and Schaper (56) also point out that in several different animal studies collateral vessels were able to recover only ~40% of the pre-occlusion maximal conductance and raise the question whether collateral growth will ever be able to completely compensate for an occluded parent artery. Subsequently, though, their group was able to demonstrate that collaterals can completely compensate for an occluded parent artery, if fluid shear stress

in the expanding collaterals is not allowed to normalize (45). In addition, Rissanen et al. (105) site Poiseuille's law to suggest that collateral blood flow is more critically dependant on vessel diameter than on vessel number and also observed that the response to femoral occlusion consisted mainly of the enlargement of pre-existing collateral vessels as opposed to increased capillary density. Similarly, White et al. (160) excluded vessels ≤20 μm from their analysis because Poiseuille's law indicates that the flow through vessels of this size or smaller is miniscule versus the flow through larger vessels.

Therefore, because: 1) experimental evidence suggests hindlimb resistance is more dependant on collateral enlargement than on capillary proliferation after femoral occlusion, 2) enlargement of collaterals can provide significantly more flow than proliferation of capillaries, 3) volume flow is directly proportional to the fourth power of the radius, and 4) the energy losses to the system are greater when adding new vessels (i.e., capillary proliferation) rather then expanding the vessels already present (i.e., collateral growth), it appears that collateral growth is more potent mechanism for maintaining hindlimb perfusion than angiogenesis.

The Shift in Focus from Collateral Growth to Therapeutic Angiogenesis

Judah Folkman "fathered" the field of angiogenesis when he proposed in the New England Journal of Medicine in 1971 that: 1) tumors depend on the active induction and continuous growth of new blood vessels (angiogenesis) to survive and expand (48, 54), 2) that tumors release diffusible factors that stimulate endothelial cell proliferation and capillary formation, and 3) that anti-angiogenesis therapy could possibly control tumor growth; as reviewed by Zetter (165). Thus, Folkman was the first to propose anti-angiogenic therapy. Based on Folkman's assertion that tumors secrete factors which

stimulate angiogenesis, many researchers set out to investigate the hypothesis that the stimulation of new capillary growth could be beneficial for the treatment or prevention of pathological clinical situations characterized by a local decreased number of blood vessels or decreased blood flow (66). Indeed, as early as 1977 there was an article in Lancet entitled "Tumour angiogenesis factor for revascularisation in ischaemia and myocardial infarction." by Svet-Moldavsky and Chimishkyan (136). This soon blossomed into an entire field of its own termed "therapeutic angiogenesis" (66). Partly due to the fact that the molecular mechanisms underlying the process of angiogenesis were becoming more clearly understood than those that mediate collateral growth, a shift in the focus of research from collateral growth to therapeutic angiogenesis began to occur. Jeffrey Isner (69), who would become one of the leaders in the field of therapeutic angiogenesis, said that angiogenesis "presumably accounts for some, if not most, collateral vessels which constitute 'auto-bypass' conduits in patients with vascular disease". Thus, according to this statement, the mechanism for collateral development is suggested to be capillary sprouting and not the enlargement of pre-existing bypass vessels as was identified by the early studies and was described in medical texts. Statements like this from well respected leaders in the field like Isner implying angiogenesis is the source of collateral vessels combined with the prevalence of angiogenic molecular therapy based studies further exacerbated the shift in focus from the study of pre-existing collaterals to therapies focused on angiogenesis. Indeed, this shift in focus led Schaper to suggest that, by 1984-85, the pioneering work of the animal physiologists on collateral growth (as outlined above) had all but been forgotten (113). However, novel therapies to enhance the growth of pre-existent collaterals are still needed because the efficacy of angiogenic

growth factor therapy for treatment of CLI is still not well established (74) and growth factor therapy is best investigated in the context of a placebo-controlled trial (64). The failures of these molecular angiogenic therapies have led investigators to question the effectiveness of therapeutic angiogenesis as a whole (74). Indeed, the evidence presented in the previous section further suggests that therapeutically enhancing collateral growth may be able to restore hindlimb perfusion more adequately than enhancing angiogenesis.

The Strict Definition of Arteriogenesis has caused some Confusion in the Field of Collateral Growth Research

Schaper and colleagues have been one of the leading groups trying to shift the focus back on to collaterals from capillary angiogenesis arguing that "Arteriogenesis is by far the most efficient adaptive mechanism for the survival of ischemic limbs or internal organs such as heart and brain because of its ability to conduct, after adaptive growth, relatively large blood volumes per unit of time… an increase in the number of capillaries, the result of stimulated angiogenesis, is unable to do that." (13). Thus, therapeutically enhancing collateral growth as opposed to the growth and/or proliferation of the distal capillaries has tremendous potential for improving the morbidity and mortality caused by arterial occlusion within PAD by enhancing tissue perfusion more effectively than angiogenesis. However, Schaper's "arteriogenesis" model of collateral growth puts forth a strict definition of what vessels can become collaterals and what characteristics these collateral vessels display: suggesting that only arterioles 10-30 μm in diameter can become collaterals, that these vessels are capable of enlarging 10-25 fold, and that collaterals will display the characteristics of increased intimal cell number, neo-intimal formation, and vascular wall thinning (14). This strict definition of

arteriogenesis/collateral growth has only added to the confusion in the field because much of what other investigators would consider to be collateral growth falls within a much more general definition: small inter-connecting arteries 100-300 μm in diameter enlarging 2-3 fold and displaying increased intimal cell number, no neo-intimal formation, and thickening of the vascular wall (24, 25, 31, 53, 63, 81, 100, 118, 120, 121, 148, 168).

What should now be done to advance the field of therapeutic collateral growth?

If the goal is to derive therapies translatable into the clinic, then scientifically rigorous translational studies carried out in appropriate (diseased) animal models will be necessary to develop and assess potential therapies before their use in humans (153, 159). Thus, to advance the field of collateral growth, future studies must: investigate collateral growth in animal models with appropriate co-morbidities (such as age, atherosclerosis, diabetes, etc.), occlude vessels in anatomical locations relevant to human disease (if feasible, and gradual occlusion would be preferred to acute ligation), focus on the process that will best enhance limb perfusion (collateral growth not angiogenesis), use appropriate methods to assess collateral growth, and functionally identify and study only the most important enlarging collateral vessels (excluding the possibly confounding process of collateral regression). In addition, a recent review by van Weel et al. (157) has suggested combining therapies to induce both angiogenesis and arteriogenesis in order to both increase gas exchange in ischemic tissues as well as enhance the delivery of blood flow to the compromised limb. Indeed, data presented later in this thesis (Chapter II) will suggest that both collateral growth as well as the preservation of the distal vasculature are necessary for adequate perfusion of the hindlimb measured via laser Doppler perfusion

imaging. Van Weel et al. (157) goes on to state that they had success (measured via ABI and improved ulcer healing) using VEGF gene transfer into diabetic patients with critical limb ischemia in a double-blinded, placebo controlled clinical trial; suggesting that the ischemic limbs of diseased patients are amenable to therapeutic intervention.

In conclusion, the current pre-clinical rodent models only roughly approximate the clinical human situation. A review of PAD identified several primary properties which should be incorporated into future animal models, including: 1) gradual onset/occlusion is more relevant (but may be more difficult to mimic, based on the animal models used), 2) infrainguinal occlusion most frequently occurs in the short segment of the femoral artery, 3) pre-existing collaterals arising from the profunda femoral artery are involved for compensating for occlusions at this site (as long as they remain intact), and 4) the patient populations most at risk for significant morbidity and mortality associated with PAD are those who are older, have diabetes, and who smoke. The three endogenous mechanisms of collateral growth, angiogenesis, and vasculogenesis were identified as potentially able to restore hindlimb perfusion upon arterial occlusion; however, experimental as well as theoretical evidence suggests collateral growth is the most important and/or most able to restore perfusion to pre-occlusion levels. In addition, 1) major limitations to the animal models and analytical methods currently utilized to study angio-/arterio-genesis and collateral growth were identified, 2) a shift of focus and resources by some but not all investigators away from collateral growth on to therapeutic angiogenesis was discussed, and 3) the strict definition of arteriogenesis has caused additional confusion regarding which vessels are the most important to focus on in regards to collateral growth. Therefore, the focus of my thesis

work will be to: 1) address the current state of confusion in the field of collateral growth using animal models and assessment methodologies appropriate for the study of collateral growth (Chapters I-III), 2) identify the most important collateral vessels in providing distal perfusion in the hindlimb (Chapter I), 3) determine what characteristic changes can be observed in the vascular wall of growing collaterals between multiple species (Chapter I), and 4) to investigate mechanisms mediating the growth of the most important collaterals in both young, healthy and aged animals (Chapter II and III) in response to femoral arterial occlusion.

CHAPTER I

Characteristics of Major Collateral Arteries in the Peripheral Circulation of Humans, Pigs, Rats, and Mice

Abstract:

Improvement in tissue perfusion after focal arterial occlusion in the peripheral circulation results mostly from increased conductance in collateral vessels rather than the distal microvasculature. Although post-occlusion tissue perfusion is determined primarily by the largest collaterals, controversy exists regarding the size of vessels which enlarge to become major collaterals, the nature of their wall remodeling, and degree of tortuosity. In addition, recent studies have suggested that important aspects of collateral growth may differ between the small animals most commonly used as experimental models and large species, including humans. To clarify these issues, collateral arteries resulting from focal arterial occlusion were analyzed and compared in four species; humans, miniature swine, rats, and mice. The presence of pre-existing collaterals in the porcine hindlimb was demonstrated by a rapid recovery of blood flow after acute superficial femoral artery occlusion. Angiograms (humans and pigs) and vascular casting (rats and mice) demonstrated that the major collaterals in all species formed from pre-existing small arteries. Tortuosity of collaterals varied considerably with major segments displaying limited convolution. The pre-existing arteries which formed major collaterals enlarged <3-fold while other pre-existing collateral pathways regressed. Collateral diameter ranged from <0.1 mm in mice to >1 mm in pigs. Histological examination of collateral cross-sections in pigs, rats, and mice revealed dramatic increases in intimal cell number. Neointimal formation was observed in regressing but not enlarging collaterals.

While the results indicate that the major collaterals in small and large species share many characteristics including development from small arteries, not arterioles, intimal cell recruitment/proliferation, and varying degrees of tortuosity; the substantial variation in size and wall thickness between species could significantly impact the diffusion of paracrine factors and thereby the remodeling process. Conflicting data in previous studies might be explained by differences between enlarging and regressing collaterals.

Key Words: arteriogenesis, Ossabaw, collateral vessel regression,

Introduction:

Vascular adaptations to arterial occlusion in the peripheral circulation involve all vessels from capillaries to collateral arteries. While there is no consensus as to which vascular adaptations are most important (17), a number of studies provide evidence that changes in collateral vessel conductance or resistance is most important, both immediately and long-term after abrupt arterial occlusion (76, 111, 112, 149, 150, 163). Certainly collateral vessels are considered to be largely responsible for the restoration of blood flow to distal tissues after abrupt arterial ligation in the human leg (37, 51). Available evidence suggests that the largest collaterals are the most important in tissue perfusion (36, 81, 121).

Controversy exists regarding the vessels from which collaterals develop, including the large (major) collaterals. It has been proposed that the tortuous portion of collaterals may represent de novo vascular formation (137). In support of new vessel formation, high resolution micro-CT has demonstrated an increased number of small-to-medium sized vessels after mouse iliac-femoral artery and vein ligation (79). Other investigators have questioned if newly formed vessels can have functional significance (55, 57) and many studies have concluded that collaterals are formed by pre-existing vessels. Potential pre-existing vessels forming collateral pathways include arcading arterioles and arteries. Early studies of vascular adaptation to arterial occlusion in the peripheral circulation focused on small arteries, but many current studies focus on capillaries or small arterioles. While early studies reported collateral growth to involve a 2-3-fold expansion of small arteries (31, 81), some recent studies and reviews report that arteriogenesis, a term commonly used as a synonym for collateral growth, involves small

arterioles ≤50 μm which enlarge 10-25-fold to become conduit collateral arteries (14, 117, 155).

The stimulus for both collateral growth and arteriogenesis in the peripheral circulation is thought to be increased shear stress. But the phenotype of outward remodeling in response to elevated shear is reported to be quite different for large through small arteries, versus the small arterioles undergoing arteriogenesis. Studies of arteries have reported endothelial proliferation and maintenance of, or increased, medial thickness (15, 83, 126, 130, 142-145, 148), while small arterioles are indicated to undergo medial thinning and intimal destruction with neointimal formation (63, 114, 119).

The objective of this study was to characterize the major collaterals which develop subsequent to focal arterial occlusion in the peripheral circulation of multiple species. To accomplish this objective, 1) human angiograms with focal infrainguinal arterial occlusion were studied to identify the type of vessels which enlarged to become major collaterals in humans; 2) common femoral artery flow in miniature pigs was measured before and after acute compression of the superficial femoral artery to assess the presence of pre-existing collaterals; 3) the arterial pathways from which major collaterals developed in the hindlimb of miniature pigs, rats, and mice after femoral artery ligation were identified from angiograms and vascular casts; and 4) arterial cross-sections of the major collaterals and similar vessels in contralateral limbs were compared. Collateral vessel tortuosity was also evaluated because some investigators utilize this trait as the primary method to identify collaterals.

Materials/methods:

Human Studies

Past angiograms of patients with lower extremity arterial occlusive disease were obtained by the physician authors with approval from the IUPUI/Clarian Institutional Review Board. After removal of all identifying information, these angiograms were used to evaluate the vessels which formed collaterals after focal occlusion. The human angiograms were obtained by means of routine angiographic protocols through a femoral access via a Seldinger approach followed by a standard lower extremity angiogram.

Animals

Male Ossabaw miniature swine that were gonadally intact were studied as fully mature adults at ~20 months of age. Male WKY rats and C57BL/6 mice purchased from Harlan (Indianapolis, IN) were studied at 3-6 months of age. Animals were housed in the Laboratory Animal Resource Center at Indiana University School of Medicine which is fully accredited through the Association for Assessment and Accreditation of Laboratory Animal Care International. The mice were part of another study (39), but no duplicate data are presented. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Indiana University School of Medicine and complied fully with recommendations outlined by the National Research Council in the *Guide for the Care and Use of Laboratory Animals* (104).

Pig Procedures

Femoral artery ligation was performed in the miniature swine during a routine procedure for cardiac stenting as previously described (44). In brief, the right superficial femoral artery (SFA) underwent double ligation (1 cm apart) using sterile silk sutures

(Figure 1). Four weeks later the pigs were prepared for lower extremity hemodynamic assessment and angiograms. The pigs were sedated with a mixture of telazol (5.0-6.0 mg/kg) and xylazine (2.2 mg/kg) via an intramuscular neck injection followed by atropine (0.05 mg/kg). Once adequate sedation was achieved, the pigs were transported to the operating room where they were pre-oxygenated and given general gaseous anesthesia using isofluorane. The anterior neck and bilateral medial groin areas were shaved using electric clippers in preparation for vascular access. After proper positioning of the pig on the operating table, a right carotid arterial cutdown was performed using Bovie cautery and blunt dissection. An arteriotomy was made in the right carotid artery and an introducer catheter was placed using the Seldinger technique. A guidewire was placed into the introducer catheter and used to insert a guiding catheter into the hindlimb arterial system. Intravascular ultrasound was used to determine common femoral arterial diameter. After accessing the artery with a guiding catheter, a 0.014-inch diameter guidewire or a ComboWire™XT (Volcano Corporation, Rancho Cordova, CA) was advanced down the artery and the intravascular ultrasound catheter (35 MHz, Ultracross, Boston Scientific/SciMed, Maple Grove, MN) was advanced over the flow wire and positioned (91, 135).

Hemodynamic measurements were performed using the guiding catheter that provided proximal (typically aortic) blood pressure and the ComboWire™XT which provided continuous distal pressure and blood flow velocity readings. These measurements were recorded with the tip of the ComboWire advanced out of the guiding catheter in the common femoral artery. The Doppler signals were continuously recorded both as instantaneous peak velocity (IPV) and averaged peak velocity (APV) values.

Each APV value was calculated on-line by the ComboWire system as an average of instantaneous peak velocity over 2 consecutive cardiac cycles.

The tip of the guiding catheter was placed in the external iliac and common femoral artery to perform angiography of both proximal hindlimbs using C-arm fluoroscopy as described in detail (135). Angiograms were performed in real-time with Hypaque 60 or 75 contrast using a power injector set at 5 seconds (30 ml). At the conclusion of the experiment, the animal was euthanized by excision of the heart. Collaterals were identified from the frame by frame analysis of the angiogram as vessels through which the contrast agent was observed to enter the distal femoral artery (Figure 3). Collateral diameter was measured using the ECG electrode as a spatial calibration marker and was also expressed relative to the profunda (deep) femoral artery.

Rat and Mouse Procedures

Animals were anesthetized via 2% isofluorane. The ventral surface of their right hindlimb was shaved and sterilized by cleansing with iodine and an alcohol prep pad. Animals were placed on a heating pad to maintain body temperature throughout the procedure. Sterile surgical techniques were observed at all times.

In rats, the superficial femoral artery was carefully dissected away from the vein and nerve and ligated with sterilized silk at the level of the inguinal ligament (Figure 1). In mice, a femoral artery single ligation model to produce moderate ischemia (128) was performed in which the femoral artery was carefully dissected away from the vein and ligated with sterilized silk at a point just distal to the superficial epigastric artery (Figure 1). The incisions were closed with sterile resorbable suture and the rats and mice were given buprinorphen (0.05 mg/kg) subcutaneously for pain maintenance.

Perfusion fixation was performed two weeks post-ligation via an aortic cannula at physiologic pressure (~100 mmHg) with 4% Zn-formalin after clearing the blood and dilating the vasculature (0.9% sterile saline, 10 mM adenosine, 1 mM sodium nitroprusside, and 2.5 U/ml heparin). The vascular casting agent (Microfil®, Flow Tech Inc., Carver, MA) was then slowly injected by hand and stopped as soon as it entered the saphenous artery as visualized via intravital microscopy. After curing of the casting agent overnight at 4°C, the collateral pathway, beginning with the re-entry segment, was then dissected through the midzone region. The same pathway was identified in the contralateral limb, dissected, and utilized as the same-animal control. Arterial sections from the mid-zone region were excised.

Histology and Morphometry

The excised collateral vessels and their contralateral controls were placed in 4% Zn-formalin for 24 hours before histological processing. Vessel tissues were embedded in plastic, sectioned, and stained with Lee's Methylene Blue (rats and mice) or embedded in paraffin and stained with Hematoxylin and Eosin (pigs). Images were obtained with a Leica DM 5000B microscope with 5-40X objectives using a Diagnostic Instruments Inc. Spot RT_{KE} camera. Lumen circumference was traced using Image J and the cross-sectional diameter was calculated.

Statistical Analysis

Statistical analyses were performed via ANOVA (SigmaStat 3.0, Systat Software, Inc., San Jose, CA). When the ANOVA identified significant differences ($P \leq 0.05$), the Holm-Sidak method was used for pairwise multiple comparisons. Data are expressed as means ± SEM. Humans, n = 3; Pigs, n = 12; Rats, n = 12; and Mice, n = 7.

Results:

Hemodynamic Evidence of Pre-existing Collaterals

In the miniature swine, APV at baseline in the patent left common femoral was 24±1.6 cm/sec. With acute occlusion of the superficial femoral artery, the APV initially decreased to ~50% of baseline and then recovered to 88±3.9% of baseline within 3 minutes. This recovery in APV after the initial decrease is consistent with earlier studies in humans (84, 124, 125), dogs (42, 111) and rats (149) and is best explained by the dilation of pre-existing vessels which form alternative or collateral pathways.

Major Collateral Pathways and Tortuosity

Human angiograms with focal arterial occlusion in the lower extremity are shown in Figure 2. With superficial femoral artery (SFA) occlusion above the knee in Figure 2A, multiple collateral arteries are seen arising from proximal SFA branches and re-inserting into the distal SFA. Figure 2B shows similar bridging collaterals for popliteal artery occlusion with large collaterals originating from the proximal popliteal, bypassing the occlusion, and re-inserting into the distal popliteal artery. The angiogram in Figure 2C and D shows two focal occlusions in the superficial femoral artery with proximal collaterals arising from the profunda femoral. Major collaterals in all angiograms include the geniculate arteries. Note that some collaterals are more tortuous than others and most have segments which are relatively straight.

In pigs, major collaterals were identified as vessels which reconstituted the femoral system distal to the occlusion site. This was accomplished by viewing sequential frames of the angiogram as illustrated in Figure 3. All collaterals were observed to originate from the gluteal artery, profunda (deep) femoral artery, or proximal SFA shown

in Figure 3B. The contrast agent enters the distal SFA through three collateral pathways identified by arrows in Figure 3C and continues downstream (Figures 3D and E). There was an animal average of 1.3±0.17, 1.5±0.19 and 2.7±0.33 collaterals, respectively, which branched from the proximal SFA, gluteal, and profunda femoral arteries. The collateral branches originating from the gluteal artery consistently formed the largest collaterals in our porcine hindlimb model. As percent of the profunda femoral artery, the collateral branches from the gluteal artery averaged 37±3.0% vs. 26±2.1% and 29±1.2% for collaterals branching from the SFA and profunda femoral artery. Only a subset of these major collaterals demonstrated tortuosity. In other miniature and farm pigs with similar arterial ligation, collaterals have been reported to branch from the proximal SFA and profunda femoral, but not the gluteal artery (14), perhaps due to subspecies differences. The average diameter of the collateral mid-zone region based upon calibration with the ECG electrode was 1.1±0.05 mm, versus an average diameter of 3.6 mm for the profunda and 4.9 mm for the common femoral artery. Pre-existing stems and re-entry points but not interconnecting mid-zone regions could typically be visualized in the same-animal control limbs (Figure 3G). Such interconnections must be pre-existing (but below the angiogram resolution) or the APV would have been severely compromised after acute arterial compression.

In WKY rats, major collaterals were identified by vascular casting. We had anticipated that the major collateral pathway would involve the perforating artery as previously reported (63, 100). When initial experiments indicated this vessel was not consistently enlarged, we identified the superficial circumflex iliac artery and its proximal branch as the major collateral pathway most frequently demonstrating

enlargement (Figure 4). This anatomical pathway has previously been reported as a potential pre-existing collateral in the rat hindlimb (82). As shown in Figure 4, the superficial circumflex iliac artery and its proximal branch is observed to be present but smaller in the contralateral control limb. Similar to the major collaterals in the human and pig, the rat collaterals develop from pre-existing small arteries and have limited tortuosity.

In mice, we observed the gracilis artery to be the major collateral pathway after femoral artery occlusion. As shown in Figure 5, this vessel is both enlarged and more tortuous in the experimental than contralateral limb. This is consistent with several previous reports (25, 121, 167).

Collateral Vessel Wall Characteristics Including Collateral Size and Enlargement

Micrographs of pig, rat, and mouse control and collateral cross-sections are presented in Figure 6. A representative micrograph of a collateral branching from the porcine gluteal artery is shown in Figure 6A next to a similar sized artery from the contralateral limb. Representative micrographs are shown for the rat superficial circumflex iliac artery from control and experimental limbs in Figure 6B. Figure 6C shows typical micrographs of the rat perforating arteries in which the diameter of the perforating artery in the experimental limb was reduced relative to the same animal control limb. Gracilis arteries from the control and experimental limbs of a mouse are presented in Figure 6D.

Perhaps the most remarkable difference in the enlarged collaterals of all species is the increase in intimal cell number relative to the control limb. A neointima was not observed in any of the pig collaterals nor in any of the superficial circumflex iliac or

gracilis arteries of experimental limbs in rats and mice, respectively. In general, the media was thicker in the enlarged collaterals of the experimental limbs than the controls in the contralateral limbs. In mice the medial thickness of the control limb gracilis artery averaged 3.2±0.13 μm vs. 5.4±0.36 μm in the experimental limb. The considerable difference in the diameter and thickness of collaterals from pigs compared to rats and especially mice is striking, as illustrated in Figure 6E.

In rats and mice, diameters were determined from arterial cross-sections of the major collateral in the experimental limb and the identical pathway in the contralateral limb. In rats, diameters were measured for both the perforating artery and superficial circumflex iliac arteries as both have been reported to be collaterals. Relative to same animal control diameters, the perforating artery was enlarged in only 3 of 12 animals where paired observations were possible versus 8 of 12 rats for the superficial circumflex iliac. As shown in Figure 7A, vessel diameters in these animals averaged 199±54.3 μm and 135±13.4 μm for the perforating and superficial circumflex iliac arteries in the control limbs and were 57±23.6 ($P = 0.083$) and 88±26.2% ($P = 0.004$) larger, respectively, in the experimental limbs. Although the perforating artery formed a collateral pathway in rats, it was smaller in the experimental vs. control limbs in 9 of 12 rats, averaging 50±8.2% of the same animal control limb (Figure 7A).

Similar diameter measurements were made in mice and are reported in Figure 7B. The diameter of the gracilis artery in arterial cross-sections of control limbs averaged 55±3.4 μm. In the experimental limb, the diameter was 50±13.5% larger than in same animal control limbs.

Discussion:

This study was focused on the main collateral arteries in the human lower extremity and hindlimb of pigs, rats and mice. Because of the exponential relationship between vessel radius and vascular resistance, the largest collaterals have been considered to be the most important in terms of tissue perfusion distal to the site of occlusion. In the rabbit hindlimb, the largest collaterals have been reported to contribute nearly 100% of total resting flow (36). In mice, major differences in hindlimb perfusion observed between various strains (121) after femoral artery ligation are explained by small differences in the expansion of the pre-existing vessels which form the major collaterals. Our results from four species indicate that major collaterals develop from pre-existing, named, small arteries or their direct branches rather than arterioles, enlarge <3-fold rather than 10-25-fold, and are characterized by an increase in intimal cell number but not neo-intima formation. The results also indicate that tortuosity is not necessarily a characteristic of the major collaterals. An additional, often over-looked consideration is that some pre-existing small arteries which form collateral pathways undergo regression rather than enlargement.

Importance of Pre-existing Pathways

To our knowledge, the observations in the miniature swine of an initial drop in common femoral APV after acute SFA occlusion, followed by significant recovery represent the first demonstration in the pig hindlimb of functional pre-existing collaterals. These results are consistent with previous studies in the human leg reviewed below as well as studies in the dog (42, 111) and rat (149) hindlimb. This increase in perfusion occurs concomitant with an elevation in arterial pressure distal to the site of acute

occlusion (42, 111, 149), indicating that the primary compensation involves dilation of the pre-existing bypass collaterals rather than the distal microcirculation.

The existence and role of pre-existing "mid-zone" collateral pathways has been debated since before Longland's work in which he both reviewed and provided additional anatomical evidence for these vessels (81). The clinical importance of pre-existing collateral vessels has been recognized since the 18th century, when surgeons like Anel, Desault, and Hunter utilized the concept of a collateral circulation as the basis for limb survival after large arterial ligations for the treatment of brachial and popliteal artery aneurysms (86, 116). In 1910, Matas (84) described methods of determining the adequacy of the collateral circulation during temporary large artery occlusion, and he applied these methods to evaluate treatment options for limb aneurysms. Further evidence of the importance of pre-existing collaterals came in 1945, when Debakey and Simeone (37) published a large volume of data that examined limb salvage rates in soldiers who had suffered major arterial injury. These surgeons considered damage to the collateral circulation either by the injury or debridement to be a major factor influencing limb survival after arterial ligation. Even after emergency iliac artery ligation in humans, the collateral circulation can be adequate to maintain limb perfusion and integrity (51). In the early 1950's, human experiments by Shepherd (124, 125) demonstrated that an abrupt decrease in blood flow to the calf immediately after femoral artery compression was followed by a significant recovery to near normal levels within minutes.

Primary Collateral Artery Pathways

The above studies demonstrate the presence of pre-existing collateral pathways, but do not identify the vessels or establish that they are the ones which undergo luminal

expansion. For focal arterial occlusion near the knee, the small genicular arteries typically form the major collaterals as shown in our angiograms (Figure 2) and reported in human anatomical and surgical texts (78, 88) and reviews (122). For more proximal or extensive occlusion, pre-existing collateral pathways involve branches from the internal iliac and profunda femoris arteries as well as branches from the external iliac and common femoral arteries (51, 88). Our experiments in pigs, rats and mice demonstrate similar findings in that the major collaterals develop from small, named arteries or their direct branches. This is similar to many previous reports as summarized in Table 1.

It is important to note that variation occurs in the specific collateral pathways which become major collaterals and that regression of smaller collaterals occurs. For example, in rats we observed the superficial circumflex iliac artery and its proximal branch to be the major collateral after proximal femoral ligation. Previously published studies had identified the major collateral pathways to include the perforating artery connection to the popliteal artery (63) and a more proximal pathway that appears to connect to the muscular branch of the femoral artery proximal to the popliteal artery (100). The specific pathways which become the major collaterals are likely determined by the ligation site and the size and length of the pre-existing anastomoses. Our ligation of the femoral artery was performed proximal to the superficial circumflex iliac artery. In this case, branches between the internal iliac and superficial circumflex iliac provide a significantly shorter collateral path than the perforating artery which connects the internal iliac to the popliteal artery. At 2 weeks post-ligation we observed a decrease in the cross-sectional diameter of the perforating artery in most rats (9 of 12; Figure 7A). Herzog et al. (63) have previously reported the diameter of this artery to be increased 7 days after

femoral artery ligation and then to decrease in size. Such a regression in collateral diameter is consistent with reports that only a few of the collaterals that initially enlarge will remain as a major compensator (81, 117). This regression is thought to result from decreases in shear stress as other collaterals enlarge (45, 117). Such a regression of collaterals was first reported by Longland (81) and more recently by others (63, 97, 115).

Collateral Diameters and Percent Enlargement

Comparison of identical collateral midzones in experimental and contralateral control limbs required vascular casting and was performed only in rats and mice. Because our measurements of initial and final collateral artery size differ substantially from some recent studies (14, 119) and reviews typically indicate arterioles and not small arteries to be the "substrates" of arteriogenesis (117, 158), we identified additional studies which used angiograms or vascular casting and reported diameters of major collaterals that develop from pre-existing vessels. These results are presented in Table 1. The majority of the studies indicated the pre-existing collateral vessel to be an anatomically named small artery; the gracilis artery in mice and the perforating artery in rats. The range of initial collateral artery size was 10-75 μm for mice, 140-230 μm for rats, 50-300 μm for rabbits, 150 μm for dogs, and 10-30 μm for pigs. Only 2 of the 14 studies reviewed reported collateral diameter enlargement ≥3-fold. One of these studies reported the initial size to be 10-30 μm; values significantly lower than the other reports. Neither of these two studies indicated how the vessels examined histologically were identified as pre-existing collateral mid-zones.

Collateral Wall Characteristics

Both wall thinning (16) and thickening (77, 148) have been reported for collaterals in different vascular beds and at different stages of development (16). Studies examining pathways that develop from small arteries consistently show medial thickening occurs early (Table 1), as observed in this study. Fragmentation of the internal elastic lamina and neointimal formation have also been reported as early events in collateral remodeling in heart and hindlimb (60, 63, 114, 119). But these studies did not distinguish between major and regressing collaterals. Fragmentation and neo-intimal formation were not observed in the major collaterals of any species in the current study and are not apparent in micrographs presented in many other studies of major collaterals (Table 1). The increase in intimal cell number without neointima formation was a consistent observation in hindlimb collaterals of pigs, rats and mice (Figure 6) and similar to our previous reports in mesenteric collaterals (126, 148). This phenotype is also consistent with earlier studies of shear-mediated vascular remodeling in large and small arteries (126, 130, 144, 145, 148). The increase in intimal cell number in our vessels is most likely due to flow-induced endothelial cell proliferation (126, 129, 144), but cell recruitment may also be involved (129). The intimal cell nuclei have a size and orientation consistent with endothelial cells and immunostaining indicates the intimal cells are eNOS, CD31 positive and CD45, ED2 negative (39, 126, 144); polyploid or multinucleated cells were not observed in the intima.

Species Comparisons

Our results, together with earlier work referenced above, demonstrate that basic similarities exist between the major collateral vessels in small to large species, including

development from pre-existing small arteries, degree of luminal enlargement, and intimal cell proliferation/recruitment. However, preclinical studies of novel therapies in small species have had much greater efficacy than comparable therapies in clinical trials (see reviews (153, 159)). As a consequence, some investigators have called for improved models of peripheral arterial occlusive disease (159) and others have questioned if collateral growth in small species, especially mice, accurately represents what occurs in larger species, specifically man (14, 153).

There are multiple reasons why collateral growth might be different between small and large species. Luminal and wall dimensions in the major collaterals of mice and pigs (Figure 6) differ by ~10-fold. Based upon the differences in the amount of new tissue needed to produce fully remodeled collaterals in mice versus larger species, it has been proposed that the time course of remodeling may differ substantially (14). All wall layers are involved in the remodeling of collateral vessels. The media and adventitia of larger species are far thicker than in mice and consequently the ratio of adventitial and smooth muscles cells to endothelial cells is much greater in larger than smaller species. This could greatly impact the relative amount of growth modulators produced by various layers. The diffusion distance for paracrine factors is much greater in larger species. Reactive nitrogen and oxygen species are considered to have important roles in the promotion and impairment of collateral growth (87, 89, 101, 103, 108) and diffusion distances could have an important impact on reactive species concentrations in various wall layers. The vasa vasorum can have an important role in arterial remodeling (2). Although it is much more developed in small arteries of larger species, it is unknown if or how the vasa vasorum influences the growth of major collaterals.

While small animals offer advantages which have been previously reviewed (14, 159), there are compelling reasons why preclinical evaluation of novel therapies to promote collateral growth should include large animals models. In addition to the above arguments, therapeutic delivery and methods of assessment can be identical in large animals such as the miniature pig and humans. Pigs also have a cardiovascular system that much more closely resembles the human than smaller species. This includes hemodynamics (flow and pulse rate), vascular dimensions and branching patterns. As an example of the latter, rats do not have a profunda (deep) femoral artery (52). Lipid metabolism and serum lipoprotein distribution in pigs are also similar to humans (40). Additional advantages of large animal models such as the pig include the ability to obtain large blood samples. Hemodynamic measurements can be made which are difficult if not impossible in rodents. Chronic instrumentation and longitudinal studies are far more feasible. The same instruments and techniques (e.g. Duplex ultrasound, MRI, angiograms) can be used to evaluate disease in both man and pig.

Study Limitations

This investigation was focused upon focal occlusion of the femoral artery because short-segment total occlusion of the superficial femoral artery is the most common lesion seen with chronic obliterative atherosclerosis of the infrainguinal vessels (88). While we observed the major collaterals to develop from pre-existing small arteries in our study, the results observed could differ significantly from what might be observed in humans with extensive and diffuse obstructive disease and in animal models which use multiple arterial ligations or excision. While the data presented demonstrate the presence of pre-existing collaterals in the lower extremity and hindlimb, it is important to note that other

studies in various vascular beds have demonstrated that the number of pre-existing collaterals, as well as their ability to dilate acutely and enlarge chronically, is influenced by the presence of risk factors for vascular disease, physical activity, and genetic background (23, 89, 121, 145, 146, 162).

In conclusion, the data presented and reviewed are consistent with the development of major collaterals in the hindlimb of mice, rats, and pigs and the lower extremity of humans from small pre-existing arteries, rather than small or terminal arterioles. Except for mice, the initial size appears to be consistently >100 μm diameter. The enlargement most commonly observed is <3-fold. While many recent studies have focused on smaller vessels and neovascularization, we consider these pre-existing small arteries to represent a neglected vascular segment with significant therapeutic potential in peripheral arterial disease. For example, the capacity for shear-mediated luminal expansion in arteries is diminished or even abolished by at least some of the risk factors for vascular disease; specifically aging and hypertension (10, 89, 90, 145, 146). Recent studies have demonstrated successful therapeutic reversal of this impairment in small animal models (33, 89). Such therapies either alone or in combination with other molecular or cellular therapies might significantly impact the development of major collaterals in patients with peripheral arterial disease. Considering that collateral growth is impaired in humans with risk factors for arterial disease, the development of a large animal model of vascular disease with impaired collateral growth would be a very significant addition to animal models available for preclinical testing.

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Table 1: *Hindlimb Collateral Vessel Size and Fold Enlargement*

First Author/ Year:	Species	Method	Pathway	Duration (Wks)	Initial Size (μm)	Final Size (μm)	Fold Increase	Neo-Intima	WT or MT
Distasi/2008 (39)	Mouse	IS	GA	2	57	87	1.5*	No	Inc
	Mouse	IS		2-4	59	139	2.4*		
Scholz/2006 (120)	Mouse	CS	GA	3	42	71	1.7	No*	Inc*
Chalothorn/ 2005 (24)	Mouse	CS	GA	3	45	95	2.1*	No*	Inc (54%)
		-Angio	PA	3	75	150	2*		
Chalothorn/ 2005 (25)	Mouse	CS	GA	3	50	75	1.5*	No*	Inc (54%)
Gruionu/ 2005 (53)	Mouse	IS	GA	8	20	40	2*		
Ziegelhoeffer/ 2003 (167)	Mouse	CS	GA	1	39	77	2*	No*	Inc*
Scholz/2003 (118)	Mouse	CS	GA	1	32	49	1.5*	No*	
		CS	GA	4	32	77	2.4*		
Buschmann/ 2003 (14)	Mouse	CS	?	2	10-30	160	5*		Inc*
Scholz/2002 (121)	Mouse	CS	GA	3	36-41	76-85	2.1-2.4	No*	Inc*
Herzog/2002 (63)	Rats	CC	PA	2-8	140	300	2.1*	Yes	Inc, 32wks
Prior/2004 (100)	Rat	IV	PA	3.5	230	332	1.4*		
Scholz/2000 (119)	Rabbit	CS	?	2	50	225	4.5*	Yes	Dec
				34	50	550	10*		
Longland/ 1953 (81)	Rabbit	Angio	Multiple	12	100-300	500	1.7*		
Conrad/1971 (31)	Dog	CC	Multiple	11	150	380	2.5*		
Buschmann/ 2003 (14)	Pig	CS	?	2	10-30	620	20*		

Table 1: Abbreviations defined below by table column. Method: CS = cross-sections, IS = in situ after vascular casting, Angio = angiogram, CC = corrosion cast, IV = isolated vessels (pressurized). Pathway: GA = gracilis artery, PA = perforating artery, ? = not reported. Fold Increase: * = Estimated from reported diameters. Neointima: * = not reported by authors but our impression of included cross-section. WT or MT = wall thickness or medial thickness, * = not reported by authors but our impression of included cross-section, Inc = Increased, Dec = Decreased.

Figure Legends:

Figure 1: Diagram of the hindlimb arterial vasculature and experimental model in pigs, rats and mice. Sketches demonstrate the location of the arterial ligation site (X) in each species and identify the pre-existing vessels which form the major collaterals identified in this study; namely the profunda femoral and gluteal arteries in pigs, superficial circumflex iliac and perforating arteries in rats, and gracilis artery in mice. SFA = superficial femoral artery.

Figure 2: Major collaterals in human lower extremity angiograms with arterial occlusion. Collateral vessels are observed bypassing the site of a superficial femoral artery (SFA) occlusion (A), a popliteal artery (PA) occlusion (B), and a patch angioplasty combined with multiple SFA occlusion (C and D) in three different patients. The occlusion(s) is bounded by * and differing degrees of tortuosity, which occasionally occur within the same collateral pathway, are indicated by arrows (less tortuous) or arrowheads (more tortuous). The collaterals providing distal reconstitution arise from pre-existing, named arteries as labeled.

Figure 3: Representative porcine hindlimb angiographic series demonstrating major collateral arteries. Angiograms were reviewed frame by frame to identify true bypass collaterals responsible for the reconstitution of the distal superficial femoral artery (SFA) in each pig. (A) Experimental limb prior to contrast injection. (B) Appearance of the SFA, profunda femoral artery (PFA), and gluteal artery (GA) with contrast injection. Arrows in C indicate multiple sites of distal SFA filing and arrows in D identify

collaterals arising from branches of the SFA, PFA, and GA. Note that not all collaterals have a tortuous appearance. Panels E and F demonstrate reconstitution of the distal SFA and the exit of the contrast medium from the PFA and GA; asterisks in F indicate the ligation site of the SFA. (G) Angiogram of the same-animal control limb highlights the preexisting small arteries (identified by arrows) which form the origins of major collateral. Spatial calibration is identical for all panels.

Figure 4: Major collaterals in rat hindlimb with proximal femoral artery occlusion. Representative intravital microscopy images of vascular casting in the experimental and control limbs of the rat. The superficial circumflex iliac artery (SCIA) branches from the femoral artery (FA) and gives rise to a proximal branch (arrowheads) that undergoes collateral enlargement when the femoral artery is ligated (X). The contralateral control proximal branch vessel can be observed in the unligated, control limb.

Figure 5: Major collaterals in mouse hindlimb with distal femoral artery occlusion. Representative intravital microscopy images of vascular casting in the experimental and control limbs of the mice. The femoral artery (FA) ligation point is indicated, as are the saphenous (SA) and profunda femoral (PFA) arteries. The major collateral pathway (arrowheads) involves the pre-existing gracilis artery (arrows) passing through the anterior gracilis muscle, which is clearly evident in the control limb. The posterior gracilis artery can also contribute to collateral blood flow and is observed just below the arrowheads.

Figure 6: Cross-sections of pig, rat, and mouse control and major collateral arteries. (A) Comparison of control and collateral porcine arteries revealed an increase in intimal cell number in the collateral without apparent neointimal formation. The same result also was obtained for the enlarged collaterals in rats (B) and mice (D). For a regressing collateral pathway identified in the rat (C), neointimal formation (arrow) is apparent but not an increase in intimal cell number. (E) images of a mouse and rat collateral are inserted into the lumen of a pig collateral to emphasize the tremendous difference (>10-fold) in vessel size, wall thickness, and distance from inner to outer wall layers. Ad = adventitia, M = media, L = lumen, arrowheads indicate the intima, * = denotes artifacts from histological processing and/or Microfil ® casting agent left in the lumen. Control and collateral artery pairs from all species were imaged at the same magnification.

Figure 7: Average diameters of hindlimb collateral and control arteries from (A) rats and (B) mice. Diameter measurements were obtained from arterial cross sections. In rats, the superficial circumflex iliac (SCI) artery was consistently larger in the experimental than control limb (8 of 12 rats). Diameters of the perforating artery (PA) are divided into two groups depending upon whether the diameter in the experimental limb was larger (Enl-PA, 3 of 12 rats) or smaller (Reg-PA, 9 of 12). In mice, the gracilis artery (GA) was consistently enlarged in the experimental limb.

Figure 1

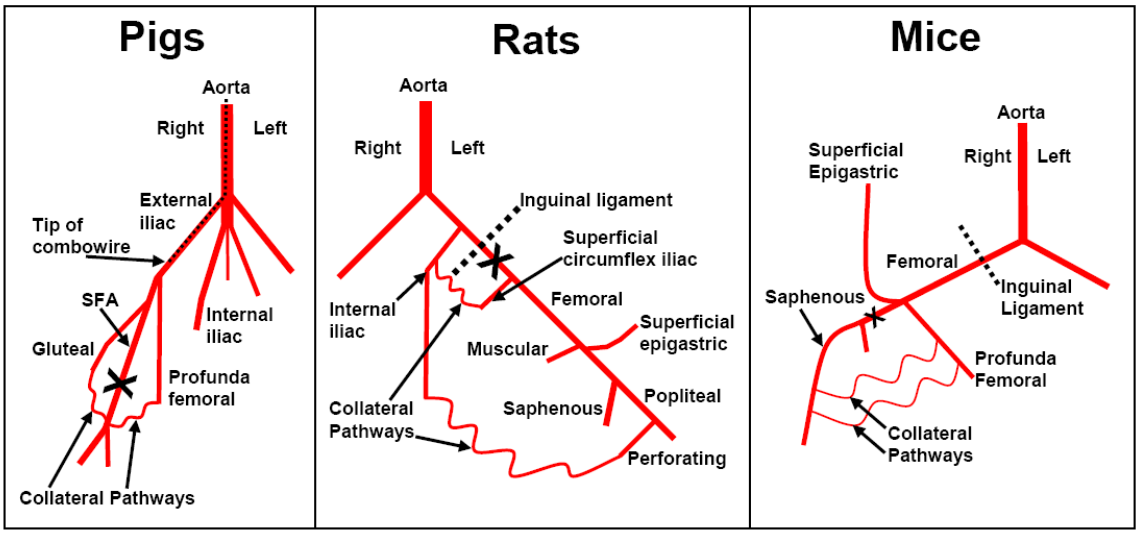

Pigs
Aorta
Right
Left
External iliac
Tip of combowire
SFA
Internal iliac
Gluteal
Profunda femoral
Collateral Pathways
Rats
Aorta
Right
Left
Inguinal ligament
Superficial circumflex iliac
Internal iliac
Femoral
Superficial epigastric
Muscular
Popliteal
Collateral Pathways
Saphenous
Perforating
Mice
Aorta
Superficial Epigastric
Right
Left
Femoral
Inguinal Ligament
Saphenous
Profunda Femoral
Collateral Pathways

Figure 2

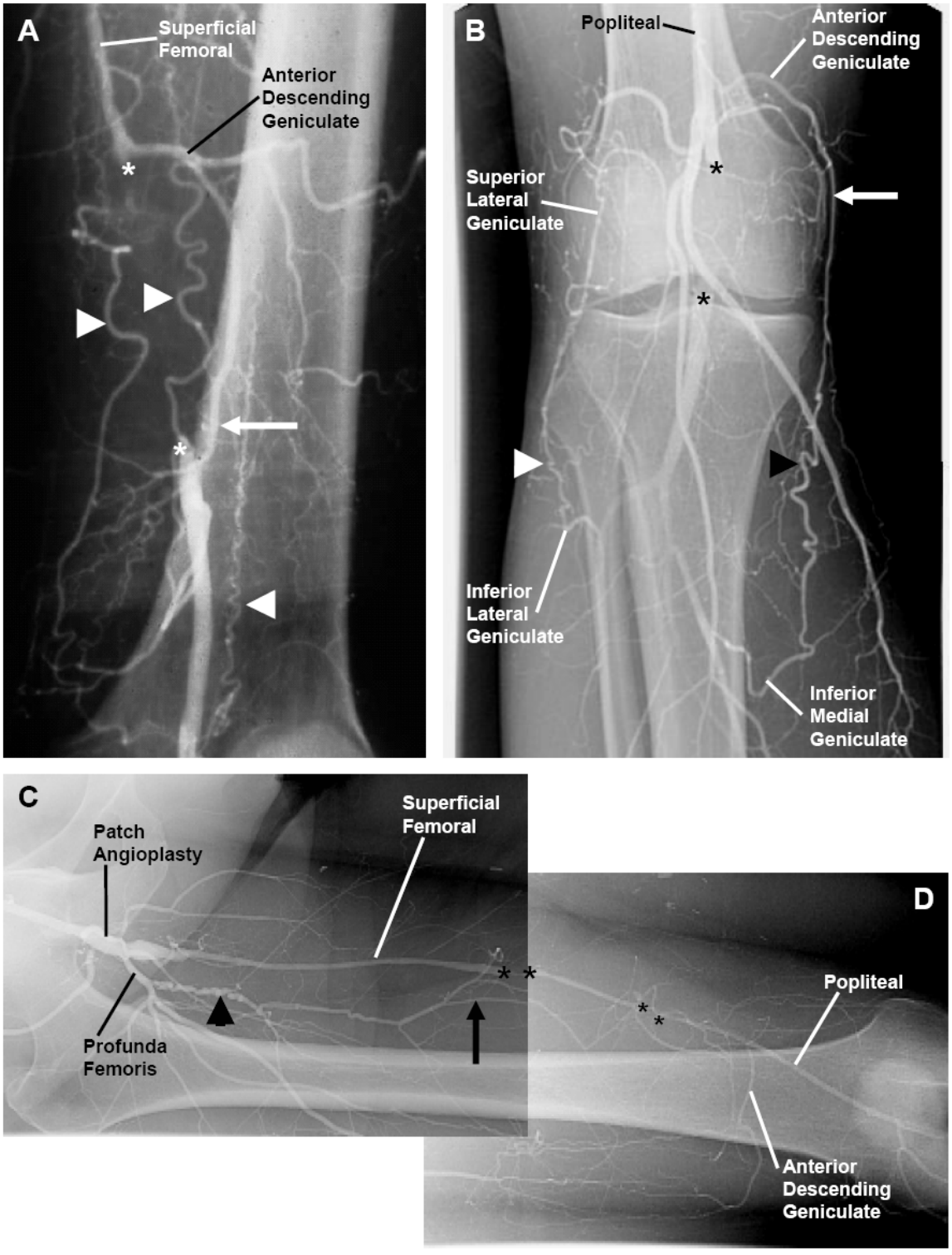

A
Superficial Femoral
Anterior Descending Geniculate
B
Popliteal
Anterior Descending Geniculate
Superior Lateral Geniculate
Inferior Lateral Geniculate
Inferior Medial Geniculate
C
Patch Angioplasty
Superficial Femoral
Profunda Femoris
D
Popliteal
Anterior Descending Geniculate

Figure 3

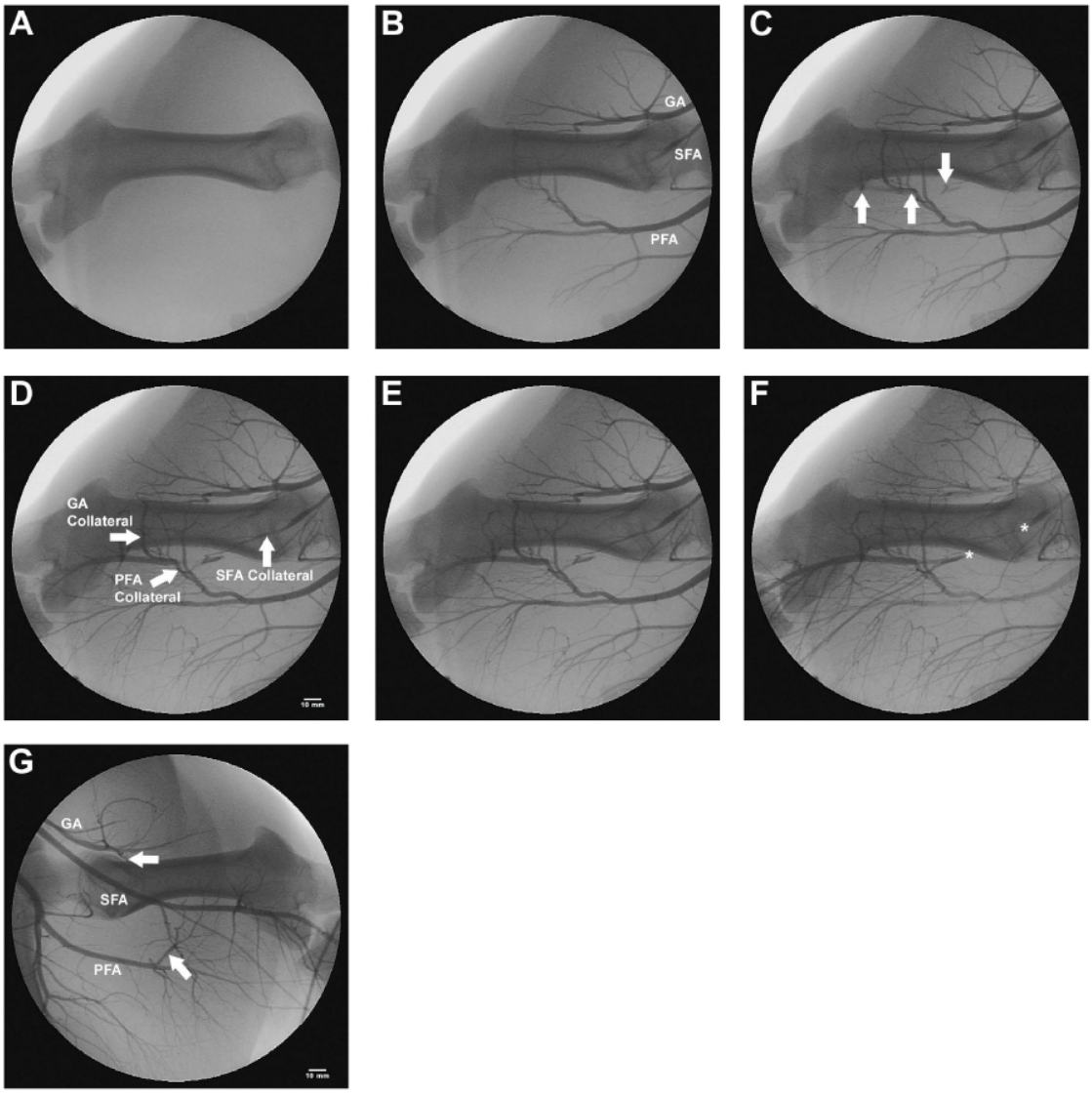

A
B
GA
SFA
PFA
C
D
GA
Collateral
PFA
Collateral
SFA Collateral
E
F
*
*
G
GA
SFA
PFA

Figure 4

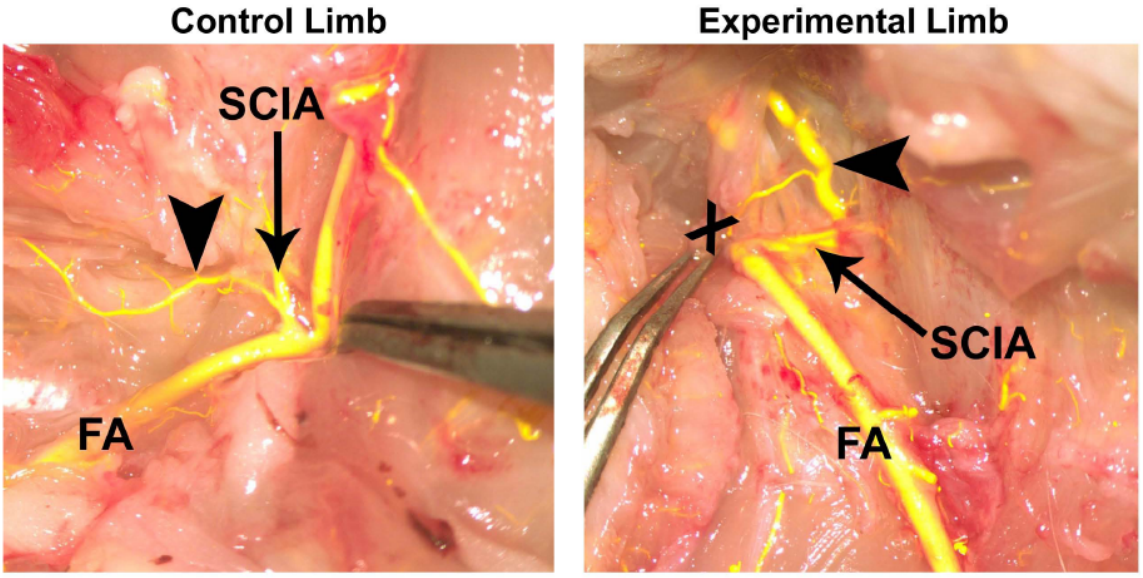


Figure 5

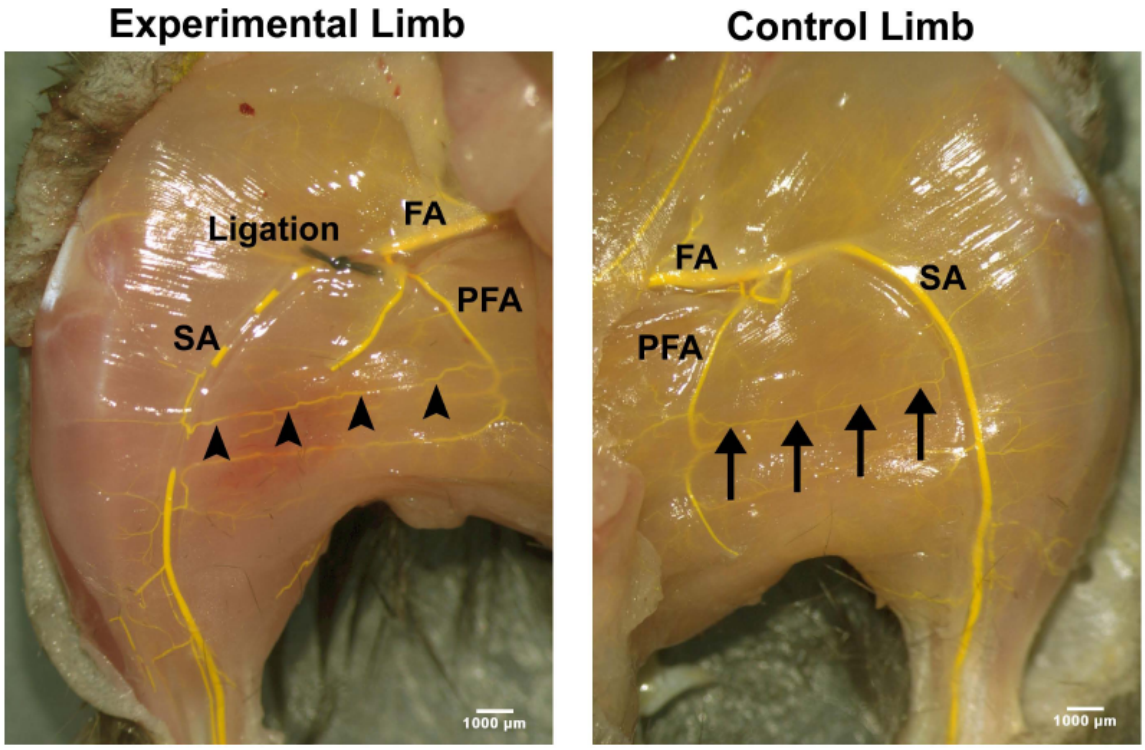

Experimental Limb
Control Limb
Ligation
FA
PFA
SA
FA
SA
PFA
1000 µm
1000 µm

Figure 6

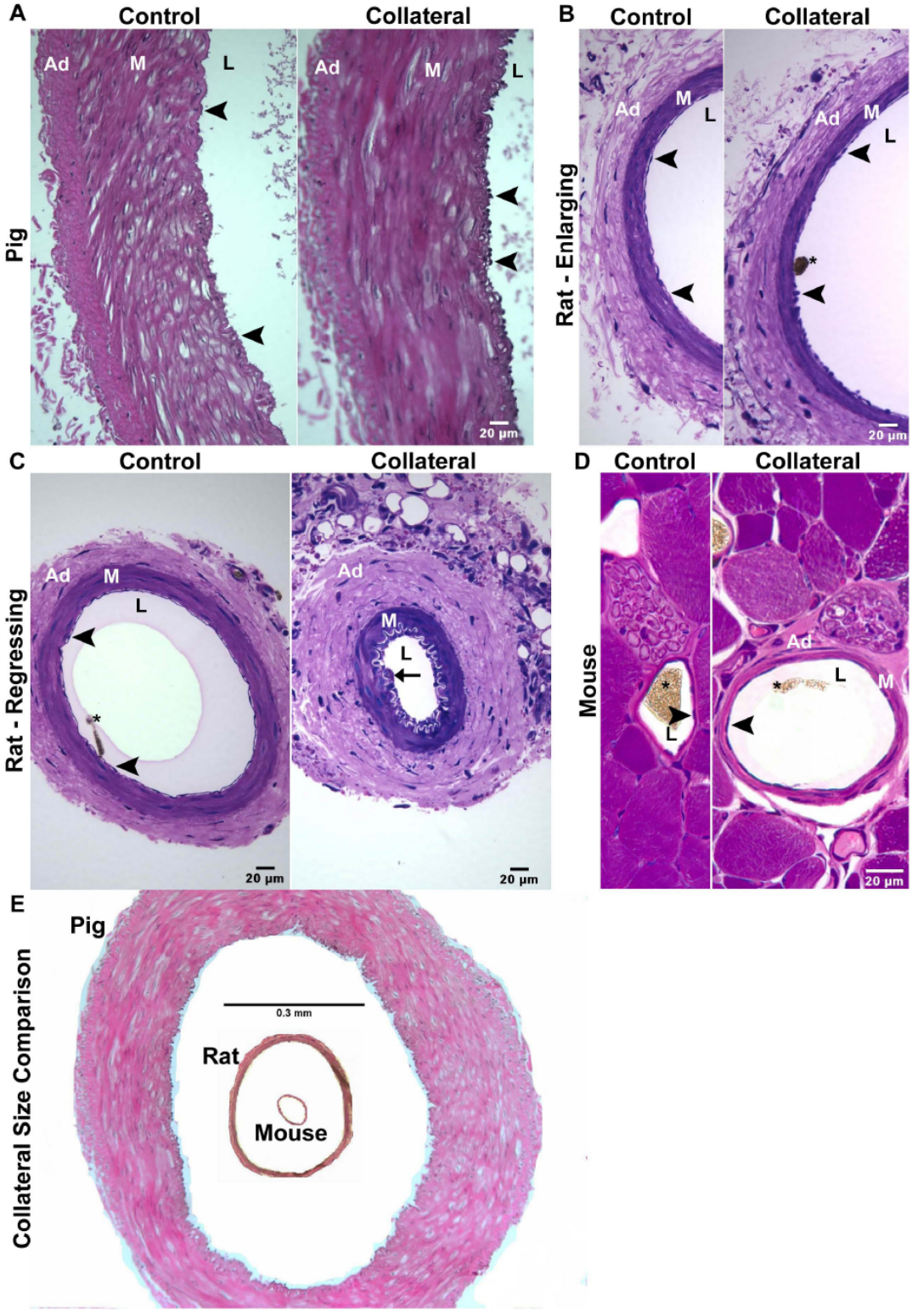
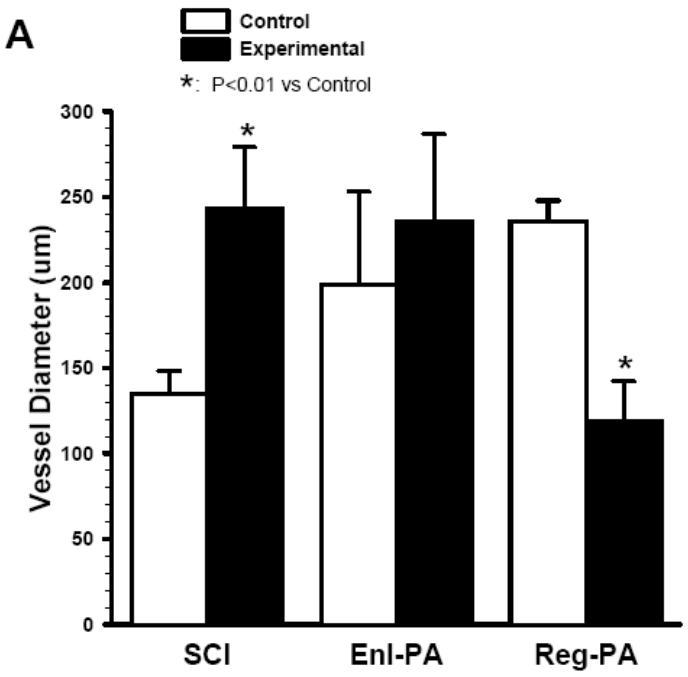
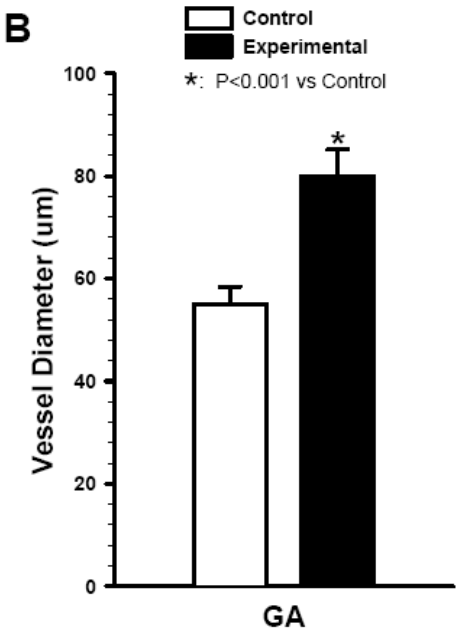

A
Control
Collateral
Pig
Ad
M
L
20 µm
B
Control
Collateral
Rat - Enlarging
Ad
M
L
*
20 µm
C
Control
Collateral
Rat - Regressing
Ad
M
L
*
20 µm
D
Control
Collateral
Mouse
Ad
M
L
*
20 µm
E
Collateral Size Comparison
Pig
0.3 mm
Rat
Mouse

Figure 7


A
Control
Experimental
*: P<0.01 vs Control
Vessel Diameter (um)
300
250
200
150
100
50
0
*
*
SCI
Enl-PA
Reg-PA


B
Control
Experimental
*: P<0.001 vs Control
Vessel Diameter (um)
100
80
60
40
20
0
*
GA

CHAPTER II

Suppressed Hindlimb Perfusion in *Rac2-/-* and *Nox2-/-* Mice Does Not Result from Impaired Collateral Growth

Abstract:

While tissue perfusion and angiogenesis subsequent to acute femoral artery occlusion are suppressed in *Nox2-/-* mice, studies have not established the role of Nox2 in collateral artery enlargement. Rac2 is a small GTPase which binds Nox2 and activates the Nox2-based NAD(P)H oxidase, but unlike Nox2 is primarily restricted to bone marrow derived cells. In this study, we utilized *Rac2-/-* and *Nox2-/-* mice with a novel method of identifying primary hindlimb collaterals to investigate the hypothesis that collateral growth requires these molecules. When initial studies performed with femoral ligation demonstrated similar perfusion and collateral growth in the *Rac2-/-*, subsequent studies were performed with a more severe ischemia model, femoral artery excision. After femoral excision, tissue perfusion was suppressed in *Rac2-/-* relative to C57BL/6 (BL6) mice. The diameters of primary collaterals identified during Microfil® injection with intravital microscopy were enlarged to a similar extent in BL6 and *Rac2-/-* mice. Intimal cell number in collateral cross-sections was also increased in both strains and the cells were CD31 positive, CD45 negative. Circulating leukocytes and $CD11b^+$ cells were increased more in *Rac2-/-* than BL6. Experiments performed in *Nox2-/-* mice to verify that the unexpected results related to collateral growth were not unique to the *Rac2-/-* gave equivalent results. The data demonstrate that, subsequent to acute femoral artery excision, perfusion recovery is impaired in *Rac2-/-* and *Nox2-/-* mice but that collateral luminal expansion and intimal cell recruitment/proliferation are normal. These novel

results indicate that collateral luminal expansion and intimal cell recruitment/proliferation are not mediated by Rac2 and Nox2.

Key Words: arteriogenesis, macrophage, reactive oxygen species, hindlimb ischemia

Introduction:

In the peripheral circulation, the dilation and enlargement of pre-existing vessels which form collateral pathways subsequent to arterial occlusion is the primary vascular compensation which preserves tissue viability and maintains function. These vessels dilate within seconds (42, 71, 81, 149), and undergo expansion for weeks (31, 42, 81, 148). In various species, the pre-existing vessels are the size of the smallest arteries and they enlarge ~100% (24, 31, 63, 81, 121). Available clinical studies indicate subsequent to arterial occlusion in the peripheral circulation, the primary vessels to enlarge as collaterals are pre-existing named arteries (8, 51, 122). The largest of the pre-existing vessels are typically those which become the dominant collaterals (63, 81, 100). Combined anatomical and modeling studies have predicted that hindlimb flow subsequent to femoral artery occlusion is primarily determined by these collaterals (36, 121). This is consistent with studies of segmental resistances which demonstrate that compensation in the collateral vessels is of significantly greater hemodynamic importance than adaptations in the distal microvasculature (76, 150, 163). Nevertheless, few studies investigating mechanisms of vascular compensation subsequent to arterial occlusion in mice have specifically identified and studied these pre-existing vessels which form the primary collateral pathways. Such investigations are needed because angiogenesis and collateral growth are initiated by different stimuli, and differences exist in the molecules and mechanisms which mediate these important processes (13, 19).

Leukocytes, especially lymphocytes (132, 156) and macrophages (6, 58, 67), have been indicated to have an important role in vascular compensation to hindlimb ischemia. Recent studies from Tojo et al. (140) and Urao et al. (151) have established that Nox-2

derived reactive oxygen species (ROS) from bone marrow derived cells (BMDC) have an important role in neovascularization in the ischemic mouse hindlimb. The initial report (140) concluded from suppressed blood flow that collateral growth was impaired, but did not specifically identify collateral bypass vessels or measure their diameters.

In order to investigate the hypothesis that Nox2 NAD(P)H oxidase mediates primary collateral growth subsequent to arterial occlusion, the current study utilized *Rac2* null (-/-) and *Nox2*-null mice and a novel method of identifying primary hindlimb collaterals. Rac2 is expressed primarily, if not exclusively, in hematopoietic cells (68, 107), and binds to and activates the Nox2-containing NAD(P)H oxidase (38, 72). In addition, leukocytes from *Rac2* and *Nox2* null mice have impaired function related to reduced ROS production (94, 140, 151). We present a method to identify a dominant or primary collateral which should be the major collateral supplying flow to the distal limb and utilize this vessel to determine if mechanisms of collateral growth are impaired by Rac2 ablation. Our results indicate that Rac2 is not required for the enlargement of these primary collateral arteries but is required for the recovery of hindlimb perfusion and the maintenance of distal tissue morphology subsequent to femoral artery ligation and excision. As these results were unexpected, primary collateral artery enlargement was also investigated in *Nox2-/-* mice. The response of *Nox2-/-* mice to arterial excision was similar to that observed in the *Rac2-/-* mice. Therefore, the results obtained in the current study indicate that enlargement of pre-existing primary collaterals in the murine hindlimb occurs independently of both Rac2 and Nox2.

Materials/methods:

Animals

Rac2-/- and *Nox2-/-* backcrossed into a C57BL/6J background for >12 generations and C57BL/6J wild type (BL6) mice were studied at age 3-6 month. *Rac2-/-* and *Nox2-/-* mice were bred and maintained at Indiana University while C57BL/6 were obtained from Harlan Industries (Indianapolis, IN). Six BL6 and 7 *Rac2-/-* were used in the initial experiments with femoral artery ligation. For femoral artery excision, 7 BL6, 14 *Rac2-/-*, and 9 *Nox2-/-* were used for perfusion studies with half of the *Rac2-/-* and *Nox2-/-* being utilized for the morphometric and histological studies. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Indiana University School of Medicine.

Murine Hindlimb Ischemia Model

Animals were anesthetized via 2% isofluorane delivered under constant oxygen flow. The right hindlimb was shaved, the hair was removed with a depilatory agent, and the area to be incised was sterilized by cleansing with iodine and an alcohol prep pad. The animal was then placed on a heating pad to maintain body temperature and aseptic surgical techniques were utilized. An approximately 1 cm incision was made from the origin of the saphenous artery to the groin. A schematic of the normal murine hindlimb vascular anatomy is presented in Supplemental Figure S1A. One of two procedures was then performed which produced a femoral artery ligation model with moderate ischemia or a femoral artery excision model with more severe ischemia (128). In the former, the femoral artery was carefully dissected away from the vein and nerve and ligated with sterile 6-0 silk suture at a point distal to the superficial epigastric artery and proximal to

the femoral's trifurcation into the saphenous, popliteal, and geniculate arteries (Supplemental Figure S1B). In the excision model, an additional ligature was placed on the femoral artery at the level of the inguinal ligament with 6-0 silk suture, all side branches were ligated with 9-0 silk suture, and the femoral artery was excised (Supplemental Figure S1C). The incision was then closed with sterile 5-0 resorbable suture and the animal was given buprinorphen 0.05 mg/kg subcutaneously for pain maintenance. In all experiments, great care was taken to keep all vessels and tissues moist and to minimize trauma to adjacent tissue in an attempt to reduce local inflammation.

Laser Doppler Perfusion Imaging

Laser Doppler perfusion imaging (LDPI) (Model LDI-2, Moor Instruments, Devon, U.K.) of the hind paw plantar surface was performed over the course of 14 days to serially and non-invasively assess the recovery of hindlimb perfusion subsequent to arterial ligation/excision. To control for temperature variation during laser Doppler scanning, the animal was placed on a heating pad set at 37°C as previously described (3). Each animal was allowed to acclimate to the heating pad/scanning environment for ≥10 minutes before scanning was performed.

Perfusion Fixation and Vascular Casting

After day 14 the animal was again anesthetized, the infrarenal abdominal aorta was cannulated in the direction of flow with pulled PE-50 tubing, and the hindlimbs were perfused at a constant pressure (100 mmHg) with 10 ml of 0.9% sterile saline with dilator (10 mM adenosine and 1 mM Na-nitroprusside) and heparin (2.5 U/ml heparin) followed by 10 ml of 4% Zn-formalin. Microfil® vascular casting agent (Flow Tech Inc., Carver,

MA) was then slowly injected through the aortic cannula while monitoring the saphenous artery through a dissecting microscope. The Microfil® injection was stopped as soon as the casting agent appeared distal to the most caudal ligation (as it entered the saphenous artery) and the primary collateral pathway was noted. Some filling of the distal vasculature continued until pressure was equilibrated. The carcasses were then wrapped in Saran Wrap® and stored at 4°C overnight prior to dissection.

Collateral Growth and Determination of Primary Compensatory Pathways

After the casting agent had cured, the previously identified collateral pathway was carefully dissected from its point of insertion to its origin. Digital images (via a Leica MZ-9_5 microscope with Diagnostic Instruments Inc. Spot Insight Image Sample camera) of the *in situ* collateral vessel (Figure 1B) were obtained at the midzone region (81). Then the identical pathway was located, dissected in the contralateral control limb as in the ligated limb (Figure 1B), and imaged at the same location. Vessels diameters were measured from the images and collateral growth was assessed by comparing diameters of the same animal control and collateral vessels.

Immunophenotyping and FACS

A HEMAVET Model 950FS cell counter (Drew Scientific Inc., Waterbury, CT) was used to determine the number of total white blood cells (WBCs) in blood samples collected from the tail vein. To assess cell-surface antigen expression, murine peripheral blood mononuclear cells were incubated with primary or isotype control antibodies, as outlined below, in 100 µl of phosphate-buffered saline (PBS)/1% fetal bovine serum (FBS) (PBS, Invitrogen, Grand Island, NY; FBS, Hyclone, Logan, UT). All incubations were performed at 4°C for 30 minutes; cells were washed with PBS/1% FBS and

analyzed using Fluorescence Activated Cell Sorting (FACS, FACSCaliber, BD Immunocytometry Systems, San Jose, CA). Primary antimouse monoclonal antibodies against CD11b were conjugated to phycoerythrin (PE, BD Pharmingen, San Diego, CA). Directly conjugated PE rat immunoglobulin $G_{2b,\kappa}$ ($IG_{2b,\kappa}$) (BD Pharmingen) was used for isotype controls.

Histology, Intimal Cell Counts, and Immunostaining

Once the primary compensatory collateral pathways were identified in the adductor muscles, the control and collateral vessels as well as the more distal control and experimental limb gastrocnemius muscles were harvested and placed in 4% Zn-formalin for 24 hrs before histoprocessing. Vessel and gastrocnemius muscle tissues were embedded in plastic, stained with either Hematoxylin and Eosin or Lee's Methylene Blue, then visualized and imaged with 5-40X objectives on a Leica DM 5000B microscope with a Diagnostic Instruments Inc. Spot RT_{KE} camera. For immunostaining, sections were blocked for endogenous peroxidase activity with 3% hydrogen peroxide in methanol following antigen retrieval either in Antigen Unmasking Solution (Vector Laboratories, Berlingame, CA) at 95°C for CD45 staining or in 20 ng/ml Proteinase K for 15 minutes at 37°C for CD31 staining. Sections were blocked in 3% bovine serum albumin (BSA, Sigma) for 1 h and were stained for CD31 or CD45 (BD Pharmingen, San Jose, CA). Purified class- and species-matched immunoglobulins (BD Pharmingen) were used for isotype controls. Sections were incubated with appropriate biotinylated secondary antibody (Vector Laboratories) followed by incubation with 3,3'-Diaminobenidine (DAB, Vector Laboratories) and counterstained with hematoxylin to permit nuclear identification.

Statistical Analysis

Statistical analyses were performed with two way repeated measures ANOVA (SigmaStat 3.0). When the ANOVA identified significant differences ($P \leq 0.05$), the Holm-Sidak method was used for pairwise multiple comparisons. Data are expressed as means ± SEM.

Results:

Effect of Rac2 Knockout on Vascular Compensation for Femoral Arterial Ligation

Isolation of the primary collateral vessels revealed four different pathways. Representative micrographs are presented in Figure 1A and the legend contains additional details of each pathway. For each collateral, an identical pathway existed in the contralateral control limb (Figure 1B). The primary collateral pathway that enlarged most frequently in response to single arterial ligation in both *Rac2-/-* and BL6 mice was the Gracilis Collateral pathway (Table 1).

Hindlimb perfusion, diameters of collaterals and contralateral control arteries, cross-sectional intimal cell number and representative micrographs are presented in Figure 2. LDPI (Figure 2A) demonstrated improved perfusion in the experimental limb by day 7 with no differences between BL6 and *Rac2-/-* at any time point. Comparison of diameters (Figure 2B) indicated similar expansion of the primary collateral vessel diameter relative to same animal control in the *Rac2-/-* mice and BL6 mice (36±10.4% vs. 55±12.3%, respectively). Histological assessment of the primary collateral vessels demonstrated a profound increase in intimal cell number in the collateral vessels versus their same animal controls which was similar in both strains (159±25.1% in BL6 and 124±30.6% in *Rac2-/-*, Figure 2C). Micrographs representative of all animals investigated in each group (Figure 2D) demonstrate the increase in collateral intimal cell number. All intimal cell nuclei have a similar phenotype (size, shape, orientation) that is consistent with endothelial cells. We performed immunostaining and observed the entire collateral intima to be CD31 positive and CD45 negative (data not shown). This is

consistent with our previous studies in rats in which the entire collateral intima is positive for eNOS (144) and negative for ED2 (126).

Micrographs of the gastrocnemius muscles after femoral artery ligation are shown in Supplemental Figure S2. Minimal if any abnormalities were apparent in the experimental limb gastrocnemius sections of BL6 and *Rac2-/-* experimental limbs as represented in Supplemental Figure S2 B and C, respectively.

The similarity of collateral growth between strains was unanticipated and seemed inconsistent with previous studies with a model of more severe ischemia (140, 151). For this reason a more severe excision model was studied. Collateral growth was evaluated after both 7 and 14 days to determine if the rate but not final magnitude of collateral growth was suppressed (139).

Effect of Rac2 Knockout on Vascular Compensation for Femoral Arterial Excision

Figure 3 presents the data for hindlimb perfusion, diameters of collaterals and contralateral control arteries, and cross-sectional intimal cell number after femoral artery excision. Analysis of the LDPI data by 2-way Repeated Measures ANOVA indicated a significant difference between strains ($P<0.001$), day ($P<0.001$), and a significant interaction ($P = 0.003$). As shown in Figure 3A, LDPI was increased by day 7 in both BL6 and *Rac2-/-*. In *Rac2-/-* there was an additional increase from day 7 to 14. The LDPI ratio was similar between strains at day 1, but was significantly less in *Rac2-/-* than BL6 at days 7 and 14. Consistent with the reduced perfusion observed in the *Rac2-/-*, micrographs of gastrocnemius muscles shown in Supplemental Figure S3 demonstrate greater tissue damage in these mice. Comparison of the day 14 LDPI ratio indicated the perfusion was significantly lower in the limbs with femoral artery excision than ligation

of both the *Rac2-/-* and BL6 mice ($P \leq 0.001$ for both strains). Collateral growth occurred in both *Rac2-/-* and BL6 mice, as indicated by a significant increase in the collateral vessel diameter compared to the same animal control vessel diameter after both day 7 and day 14 (Figure 3B). This collateral vessel expansion was similar between the *Rac2-/-* and BL6 mice at both day 7 (110±43.9 vs. 93±19.3, respectively) and day 14 (170±18.2 vs. 144±22.3, respectively). The collateral cross-sections demonstrated similar morphology as observed with the more moderate model; collateral intimal cell number relative to same animal controls was increased 129±32.0% in BL6 and 213±45.0% in *Rac2-/-* (Figure 3C).

Assessment of Bone Marrow Derived Cell (BMDC) Response

In order to determine if a BMDC mobilization defect was present in the *Rac2-/-* mice, circulating cell counts of total white blood cells (WBCs) and $CD11b^+$ cells, which have been previously shown to mediate improved perfusion in the mouse hindlimb (18), were performed via HEMAVET and FACS analysis. Figure 4 demonstrates there were significantly more total WBCs (Figure 4A) and $CD11b^+$ (Figure 4B) cells circulating in the bloodstream of *Rac2-/-* mice. In addition, WBCs were increased at day 3 in BL6 and *Rac*2-/- mice and remained elevated in *Rac2-/-* animals. $CD11b^+$ cells were increased at days 3 and 7 only in *Rac2-/-* mice. This may indicate either an increased production/ release of BMDCs in the *Rac2-/-* mice (1) or a decreased ability of the *Rac2-/-* monocytes/macrophages to migrate from the bloodstream into the distal tissues.

Effect of Nox2 Knockout on Vascular Compensation to Femoral Artery Excision

Our observation of normal collateral growth in the *Rac2-/-* mice was again the opposite of what we had anticipated based upon previous studies in *Nox2-/-* mice. Since

Rac2 ablation may have effects not mediated through the binding and activation of the Nox2 NAD(P)H oxidase, we performed additional experiments in *Nox2-/-* mice with femoral artery excision to determine if the phenotype we had observed was unique to the *Rac2-/-* mice.

Hindlimb perfusion, collateral growth, and typical skeletal muscle histology observed after femoral artery excision in the *Nox2-/-* mice are reported in Figure 5 and Supplemental Figure S4. The pattern of hindlimb perfusion (Figure 5A) was similar to that of the *Rac2-/-* mice with limited recovery at day 7. Very significant collateral growth occurred in the *Nox2-/-* mice, as indicated by the greater collateral diameter compared to the same animal control vessel diameter at day 14 (Figure 5B). The collateral blood vessel enlargement in the *Nox2-/-* mice relative to same animal control (158±36.3%) was similar to that observed in *Rac2-/-* and BL6 mice. Collateral intimal cell number increased and lipid deposition occurred in the *Nox*2-/- experimental limbs similar to that observed in the *Rac2-/-* mice (Supplemental Figure S4).

Discussion:

Novel and significant aspects of the current study include: 1) the presentation of an improved method of identifying primary collaterals in the mouse hindlimb for the assessment of collateral artery growth, 2) an increase in intimal cell number in the primary collaterals of *Rac2-/-* and BL6 mice consistent with elevated shear, 3) greater leukocyte mobilization in *Rac2-/-* mice than BL6 after femoral artery excision, and 4) normal enlargement of primary collaterals in *Rac2-/-* and *Nox2-/-* mice despite impaired tissue perfusion and increased skeletal muscle injury in the distal limb.

Assessment of Collateral Growth

While many previous studies have compared diameters of specific vessels in the mouse hindlimb to assess collateral growth (16, 23, 24, 53, 97, 120, 121), the method presented in this study provides a number of advantages. Controlled perfusion fixation with dilator preserves the vasculature in a consistent state. Slow injection of vascular casting material during microscopic observation helps ensure reliable filling, permits identification of the first vessels through which the casting agent enters the distal vasculature, and facilitates dissection of the collateral pathway for diameter measurement. With this method we established that four primary pre-existing collateral pathways exist in the hindlimb (Figure 1). The fact that these pathways are the first to provide casting material to the distal vasculature suggests that they are the pathways of least resistance and represent the dominant or primary collaterals. These are the vessels which others have concluded are primarily responsible for distal limb perfusion (36, 121). This is similar to the clinical situation as the major collaterals in the lower extremity are identified as pre-existing named arteries (37, 41, 51, 78, 122). We also demonstrated that

the primary pathway varies not only between models, but even among different animals within the same model (Figure 1, Table 1). Similar variation in the collateral pathway exhibiting the greatest luminal expansion also occurs in the rat hindlimb, based upon comparison of angiograms in existing studies (63, 100) and our preliminary observations. Because of this variation, failure to identify the primary collateral pathway(s) can result in significant underestimation of collateral growth. The identification of primary collaterals and their isolation permits the investigation to be performed at the vessel/local tissue level where stimuli and structural and molecular remodeling should be more uniform than the isolation of whole muscle sections such as adductors which could contain not only enlarging collaterals but also static and even regressing vessels.

Intimal Response in Primary Collaterals

In the primary hindlimb collaterals of BL6, *Rac2-/-*, and *Nox2-/-* mice, we observed a profound increase in intimal cell number (Figures 2C, 3C, S4). This response was uniform along the circumference and, unlike previous reports (63, 119), did not involve apparent neointimal formation. To our knowledge, this is the first report of such an intimal response in mouse hindlimb collaterals and it was typical of all primary collaterals we observed. The intimal cells were CD31 positive and CD45 negative indicating they are endothelial rather than myeloid or epithelial cells (65). In preliminary studies we have observed equivalent responses in primary collaterals in the rat and pig hindlimb. This intimal response is similar to that observed prior to luminal expansion in carotid arteries (83, 129) and small mesenteric arteries subjected to chronically elevated flow (126, 144, 146, 148). Such an increase in intimal cell number can be explained by

endothelial cell proliferation/recruitment (126, 129, 144) and is suppressed in collateral vessels which do not enlarge (126, 146).

Role of Bone Marrow Derived Cells

Tissue resident progenitor and bone marrow derived cells have been shown to be critically important in vascular and skeletal muscle adaptations subsequent to femoral artery occlusion. Available studies indicate that both *Rac*2 and *Nox*2 null mice have major defects in BMDC function including ROS production, homing, and chemotaxis (94, 107, 151, 152). Indeed, bone marrow cell transplantation studies performed by Urao et al. (151) provide compelling evidence that the effects of Nox2 on perfusion and capillary density in the ischemic hindlimb are mediated by bone marrow-derived cells. Skeletal muscle fat accumulation, as observed in the micrographs of *Rac2-/-* and *Nox2-/-* mice after arterial excision (Supplemental Figures S3 and S4), has been associated with an impaired inflammatory cell response (32). Yet, in spite of this, collateral luminal expansion and intimal cell proliferation/recruitment were not impaired in the *Rac2-/-* or *Nox2-/-* mice. The simplest interpretation of the data is that the growth of primary collaterals is not mediated by Rac2 or Nox2.

Vascular Compensation to Arterial Occlusion

Tissue perfusion assessed by LDPI increased significantly in all animals after day 1. Relative to BL6, this increase was suppressed in *Rac2-/-* and *Nox2-/-* mice with femoral artery excision (Figures 3A and 5A). The reduced perfusion is consistent with the increased lower limb injury (Supplemental Figures S3 and S4) observed in the *Rac2 -/-* and *Nox2-/-* and previous reports in the hindlimb of *Nox2* null mice (140, 151). Subsequent to femoral artery ligation, the perfusion of the distal tissues is determined by

the vascular resistance of the bypass collaterals and the distal microcirculation (76, 150, 163). The majority of the vascular resistance distal to the occlusion is in the bypass collateral vessels (76, 150, 163). As compensation occurs and perfusion is increased, the major decrease in resistance occurs in the collaterals with minimal if any decrease in distal microvascular resistance (76, 150, 163). Comparable studies after femoral artery excision are not available, but we would expect that the collateral resistance would be even greater with femoral artery excision. Based upon this reasoning, we were surprised that perfusion was compromised in the *Rac2* and *Nox2* null mice when the growth of primary collaterals was not suppressed. Potential explanations include abnormal collateral function, impaired growth of smaller collaterals, or microvascular abnormalities. This work was focused on collateral growth assessed by luminal expansion and did not assess collateral tone or the ability of the enlarged collaterals to dilate and constrict. Functional differences in the collaterals could result in a perfusion deficit. While it is possible that there could be a difference in the number of enlarging collaterals, the similar enlargement of the major collaterals suggests that the mechanisms responsible for collateral growth are not impaired. Previous studies in the *Nox2-/-* mouse have shown clear impairment of angiogenesis, but capillaries comprise such a small component of skeletal muscle microvascular resistance (7, 49) that it is not clear that the difference in perfusion between hindlimbs of BL6 and *Rac2* and *Nox2* null mice after femoral excision can be attributed to differences in capillarity. Other studies have shown that perfusion subsequent to femoral artery occlusion is not correlated with capillarity (62, 121). Studies have shown that chronic reductions in flow and pressure can result in enhanced constriction and inward remodeling characterized by wall thinning (5, 9, 15, 28,

30, 73, 98, 99, 133, 144); characteristics which occur in human critical limb ischemia (28-30). Thus, the profound decrease in distal microvascular flow and pressure could result in similar decompensatory events in the precapillary arterioles and small arteries which control the majority of microvascular resistance (7, 49, 75, 169). Such a decompensatory response could be exaggerated in the *Rac2* and *Nox2* null mice. Certainly more work is warranted to identify the specific vascular segment and mechanisms responsible for the reduced perfusion observed with severe ischemia in the *Rac2* and *Nox2* null mice.

In conclusion, *Rac2* and *Nox2* null mice were utilized with a novel method of identifying primary hindlimb collaterals to investigate the hypothesis that the growth of primary collaterals subsequent to arterial occlusion is mediated by Rac2 and Nox2. Results obtained suggest that enlargement of pre-existing primary collaterals in the murine hindlimb is independent of both endothelial and leukocyte Nox2. Since collateral growth is modulated if not mediated by elevated flow (45, 96), this interpretation is consistent with the observation that $p47^{phox}$ but not Nox2 mediates flow-induced outward remodeling (21). These results provide further evidence that different stimuli initiate angiogenesis and collateral growth and that differences exist in the molecules and mechanisms that mediate these important processes. Additional studies are warranted to clarify how the pre-capillary microcirculation is influenced by Rac2 and Nox2 in hindlimb ischemia and how the presence of vascular disease and risk factors alter Nox2 dependent compensation.

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Table 1: *Summary of the Primary Collateral Pathway Locations*

	BL6 Ligation	***Rac2-/-* Ligation**	**BL6 Excision**	***Rac2-/-* Excision**	***Nox2-/-* Excision**
Gracilis Collateral	12 (100%)	9 (82%)	7 (50%)	2 (22%)	4 (80%)
Deep Adductor	2 (17%)	1 (9%)	3 (21%)	3 (33%)	1 (20%)
Middle Dorsal	1 (8%)	0	3 (21%)	0	1 (20%)
Superficial Dorsal	1 (8%)	3 (27%)	7 (50%)	5 (56%)	2 (40%)
Sciatic Nerve Comp	0	0	1 (7%)	0	0
# Animals with Multiple Paths:	3 (25%)	1 (9%)	6 (43%)	1 (11%)	3 (60%)
Total # of Animals:	12	11	14	9	5

Table 1: The number of mice having primary collaterals in the specific pre-existing pathway is indicated as well as percent of total in parentheses. The counts were made after perfusion fixation and Microfil injection 14 days after femoral artery ligation/ excision. The Gracilis Collateral pathway is the primary pathway that enlarges in response to femoral arterial ligation in both BL6 and *Rac2-/-* mice while both Adductor (including gracilis) and Dorsal pathways contribute equally in response to femoral arterial excision in both strains of mice. In one instance, the companion artery to the Sciatic Nerve appeared to be the primary compensating collateral pathway.

Figure Legends:

Figure 1: Primary collateral pathways in the mouse hindlimb after 14 days of femoral artery ligation or excision. (A) Intravital microscopy and Microfil® vascular casting in the experimental limbs of BL6 and *Rac2-/-* mice identified four primary pathways that provided a collateral bypass. The first pathway identified was the Gracilis Collateral artery(s) (24), which originates from the profunda femoral (aka. muscular branch) artery and passes primarily through the superficial anterior and posterior gracilis muscles of the adductor region of the thigh to re-insert into the distal saphenous artery. This collateral path has been previously described in several studies (25, 26, 53, 58, 79, 121). The Deep Adductor pathway originates as a branch of the internal iliac artery, passes along the ventral portion of the pelvis, and then penetrates the deeper adductor musculature to re-insert into the saphenous artery proximal to the re-insertion point of the Gracilis Collateral path. This path can occasionally come off the profunda femoral artery to penetrate the deeper adductor musculature before re-inserting into the saphenous artery, as described by Scholz et al. (121). The Middle Dorsal path originates from a different branch of the internal iliac than the Deep Adductor path (a branch that passes through the pelvis), then passes down the dorsal surface of the back to the lower limb where it invades the deeper adductor musculature to re-insert into the popliteal artery and may be evident in the angiograms of Heil et al. (58). The Superficial Dorsal path originates from the same internal iliac arterial branch as the Middle Dorsal path, but upon reaching the lower limb it passes along the dorsal surface of the adductor musculature just underneath the skin (instead of penetrating the adductor muscles) to re-insert into the popliteal artery at a point distal to the re-insertion of the Middle Dorsal path. This path has been

observed in the angiograms of Bergmann et al. (6) as well as Shireman and Quinones (128). (B) For all collateral pathways in the experimental limbs, an identical pathway composed of the same pre-existing vessels could be identified in the contralateral control limbs of the same animal (identified by arrowheads), as shown in the upper left versus right panels. Diameter measurements were made from high magnification digital images of the collateral and control vessels (lower left and right panels, respectively). Insets indicate areas enlarged in bottom panels. FA = Femoral artery, PF = Profunda Femoral artery, P = Popliteal artery, S = Saphenous artery, II = Internal Iliac artery branch, Arrows = Collateral pathway.

Figure 2: Vascular compensation to femoral arterial ligation in BL6 and *Rac2-/-* mice. (A) The perfusion of the experimental limb in BL6 and *Rac2-/-* mice assessed by LDPI is expressed relative to same animal control at days 1, 7, and 14 post-ligation. In both BL6 and *Rac2-/-,* the LDPI increased after day 1. Perfusion ratios were similar between strains at all days, (BL6, n = 6; *Rac2-/-*, n = 7). (B) Assessment of control and collateral artery diameters 14 days after femoral arterial ligation in BL6 and *Rac2-/-* mice indicated a significant increase in collateral vessel diameter vs. same animal control vessel diameter (BL6, n = 6; *Rac2-/-*, n = 7). (C) Quantification of intimal cell number in BL6 and *Rac2-/-* obtained from micrographs of control and collateral cross-sections, as illustrated in panel *D*, demonstrates an increase of >100% (BL6, n = 6; *Rac2-/-*, n = 7). (D) Vessel cross-sections (stained w/ Lee's Methylene Blue) from BL6 and *Rac2-/-* mice 14 days post-ligation demonstrate typical luminal expansion and increased intimal cell numbers (micrographs representative of all observed in each animal strain). Arrows

identify some of the intimal cell nuclei and * identifies Microfil in the lumen of the vessels, which is sometimes lost during sectioning (lower right panel).

Figure 3: Vascular compensation to femoral arterial excision in BL6 and *Rac2-/-* mice. (A) LDPI perfusion ratios demonstrate an increase in perfusion by day 7 that is greater in BL6 than *Rac2-/-* mice (BL6, n = 7; *Rac2-/-*, n = 14). (B) Relative to same animal controls, collateral diameter in BL6 and *Rac2-/-* mice was similarly increased at 7 and 14 days after arterial excision (BL6, n = 6 after 7 days and n = 8 after 14 days; *Rac2-/-*, n = 4 after 7 days and n = 5 after 14 days). (C) Quantification of intimal cell number after 14 days of femoral excision indicates a significant increase in collaterals relative to same animal controls of both BL6 and *Rac2-/-* (BL6, n = 5; *Rac2-/-*, n = 5).

Figure 4: HEMAVET and FACS analysis of total white blood cells (WBCs) (A) and $CD11b^+$ cells (B). There were significantly more total WBC and $CD11b^+$ cells in *Rac2-/-* than BL6 both before surgery and during the 14 days following femoral artery excision. The number of WBC was increased from baseline at day 3 in BL6 and day 3-14 in *Rac2 -/-*. The number of CD11b+ cells was increased from baseline at days 1 and 3 post-ligation in *Rac2-/-* but not in BL6. (BL6, n = 7; *Rac2-/-*, n = 6).

Figure 5: Vascular compensation to femoral arterial excision in *Nox2-/-* mice. (A) LDPI perfusion ratios for *Nox2-/-* mice (n = 9). (B) Enlargement of the primary collateral vessel diameter occurred in the *Nox2-/-* and the percent increase from control is similar to the increase observed in both the BL6 and *Rac2-/-* mice (n = 5).

Figure 1: *Primary Collateral Pathways in the Mouse Hindlimb*

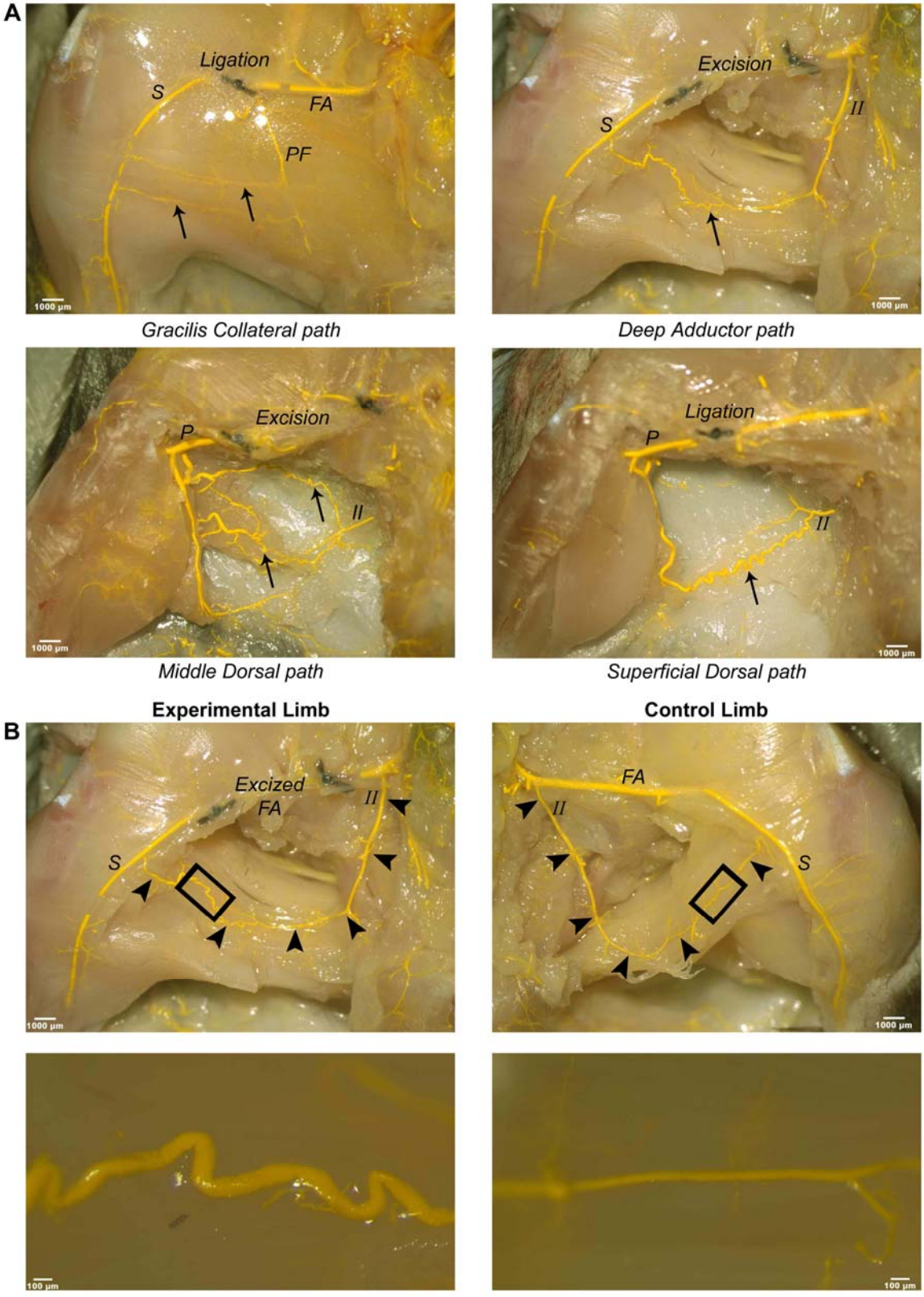


Figure 2

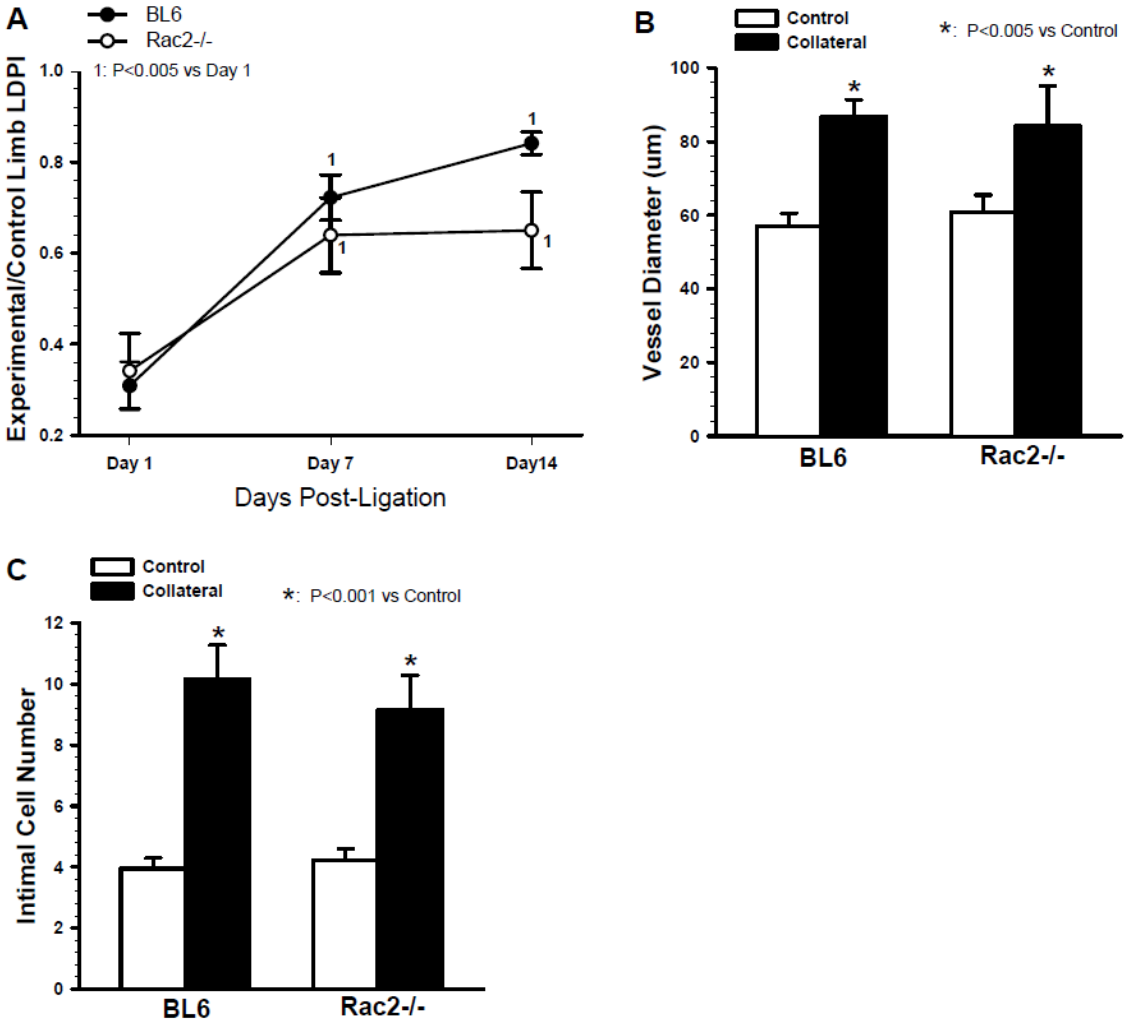

A
BL6
Rac2-/-
1: P<0.005 vs Day 1
Experimental/Control Limb LDPI
1.0
0.8
0.6
0.4
0.2
Day 1
Day 7
Day14
Days Post-Ligation
B
Control
Collateral
*: P<0.005 vs Control
Vessel Diameter (um)
100
80
60
40
20
0
BL6
Rac2-/-
C
Control
Collateral
*: P<0.001 vs Control
Intimal Cell Number
12
10
8
6
4
2
0
BL6
Rac2-/-

Figure 2D

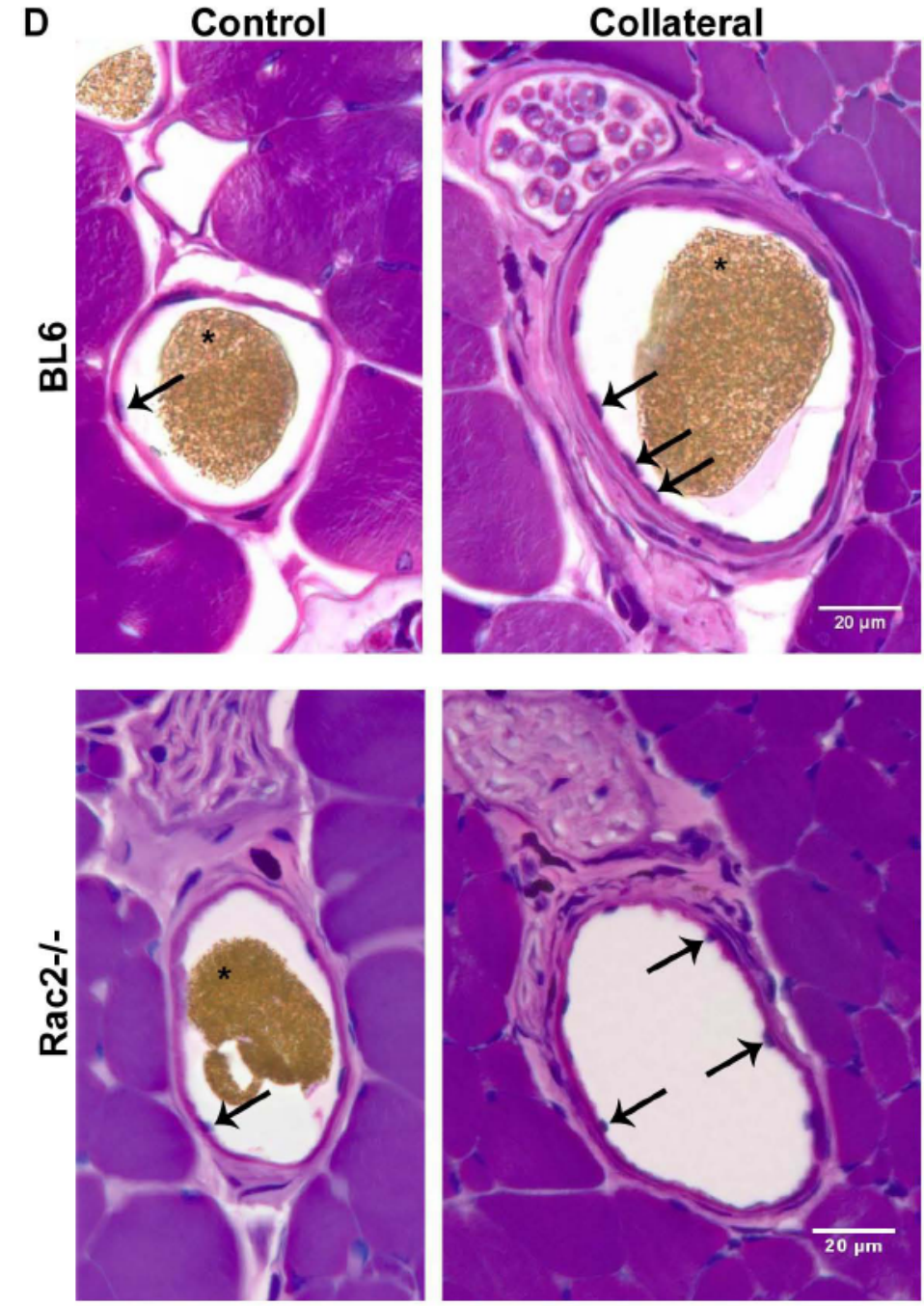


Figure 3

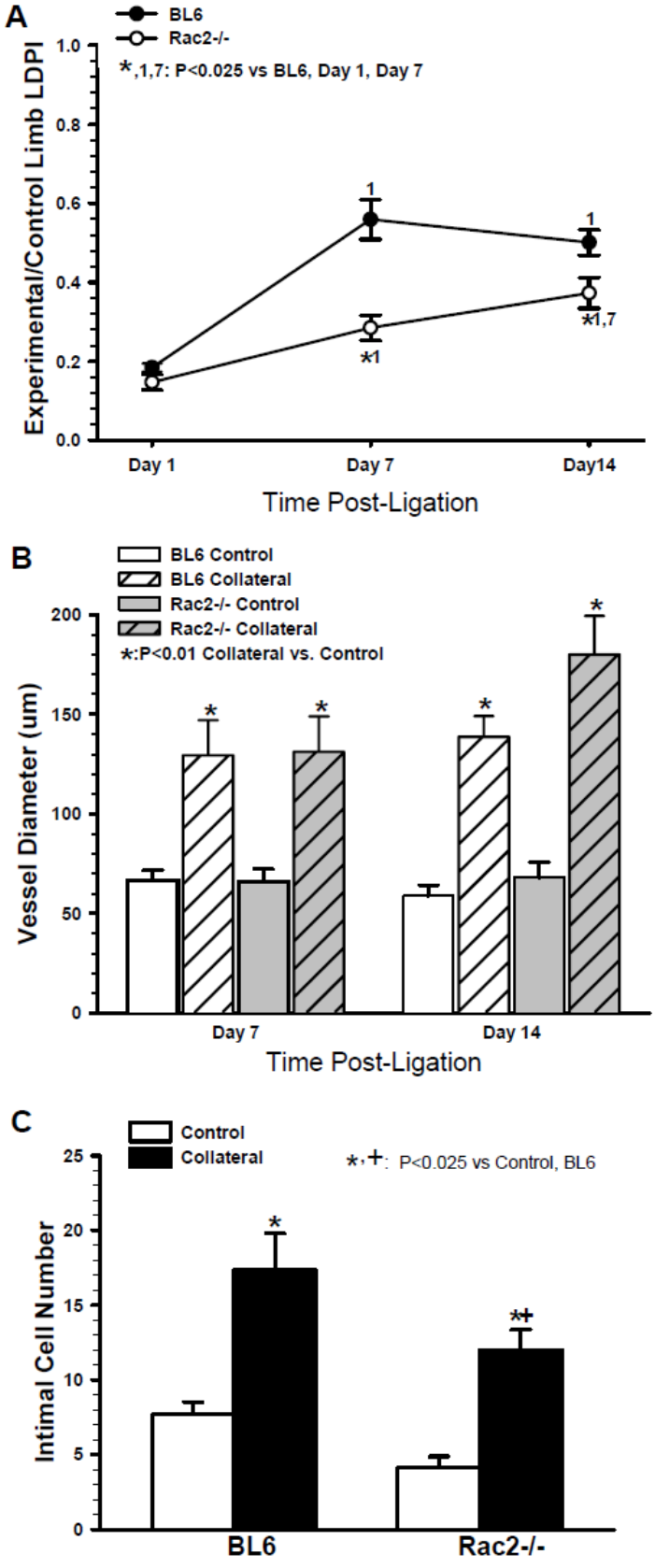

A
BL6
Rac2-/-
*,1,7: P<0.025 vs BL6, Day 1, Day 7
Experimental/Control Limb LDPI
1.0
0.8
0.6
0.4
0.2
0.0
1
1
*1,7
*1
Day 1
Day 7
Day14
Time Post-Ligation
B
BL6 Control
BL6 Collateral
Rac2-/- Control
Rac2-/- Collateral
*:P<0.01 Collateral vs. Control
Vessel Diameter (um)
200
150
100
50
0
*
*
*
*
Day 7
Day 14
Time Post-Ligation
C
Control
Collateral
*,+: P<0.025 vs Control, BL6
Intimal Cell Number
25
20
15
10
5
0
*
*+
BL6
Rac2-/-

Figure 4

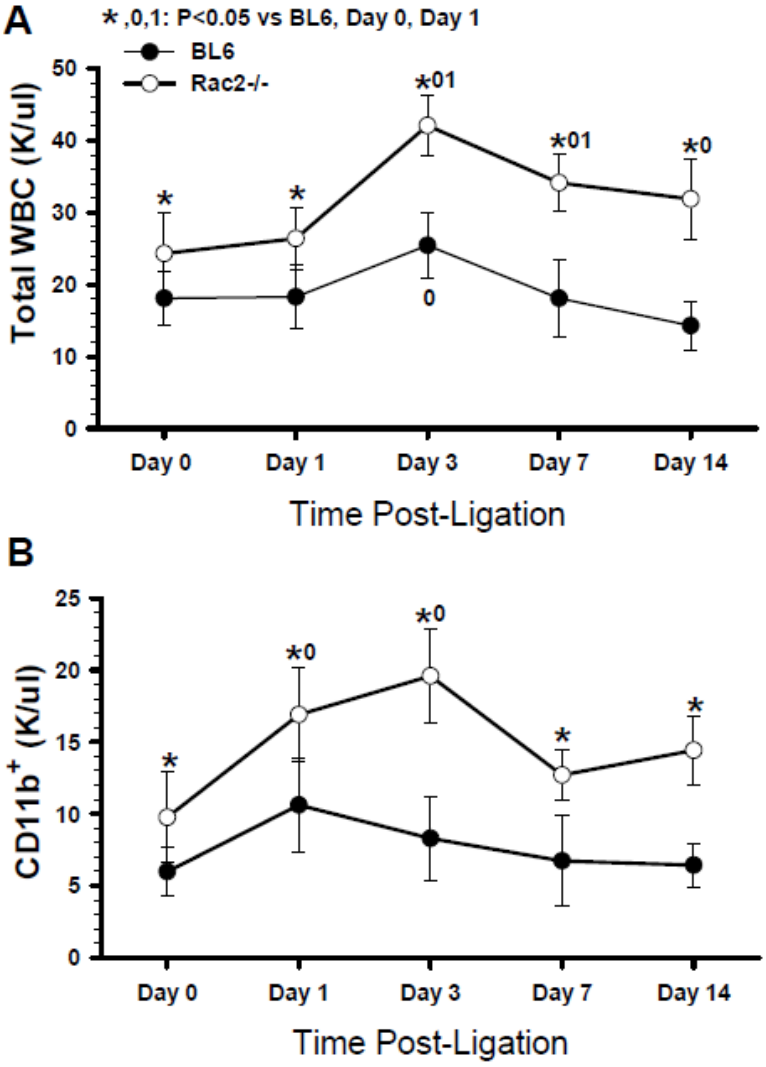

A
*,0,1: P<0.05 vs BL6, Day 0, Day 1
BL6
Rac2-/-
Total WBC (K/ul)
50
40
30
20
10
0
*
*
*01
0
*01
*0
Day 0
Day 1
Day 3
Day 7
Day 14
Time Post-Ligation
B
CD11b+ (K/ul)
25
20
15
10
5
0
*
*0
*0
*
*
Day 0
Day 1
Day 3
Day 7
Day 14
Time Post-Ligation

Figure 5

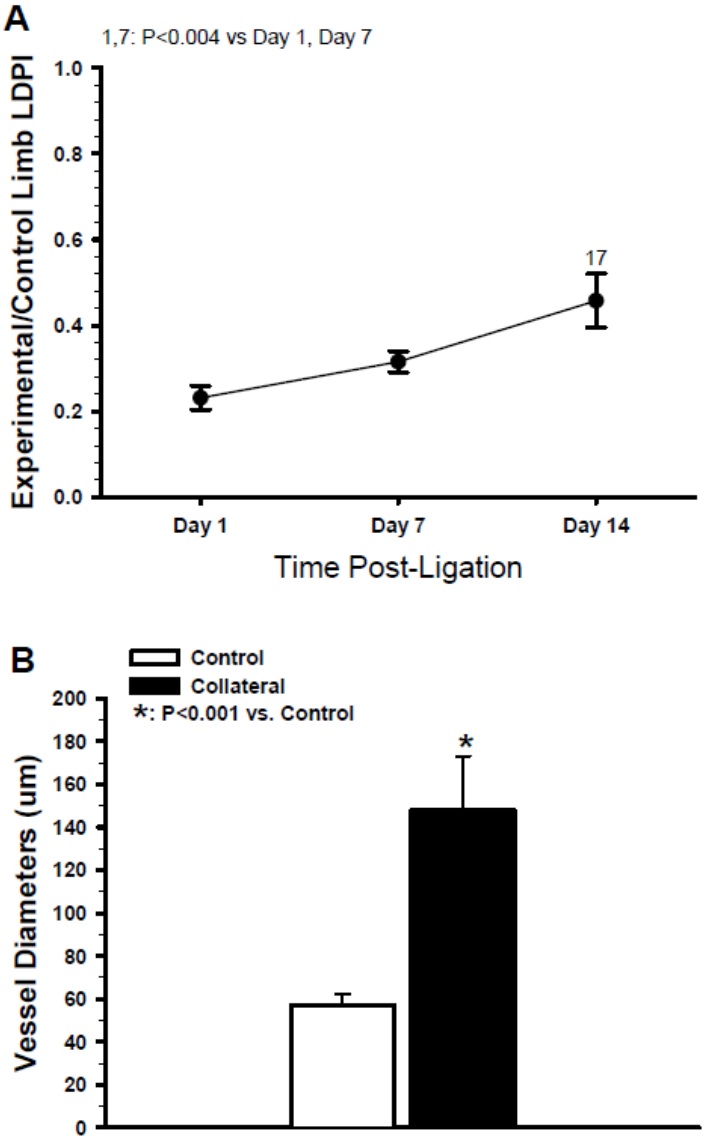

A
1,7: P<0.004 vs Day 1, Day 7
Experimental/Control Limb LDPI
1.0
0.8
0.6
0.4
0.2
0.0
17
Day 1
Day 7
Day 14
Time Post-Ligation
B
Control
Collateral
*: P<0.001 vs. Control
Vessel Diameters (um)
200
180
160
140
120
100
80
60
40
20
0
*

Table S1: *Laser Doppler Flux Values in Control and Experimental Limbs in All Groups*						
Raw	**Day 1**		**Day 7**		**Day 14**	
	Control	**Experimental**	**Control**	**Experimental**	**Control**	**Experimental**
BL6 Moderate	1091.2±71.5	344.1±78.8	1227.8±57.7	888.2±78.1	1298.7±10.5	1092.7±35.5
***Rac2* -/- Moderate**	1259.8±46.0	436.8±110.2	1294.7±24.7	827.8±105.4	1251.5±35.1	806.8±95.2
BL6 Severe	1066.0±23.4	194.4±13.3	1078.7±41.4	585.9±62.6	1112.0±39.6	540.2±35.6
***Rac2* -/- Severe**	1222.2±133.5	168.6±20.8	1338.1±142.5	361.5±40.6	1586.4±192.1	628.2±128.7
***Nox2*-/- Severe**	992.2±28.8	223.9±22.5	893.6±29.2	283.2±26.3	964.1±31.8	424.6±62.3
(Relative Units, Average ± SEM)						

Table S1: The average Laser Doppler flux units are reported. The values from individual animals were used to calculate the ratios reported in Figures 1, 3, and 5.

Supplemental Figure Legends:

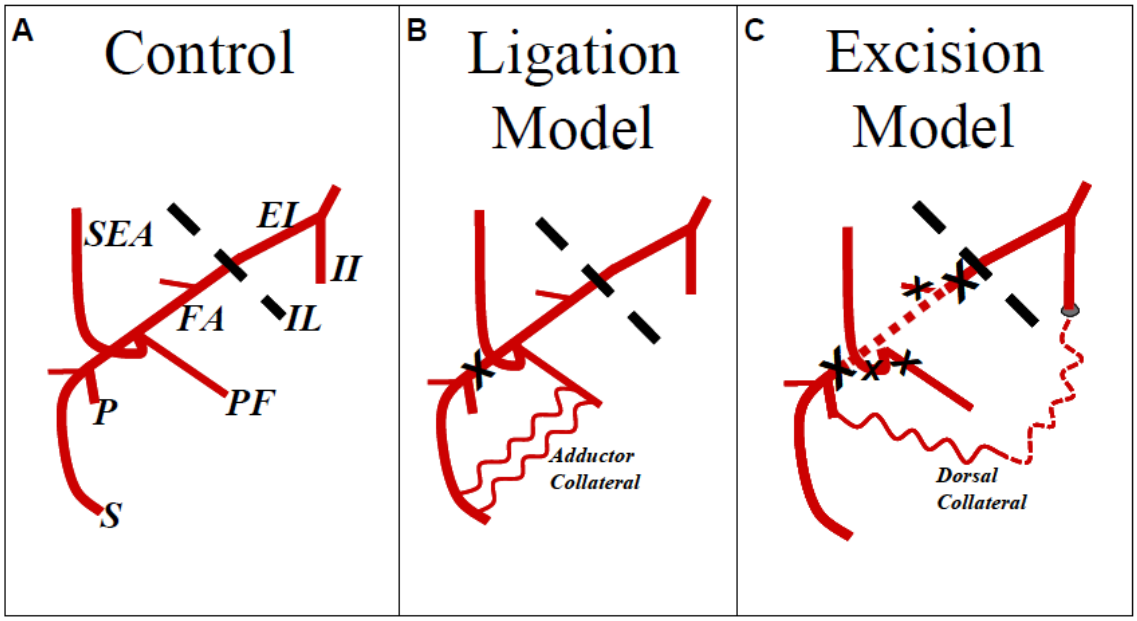
Supplemental Figure S1: Schematic of the murine hindlimb vascular anatomy (A) as well as the femoral artery ligation (B) and excision (C) models used in this study. EI = External Iliac artery, II = Internal Iliac artery, IL = Inguinal Ligament, FA = Femoral artery, PF = Profunda Femoral artery, SEA = Superficial Epigastric artery, P = Popliteal artery, S = Saphenous artery, Tortuous Line = Collateral pathway, X = Ligation site, Dotted red line indicates excised portion of femoral artery, Dashed red line indicates unspecified portion of collateral pathway connecting the internal iliac artery to the dorsal collateral artery.

Supplemental Figure S2: Control and experimental limb gastrocnemius muscles from BL6 and *Rac2-/-* mice after 14 days of femoral arterial ligation. The BL6 control limb gastrocnemius muscle in panel A is representative of all control gastrocnemius muscles observed in both animal strains. The BL6 experimental limb gastrocnemius muscle in panel B and the *Rac2-/-* experimental limb gastrocnemius muscle in panel C are representative of those observed in both strains, respectively, and demonstrate little, if any, lipid deposition relative to their respective controls. A band of connective tissue is seen spanning from the upper left to lower right corners of panel C. (BL6, n = 6; *Rac2-/-*, n = 7).

Supplemental Figure S3: Control and experimental limb gastrocnemius muscles from BL6 and *Rac2-/-* mice after 14 days of femoral arterial excision. The BL6 control gastrocnemius muscle in panel A is representative of all control gastrocnemius muscles

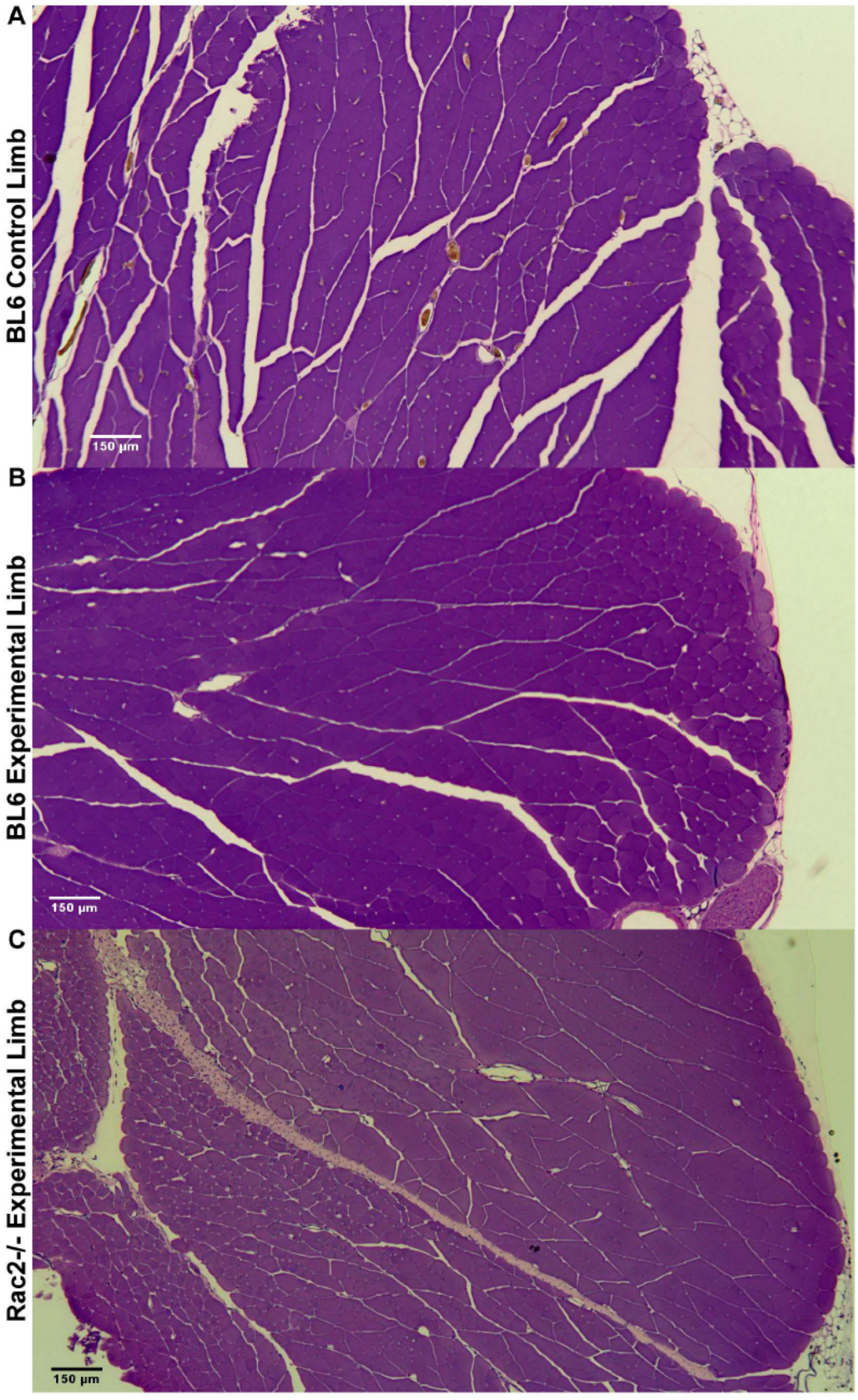
observed in both animal strains. The BL6 experimental gastrocnemius muscle in panel B exhibited the greatest amount of lipid accumulation observed in all of the BL6. Panel C is a representative micrograph of the *Rac2-/-* experimental limb gastrocnemius muscles and demonstrates the extensive lipid accumulation evident in the *Rac2-/-* mice. The lipid deposition in B and C results from adipocyte formation (32). Arrows indicate representative adipocytes characterized by the clustered, clear circular structures. (BL6, n = 5; *Rac2-/-*, n = 6).

Supplemental Figure S4: Representative control and collateral vessels as well as control and experimental limb gastrocnemius muscle micrographs from *Nox2-/-* mice. Arrowheads in vessel sections identify intimal cell nuclei, * indicates Microfil® in the lumen of the vessels, and arrows indicate representative adipocytes responsible for the extensive lipid accumulation that occurred in *Nox2-/-* experimental limb gastrocnemius muscles (similar to the *Rac2-/-* mice, n = 3).

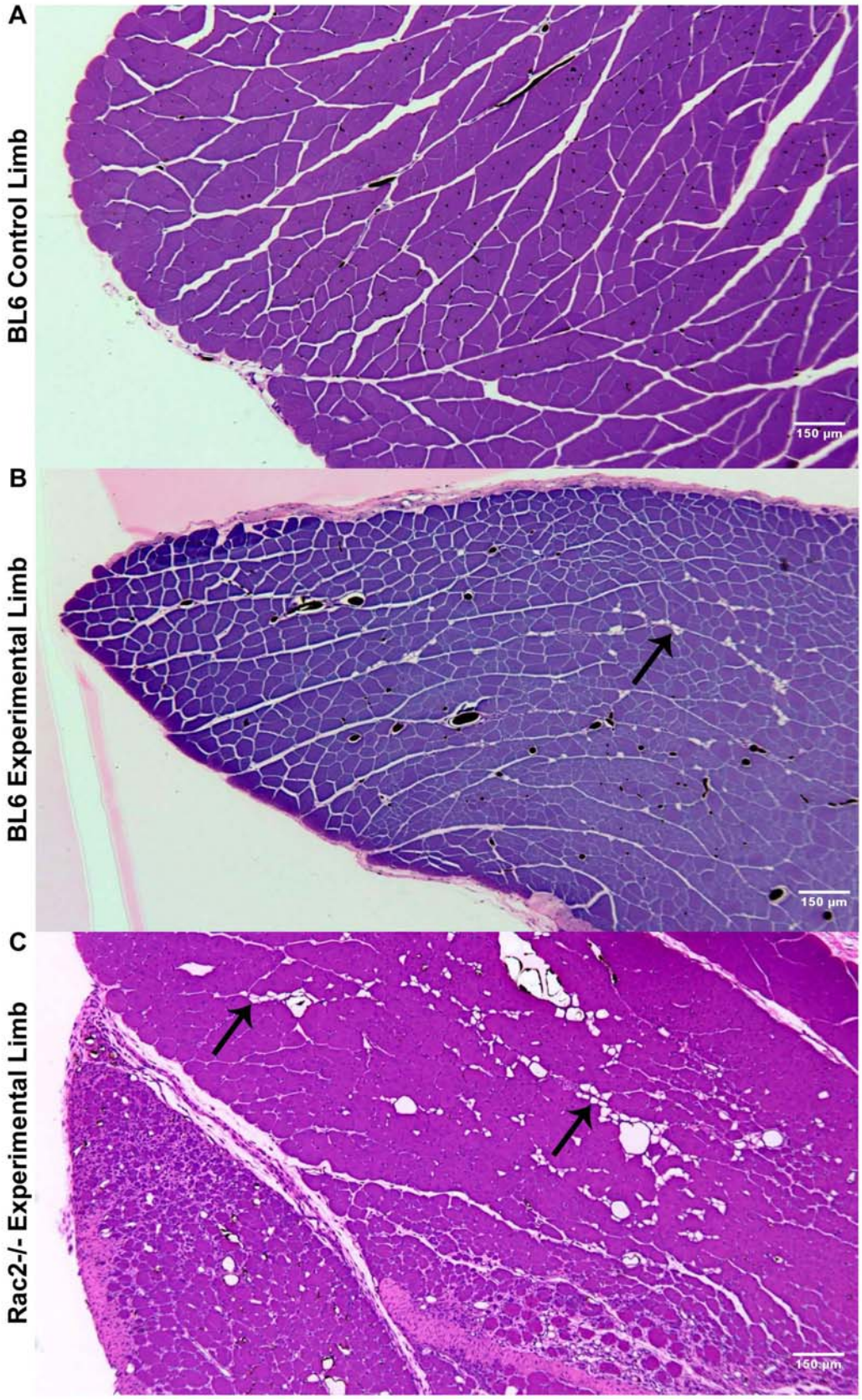


Supplemental Figure S-1

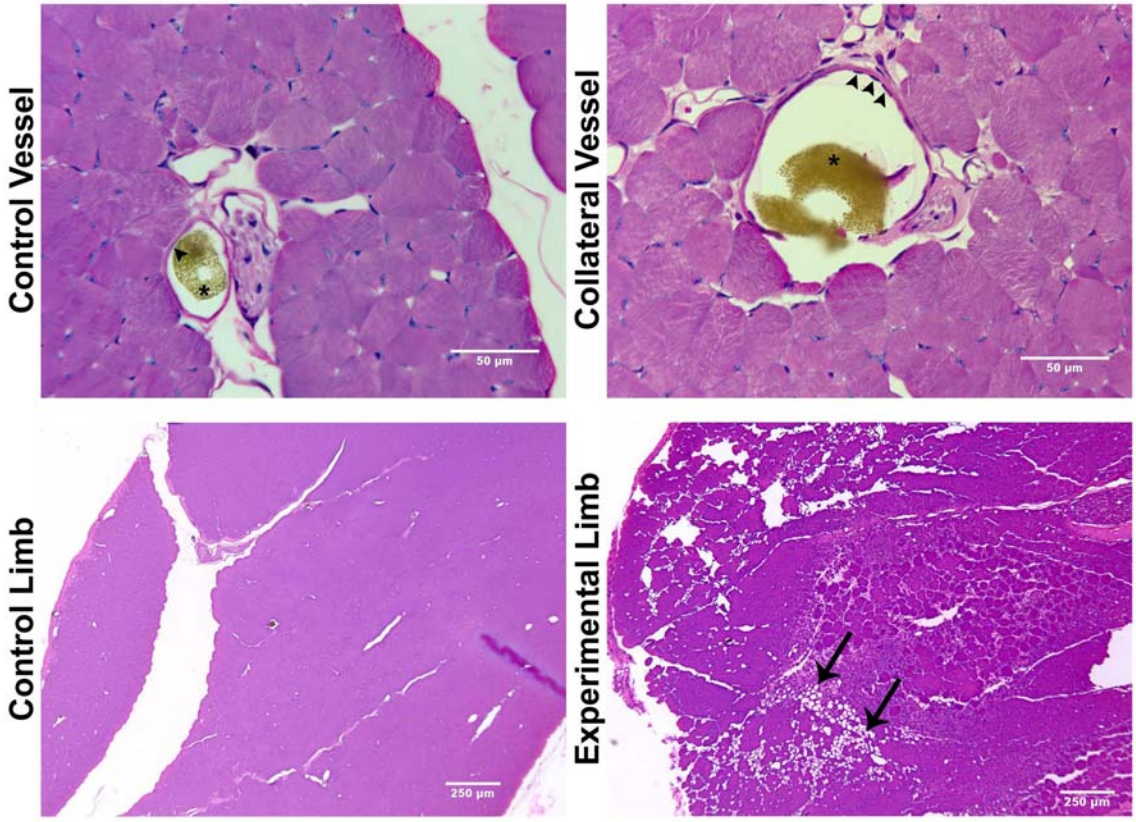
Supplemental Figure S-2



Supplemental Figure S-3



Supplemental Figure S-4



CHAPTER III

Pilot Study for Future Experiments: Effect of Bone Marrow Cell and Antioxidant Therapy on Age-Related Collateral Growth Impairment

Introduction:

Collateral growth has been shown to be impaired in the presence of risk factors for cardiovascular disease, as reviewed by van Oostrom et al. (153). Specifically, collateral growth is impaired with aging in both humans (93) and rodents (106, 145); however, the mechanism mediating this impairment is unknown. The current prevailing paradigm suggests that collateral growth is mediated by both the increased shear stress experienced in the vessels bypassing the parent artery occlusion as well as through the recruitment, incorporation, and subsequent growth factor production by bone marrow derived monocytes (12).

Capoccia et al. recently demonstrated that injection of bone marrow cells (BMCs) enhances hindlimb perfusion and angiogenesis in mice; but their study did not directly assess collateral growth (18). While Capoccia et al.'s study and others have demonstrated improvements in hindlimb reperfusion and/or angiogenesis via BMC therapy, the majority of these studies have been performed in young healthy animals experiencing an acute, surgically induced arterial occlusion. Conversely, arterial occlusion in patients happens gradually over time and in the presence of significantly diseased vasculature. In addition, many of the clinical trials aimed at enhancing angiogenesis in patients with peripheral arterial disease (PAD) in order to increase perfusion have failed to show a significant degree of improvement. One reason for this

discrepancy is probably due to the presence of significant vascular disease and other co-morbidities (such as diabetes, hypercholesterolemia, etc.) in the aged patients treated in these trials that was absent in the young healthy animals utilized in the pre-clinical studies (131). Indeed, if the field of therapeutic interventions aimed at enhancing collateral growth is to advance, then translational studies in appropriate animal models are necessary to develop and assess the potential of such interventions (159).

Previous studies in our laboratory demonstrated that mesenteric collateral growth is impaired with age (145). However, this study in aged rats did not investigate hindlimb collaterals (145) and a previous study in mice demonstrating an age-related impairment in hindlimb reperfusion focused primarily on angiogenesis (capillary proliferation), not adequately assessing hindlimb collateral growth (106). Studies in rats have shown that ~70-80% of total limb resistance in the rodent hindlimb (upon acute femoral artery ligation) is mediated by the bypass collateral arteries (76, 150, 163). Thus, collateral growth is the primary means to compensate for a peripheral arterial occlusion.

One possible mechanism for the age-related impairment in collateral growth is that the BMCs of the aged animals are unable to mediate collateral growth. Since monocyte recruitment is believed to be an essential step in the mechanism mediating collateral growth (12), and because injection of young BMCs led to increased hindlimb perfusion after femoral arterial ligation in young healthy animals (18), these data suggest that the injection of young BMCs into aged mice may enhance hindlimb collateral growth. Furthermore, Edelberg et al. (43) demonstrated transplantation of young bone marrow-derived endothelial progenitor cells (EPCs) into aged mice improved cardiac allograft viability and Heiss et al. (59) has shown age-related endothelial dysfunction to

be accompanied by functional impairments in EPC proliferative activity and migratory response. In addition, many studies have suggested this age-related endothelial dysfunction is associated with increased oxidative stress. For example, Mayhan et al. (85) recently published data suggesting the impairment in eNOS-dependant cerebral arteriole reactivity observed with aging is due to an increased production of superoxide from activated NAD(P)H oxidase. Thus, another potential mechanism for the age-related impairment in collateral growth could be due to the increased reactive oxygen species (ROS) known to be present with advancing age (35) altering the functional capacity of the endothelial cells, BMCs, or EPCs to mediate collateral growth. Indeed, previous studies in our lab have shown ROS to be implicated in the impairment of collateral growth in the spontaneously hypertensive rat (SHR) (89, 166).

Therefore, the primary objectives of this pilot study were to evaluate a mouse model of aging for impaired collateral growth in the hindlimb and, once this impairment has been established, determine if the impairment can be reversed by BMC-injection or antioxidant therapy. We hypothesize that: there will be an age-related impairment of collateral growth in the mouse hindlimb, the injection of young BMCs into aged mice will enhance hindlimb collateral growth, ROS balance will be associated with the age-related impairment of collateral growth, and that the age-related impairment of collateral growth in the mouse hindlimb will be characteristically similar to the age-related impairment of collateral growth in the rat mesentery.

Materials and Methods:

Animals

Male C57BL/6J mice were studied at 3 (young) and 18 (old) months of age. Mice were obtained from the NIA colony maintained by Harlan Industries (Indianapolis, IN) and housed at Indiana University. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Indiana University School of Medicine.

Murine Hindlimb Ischemia Model

The same procedure as outlined in Chapter II for moderate femoral arterial ligation was performed in all animals in this study.

Laser Doppler Perfusion Imaging

Laser Doppler perfusion imaging (LDPI) (Model LDI-2, Moor Instruments, Devon, U.K.) of the plantar surface of the hind paws was measured over the course of 14 days as outlined in Chapter II. In some animals, LDPI was continued for an additional 14 days following bone marrow-derived cell injection. Perfusion was calculated and expressed as the flux ratio between the non-ischemic and ischemic limbs.

Collateral Growth Assessment and Determination of Primary Compensatory Pathways

Perfusion fixation, vascular casting, collateral growth assessment, and determination of primary compensatory collateral pathways were all performed as outlined in Chapter II.

Immunophenotyping and FACS

HEMAVET and FACS quantification of total circulating white blood cells (WBCs) and $CD11b^+$ cells in blood samples collected from the mouse tail vein is the same as was previously outlined in Chapter II.

Bone Marrow Cell Isolation and Injection

To obtain donor cells, C57BL/6 mice 3 or 18 months old were euthanized by CO_2 inhalation and pinned in the supine position to a sterilized dissecting tray. The hind limbs were removed and placed in minimal essential medium plus Earle's salts (MEM) (Gibco, Grand Island, NY). Adipose and muscle tissue were carefully removed and discarded. Bone marrow (BM) was flushed with MEM from the medullary cavities of tibias and femurs using a 27-gauge needle. BM MNCs were obtained by Ficoll density gradient centrifugation. Briefly, BM was diluted 1:1 with phosphate-buffered saline (PBS, Invitrogen, Grand Island, NY) and an equivalent volume of Ficoll-Paque™ PLUS (GE Healthcare Biosciences Corporation, Princeton, NJ) was then underlaid. Cells were centrifuged for 30 minutes at room temperature at 740 x g. MNCs were isolated and washed 2 times with PBS supplemented with 2% fetal bovine serum (Hyclone, Logan, UT), 1% penicillin/streptomycin (Invitrogen) and 0.25 μg/ml amphotericin B (Invitrogen). Cell count and viability was assessed by Trypan blue (Sigma, St. Louis, MO) staining. 5×10^6 BM MNCs re-suspended in 200 μl of PBS, or PBS alone, were injected into the lateral tail vein either 24 hrs or 14 days after surgical induction of hind-limb ischemia.

Apocynin Therapy

Starting one week before surgical ligation, animals were given a continuous dose of 1 mM apocynin via their drinking water (water was changed ~1-2 times a week) throughout the duration of their 28 day recovery period.

Histology and Intimal Cell Counts

Vessel tissues were embedded in plastic, sectioned, stained with either Hematoxylin and Eosin or Lee's Methylene Blue, then visualized and imaged with 5-40X objectives (Leica DM 5000B with Diagnostic Instruments Inc. Model 7.4 Slider, Spot RT_{KE} camera) as described in detail in Chapter II.

Statistical Analysis

Prior to statistical analysis, any values >1.5 standard deviations from the mean were excluded. ANOVA was then performed with SigmaStat 3.0 to evaluate all data. The Holm-Sidak method was used for pairwise multiple comparisons. Data are expressed as means ± SEM.

Results:

Recovery of Hindlimb Perfusion

As can be seen in Figure 1, Laser Doppler perfusion imaging (LDPI) demonstrated a significant recovery of hindlimb perfusion from the baseline established at Day 1 post-ligation over the course of 14 days in both young and old mice ($P \leq 0.001$ for young and old vs. day 1); however, hindlimb perfusion was significantly greater in the young than in the old at all time points ($P \leq 0.016$ at day 1, $P \leq 0.001$ at day 7 and day 14 vs. old).

After verification of a post-ligation perfusion impairment through 14 days, either bone marrow-derived mononuclear cells (BM-MNCs) obtained from young or old mice suspended in PBS or a PBS-only control were injected into old mice via the tail vein and LDPI was assessed over the course of an additional 14 days (i.e., measurements were made at days 21 and 28 post-ligation). As can be seen in Figure 2, treatment of aged mice with either young or old BM-MNCs did not result in a significant increase in the hindlimb perfusion at either day 21 or day 28 post-ligation versus that observed at day 14 in the untreated aged animals ($P = 0.133$ and $P = 0.097$ for young and old BM-MNC injections, respectively). However, the hindlimb perfusion ratios at days 21 and 28 post-ligation in aged mice following PBS-only injection were significantly increased versus untreated aged mice at day 14 ($P \leq 0.001$, Figure 2). Because these results were unexpected we evaluated injection of BM-MNCs at day 1 post-ligation. As can be seen in Figure 3, injection of young BM-MNCs at day 1 post-ligation did not result in a significant enhancement of hindlimb perfusion versus old untreated mice at any time point from day 7 through day 28 post-ligation ($P = 0.126$).

Lastly, as recent studies have suggested collateral growth and hindlimb perfusion recovery are associated with a specific balance of reactive oxygen species (ROS) (108, 140, 151), and since ROS are known to be increased with age (35), we assessed the recovery of hindlimb perfusion in aged mice pre-treated with the antioxidant apocynin. As can be seen in Figure 4, apocynin therapy in the aged mice produced a significant increase in the hindlimb perfusion at day 14 post-ligation ($P \leq 0.001$ vs. untreated aged mice at day 14). However, this enhanced perfusion was lost by day 21 post-ligation, since the hindlimb perfusion ratios of the treated animals at days 21 and 28 post-ligation were not significantly different from untreated aged mice at day 14 ($P = 0.355$, Figure 4).

As these results were also unexpected, we questioned whether or not recovery of hindlimb perfusion had actually plateaued by day 14 post-ligation, or if it was possibly still fluctuating between days 14 and 28 post-ligation. Therefore, a second group of young and old mice that remained untreated throughout the entire 28 day post-surgery recovery period were evaluated. Unfortunately, Figure 5 demonstrates that this second (2^{nd}) group of young and old mice gave us completely different hindlimb perfusion recovery profiles than the initial group of young and old animals we assessed. Similar to our initial group, the 2^{nd} group of young mice showed significant perfusion recovery by day 14 ($P \leq 0.001$ vs. young day 1). The 2^{nd} group of old mice eventually showed significant recovery as well ($P \leq 0.001$ vs. old day 1); however, the recovery in the 2^{nd} old mice did not occur until day 21 while it had already reached significance by day 14 in the initial group. Most importantly, and in contrast to the initial group which showed a significant difference in hindlimb perfusion between young and old mice at all time points, there was no significant difference in the hindlimb perfusion ratios between the

young and old mice at any time point in the 2nd group (at day 14, P = 0.004 but the confidence interval of the analysis was 0.002; at all other time points, P≥0.188).

Growth of Primary Collateral Vessels

As can be seen in Figure 6, young mice exhibited a significantly greater degree of enlargement in their primary collateral vessels than did the aged mice 14 days after arterial ligation, indicated by a significantly larger percent increase of collateral vessel diameter in the young versus old (55±8.2% vs. 14±9.7%, respectively, P≤0.011). This finding is consistent with the increased perfusion observed in the young versus the old mice at Day 14 post-ligation shown in Figure 1. However, Figure 6 also demonstrates both young and old mice exhibited the same degree of collateral vessel enlargement by 28 days post-ligation (102±18% vs. 99±29%, respectively). This finding is consistent with the similar perfusion observed in the young versus the old mice at Day 28 post-ligation shown in Figure 5. Therefore, the aged mice do not demonstrate a complete lack of collateral growth capacity, but instead have a delayed ability to develop collateral vessels in response to femoral arterial ligation.

Ligations were created in additional aged mice and these animals were then treated with either a PBS-only control injection (iv.), an injection of BM-MNCs on day 1 or day 14 post-ligation, or pre-treated with apocynin, and then sacrificed 28 days after surgical induction of collateral development. Table 1 demonstrates that neither injection of BM-MNCs (at either day 1 or day 14 post-ligation) nor apocynin pretreatment resulted in an enhanced capacity for collateral development, since there was not a significant increase in the percent of collateral enlargement in any of the treatment groups versus the untreated aged mice at 28 days post-ligation (Table 1). In fact, injection of young BM-

MNCs at day 1 post-ligation significantly reduced the percent increase in collateral diameter that should have occurred by day 28 post-occlusion ($P \leq 0.001$, Table 1). Again, these results were contrary to our expectation that either BM-MNC injection or antioxidant treatment would significantly enhance collateral development in aged mice.

Assessment of Bone Marrow Derived Cell (BMDC) Response

Since white blood cells (WBCs) in general, and $CD11b^+$ WBCs in particular, have been previously shown to mediate improved perfusion in the mouse hindlimb (18), the number of circulating white blood cells (WBCs) and $CD11b^+$ cells was determined in untreated young and old mice via HEMAVET and FACS analysis (respectively) to investigate whether a defect in the number of circulating BMDCs was present in the aged mice. Figure 7 demonstrates that, while there are no significant differences in the number of total WBCs between young and old mice (Figure 7A), the old mice start out with a significantly higher number of $CD11b^+$ cells circulating in the bloodstream than do the young mice ($P \leq 0.008$, Figure 7B). However, this difference is lost between days 3 and 7 post-ligation. This sharp decline in the number of circulating $CD11b^+$ cells in the aged animals could indicate that these cells are leaving the bloodstream and migrating into the tissues, possibly to effect collateral growth. Previous studies have shown that monocyte/macrophage accumulation around growing collateral vessels in young healthy animals is maximal three days post-occlusion (119). In the current study, this emigration of monocytes in young mice from the bloodstream could be indicated by the slight decrease in circulating number of $CD11b^+$ observed between Days 1 and 3 post-ligation in Figure 7B. Thus, if these cells are the necessary prerequisite of collateral growth, the sharp decline in $CD11b^+$ cells between Days 3 and 7 post-ligation in the old mice (Figure

7B) would be consistent with the delay in collateral growth suggested by the LDPI and diameter data (Figures 5 and 6, respectively). However, additional experiments are needed to determine where these $CD11b^+$ cells are going and whether or not they are truly effecting collateral growth. It can be concluded from the current data that there is no defect in the number of circulating BMDCs in the aged mice versus young; indeed, there were significantly more $CD11b^+$ cells at baseline in the aged mice than in the young.

Histological Assessment of Primary Collaterals

Histological assessment of primary collateral cross-sections (Figure 8A) demonstrated similar morphology as was observed in the Chapters I and II; specifically, increased intimal cell number without neointimal formation and apparently increased vascular wall thickness at 14 days post-ligation, despite the observed impairment in luminal expansion. Figure 8B depicts the average intimal cell number of collateral and control arteries. While there was a significant increase in the number of intimal cells in the collaterals versus the controls in both young and old mice (14±1.4 vs. 4.8±0.4 in young and 14±1.6 vs. 4.8±0.5 in old, $P \leq 0.001$), there was not a significant difference between age groups ($P = 0.837$). This data is consistent with our previous work in the aged rat mesentery (145) and suggests that the age-related impairment in collateral growth is similar between different vascular beds in different species.

Discussion:

We hypothesized that: there would be an age-related impairment of collateral growth in the mouse hindlimb, the injection of young BMCs into aged mice would enhance hindlimb collateral growth, ROS balance would be associated with the age-related impairment of collateral growth, and that this impaired collateral growth would be characteristically similar to the age-related impairment of collateral growth in the rat mesentery. The most significant findings of the current study are: 1) verification of a transient perfusion and collateral growth impairment in the aged mice at 14 days post-ligation that was attenuated by 28 days post-ligation (Figures 1, 5, and 6); 2) in contrast to studies in younger mice, bone marrow cell (BMC) injection did not enhance limb perfusion or collateral growth (Figures 2 and 3, and Table 1); 3) if the impairment in collateral growth observed with aging is due to altered BMC function, the problem is not related to circulating BMC number (Figure 7); 4) antioxidant therapy showed potential for alleviating the impairment in hindlimb perfusion exhibited by aged mice after arterial occlusion but had no effect on collateral growth (Figure 4 and Table 1); and 5) the characteristics of increased intimal cell number, without neointimal formation, and increased vascular wall thickness upon induction of collateral growth are preserved despite the impairment in luminal expansion observed in the aged mice at 14 days post-ligation (Figure 8), consistent with our previous findings in the aged rat mesentery and suggesting that the age-related impairment in collateral growth is similar between different vascular beds in different species (145). If the observed increase in intimal cell number is taken as a functional assessment, then this data also suggests the endothelial

cells (and/or the EPCs) retain some of their functional capacity in the context of advancing age.

The current study verifies our previous finding of an age-related impairment in the luminal expansion of collateral arteries in the rat mesentery (145), and extends this finding to include the mouse hindlimb vasculature (Figure 6). Our findings are also consistent with those of Brownlee and Langille as well as Miyashiro et al. (10, 90) who found a decrease in flow-mediated arterial diameter expansion with advancing age. It has been shown that angioscores derived from collateral vessel density in angiograms does not necessarily correlate with collateral flow and does not reflect tissue perfusion as assessed by microsphere techniques (50). Thus, by means of a much more direct assessment of primary collateral arteries, the current study verifies and extends the findings of Rivard et al., who used only an angioscore in rabbits and capillary density measurements in mice to demonstrate an age related impairment in collateral growth and angiogenesis (106).

Our results of an inability of BMC injection therapy to enhance collateral growth (Table 1) are in contrast to those of Capoccia et al. (18), despite using the same BMC subfraction for our injections. One explanation is that the vasculature of the young mice used in their study may have been receptive to the increased number of BMCs injected, while the vasculature of the aged animal may not have been as receptive to injected BMCs. Further studies in aged mice are needed to verify this possibility. Another important difference between the two studies is that Capoccia et al. (18) focused primarily on angiogenesis, assessing hindlimb perfusion with LDPI and capillary density as the endpoints, while the current study is focused primarily on growth of pre-existing

collateral arteries. Lastly, there could be a stimulus effect difference between our study and that of Capoccia et al. as they utilized a much more severe femoral arterial ligation model (with ligation and excision of both the femoral artery and vein down to the saphenous artery and vein) than was used in the current study (18). The more severe model used by Capoccia et al. may have resulted in the elimination of arterial and venous collaterals that would have remained present in the current study and, thus, microvascular growth may be more important when the surgical model utilized is more severe. In addition, Shepherd (124) has shown simultaneous arterial and venous occlusion in humans has a detrimental effect on collateral growth, most likely due to increased venous pressure. This would be consistent with the findings of our earlier study that showed decreasing hindlimb perfusion with increasing model severity in young C57BL/6 mice (Chapter II).

One limitation of the current study is that we observed a large degree of inter-animal variation within treatment groups, especially in the LDPI measurements. Indeed, Figure 5 suggests either a very low degree of reproducibility between LDPI measurements or a very high degree of variation between animals purchased from the same supplier but ordered several months apart. Shireman (128) pointed out that several other previously published reports experienced considerable variation in LDPI. Possible causes of this LDPI variability include: 1) LDPI is not sensitive enough to detect small differences in perfusion because LDPI is based more on the number of red blood cells moving through a given tissue region of interest rather than on direct measurement of blood flow, 2) LDPI has a penetration depth of only 1 mm which is not deep enough to reach the collateral vessels providing the primary bypass for femoral arterial occlusion,

and 3) LDPI is very temperature sensitive and can be greatly effected by the vasoconstriction that occurs in the mouse extremities upon changes in ambient temperature. In addition, Epstein's group has demonstrated that interanimal variability can greatly affect the outcome of a study: when all animals were grouped together a significant difference was observed in LDPI between those that were treated with mesenchymal stem cells and those that were not; however, when the animals were subdivided into different groups based on their recovery during the first 24 hours after surgery, the significant difference between treatment groups disappeared (164).

Another limitation in the current study is that the observed LPDI-measured hindlimb perfusion does not always agree with the observed collateral development. For example, Table 1 demonstrates that collateral growth in aged mice at 28 days is equivalent between those injected with BMCs/PBS at Day 14 or treated with apocynin and those left untreated. However, if LDPI measured hindlimb perfusion is truly dependant on the size of the primary collaterals, then the primary collaterals observed in the BMC/apocynin treatment groups should have been the size of those observed in the untreated aged mice at 14 days, since the LDPI measured perfusion ratios of the treated groups (except the PBS treated group) were all statistically similar to the perfusion ratio of the untreated aged mice at 14 days post-ligation (Figures 2, 3, 4, and Table 1). This is in contrast to previous studies that have suggested LDPI correlates well with collateral development (34, 121). One possible explanation for this discrepancy is that LDPI was used to measure resting flow (while the vasculature is maintaining vasomotor tone), in contrast to the diameter measurements which were obtained at maximal dilation of the vasculature at the time of sacrifice. In addition, collateral dependent perfusion was not

directly assessed due to the limited penetration depth of LDPI. Another possible explanation for the discrepancy between studies is that in the initial study by Couffinhal et al. the "collateral" development investigated was actually angiogenesis as opposed to the enlargement of primary collaterals performed in the current study (34). Nevertheless, the current results, as well as those from the previous study in Chapter II, suggest LDPI of the hind paws is not an adequate method of assessing collateral dependant perfusion.

A third limitation of the current study relates to the time point of collateral growth assessment. We first verified a collateral growth impairment at day 14, then planned to provide a therapy and measure its effectiveness after an additional 14 days. However, we did not discover until additional experiments were completed that the impairment observed at day 14 was attenuated by day 28. Therefore, the most appropriate comparison would have been to provide our injections earlier and then to assess collateral growth at day 14 when the verified collateral growth impairment was still present.

One final limitation to the current study was the questionable effectiveness of the apocynin treatment. Previous studies in our lab with apocynin used a protocol where medicated water was replaced every 48 hours. However, the medicated water in the current study was only replaced every 3-4 days, thus its effectiveness is in question. This may be the reason for the initial effectiveness of the therapy on restoring hindlimb perfusion in the aged animals at Day 14 that was eventually lost over the course of the 28 day recovery period. Therefore, it is these significant limitations that have led to our decision not to submit this data for publication, but, rather, to use it as preliminary data for a grant submission.

In order to complete this study, the following additional experiments would need to be performed: 1) the BMC injection experiments should be repeated with an earlier time point of injection and collateral growth assessment at day 14 post-ligation, 2) the apocynin experiment should be repeated with collateral growth assessment at day 14 post-ligation and strict adherence to the previously described dosing protocol, 3) a more accurate method of measuring collateral dependant blood flow should be utilized, such as the use of properly sized microspheres or chronic flow probes measuring femoral vein return flow, and 4) in order to more directly assess the BMC hypothesis, bone marrow transplantation studies generating chimeric animals with GFP+/traceable BMCs (Old animals with Young bone marrow and Young animals with Old bone marrow, with the appropriate controls) should be utilized with the hindlimb ischemia model to directly assess the contribution of BMCs to collateral growth. In addition, assays to determine the functionality of the aged bone marrow cells should also be performed. Lastly, the safety and efficacy of a combined cell and antioxidant therapy should be investigated.

In conclusion, we believe our study has allowed us to make some significant observations, the most important being that bone marrow cell therapy did not enhance the growth of primary collaterals in aged mice. Indeed, numerous studies in animals and even humans are currently being conducted to assess the effectiveness of BMC treatment. Our data suggests that the problem may be inherent to the vasculature itself and not lie with the circulating cells. In addition, we believe our aged mouse model is more relevant to the clinical situation than young animal models and completion of additional studies utilizing aged animals would make a significant contribution to the fields of therapeutic angio- and arterio-genesis.

Table 1: *Collateral Growth in Treated and Untreated Aged Mice 28 Days Post-Ligation*

	UnTx	BMI-Yd14	BMI-Od14	PBS-d14	BMI-Yd1	Apocynin
% Increase in Collateral Vessel Diameter:	99±29	58±12	40±12	49±11	17±5.0*	59±9.3

Table 1: Collateral Growth in Aged Mice Injected with either BM-MNCs or PBS, or Pre-treated with Apocynin. Measurement of in situ gracilis artery collateral diameter indicated injection of either young or old BM-MNCs at either day 1 or day 14 post-ligation did not enhance collateral growth in the aged mice at 28 days post-ligation vs. untreated aged mice at 28 days post-occlusion. UnTx = Untreated aged mice allowed to recover for 28 days after ligation; BMI-Yd14 = Old animals injected with young BM-MNCs at day 14, n = 11; BMI-Od14 = Old animals injected with old BM-MNCs at day 14, n = 9; PBSd14 = Old animals injected with PBS at day 14, n = 8; BMI-Yd1 = Old animals injected with young BM-MNCs at day 1, n = 5; Apocynin = Old animals treated with apocynin for one week prior to arterial occlusion and throughout the course of the recovery period, n = 5. *P≤0.001 vs. Old Untreated Day 28.

Figure Legends:

Figure 1: Hindlimb perfusion in young and old mice. The perfusion of the experimental limb is expressed relative to same animal control at days 1, 7, and 14 post-ligation. Hindlimb perfusion increased over time from Day 1 through Day 14 post-ligation in both young and old mice; however, the perfusion in the old mice was significantly reduced compared to that of the young mice. Young, n = 6-8; Old, n = 33-41.

Figure 2: Hindlimb perfusion after bone marrow mononuclear cell (BM-MNC) injection therapy. Aged mice were intravenously injected 14 days after femoral artery ligation with either young or old BM-MNCs, or PBS and allowed to recover an additional 14 days (i.e., 28 days after the original occlusion). Neither aged mice injected with young BM-MNCs nor old BM-MNCs exhibited a statistically significant increase in hindlimb perfusion compared to the pre-injection perfusion measured at Day 14. BMI-Y = Old animals injected with young BM-MNCs, n = 9-10; BMI-O = Old animals injected with old BM-MNCs, n = 10; PBS = Old animals injected with PBS, n = 8-9.

Figure 3: Hindlimb perfusion after injection of BM-MNCs at day 1 post-ligation. Aged mice were injected with young BM-MNCs ~24hrs after femoral arterial ligation and allowed to recover an additional 27 days (compare to untreated aged animals). Injection of young BM-MNCs at Day1 post-ligation did not significantly increase the perfusion of the hindlimb in aged mice compared to untreated aged mice. BMI-Y = Old animals injected with young BM-MNCs, n = 5.

Figure 4: Hindlimb perfusion in aged mice treated with apocynin. Apocynin therapy was initiated 1 week prior to ligation and continued throughout the recovery period. Enhanced perfusion in the apocynin treatment group was short-lived as the perfusion values returned to levels similar to untreated aged mice by day 21 post-occlusion. Apocynin, n = 5-6.

Figure 5: Hindlimb perfusion in two sets of young and old mice. The second (2^{nd}) group of old and young mice did not reproduce the observations from the initial group because 1) there was no difference in the recovery of hindlimb perfusion between the 2^{nd} young and 2^{nd} old mice at all timepoints investigated and 2) both the 2^{nd} old and 2^{nd} young mice demonstrated significantly reduced perfusion ratios vs. the initial group of young and old mice. 2^{nd} Young, n = 4-5; 2^{nd} Old, n = 4-5.

Figure 6: Percent increase in collateral vessel diameter in young and old mice 14 and 28 days post-occlusion. Diameters for young and old mice at days 14 and 28 post-ligation were obtained from digital images of the gracilis artery collaterals in the superficial adductor compartment of the hindlimb subsequent to vascular casting with Microfil®. Aged mice collateral diameters were significantly smaller than young mice at day 14 post-collusion but were not different by 28 days after surgery. Young and Old d14, n = 5; Young d28, n = 5; Old d28, n = 4.

Figure 7: Analysis of total white blood cells (WBCs) by HEMAVET (*A*) and $CD11b^{+}$ cells by FACS (*B*). While there was no difference in the number of total WBCs, there

were significantly more $CD11b^+$ cells in old mice than in the young from before arterial ligation through 3 days post-occlusion. The number of CD11b+ cells in the old mice then fell to match the level observed in the young mice. In addition, there were significantly more $CD11b^+$ cells in the young mice at Day 14 tan there were in the young mice at Day 0. Young, n = 3; Old, n = 6.

Figure 8: Assessment of intimal cell number in young and old mice. As demonstrated by the micrographs in panel A, the gracilis collateral arteries of aged mice still display some of the characteristics of flow-induced outward remodeling observed in young mice: increased intimal cell number, no neointima, and increased wall thickness (vs. control, compare to Figure 2D in Chapter II). Quantitation of intimal cell number from the old and young mice is presented in panel B. Young, n = 6; Old, n = 5.

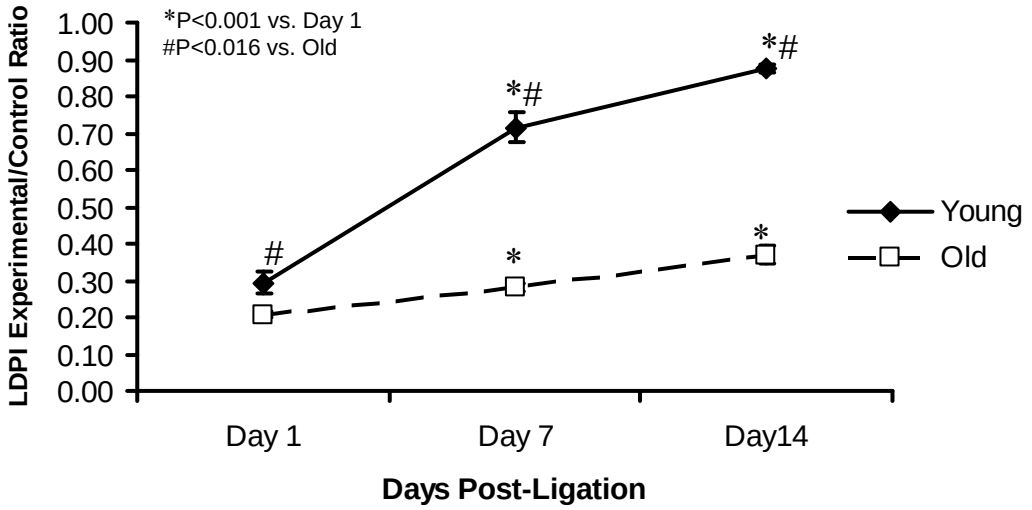

*P<0.001 vs. Day 1
#P<0.016 vs. Old
LDPI Experimental/Control Ratio
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
*#
*#
#
*
*
Young
Old
Day 1
Day 7
Day14
Days Post-Ligation

Figure 1

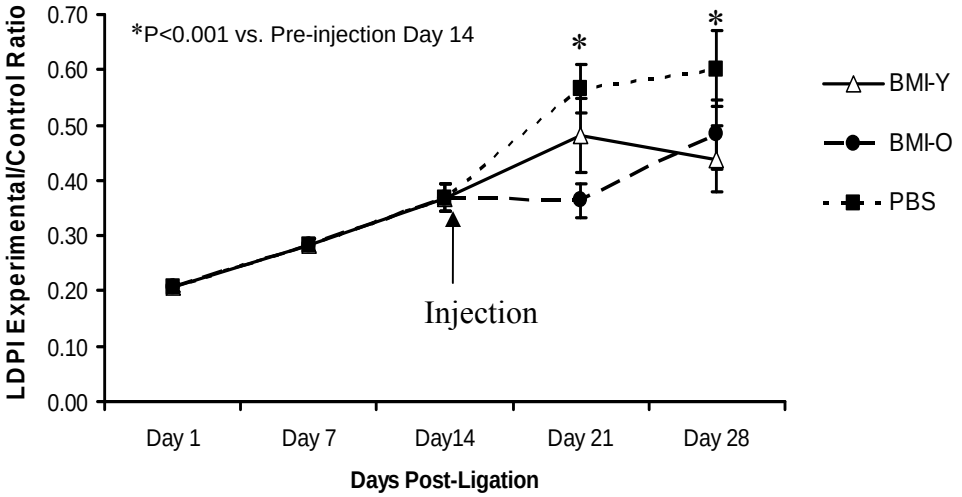

*P<0.001 vs. Pre-injection Day 14
LDPI Experimental/Control Ratio
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Day 1
Day 7
Day14
Day 21
Day 28
Days Post-Ligation
Injection
*
*
BMI-Y
BMI-O
PBS

Figure 2

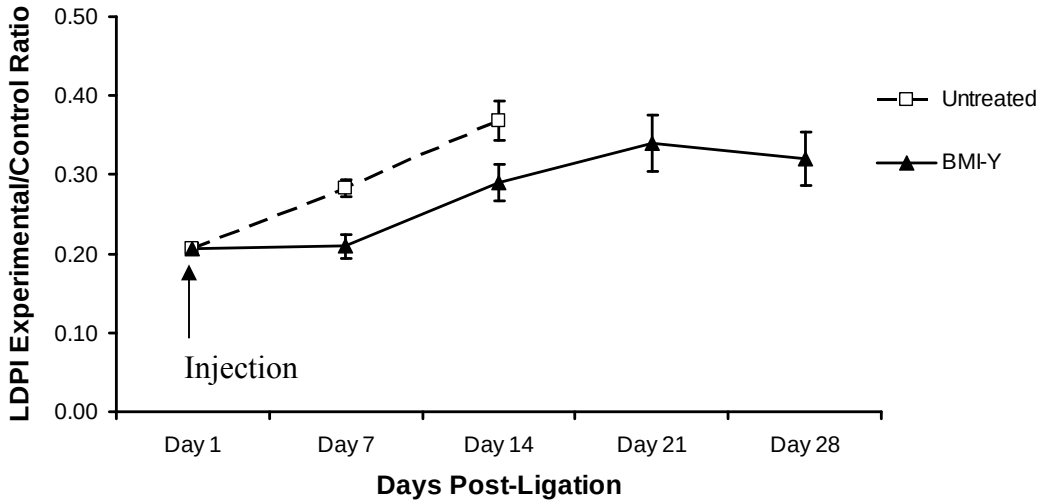

LDPI Experimental/Control Ratio
0.50
0.40
0.30
0.20
0.10
0.00
Injection
Day 1
Day 7
Day 14
Day 21
Day 28
Days Post-Ligation
Untreated
BMI-Y

Figure 3

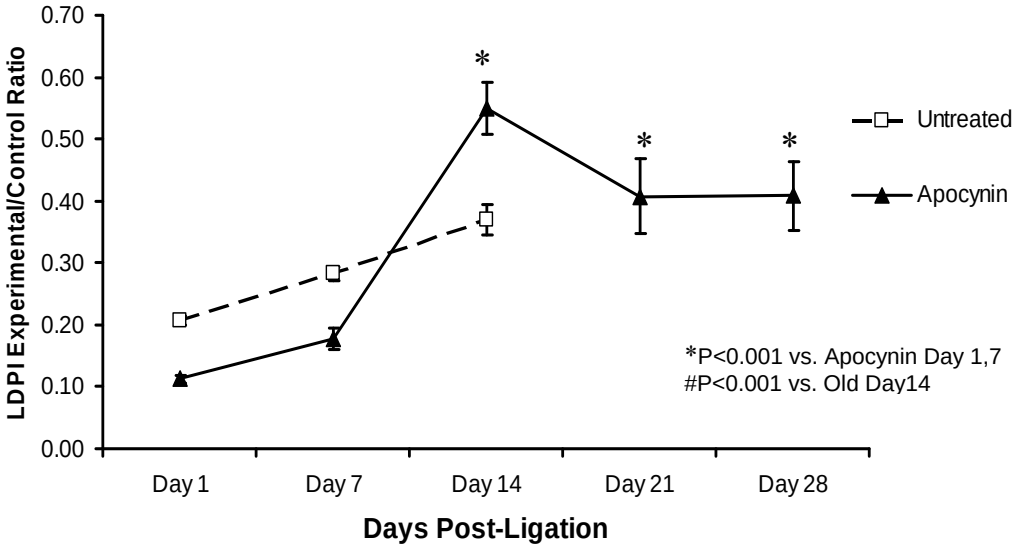

0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
LDPI Experimental/Control Ratio
Day 1
Day 7
Day 14
Day 21
Day 28
Days Post-Ligation
Untreated
Apocynin
*P<0.001 vs. Apocynin Day 1,7
#P<0.001 vs. Old Day14

Figure 4

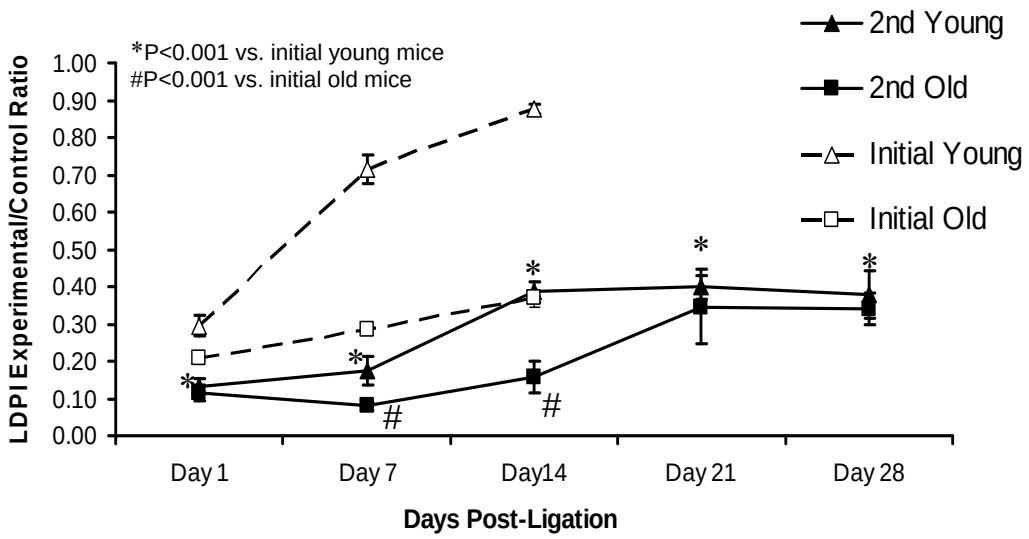

*P<0.001 vs. initial young mice
#P<0.001 vs. initial old mice
2nd Young
2nd Old
Initial Young
Initial Old
LDPI Experimental/Control Ratio
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Day 1
Day 7
Day14
Day 21
Day 28
Days Post-Ligation

Figure 5

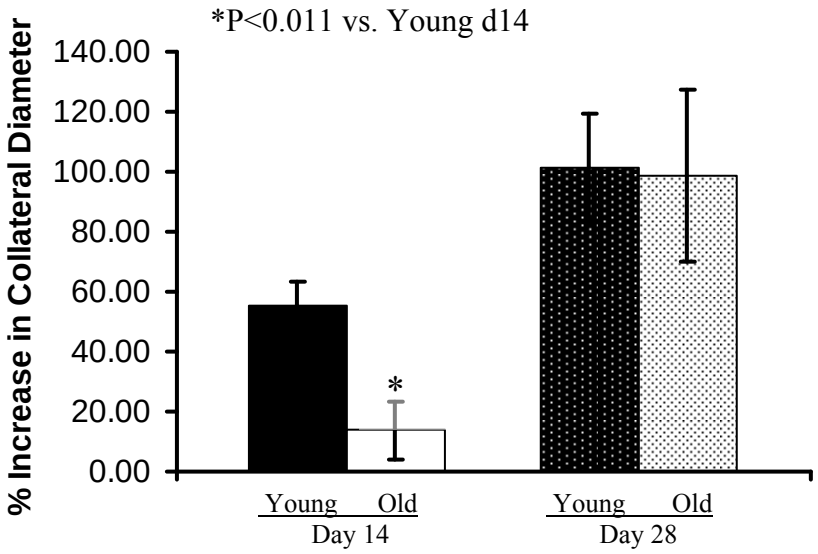

*P<0.011 vs. Young d14
% Increase in Collateral Diameter
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
*
Young
Old
Day 14
Young
Old
Day 28

Figure 6

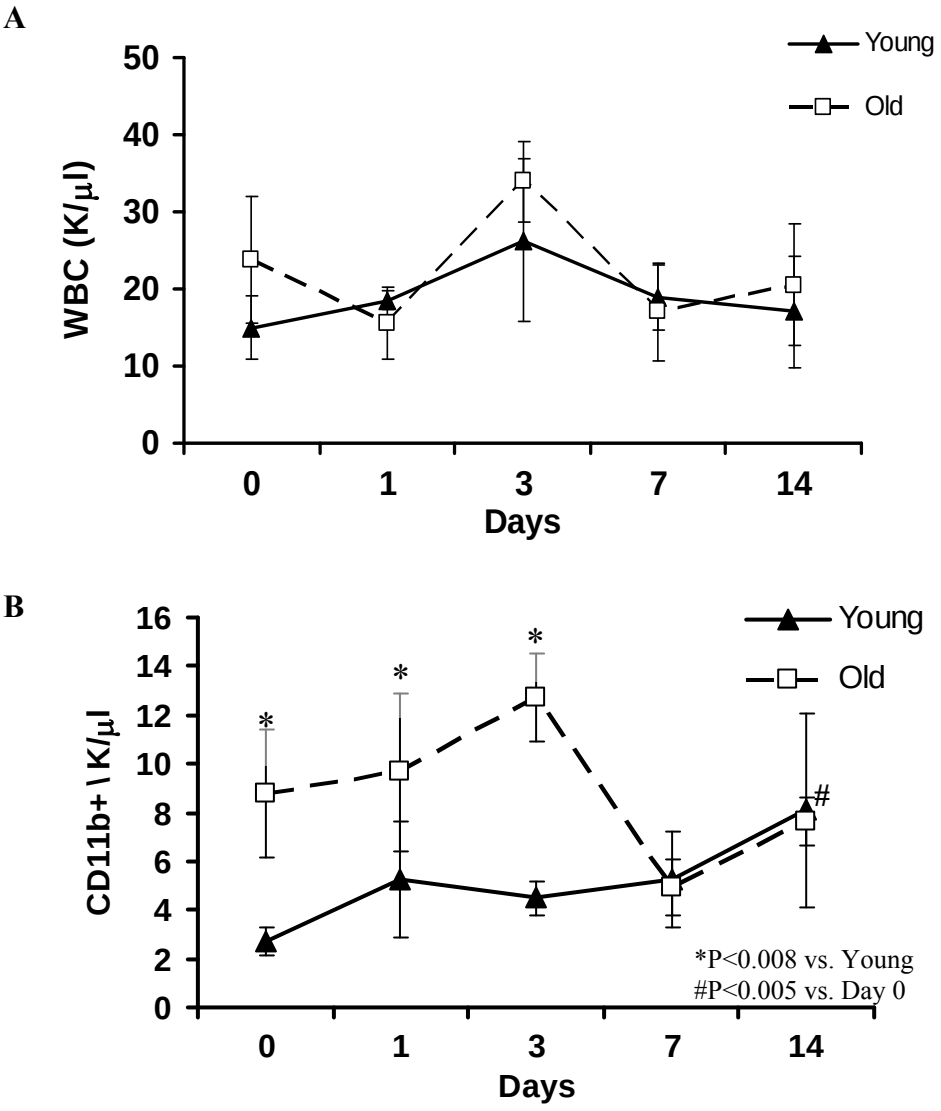

A
Young
Old
WBC (K/μl)
50
40
30
20
10
0
0
1
3
7
14
Days
B
Young
Old
CD11b+ \ K/μl
16
14
12
10
8
6
4
2
0
*
*
*
#
*P<0.008 vs. Young
#P<0.005 vs. Day 0
0
1
3
7
14
Days

Figure 7

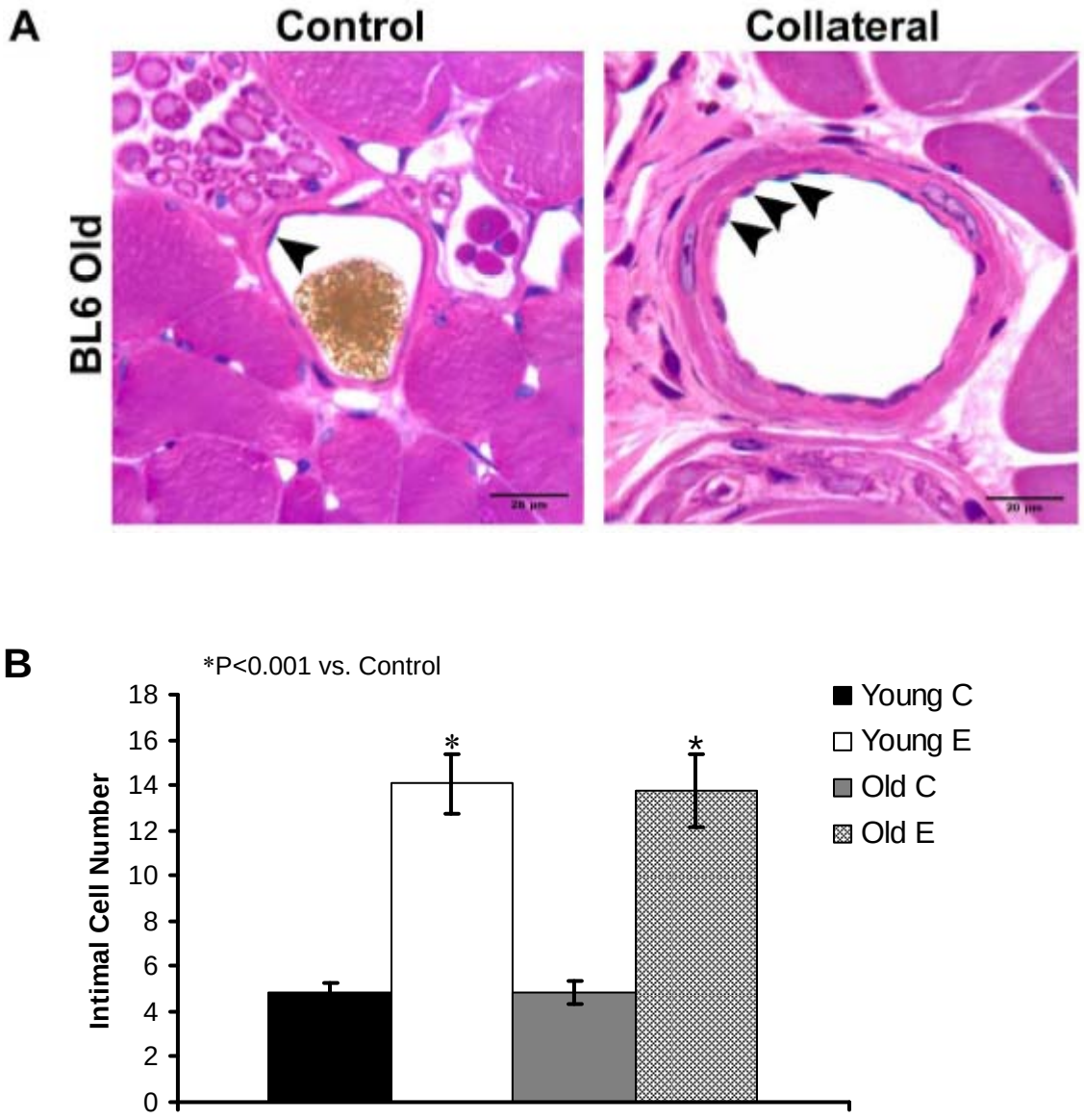

A
Control
Collateral
BL6 Old
B
*P<0.001 vs. Control
Intimal Cell Number
18
16
14
12
10
8
6
4
2
0
*
*
Young C
Young E
Old C
Old E

Figure 8

SUMMARY/DISCUSSION OF THESIS WORK AND FUTURE EXPERIMENTS

Summary/Discussion:

There is little consensus in recent literature regarding the adaptations to arterial occlusion, even at the most fundamental levels: what is a collateral, what type of vessel can become a collateral, what size are those vessels, how large can they grow, what cells are involved, what signaling molecules are involved, why is the process inhibited in the presence of cardiovascular disease, and is there a way to enhance collateral growth? Through the course of the three studies presented here, I was able to address a number of these areas.

The simplest definition of a collateral is a natural bypass vessel that provides for the flow of blood around a site of occlusion and allows for continued perfusion of the distal tissues. The collateral circulation may consist of a readily identifiable vessel providing an immediate bypass or it can be a circuitous path through many vessels. For example, one carotid artery can provide collateral flow to the other carotid via the Circle of Willis at the base of the brain. In order to bypass a site of occlusion, a collateral will generally have a proximal stem, a midzone comprised of communicating vessels, and then a distal re-entrant point that re-establishes the blood flow in the occluded parent artery (81). While this definition of a collateral is amenable to pre-existent vessels of any size, recent studies have purported to show that only specific types of vessels within a certain initial size range are capable of experiencing exorbitant degrees of enlargement and thus developing into collaterals. These studies suggest that only pre-existent arterioles initially <50 μm in diameter enlarge 10-25X and display neointimal formation and wall thinning, via a process now known as arteriogenesis, to become collaterals that

compensate for a parent artery occlusion (14). However, this definition is inconsistent with the results of many previous and current studies as numerous groups, our own included, have observed small arteries much larger than arterioles contributing to a collateral pathway and demonstrating luminal expansion with vascular wall thickening and no neointimal formation (i.e., experiencing collateral growth) (24, 25, 31, 53, 81, 100, 148). Thus, the question remains which pre-existing vessels are the most relevant in regards to the study of collateral growth? This controversy was the impetus for my first study, which was a survey of the collateral growth occurring in response to femoral arterial occlusion in pigs, rats, mice, and humans. In this study, I observed that the major collateral vessels that enlarge in response to femoral arterial occlusion in pigs, rats, and mice: 1) were anatomically at the level of the small arteries as opposed to arterioles, 2) typically enlarged <3X in contrast to 10-25X, 3) may or may not be tortuous, and 4) display wall remodeling characteristics similar to large and small arteries experiencing flow-mediated outward remodeling (namely, increased intimal cell number without neointimal formation, luminal expansion, and increased wall thickness) instead of displaying neointimal formation and wall thinning. While all these characteristics were similar between species, we did observe a 10-100X difference in vessel size, wall thickness, and distance from inner to outer wall layers between pigs and mice which we hypothesize may drastically effect the diffusion of reactive oxygen and nitrogen species as well as paracrine factors within the collateral wall.

My second study addressed what cells and signaling molecules may be involved in the process of collateral growth. The prevailing paradigm for collateral growth, based on the definition of collaterals as enlarging arterioles, suggests that the recruitment of

monocytes/macrophages is essential for the remodeling to occur (158). However, flow-mediated vascular remodeling in large and small arteries occurs without the recruitment of these cells and some studies suggest that increased shear stress (widely regarded as the stimulus initiating collateral growth) is actually anti-inflammatory (27, 141). Indeed, previous studies in our own lab on rat mesenteric artery collateral growth were inconsistent with recent studies in regards to macrophage accumulation in collateral arteries which have enlarged; immunohistochemically demonstrating macrophage accumulation in the adventitia only sporadically. Several studies have shown that macrophages are only present transiently after abrupt occlusion and inflammation is not noted with gradual occlusion (138). Also, the final endpoints of collateral vessel diameter enlargement are similar in some studies of suppressed macrophage function (67). Together these studies raise questions about the clinical relevance of macrophages in collateral development.

Other studies have suggested that reactive oxygen species (ROS), and specifically those coming from Nox2-NAD(P)H oxidase, are required for the recovery of hindlimb perfusion, angiogenesis, and collateral growth subsequent to femoral arterial occlusion (140, 151). One of the most recent studies links Nox2-NAD(P)H oxidase with bone marrow derived cells (BMCs), suggesting that the BMCs are the source of the Nox2-NAD(P)H oxidase involved in the vascular remodeling response (140, 151). However, despite adequate assessment of hindlimb reperfusion and angiogenesis, the study linking Nox2-NAD(P)H oxidase with BMCs did not specifically investigate collateral growth. Therefore, we specifically addressed the link between BMC Nox2-NAD(P)H oxidase and collateral growth using our technique of major collateral identification in combination

with mouse models of impaired Nox2-NAD(P)H oxidase function (Nox-2 and Rac2 knockout mice). A Rac family member is required for Nox2-NAD(P)H oxidase activation and Rac2 has been shown to bind to and activate Nox2-NAD(P)H oxidase (38, 72). Also, Rac2 is exclusively expressed in the BMCs (68, 107). Thus, Nox2-NAD(P)H oxidase should have been impaired in both vascular and bone marrow-derived cells in the *Nox2-/-* while Nox2-NAD(P)H oxidase should have only been impaired in BMCs in the *Rac2-/-*. In my second study, I observed both *Nox2-/-* and *Rac2-/-* mice demonstrating the same phenotype upon femoral arterial ligation: 1) impaired hindlimb reperfusion assessed via laser Doppler perfusion imaging, 2) impaired preservation of distal tissue morphology, and 3) no impairment in primary collateral growth. Therefore, I conclude that Nox2-NAD(P)H oxidase in either endothelial or bone marrow-derived cells is not required for collateral growth but is required for recovery of hindlimb perfusion and the preservation of distal tissue morphology. In addition, this study provides further evidence that the processes of angiogenesis and collateral growth are mediated by different molecules and mechanisms (13, 19).

My final study addresses the impairment of collateral growth in the presence of a risk factor of cardiovascular disease and the possibility of enhancing collateral growth in the presence of advancing age. This study was undertaken because previous studies primarily used young, healthy mice. In this study I confirmed an impairment in the recovery of hindlimb perfusion as well as a delay in the degree of enlargement experienced by the primary collateral vessels at 14 days post-ligation in aged wild-type mice versus young controls. Upon verification of this impairment, I then assessed the ability of BMC injection or antioxidant treatment to attenuate the impaired collateral

growth. I observed that neither BMC injection nor antioxidant therapy enhanced collateral growth. While these results are contrary to previous studies with young healthy animals, they may more accurately reflect the situation in aging humans. However, due to problems with interanimal variability and questions of the effectiveness of the antioxidant treatment, more experiments are needed before firm conclusions can be made.

Future Experiments:

In order to complete the study on collateral growth impairment with aging, additional old animals should be either injected one day post-ligation or treated with apocynin (starting one week prior to ligation and then continuing through the recovery period) and collateral growth assessed at day 14 when the collateral growth impairment was observed (instead of at day 28, when the impairment may no longer be present). Bone marrow transplantation studies to generate chimeric animals (Old animals with Young bone marrow and Young animals with Old bone marrow, with the appropriate controls) with GFP+/traceable BMCs should be utilized with the hindlimb ischemia model to directly assess the contribution of BMCs to collateral development. In addition, assays to determine the functionality of the aged bone marrow cells should also be performed. Lastly, combined BMC and antioxidant therapy should be investigated because correction of the altered balance of ROS known to be present in aged animals may be necessary for injected BMCs to exert a beneficial effect.

In order to more directly assess the role of macrophages in collateral development, bone marrow transplantation studies should be performed to generate

chimeric animals using an animal model of inducible macrophage impairment, such as the macrophage Fas-induced apoptosis (Mafia) transgenic mice (11). Since we have previously shown C57BL/6 (BL6) mice display normal collateral growth upon femoral arterial ligation, the first experiment would be to transplant Mafia mouse bone marrow into the BL6 mice and verify there is no impairment in collateral growth after transplantation. The next experiment would then be to transplant the Mafia bone marrow into the BL6 mice, perform the femoral ligation, systemically deplete the macrophages in the transplanted animals using a synthetic dimerizer (AP20187) (11), and then assess whether the growth of primary collaterals is impaired.

One potential problem with these proposed transplantation studies is the negative effect the transplantation procedure (specifically, the lethal irradiation) may have on the endothelium of the recipient animal blood vessels. Indeed, we conducted some preliminary transplantation experiments and, while young BL6 animals transplanted with young BL6 bone marrow did grow collaterals, their hindlimb perfusion recovery measured via laser Doppler was impaired versus young BL6 that did not receive transplantation. Also, when GFP+ animals were investigated, transgenic animals expressing GFP would grow collaterals but their hindlimb perfusion recovery was less than wild-type. In addition, wild-type animals transplanted with GFP+ bone marrow had reduced hindlimb perfusion recovery and their collaterals did not enlarge as much as wild-type un-transplanted controls. The problems with hindlimb perfusion recovery may be related to the variability issues we had with some of our laser Doppler measurements and may not be apparent if a more direct measurement of collateral dependant flow was utilized. Indeed, all future studies would benefit from using a more direct measurement

of collateral dependant flow than that obtained via laser Doppler perfusion imaging. Nevertheless, the potential issue of irradiation affecting the ability of the endothelial cells to stimulate collateral development would need to be addressed if transplantation studies were to be utilized.

Lastly, continued investigation of collateral growth in both large, healthy animal models as well as in large and small animal models of disease are desperately needed to advance the field of therapeutic collateral vessel enhancement. These models are needed since the large animal models would anatomically resemble the human vasculature more precisely and because animals possessing comorbidities consistent with human cardiovascular disease would more closely resemble the state of the aged, diseased human vasculature that is in need of collateral growth therapy.

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169. **Zweifach BW, Kovalcheck S, DeLano F, and Chen P**. Micropressure-flow relationships in a skeletal muscle of Spontaneously Hypertensive Rats. *Hypertension* 3: 601-614, 1981.

CURRICULUM VITAE

Matthew Robert DiStasi

Education:

Ph.D. Cellular & Integrative Physiology	Indiana University-Purdue University, Indianapolis, IN	2008
M.S. Physiology	Ball State University, Muncie, IN	2003
B.A. Biology	Taylor University, Upland, IN	2000

Professional Experience:

Doctoral Student 2003-2008
Department of Cellular & Integrative Physiology
Indiana University School of Medicine

Graduate Assistant 2001-2003
Department of Physiology
Ball State University

Laboratory Technician 2000-2001
Department of Pathology and Laboratory Medicine
Indiana University School of Medicine

Honors, Awards, and Memberships:

Leadership Merit Award, Taylor University 2000

English-Bonter-Mitchell Grant, Taylor University 2000

Homer D. Paschal Outstanding Graduate Assistant Award, Ball State University 2003

Spring Travel Award, from IUPUI Fellowship Committee. 05/2005. Used to attend North American Vascular Biology Organization (NAVBO) meeting, 06/2005.

American Association of Anatomists (AAA) 2008 Student Travel Award, from American Association of Anatomists (AAA) and the Advisory Committee for Young Anatomists (ACYA), 12/2007. Used to attend Experimental Biology, 04/2008.

North American Vascular Biology Organization Member since 03/2005

American Association of Anatomists Member since 04/2006

American Physiological Society Member since 06/2008

Teaching Experience:

Undergraduate Courses:

PHYSL 210L Human Physiology 1	Laboratory Instructor	2001-2003
PHYSL 211L Human Physiology 2	Laboratory Instructor	2001-2003
PHYSL 211 Human Physiology 2	Course and Laboratory Instructor	2002
BIO 307 Vertebrate Natural History	Laboratory Assistant	2000
BIO 244 Human Anatomy and Physiology	Laboratory Assistant	1999

Invited Lectures, Seminars and Symposia:

Cardiovascular Section Young Investigator Featured Topic: Molecular Regulation of eNOS Activity and Vascular Reactivity. Sponsored by the American Physiological Society at Experimental Biology 2006, San Francisco, CA, April 2006.

> **M.R. Distasi**, S.J. Miller, J.L. Unthank. Role of eNOS and DDAH1 in the impairment of collateral growth in spontaneously hypertensive rats (SHR) and its reversal by captopril.

North American Vascular Biology Organization: Highlights in Trainee Research and Welcome Reception. Sponsored by the North American Vascular Biology Organization at Experimental Biology 2006, San Francisco, CA, April 2006.

> Norton LE, **Distasi MR**, Dalsing MC, Miller SJ, Unthank JL. Impairment of collateral growth in spontaneously hypertensive rats: A role for NADPH oxidase?

Grants, Fellowships, and Awards:

Indiana University School of Medicine Graduate Minor in Aging Fellowship (Institutional). 03/2005-05/2006. Gain preliminary data in support of thesis project. Role: Principal Investigator.

Indiana University School of Medicine Graduate Fellowship in Translational Research (Institutional). 07/2006-06/2007. Investigate the role of extramural cells in the impairment in collateral growth observed in the Spontaneously Hypertensive rat and its reversal via antioxidant therapy. Role: Principle Investigator.

Publications:

M.S. Thesis: The 3D Characterization of the Annulate Lamellae. June, 2003.

Peer Reviewed Publications:

1. **Distasi MR**, Case J, Ziegler MA, Dinauer MC, Yoder MC, Haneline LS, Dalsing MC, Miller SJ, Murphy MP, Ingram DA, and Unthank JL. Suppressed hindlimb perfusion in *Rac2-/-* and *Nox2-/-* mice does not result from impaired collateral growth. *American Journal of Physiology - Heart and Circulatory Physiology* 2008 (In Revision).

2. Ziegler MA*, **Distasi MR***, Miller SJ, Alloosh M, Murphy MP, Sturek M, Dalsing MC, and Unthank JL. Characteristics of major collateral arteries in the peripheral circulation of humans, pigs, rats, and mice. *Journal of Applied Physiology* 2008 (Submitted). *=Authors contributed equally to this work.

3. Sheridan KM, Ferguson MJ, **Distasi MR**, Witzmann FA, Dalsing MC, Miller SJ, and Unthank JL. Impact of genetic background and aging on mesenteric collateral growth capacity in Fischer 344 (F344), Brown Norway (BN), and F344xBN rats. *American Journal of Physiology - Heart and Circulatory Physiology* 2007 Sep 28; [Epub ahead of print].

4. Haas TL, Doyle JL, **Distasi MR**, Norton LE, Sheridan KM, and Unthank JL. Involvement of MMPs in the outward remodeling of collateral mesenteric arteries. *American Journal of Physiology - Heart and Circulatory Physiology* 2007 Oct;293(4): H2429-37. Epub 2007 Jul 20.

Research Abstracts Presented at Annual Meetings:

1. **DiStasi MR** and Reber JM. Oxidative stress as a mechanism of photo-enhanced toxicity in *Ceriodaphnia dubia*. Indiana Academy of Science, 114th meeting: 71, 1998.

2. **DiStasi MR**, Norton LE, Haas TL, and Unthank JL. Matrix metalloproteinase (MMP) inhibition limits luminal expansion but not endothelial proliferation in rat collateral arteries. *Federation of American Societies for Experimental Biology Journal* 19(5): A1271, 2005.

3. **Distasi MR**, Miller SJ, and Unthank JL. Role of eNOS and DDAH1 in the impairment of collateral growth in spontaneously hypertensive rats (SHR) and its reversal by captopril. *Federation of American Societies for Experimental Biology Journal* 20(4): A720, 2006.

4. **Distasi MR**, Miller SJ, Ingram DA, and Unthank JL. Normal hindlimb collateral blood vessel development occurs in a mouse model of impaired macrophage migration (*Rac2-/-*). *Federation of American Societies for Experimental Biology Journal* 21: 897.6, 2007.

5. **Distasi MR**, Miller SJ, Ingram DA, Murphy MP, and Unthank JL. Sustained age-related impairment of vascular compensation to hindlimb ischemia in C57BL/6 mice. *Microcirculation* 2007,14: 442; AVD-8.

6. **Distasi MR**, Ziegler MA, Miller SJ, Murphy MP, Dalsing MC, Sturek M, and Unthank JL. Species differences in collaterals arising from femoral artery occlusion: A comparison from mice to men. *Federation of American Societies for Experimental Biology Journal* 22: 1147.4.

7. Murrell J, **DiStasi M**, Lane K, Gao S, Farlow M, and Hendrie HC. The interleukin 1A gene polymorphism does not affect risk of Alzheimer Disease in African Americans. *Neurobiology of Aging* 23(Supp. 1): S331-332, 2002.

8. Norton LE, **Distasi MR**, Bills RG, and Unthank JL. Impairment of collateral artery growth in the Spontaneously Hypertensive Rat (SHR) is mediated via reactive oxygen species (ROS). *Federation of American Societies for Experimental Biology Journal* 19(4): A1271, 2005.

9. Norton LE, **Distasi MR**, Bills RG, and Unthank JL. Therapeutic enhancement of collateral artery growth in the Spontaneously Hypertensive Rat (SHR). *Vascular Medicine* 2005;10: 137:P-7.

10. Norton LE, **Distasi MR**, Bills RG, Unthank JL, and Dalsing MC. Collateral artery growth in the spontaneously hypertensive rat (SHR) is impaired by NADPH oxidase. *Journal of the American College of Surgeons* 201: S99, 2005.

11. Norton LE, **Distasi MR**, Dalsing MC, Miller SJ, and Unthank JL. Impairment of collateral growth in spontaneously hypertensive rats: A role for NADPH oxidase? *Federation of American Societies for Experimental Biology Journal* 20(5): A1099, 2006.

12. Ziegler MA, Bills RG, **Distasi MR**, Miller SJ, and Unthank JL. Perforating artery contribution to vascular compensation in the ischemic hindlimb of spontaneously hypertensive rats (SHR). *Federation of American Societies for Experimental Biology Journal* 21: 897.7, 2007.

13. Goel MC, Jiang H, **Distasi MR**, and Galis ZS. Matrix metalloproteinases (MMPs) are necessary for flow-induced arterial remodeling. *Federation of American Societies for Experimental Biology Journal* 21: 383.6, 2007.