PROJECT NARRATIVE

Recent studies have uncovered the importance of the adventitia as well as resident and circulating stem and progenitor cells in mediating the arterial injury response that leads to the development of neointimal hyperplasia. Nitric oxide (NO) is a potent inhibitor of neointimal hyperplasia, but little is known about how NO affects the adventitial layer of the arterial wall following injury. The studies in this proposal will elucidate the role of adventitial stem and progenitor cells in mediating the beneficial effects of NO in the vasculature and will result in a change in how we think about the adventitia and the vascular biology of NO thereby allowing the development of new strategies to prevent neointimal hyperplasia and restenosis following vascular interventions.

PROJECT SUMMARY/ABSTRACT

Neointimal hyperplasia is a significant problem that results in the failure of vascular interventions. The classic arterial injury response that leads to the development of neointimal hyperplasia describes processes involving the intima and media with a relative lack of involvement of the adventitia. However, the adventitia is no longer considered a simple structural component of the arterial wall. In the last decade, research has shown that many cell types from the adventitia actively regulate and contribute to the development of neointimal hyperplasia, including resident and circulating adventitial stem and progenitor cells. We, and others, have demonstrated that NO is a potent inhibitor of neointimal hyperplasia through regulating different aspects of the classic arterial injury response. **However, little is known about how NO affects the adventitial layer of the arterial wall following injury.** We have recently assessed the cellular response throughout the arterial wall to injury and exposure to NO. Interestingly, while NO prevents the development of neointimal hyperplasia and delays repopulation of the media, we were surprised to find that NO actually increases cellularity in the adventitia. This increase in cellularity is not due to an increase in vascular smooth muscle cells, fibroblasts, myofibroblasts, or inflammatory cells, but may in fact be due to an increase in Sca1+ progenitor cells, among others. Thus, given the important role of the adventitia in regulating the arterial injury response, recent discoveries of the role of stem and progenitor cells in this process, and our preliminary data, **we hypothesize that NO inhibits neointimal hyperplasia by regulating recruitment and phenotypic differentiation of resident and circulating adventitial stem and progenitor cells following injury. Furthermore, we hypothesize that NO supports differentiation of adventitial stem and progenitor cells into endothelial-like cells, contributing to adventitial neovascularity.** To investigate these hypotheses, our specific aims are: 1) to characterize the effect of NO on Sca1+, CD34+, and flk-1+ adventitial stem and progenitor cell populations following arterial injury *in vivo; 2)* to determine the effect of NO on Sca1+, CD34+, and flk-1+ adventitial cell populations *in vitro;* and 3) to manipulate Sca1+, CD34+, and flk-1+ adventitial cell populations *in vivo* to determine if NO-mediated inhibition of neointimal hyperplasia following arterial injury is dependent on these adventitial cell populations. The innovative studies described in this proposal will result in a change in how we think about the adventitia and the vascular biology of NO, and provide a novel mechanism by which NO regulates neointimal hyperplasia that will lead to the development of new strategies to prevent neointimal hyperplasia and restenosis following vascular interventions.

A. SPECIFIC AIMS

Neointimal hyperplasia is a significant problem that results in the failure of vascular interventions such as bypass grafting, angioplasty and stenting, and endarterectomy. The classic arterial injury response that leads to the development of neointimal hyperplasia describes processes involving the intima and media with a relative lack of involvement of the adventitia. However, the adventitia is no longer considered a simple structural component of the arterial wall. In the last decade, research has shown that many cell types from the adventitia actively regulate and contribute to the development of neointimal hyperplasia, including adventitial fibroblasts and macrophages. In fact, we now know that cellular infiltration and proliferation occurs in the adventitia before the media. The adventitial microvasculature has been shown to increase in density following arterial injury and correlates with the development of neointimal hyperplasia. The adventitia has also been found to host resident adventitial progenitor cells (Sca1+), as well as recruit circulating bone marrow-derived stem cells (CD34+, flk-1+), and these cells have been shown to contribute significantly to the neointima.

We, and others, have demonstrated that NO is a potent inhibitor of neointimal hyperplasia through regulating different aspects of the classic arterial injury response. **However, little is known about how NO affects the adventitial layer of the arterial wall following injury.** We have recently identified distinct differences in how the layers of the arterial wall respond to NO with respect to reactive oxygen species. Additionally, a detailed time course analysis of the cellular response in the arterial wall to injury and exposure to NO was performed. Interestingly, while NO prevents the development of neointimal hyperplasia and delays repopulation of the media, we were surprised to find that NO actually increases cellularity in the adventitia. This increase in cellularity is not due to an increase in vascular smooth muscle cells (VSMC), fibroblasts, myofibroblasts, or inflammatory cells, but may in fact be due to an increase in Sca1+ progenitor cells, among others.

Thus, given the important role of the adventitia in regulating the arterial injury response, recent discoveries of the role of stem and progenitor cells in this process, and our preliminary data, **we hypothesize that NO inhibits neointimal hyperplasia by regulating recruitment and phenotypic differentiation of resident and circulating adventitial stem and progenitor cells following injury. Furthermore, we hypothesize that NO supports differentiation of adventitial stem and progenitor cells into endothelial-like cells, contributing to adventitial neovascularity.** To investigate these hypotheses, the specific aims are as follows:

Specific Aim 1 - Characterize the effect of NO on Sca1+, CD34+, and flk-1+ adventitial stem and progenitor cell populations following arterial injury *in vivo.* Specifically, we will evaluate and characterize adventitial stem and progenitor cell recruitment, phenotypic differentiation, proliferation, migration, and neovascularity following arterial injury and exposure to NO.

Specific Aim 2 - Determine the effect of NO on Sca1+, CD34+, and flk-1+ adventitial cell populations *in vitro.* Specifically, we will isolate and identify Sca1+, CD34+, and flk-1+ adventitial stem and progenitor cell populations and determine how NO affects phenotypic differentiation, proliferation, migration, and reactive oxygen species (ROS) production when exposed to different growth conditions (i.e., starvation, hypoxia, fibronectin, collagen, co-culture, etc) and stimuli (VEGF, PDGF, TGFβ, bFGF, etc).

Specific Aim 3 - Manipulate Sca1+, CD34+, and flk-1+ adventitial cell populations *in vivo* to determine if NO-mediated inhibition of neointimal hyperplasia following arterial injury is dependent on these adventitial cell populations. Specifically, we will perform gain- and loss-of-function experiments by evaluating the role of 1) adventitial removal and selective overexpression of Sca1+, CD34+, and flk-1+ cells, 2) stimulation of circulating stem and progenitor cells with granulocyte-colony stimulating factor (G-CSF), and 3) Sca1+, CD34+, and flk-1+ cell populations in iNOS and eNOS deficient mice following arterial injury.

The adventitia can no longer be considered a passive, structural component of the arterial wall. Much is known about how NO inhibits neointimal hyperplasia with respect to the media and intima, but little is known about the adventitia. An innovative aspect of this proposal is that our focus is shifted away from the media as the central mediator of the arterial injury response, to the adventitia, as the adventitia appears to be a portal that directs cell trafficking and differentiation. The studies described in this proposal will result in a change in how we think about the adventitia and the vascular biology of NO, and provide a novel mechanism by which NO regulates neointimal hyperplasia. Ultimately, these studies may lead to the development of new strategies to prevent neointimal hyperplasia and restenosis following vascular interventions.

B. SIGNIFICANCE

Scope of the problem. Atherosclerosis is prevalent in all developed nations and is the leading cause of death and disability in the United States. In 2006, more than 7 million inpatient cardiovascular operations and procedures were performed in the United States alone.[1] These operations and procedures include percutaneous balloon angioplasty with and without stenting, endarterectomy, bypass grafting, or plaque debulking. Unfortunately, the long-term durability of these procedures is limited due to the development of neointimal hyperplasia, which results in luminal narrowing, restenosis, and ultimately, vascular occlusion. For example, for balloon angioplasty and stenting, approximately 27% of sites develop angiographic restenosis at 1 year.[2] For coronary artery bypass grafting, approximately 32% of grafts fail by 10 years, and 41% of patients require repeat coronary revascularization by 20 years.[3] For peripheral arterial revascularization, the patency for infrainguinal vein grafts is approximately 70% at 5 years. However, for expanded polytetrafluoroethylene (ePTFE) grafts, the 1-, 2-, and 4-year primary patency rates are only 65%, 30%, and 12% respectively.[4] Therefore, neointimal hyperplasia, the root cause of restenosis and poor patency, is an alarming problem that causes significant morbidity and mortality.

The classic arterial injury response. The classic description of the arterial injury response by [redacted] and [redacted] involves injury to the arterial wall leading to endothelial denudation.[5-9] The underlying internal elastic lamina and vascular smooth muscle cells (VSMC) are exposed to circulating blood elements. Platelets immediately aggregate and adhere to the site of injury.[10] An inflammatory response follows, with the infiltration of neutrophils, macrophages, and leukocytes.[11, 12] Twenty-four hours following injury, under the influence of growth factors and cytokines, medial VSMC convert from a contractile to a synthetic phenotype and begin to proliferate.[13] VSMC migration to the neointima where they continue to proliferate for weeks.[5, 14, 15] Concurrently, endothelial cell regeneration occurs through the stimulation of basic fibroblast growth factor (bFGF) within 24 hours after injury, and can continue for 6-10 weeks.[16] Lastly, transforming growth factor beta (TGFβ) stimulates extracellular matrix deposition.[17, 18]

Role of the adventitia following arterial injury. The classic arterial injury response described above includes no mention of the adventitia. However, the adventitia is now thought to be more of a driving force in the development of neointimal hyperplasia than the media. [redacted] et al and [redacted] et al have both characterized the proliferative response following arterial injury in rat and pig arteries.[19, 20] Common to both is that the proliferative response in the adventitia is much greater at almost all time points compared to the intima and media. While [redacted] et al reported 1.5-2-fold more proliferation in the adventitia compared to the media in rat carotid arteries (Table 1), [redacted] reported nearly 7-fold more proliferation in the adventitia than the media in pig coronary arteries. Furthermore, upon examining early time points, [redacted] et al found that proliferation in the adventitia actually occurs before proliferation in the media, at time points as early as 4 hours (Table 1). It has also been recognized that the adventitia is host to many cell types, resident and circulating, that regulate or participate in the development of the neointima. Below is a summary of the role of each of these adventitial cell types in the arterial injury response.

Table 1. Proliferating cell number/mm^2 (prolif. index) after arterial injury.[19]

Time point	Media	Adventitia
Non-injured	0.2	0.1
4 hour	42	286
8 hour	37	510
12 hour	38	531
24 hours	369	582
48 hours	972	691
72 hours	930 (29%)	1164 (53%)
7 days	576 (14%)	1424 (41%)
14 days	420 (13%)	810 (23%)
30 days	22 (0.4%)	75 (2%)

Adventitial inflammation. An important aspect of the arterial injury response has always included inflammation, but has traditionally been described as an "inside-out" response. It is now recognized that much of the inflammation occurs in the adventitia after injury, following an "outside-in" approach.[21] [redacted] et al characterized the time course of the inflammatory infiltrate throughout all three layers of the arterial wall in porcine coronary arteries following balloon injury.[22] Interestingly, they found that macrophages had a 20-fold greater presence than neutrophils, and that most of the inflammatory infiltrate was found in the adventitia, and not in the media and intima. Macrophages have also been noted to phenotypically differentiate into myofibroblasts following arterial injury and contribute to the development of neointimal hyperplasia.[23] [redacted] et al demonstrated that 14 days following arterial injury to the pig coronary artery 42% of the neointimal cells expressed the macrophage-specific antigen SWC3, and 9% of the cells co-expressed SWC3 and alpha smooth muscle actin (αSMA).[23] Studies have also shown that in association with this adventitial inflammatory infiltration, there is an increase in the expression of adhesion molecules by the microvascular endothelial cells of the vasa vasorum, such as VCAM-1, P-selectin, E-selectin, etc, suggesting that the adventitial microvasculature actively recruits inflammatory cells following injury.[22] Thus, these studies highlight the role of

the adventitia as the site of initial inflammation following arterial injury and the importance of macrophages as contributors to neointimal hyperplasia.

Adventitial fibroblasts. Our concept of the role of adventitial fibroblasts in the development of neointimal hyperplasia has also evolved over the past decade. [redacted] et al were one of the first groups to track the kinetics and differentiation of adventitial fibroblasts following arterial injury.[24] These investigators demonstrated, using a porcine coronary artery injury model, that adventitial fibroblasts proliferate at early time points (2-3 days), and then migrate to the neointima following phenotypic differentiation. By 7-8 days, 76% of neointimal cells were transformed fibroblasts, or myofibroblasts, as they stained positive for αSMA but not desmin, differentiating them from medial VSMC and blood-born cells. Later that same year, [redacted] et al confirmed these results and suggested that the increase in myofibroblasts following injury may actually constrict the injured artery and contribute to arterial remodeling and late lumen loss observed after injury.[25]

Circulating stem and progenitor cells. In the past several years, it has become apparent that the adventitia is an active site of recruitment of circulating stem and progenitor cells. Hematopoietic stem cells (HSC), mesenchymal stem cells (MSC), and endothelial progenitor cells (EPC) have all been shown to contribute to the formation of the neointima following arterial injury, resulting in a paradigm shift of what occurs in the arterial wall following injury.[26-29] [redacted] et al and [redacted] et al were the first investigators to independently demonstrate, using bone marrow irradiated mice transplanted with labeled or sex-specific bone marrow, that bone marrow-derived cells constituted approximately 50-60% of the neointima following injury.[26, 30] Some cells stained positive for CD31 while others stained positive for αSMA. Taking this one step further, [redacted] et al studied the response to injury in mice deficient in stem cell factor (SCF) or c-Kit, both important molecules to stem cell signaling. Interestingly, they found a near complete lack of neointima in these mice following arterial injury compared to wild-type controls, confirming the importance of stem cells to the formation of the neointima.[31] Further studies by [redacted] et al demonstrated that the degree of incorporation of bone marrow-derived cells into the neointima was dependent on the type of injury model used, with the wire-mediated injury resulting in the largest percentage of bone marrow-derived cells in the neointima.[32] Thus, while it is now clear that stem and progenitor cells are mobilized and recruited to the site of arterial injury and undergo phenotypic differentiation to endothelial or VSMC, much remains to be learned about forces regulating these processes.

Resident adventitial stem and progenitor cells. The adventitia is also home to resident adult pluripotent cells, including Sca1+ cells, CD34+ cells, and pericytes. [redacted] et al identified that Sca1+ cells, which are integral to the developing artery, are present in adult arteries in the adventitia, near the media/adventitia interface.[33] In an effort to determine how the adventitial Sca1+ (AdvSca1) cells are targeted to, and maintained within, the adventitia, they identified a layer of sonic hedgehog protein (shh) located between the media and the adventitia.[33] Shh is known to be important in the retention of other progenitor cell populations in areas such as the skin, nervous system, and lymphoid tissue.[34-36] Shh knockout mice were also found to have significant less AdvSca1+ cells compared to wild-type mice. In a quiescent state, AdvSca1 cells do not express VSMC or endothelial cell markers *in vivo*. However, these cells can readily differentiate into VSMC or endothelial cells *in vitro* depending on the stimulus.[33, 37] For example, [redacted] et al demonstrated that LacZ labeled Sca1+ cells transferred to the adventitial surface of vein grafts in ApoE-deficient mice contributed to the atherosclerotic vein graft intimal hyperplasia *in vivo*.[37] Pericytes, which incompletely surround the endothelial cells of the microvasculature, have traditionally been described and characterized by their supportive role. However, it has now been demonstrated that pericytes can differentiate into multiple different cell types depending upon the stimuli, including adipocytes, chondrocytes, osteoblasts, VSMC, macrophages, and fibroblasts.[38-41] By having this pluripotent ability, pericytes may serve as a reservoir of precursor cells, giving rise to cells of multiple different lineages. Lastly, [redacted] et al recently identified the presence of resident adventitial CD34+ cells in the wall of adult human arteries.[42] These cells were located in the same zone as the Sca1+ cells described above, at the media/adventitia interface, and demonstrated potential to differentiate into endothelial cells and contribute to the vasa vasorum. Thus, it is clear that the adventitia contains a "vasculogenic" zone of pluripotent cells capable of differentiating into a variety of cell types.

Characterization of stem and progenitor cell contribution to neointimal hyperplasia. [redacted] et al characterized the involvement of precursor cells in the arterial injury response.[43] Following rat arterial injury, there was a 5-fold increase in adventitial cells which preceded the formation of the neointima. This was accompanied by a 4-fold increase in the adventitial microvasculature which peaked at day 14. The cytoskeletal marker vimentin, which stains VSMC, fibroblasts, and myofibroblasts, mostly stained positive in the adventitia at day 3-4; thereafter, the staining declined in the adventitia and shifted to the neointima. Flk-1

staining, a stem cell marker, was noted in the first cells which localized to the neointima and adventitia. By day 14, all layers of the vascular wall expressed flk-1. These findings support the assertion that cells in the adventitia are activated or recruited very early in the injury response and contribute to neointima formation. To further characterize the adventitial cell types contributing to neointima formation, outgrowth assays were performed from aortic explants. Cells from these outgrowth assays co-expressed markers of multiple cell lineages including VSMC, endothelial cells, and myofibroblasts, and a high percentage of these cells were also positive for the stem cell markers flk-1, Sca1, and CD34. Thus, these data suggest that stem and progenitor cells contribute significantly to the development of neointimal hyperplasia following arterial injury.

NO and stem and progenitor cells. NO has also been shown to be an important regulator of stem and progenitor cells in different animal models, but data regarding regulation of CD34+ EPC versus Sca1+ cells appears to be contradictory. Early work by [redacted] et al demonstrated that eNOS deficient mice exhibited reduced VEGF-stimulated mobilization of EPC, and impaired neovascularization in a mouse model of hindlimb ischemia.[44] Supporting these data, both Iwakura et al and [redacted] at al demonstrated impaired recruitment of EPC in eNOS deficient mice following arterial injury, and this was associated with impaired re-endothelialization and greater neointimal hyperplasia.[45, 46] However, [redacted] et al evaluated the contribution of Sca1+ cells in eNOS deficient mice using the carotid artery ligation model.[47] They reported increased stromal cell-derived factor-1a (SDF-1α) expression, a chemokine known to regulate hematopoietic progenitor cell mobilization, increased circulating Sca1+ cells, and increased Sca1+ cells in the adventitia 1 week following carotid artery ligation in eNOS deficient compared to wild-type control mice. These data correlated with increased adventitial proliferation and neointimal hyperplasia. Together, these studies clearly demonstrate a role for NO in regulating stem and progenitor cell recruitment and activity following arterial injury, but the effects may differ according to the stem and progenitor cell type and the injury model being utilized.

Adventitial microvasculature and hypoxia. The role of the adventitial microvasculature, or vasa vasorum, in the arterial injury response has also evolved in recent years. [redacted] et al characterized the effect of arterial injury on the vasa vasorum in balloon-injured pig coronary arteries using three-dimensional computed tomography.[48] They reported an increase in the density of the vasa vasorum, an increase in size of the vasa vasorum, and a change in the special distribution of the vasa vasorum with more secondary vasa following arterial injury. They also reported that the density of the vasa vasorum was proportional to the degree of stenosis following injury. However, while there was an increase in the overall density of the vasa vasorum, there was a decrease in the actual percent of vessel wall area comprised of vasa following arterial injury. Thus, it is possible that the increase in vasa vasorum following arterial injury is not sufficient for the existing increase in tissue mass, resulting in ischemic tissue zones, further stimulating the injury response. [redacted] et al suggested this concept in 1994 after stripping the adventitia from carotid arteries of rabbits and demonstrating increased neointimal lesions.[49] Furthermore, [redacted] et al demonstrated that adventitial fibroblasts respond rapidly to hypoxic conditions by proliferating, differentiating into myofibroblasts, and migrating to the neointima.[50-53] Thus, much remains to be learned regarding the role of the vasa in the arterial injury response.

C. INNOVATION

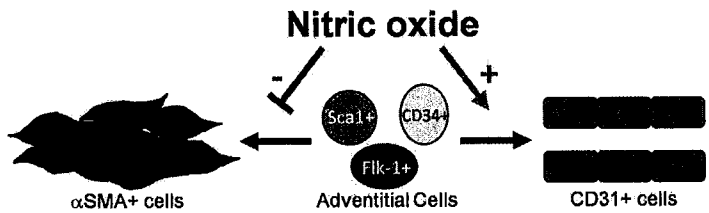


Figure 1. Schematic of our hypothesis.

From the studies described above, it is clear that the adventitia provides much more than a supportive, passive role following arterial injury, but in fact, is the central regulator of the arterial injury response, serving as a portal directing cell mobilization, recruitment, and trafficking. The role of NO in this process is not known. Thus, the goal of the studies described in this proposal is to elucidate the role of NO in regulating adventitial stem and progenitor cell populations following arterial injury, and to determine if NO-mediated inhibition of neointimal hyperplasia is dependent upon these cell populations. Furthermore, we aim to determine if NO supports differentiation of these adventitial stem and progenitor cells away from VSMC-like cells, and toward endothelial-like cells that contribute to the microvasculature (Figure 1). We are focusing our efforts on Sca1+, CD34+, and flk-1+ stem and progenitor cells based on our preliminary data as well as recently published data demonstrating a significant role for these stem cell populations in the adventitial and during the arterial injury response.[26, 30, 32, 33, 37, 42, 43]

Data from this proposal will challenge the existing paradigm of how NO inhibits neointimal hyperplasia. Currently, the model of NO-based inhibition of neointimal hyperplasia focuses on aspects of the classic arterial

injury response, with no consideration of the effects of NO on adventitial cell populations. The role of the adventitia in regulating the beneficial effects of NO can no longer be dismissed. Furthermore, given the role of adventitial stem and progenitor cells and the evidence that more than half of neointimal cells express markers consistent with stem or progenitor cell origins, the role of NO in regulating this process must be studied.

We have put together a multidisciplinary team of investigators with the required expertise to sufficiently perform, analyze, and understand the studies described in this proposal. This includes: 1) [redacted], MD, Professor of Surgery, who originally characterized the classic model of the arterial injury response; 2) [redacted], PhD, Professor of Pediatrics and Co-Director of the Center for Tissue and Cell Sciences, an adventitial and vascular stem cell embryologic researcher; 3) [redacted], MD, Professor of Medicine and Director of the Feinberg Cardiovascular Research Institute, an expert in endothelial progenitor cell vascular biology; and 4) [redacted], PhD, Chief of the Laboratory of Comparative Carcinogenesis at the NCI, a prominent nitric oxide chemist. Data generated from this proposal will result in a paradigm shift in how NO prevents the arterial injury response. By improving our scientific knowledge of NO vascular biology, data generated from this proposal will lead to the development of novel strategies to inhibit neointimal hyperplasia following vascular interventions, thereby having a significant beneficial impact on patients with cardiovascular disease requiring vascular interventions.

D. APPROACH - Preliminary Data

NO inhibits neointimal hyperplasia. We have demonstrated that multiple different NO donors, each with a different half-life of NO release, inhibit the development of neointimal hyperplasia.[54-56] Using the rat carotid artery injury model, PROLI/NO (a short half-life donor) or PAN/NO (a long half-life donor), 20 mg each, was applied to the periadventitial surface of the carotid artery following balloon injury (2F Fogarty at 5 atm x 5 min).[54] Surprisingly, morphometric analysis revealed that PROLI/NO was more effective at inhibiting neointimal hyperplasia compared to PAN/NO at 2 weeks, however both were effective (86% vs. 67%, P<0.05, respectively, Figure 2).[54] Given that PROLI/NO results in a short, high burst release of NO and PAN/NO results in a longer, lower level of NO release, we concluded that it is important to have NO present immediately following injury to inhibit early processes in the arterial injury response.

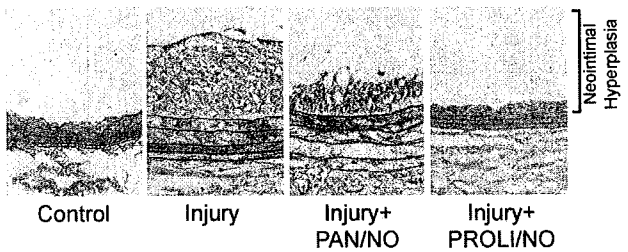


Figure 2. Periadventitial application of the NO donors PAN/NO and PROLI/NO inhibit the development of neointimal hyperplasia following arterial injury.

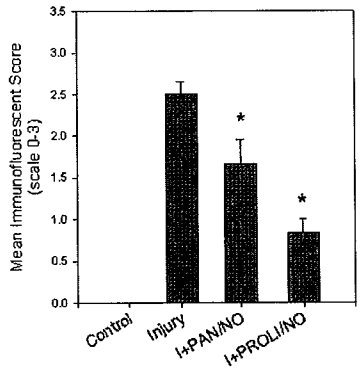


Figure 3. NO prevents macrophage infiltration (ED1 staining) following arterial injury. *p<0.05 vs. injury alone.

NO inhibits vascular inflammation. We also evaluated the effect of NO on inflammation following arterial injury. At 3 days following injury and application of NO to the periadventitial surface, we found that NO significantly inhibited macrophage (ED1 positive) infiltration into the arterial wall in two entirely separate studies (Figure 3).[54, 56] We did not observe an appreciable effect on CD45 leukocytes in either study (not shown).

The inhibition of neointimal hyperplasia by NO is durable. Next, we wanted to determine if the effect of NO on neointimal hyperplasia was durable. Thus, we evaluated the effect of periadventitial application of the NO donor PROLI/NO at inhibiting neointimal hyperplasia at both 2 and 8 weeks. Interestingly, we determined that the efficacy of NO was equally effective at 8 weeks as it was at 2 weeks (Figure 4). NO (20 mg) reduced intimal area (90% and 85%, p<0.001), medial area (46% and 47%, p<0.001), and the intima/media area ratio (90% and 85%, p<0.001) at 2 and 8 weeks, respectively, with no statistical difference between time points. Of note, 10 mg was as effective as 20 mg.

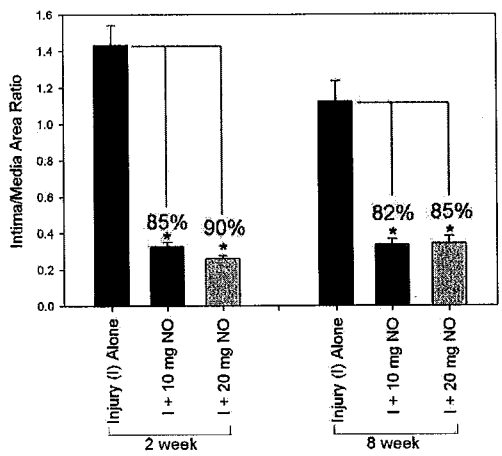


Figure 4. NO-mediated inhibition of neointimal hyperplasia is durable out to 8 weeks. *p<0.05 vs. injury alone.

Inhibition of neointimal hyperplasia by NO is due to NO and not its metabolic end products, nitrite or nitrate. To determine if the inhibition of neointimal hyperplasia is due to the NO release

from the diazeniumdiolate, or the metabolic end products, nitrite and nitrate, we evaluated the efficacy of the molar equivalents of each compound at inhibiting VSMC proliferation *in vitro* and at inhibiting neointimal hyperplasia, proliferation, and inflammation *in vivo*. *In vitro*, only NO inhibited VSMC proliferation (80% inhibition, DETA/NO 1mM, $p<0.05$). VSMC exposed to nitrite or nitrate proliferated at a rate similar to control. *In vivo*, NO inhibited neointimal hyperplasia significantly more than either nitrite or nitrate (Figure 5). Morphometric analysis revealed that NO produced greater inhibition than either nitrite or nitrate in intimal (82% vs. 45% and 41%, resp., $p<0.05$) and medial area (44% vs. 31% and 29%, resp., $p<0.05$). The intima/media area ratio was significantly decreased (63%, $p<0.05$; Figure 6) and lumen area was significantly increased (16%, $p<0.05$) in rats treated with NO, but not nitrite or nitrate. Only NO inhibited BrdU incorporation following arterial injury (59%, $p<0.05$). Leukocyte (CD45) and monocyte/macrophage (ED1) infiltration were significantly inhibited by all treatment groups studied compared to injury alone ($p<0.05$) and there were no significant differences between groups treated with NO, nitrite, or nitrate. Thus, these data demonstrated that NO is much more efficient at inhibiting neointimal hyperplasia compared to nitrite and nitrate, but that nitrite and nitrate do modestly inhibit neointimal hyperplasia, possiblly through inhibition of inflammation and not proliferation.

Figure 5. NO inhibited the development of neointimal hyperplasia to a greater extent than the molar equivalent dose of nitrite or nitrate.

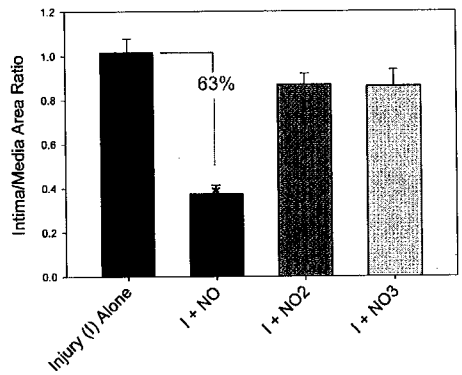


Figure 6. NO inhibited the intima/media area ratio compared to nitrite (NO2) or nitrate (NO3). $p<0.05$ compared to injury alone.

Effects of NO throughout the arterial wall. To further evaluate the mechanism by which NO inhibits neointimal hyperplasia, we performed a detailed time-course analysis throughout each layer of the arterial wall for: collagen content, cellularity, proliferation, αSMA expression, and desmin expression. Qualitatively, NO decreased collagen content compared to injury alone by 33% and 47% at 2 and 8 weeks, respectively ($p<0.05$; Figure 7).

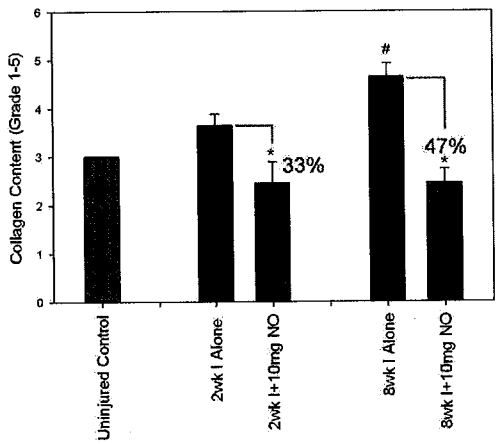


Figure 7. NO inhibited collagen content throughout the arterial wall at 2 and 8 wks. *$p<0.05$ vs. injury; #$p<0.05$ vs. uninjured control.

An interesting finding was the effect of NO on cellularity throughout the arterial wall. Nuclei were counted in four high power fields (HPF) per each section and averaged. NO reduced total cellularity at all time points studied ($p<0.05$, Figure 8). Evaluation of each layer revealed that NO reduced cell number following injury in the intima by 86% and 85% at 2 and 8 weeks, respectively ($p<0.05$) and in the media by 89% and 84% at 2 and 8 weeks, respectively ($p<0.05$). However, while NO reduced cellularity in the adventitia at early time points, NO actually increased cellularity in the adventitia by 20% and 44% at 2 and 8 weeks, resp. ($p<0.05$, Figure 8).

To identify the cell types that NO is increasing in the adventitia at the later time points, we performed additional analysis for αSMA and desmin expression at multiple time points in carotid arteries that underwent injury or

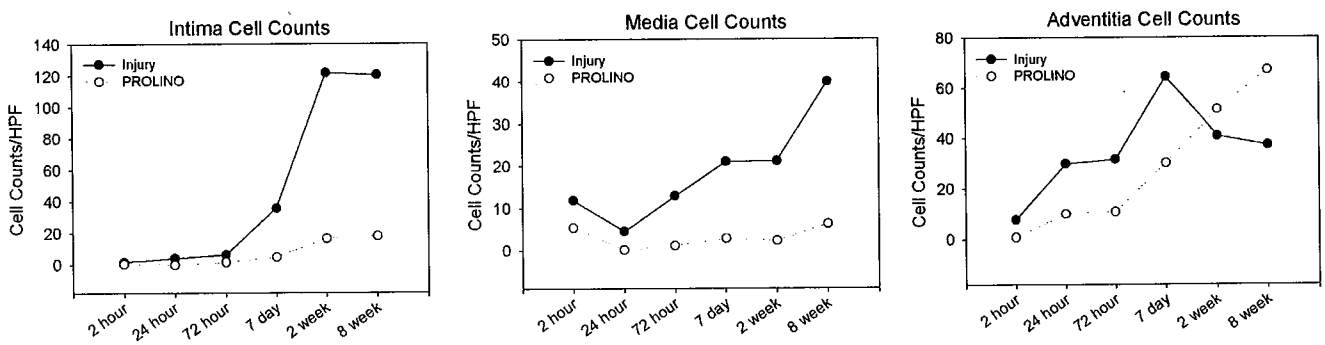


Figure 8. NO reduced cellularity following arterial injury in the intima and media at all time points, but increased cellularity at 2 and 8 weeks in the adventitia compared to rat carotid arteries that underwent injury alone ($p<0.05$).

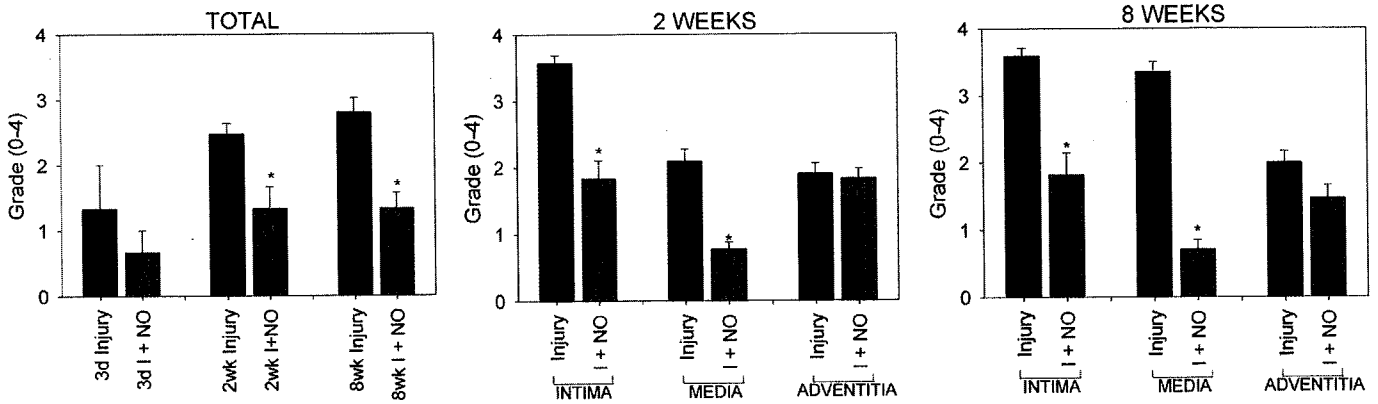


Figure 9. Quantification of immunohistochemical staining for αSMA in rat carotid arteries that were injured or injured and exposed to PROLI/NO (NO, 10mg). Expression was assessed for the whole artery (TOTAL), and also assessed by arterial layer at 2 and 8 weeks. *p<0.05 vs. injury alone.

injury + NO. For cytoplasmic stains, quantification was performed by grading on a scale of 0-4 by a blinded investigator. For nuclear stains, quantification was performed by counting positively stained nuclei per HPF.

αSMA expression, which is detected primarily in VSMC, myofibroblasts, and stem/progenitor cells that have differentiated to mural-type cells, was sparse at the 3-day time point. However, at 2 and 8 weeks following injury, NO significantly decreased total αSMA expression (Figure 9). Evaluation of the different layers of the arterial wall at 2 and 8 weeks revealed that the greatest effect of NO on αSMA expression was in the intima (~50% inhibition) and media (60-80% inhibition), and not the adventitia (Figure 9, $p<0.05$).

Desmin expression, which is detected primarily in contractile VSMC but will also detect synthetic VSMC, was similar to that observed for αSMA. NO reduced total desmin expression throughout the arterial wall at 2 and 8 weeks, but not at 3 days (data not shown). Most of the inhibition at 2 and 8 weeks was seen in the intima (~50% inhibition) and in the media (80-100% inhibition), with inhibition in the media being nearly complete. Similar to αSMA, there was no significant effect of NO on desmin expression in the adventitia.

Next, we evaluated the effect of NO on actively proliferating cells by administering BrdU (100 mg/kg, intraperitoneal [IP]) to animals 24 and 1 hour prior to sacrifice. An interesting pattern emerged. NO inhibited active proliferation in the intima and media following arterial injury at both 2 and 8 weeks (Figure 10, $p<0.05$). However, NO stimulated proliferation at the 2-week time point in the adventitia (Figure 10). By 8 weeks, NO no longer affected proliferation. Note that most of the proliferation occurred in the intima and adventitia and that very little occurred in the media (y-axis scale). Lastly, we evaluated macrophage infiltration (anti-ED1). Similar to our earlier studies (Figure 3), NO inhibited macrophage infiltration following injury (not shown).

Sca-1 expression. Given that NO treatment after arterial injury increased cellularity in the adventitia at 2 weeks, and that this increase was independent of VSMC, myofibroblast, and macrophages, we began to explore our hypothesis that the increased cell population in the adventitia is due to stem and/or progenitor cell recruitment and/or proliferation. This hypothesis is supported by our BrdU data. Thus, to explore this further, we performed additional experiments using the rat carotid artery injury model with and without exposure to

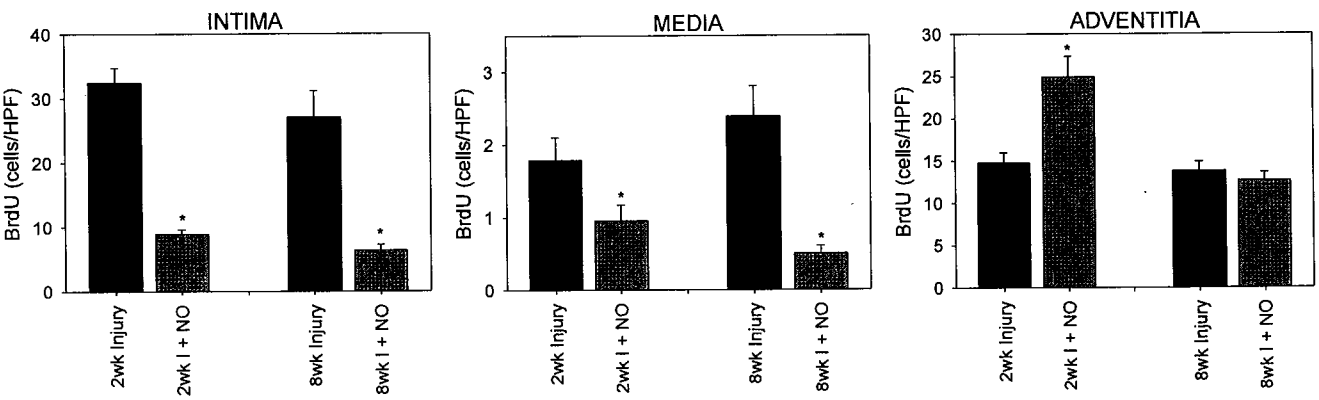


Figure 10. Quantification of immunohistochemical staining for BrdU incorporation into actively proliferating cells in rat carotid arteries that were injured or injured and exposed to PROLI/NO (NO, 10mg). Proliferation was assessed separately for the intima, media and adventitia at 2 and 8 weeks. *p<0.05 vs. injury alone.

periadventitial application of PROLI/NO (10mg). At 2 hours, 24 hours, 3 days and 7 days following injury and exposure to NO, carotid arteries were harvested fresh, snap frozen, mechanically lysed, and resuspended in a lysis buffer with appropriate protease inhibitors. Approximately 4-6 carotid arteries were combined for each treatment group. Western blot analysis was performed for αSMA, desmin, nonmuscle myosin heavy chain (NM-MHC), smooth muscle myosin heavy chain (SM-MHC) as well as the stem cell marker Sca1. Equal protein was loaded per group and confirmed with β-actin. Surprisingly, while we observed a decrease in αSMA, desmin, NM-MHC, and SM-MHC, we observed a dramatic increase in Sca-1 expression 3 days following injury and exposure to NO (Figure 11). Evaluating this at multiple time points following injury, this increase in Sca1 by NO was prominent as early as 2 hours following injury (Figure 12). These data support our hypothesis that NO is actively recruiting or stimulating progenitor cell populations in the adventitia following arterial injury.

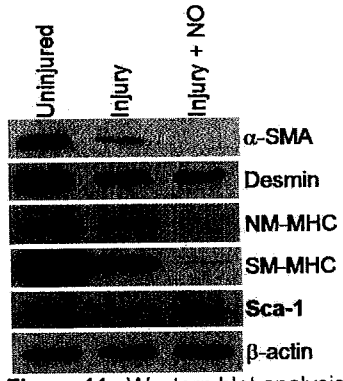


Figure 11. Western blot analysis of carotid artery lysate 3 days after injury. Note the increase in Sca1+ cells despite decreases in αSMA, desmin, and NM-MHC. NO = PROLI/NO (10mg).

Summary. In conclusion, these preliminary data demonstrate that periadventitial delivery of NO results in durable and effective inhibition of neointimal hyperplasia, but that NO affects cellular populations in the intima, media, and adventitia differently. Our data also suggest that NO inhibits neointimal hyperplasia by preventing phenotypic differentiation and/or proliferation of cell populations throughout the arterial wall, while stimulating recruitment and/or proliferation of stem or progenitor cells. It is also possible that NO may prevent differentiation of recruited stem cells, thereby accounting for the dramatic increase in Sca1+ expression we observed following injury and exposure to NO.

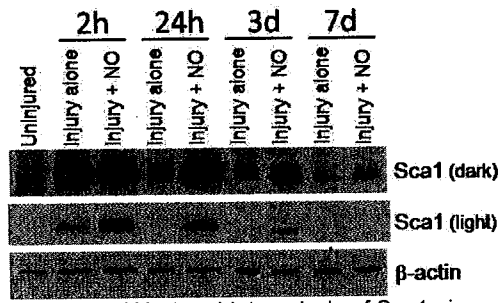


Figure 12. Western blot analysis of Sca1+ in carotid artery lysates. Dark and light exposures of Sca1 are shown to demonstrate the differences between the groups. NO = PROLI/NO (10mg).

Proposed Experiments

Specific Aim 1 - Characterize the effect of NO on Sca1+, CD34+, and flk-1+ adventitial stem and progenitor cell populations following arterial injury *in vivo.*

Overall Strategy. NO is a potent inhibitor of neointimal hyperplasia, and we have demonstrated that NO has differential effects on the cellular populations throughout the arterial wall, with an increase in cellularity observed in the adventitia of NO-treated arteries at later time points, and an increase in Sca1 expression. Research now shows that the adventitia is an active regulator of neointimal hyperplasia, and has been shown to contain resident Sca1+ and CD34+ stem cells, as well as recruit circulating bone marrow-derived stem cells that phenotypically differentiate into myofibroblast-like cells and contribute to neointimal hyperplasia.[19, 20, 26-33] The adventitia is also home to the vasa vasorum, which increases in density following injury, but out of proportion to the increase in tissue mass, possibly leading to possible zones of hypoxia.[48, 49] Lastly, NO is known to stimulate angiogenesis via mobilization and recruitment of CD34+ EPC.[57-59] Thus, we hypothesize that NO inhibits neointimal hyperplasia by regulating recruitment and phenotypic differentiation of resident and circulating adventitial stem and progenitor cells following injury. Furthermore, we hypothesize that NO supports differentiation of adventitial stem and progenitor cells into endothelial-like cells, contributing to adventitial neovascularity. To investigate this hypothesis, in this aim we will evaluate and characterize the effect of NO on adventitial Sca1+, CD34+ and flk-1+ cell recruitment, phenotypic differentiation, proliferation, and migration following arterial injury *in vivo.* We will also relate these processes to the development of adventitial neovascularity following injury, to determine if NO promotes differentiation of these adventitial cell types into endothelial-like cells thereby contributing to increased neovascularity following injury. We are focusing on Sca1+, CD34+, and flk-1+ cells because these are the stem and progenitor cells that have been identified in the adventitia and have been shown to differentiate or have the potential to differentiate into αSMA-positive or endothelial-like cells following arterial injury and contribute to neointimal hyperplasia.

Methodology and Analysis. To study the effect of NO on **recruitment** of circulating bone marrow-derived stem and progenitor cells following arterial injury, we will lethally irradiate (12 Gy) the bone marrow of wild-type recipient mice and transplant $1x10^6$ donor bone marrow cells of C57BL/6-Tg(CAG-EGFP) or FVB/N-Tgn(TIE2-

lacZ) mice into the recipient mice via tail vein injection using the method described by our collaborator [redacted][45, 60] C57BL/6-Tg(CAG-EGFP) mice ([redacted]) are a transgenic mouse line that express GFP in all cells. FVB/N-Tgn(TIE2-lacZ) mice ([redacted]) are a transgenic mouse line that carry the beta-galactosidase reporter gene under the control of the murine Tie2 promotor.[61] LacZ is expressed in embryonic and adult vascular endothelial cells. Thus, by transplanting the bone marrow from these two different transgenic mouse models into wild-type recipient mice, we will be able to determine the extent of recruitment of circulating bone marrow-derived stem cells and specifically CD34+ progenitor cells to the arterial wall following arterial injury, and if NO regulates this recruitment. Four weeks after bone marrow transplantation (BMT), a time sufficient for bone marrow regeneration, the standard femoral artery wire injury model will be performed as previously described.[45, 60] Briefly, the femoral artery of anesthetized mice will be dissected and controlled proximally and distally. A 0.014" guidewire will be advanced into the femoral artery via a side branch and passed three times. After removal of the wire, the branch will be ligated and flow restored. Treatment groups will include: 1) control, 2) injury alone, and 3) injury + PROLI/NO (1mg). For the NO treatment groups, PROLI/NO will be applied to the periadventitial surface of the injured artery. Contralateral uninjured arteries will serve as controls. n=6/treatment group. Arteries will be harvested a 2h, 6h, 1d, 3d, 7d, 1wk, 2wk, and 8wk. To identify recruitment of bone marrow stem cells, arteries of mice transplanted with bone marrow from C57BL/6-Tg(CAG-EGFP) mice will be assessed using immunofluorescence to detect recruited cells. Arteries of mice transplanted with bone marrow from FVB/N-Tgn(TIE2-lacZ) mice will be assessed for recruitment of CD34+ endothelial like cells using double staining for beta-galactosidase and the murine-specific endothelial marker, isolectin B4, as described by Dr. Losordo.[45] Each layer of the arterial wall will be assessed separately and the number of recruited cells will be counted in four HPF per arterial section.

To determine if NO regulates **phenotypic differentiation** of Sca1+, CD34+, and flk-1+ stem and progenitor cells following arterial injury, we will assess the arteries from the mice experiments described above immunohistochemically for Sca1, CD34, and flk-1 at all time points (n=6/group). We will also co-localize the immunofluorescent signal from the stem and progenitor cells with markers for differentiation: αSMA, desmin, NM-MHC, SM-MHC, CD31, and von Willebrand factor. This will allow us to fully characterize the time course of expression of the stem cell markers following arterial injury, with and without exposure to NO, and link the presence or disappearance of these stem cells to markers of differentiation into VSMC or endothelial like cells. Each layer of the arterial wall will be assessed separately and expression will be quantified. Cytoplasmic stains will be graded by a blinded observer using a standard scale (0-4). Nuclear stains will be quantified by counting positively stained nuclei in four HPF per arterial section.

To evaluate **proliferation** and **migration** of the Sca1+, CD34+ and flk-1+ cells following arterial injury, we will perform BrdU pulse chase experiments. In these experiments, we will administer BrdU to the wild-type mice via IP injection (100 mg/kg) at different time points. Cells that are proliferating at the time of BrdU administration will incorporate the BrdU into their cells. The arteries will be harvested at a subsequent time to assess the location of these BrdU-positive cells. For example, if the majority of cells proliferating at an early time point are in the adventitia, these cells will incorporate BrdU that is administered early. Harvest of these arteries at 2 weeks will demonstrate the ultimate location of these early proliferative labeled adventitial cells, thereby providing critical information on both cell migration and proliferation. To determine the phenotype of these cells, double labeling will be performed for BrdU and markers for Sca1+, CD34+ and flk-1+, as well as αSMA, desmin, NM-MHC, SM-MHC, CD31, and von Willebrand factor. Time points of BrdU administration will include 2 hr, 1d, 3d, 7d, 1wk, and 2wk. At each time point, treatment groups will include injury alone or injury + PROLI/NO (1mg). n=6/treatment group. Arteries will be harvested at 2 weeks.

To determine the effect of NO on **neovascularity** following arterial injury, we will quantify the expression of CD31 and von Willebrand factor in the arteries harvested above by counting the number of microvascular vessels present in the adventitia. We will also determine the density of the microvasculature in comparison to overall tissue area. These studies will directly determine if NO enhances neovascularity in the adventitia following arterial injury, and if this increase results in a greater density of microvasculature per tissue area.

Potential Problems and Alternative Strategies. With respect to potential technical problems, we may experience problems with histological analysis of the arteries from the bone marrow transplanted transgenic animals described above. If we experience difficulties in detecting the fluorescent bone marrow derived stem cells from the C57BL/6-Tg(CAG-EGFP) transgenic donor mice, we will use the ROSA26 mice, which are a commonly used transgenic strain that expresses beta-galactosidase in all cells.[62] Another possible challenge

may be the use of the mouse model. Although this is a well-described model that has been used in many of the landmark papers describing the role of adventitial cells in the arterial injury response, we recognize that the adventitia of the mouse is small in comparison to larger animal models. Thus, if we experience difficulties with assessing these adventitial cell populations in the mouse model, we will use the rat carotid artery injury model together with the "Y chromosome FISH method" to detect the effect of NO on the recruitment and phenotypic differentiation of these stem cells. Briefly, bone marrow of donor male rats will be transplanted into age-matched recipient female rates. After sufficient engraftment (approximately 4 weeks), we will perform the carotid artery injury model with and without periadventitial exposure to NO. At designated time points, the carotid arteries will be harvested. Donor cells, which will represent the contribution from the bone marrow, will be identified by detection of the Y chromosome by fluorescence *in situ* hybridization (FISH) according to the modified protocol of [redacted] et al.[63] This method preserves cell proteins thereby allowing detection of cell-specific markers by immunohistochemistry. Hence, we will be able to also stain for Sca1, flk-1, and CD45. Of note, [redacted] our collaborator, has experience with this bone marrow transplant model and the FISH assay. Alternatively, if both the mouse and rat models prove to be problematic, we will utilize the pig angioplasty model, with which the PI is experienced. While reagents are plentiful for the mouse model, we also recognize that reagents are more limited for rat and pig species. However, our preliminary data was conducted in the rat. For determination of new microvascular vessel growth, we may have to stain for CD34 at early time points (as opposed to CD31 or von Willebrand factor) as Zengin et al demonstrated that early adventitial vasa vasorum growth stains positive for CD34 but not CD31 or von Willebrand factor.[42] We may also need to assess for cell adhesion markers specific for endothelial cells, such as VEGF receptor, TIE2, VE-cadherin, and CEACAM1.

With respect to our hypothesis, we may find that NO increases recruitment of stem cells to the adventitia, which is supported by our preliminary data, but maintains them in an immature non-differentiated state. This would disprove our hypothesis that NO prevents differentiation of these stem cells into αSMA bearing cells but stimulates them to differentiate into endothelial-like cells that contribute to the microvasculature. If this is the case, we will redirect our attention to the effect of NO on cytokine and growth factor production from these adventitial stem cell and progenitor cells. It is possible that NO effects downstream processes in the arterial injury response by either preventing secretion of certain factors, or stimulating release of other factors from these cells. To investigate this, we will assay carotid artery tissue lysates for cytokines and growth factors, including SCF, TGFβ, PDGF, bFGF, TNFα, interleukins (14 on the panel), IFNγ, G-CSF, VEGF, adiponectin, IFNα, and IFNβ. We will use the Procarta Cytokine Assay Kit by Panomics. This bead-based multiplex assay kit can quantitatively measure multiple cytokines from as little as 25 μL of cell lysate with a limit of detection of 1 pg/ml/cytokine or less. It is also possible that NO modulates neointima formation by altering how different cell populations react to exogenous NO, leading to vastly different ROS profiles following arterial injury. Since ROS are intimately linked to proliferation, this avenue will be pursued. To investigate this, we will evaluate the effect of NO on the expression of different pro- and antioxidant proteins (i.e., NOX, SOD, PRx, TRx, GPx, etc.) throughout the arterial wall, along with superoxide, hydrogen peroxide, and peroxynitrite formation. We will also co-localize the expression of these proteins to the stem and progenitor cells, as well as the adventitial fibroblasts, macrophages, and VSMC.

Specific Aim 2 - Determine the effect of NO on Sca1+, CD34+, and flk-1+ adventitial cell populations *in vitro*.

Overall Strategy. For this aim, we will isolate and identify Sca1+, CD34+, and flk-1+ adventitial stem and progenitor cell populations and determine how NO affects phenotypic differentiation, proliferation, migration, and ROS production when exposed to different growth conditions (i.e., starvation, hypoxia, fibronectin, collagen, co-culture, etc) and stimuli (VEGF, PDGF, TGFβ, FGF, etc) *in vitro*.

Methodology and Analysis. To **isolate** stem and progenitor cells from the adventitia, we will follow the method described by our collaborator [redacted] that uses the Miltenyi system.[33] Briefly, the common femoral artery of adult mice will be harvested and placed in PBS on ice. Endothelial cells will be removed with a sterile cotton swab. The adventitia will be dissected and rinsed in HBSS, digested with 14 mg/ml collagenase type 2 and 0.75 mg/ml elastase in HBSS for 2 hours at 37°C with gentle rocking, and filtered (70 μm). Cells in the filtrate will be pelleted at 300 x g then rinsed in PBS with 0.5% BSA. Femoral artery Sca1+ cells will be isolated by using anti-Sca-1 immunomagnetic MicroBeads and a MACS cell separation system from Miltenyi. While CD34+ EPC can also be isolated with the anti-Sca-1 MicroBeads, to achieve a more successful isolation, we will use the anti-CD117 MicroBeads to specifically isolate the CD34+ EPC. To isolate flk-1+ cells, we will use an anti-flk-1 antibody and the anti-immunoglobulin MicroBeads from Miltenyi. Cells will be passed over two

consecutive columns to increase purity of the isolation. According to [redacted], a typical Sca1+ fraction prepared as described is 1–4% of total cells, with 80–95% of isolated cells positive for Sca1 antigen, and <1% of isolated cells positive for αSMA by immunostaining. Isolated stem and progenitor cells will be cultured using Miltenyi's HSC-CFU basic media for the stem and progenitor cells. This is a basic media that does not contain additional cytokines or growth factors that stimulate differentiation into certain cell types. We will confirm the purity of all cell isolates by staining for the marker of interest, as well as αSMA.

After cells are isolated, identified, and maintained in cell culture, we will determine the effect of NO on phenotypic differentiation, proliferation, migration and ROS production. To assess **phenotypic differentiation**, we will plate cells onto sterile glass coverslips that are placed at the bottom of 12- or 24-well plates. After treatment in different growth conditions and with different stimuli for 24 hours (described below), the cells will undergo immunohistochemical staining for various markers to identify cell phenotype as we have previously described.[64] This will include antibodies to Sca1, CD34, flk-1, αSMA, desmin, NM-MHC, SM-MHC, CD31, and von Willebrand factor. To assess **proliferation**, we will use ^{3}H-thymidine incorporation and MTT assays. Briefly, to assess ^{3}H-thymidine incorporation we will plate cells in 12- or 24-well plates, starve the cells in media with 0% FBS for 24 hours to induce cell cycle synchronization, treat the cells in media containing 10% FBS and 5 μCi/ml ^{3}H-thymidine for 24 hours, then assess ^{3}H-thymidine incorporation in trichloroacetic acid-precipitated DNA using a scintillation counter as we have previously published.[55, 56, 65] We will also assess proliferation using the MTT assay according to manufacturer's instructions (Promega). To assess **migration**, we will use Boyden chambers and the scratch assay. For the Boyden chambers, we will use 48-well modified Boyden microchemotaxis chambers and Poretics polycarbonate PVC-free membranes with 8-μm pores to assess the effect of NO on migration. Briefly, after cells are synchronized they will be placed in the top well of the chamber and different stimuli will be placed in the bottom well of the chamber in the presence or absence of NO. After 4 hours at 37°C, the membrane will be stained with hematoxylin. Cells that migrated will be counted in four HPF (400X) per membrane. For the scratch assay, cells will be plated in 6-well plates and growth arrested for 24 hours. Cell monolayers will be injured by a single scrape with a 1000 μL pipet tip, photographed, then treated in media with 10% FBS. Cells will be photographed again after 24 hours of treatment. Blinded counting of nuclei of cells that migrated into the empty space created by the scrape will be performed at both time points using Adobe Photoshop, and quantification will be performed using ImageJ as we have previously published.[66] To quantify intracellular **ROS production**, we will use the detection reagents CM-H2DCFDA, which detects H_2O_2, peroxynitrite, peroxyl radical, and hydroxyl radical, and dihydroethidium (DHE), which detects O_2^- as we have previously described.[55, 67] Briefly, cells will be plated in six-well plates for 24 hours after which they will be treated with media containing 10% FBS for 24 hours. Cells will be rinsed with ice-cold PBS, trypsinized, collected, and pelleted. Cells will be resuspended in either 5 μM CMH2DCFDA or 5 μM DHE and incubated at 37°C for 30 or 5 minutes, respectively. After incubation, the cell samples (10,000 cells/sample) will be analyzed on a Coulter Epics XLFlow Cytometer using excitation and emission of 488 and 535 nm for DCF, and 488 and 585 nm for DHE.

For each of the assays above (phenotypic differentiation, proliferation, migration, ROS production), we will assess the effect of NO (DETA/NO 10 μM – 1000 μM) on each of the three cell types (Sca1+, CD34+, and flk-1+) in different growth conditions and in the presence or absence of different stimuli. The different **growth conditions** we will assess include: 1) starvation (0% FBS), 2) hypoxia (2% oxygen), 3) growth on fibronectin, and 4) growth on collagen plates. We will also expose the cells to the following **stimuli**: 1) VEGF (10 ng/ml), 2) PDGF (10 ng/ml), 3) TGFβ (5 ng/ml), and 4) FGF(20 ng/ml). Lastly, we will assess the effect of NO on the Sca1+, CD34+, and flk-1+ using **co-culture** studies with mature adult endothelial cells, VSMC, or adventitial fibroblasts. These latter cells are routinely harvested from the aorta of mice or rats in our laboratory.[56, 64, 66-68]

Potential Problems and Alternative Strategies. With respect to technical problems, we may encounter difficulties isolating the Sca1+, CD34+, and flk-1+ cells from the adventitia using the Miltenyi cell isolation and separation method. If we do, we will use FACS analysis and cell sorting as previously described.[37] If yield is too low with the mouse artery, we will use rats. However, four mouse arteries yield approximately 500,000 Sca1+ cells. With respect to our hypothesis, as discussed above, it is possible that NO does not stimulate differentiation of stem and progenitor cells into endothelial cells. If we find this to be the case with the experiments in this Aim, we will study the effect of NO on cytokine and growth factor production from these cells *in vitro* using the Procarta Cytokine Assay Kit as described above. However, we will take this one step further and use the conditioned media from each of the three adventitial stem cells (Sca1, CD34, and flk-1) and determine the effect of this conditioned media on the proliferation, migration, and phenotypic differentiation of

adventitial fibroblasts and macrophages. We will also assess the effect of NO on ROS production from each of these cell types, and perform experiments with conditioned media similar to that described above but evaluating ROS production and expression of pro- and anti-oxidant proteins.

Specific Aim 3 – Manipulate Sca1+, CD34+, and flk-1+ adventitial cell populations *in vivo* to determine if NO-mediated inhibition of neointimal hyperplasia following injury is dependent on these adventitial cell populations.

Overall Strategy. For this aim, to determine if the NO-mediated inhibition of neointimal hyperplasia is **dependent** on regulating the Sca1+, CD34+, or flk-1 cell populations, we will perform gain- and loss-of-function experiments *in vivo*. Specifically, we will evaluate the role of 1) adventitial removal and selective overexpression of Sca1+, CD34+, and flk-1+ cells, 2) stimulation of circulating stem and progenitor cells with G-CSF, and 3) Sca1+, CD34+, and flk-1+ cell populations in iNOS and eNOS deficient mice following arterial injury. With each of these manipulations, we will determine if the efficacy of NO-mediated inhibition of neointimal hyperplasia is abated or enhanced.

Methodology and Analysis. To determine the contribution of each cell type (i.e., Sca1, CD34, flk-1) to NO-mediated inhibition of neointimal hyperplasia, we will **remove the adventitia and selectively reintroduce each cell type** to the outer wall of the artery after performing the femoral artery wire injury model in C57BL/6 wild-type mice. Briefly, immediately prior to wire injury, the adventitia and periadventitial adipose fat will be surgically removed as described by [redacted] et al.[49, 69] Following removal of the adventitia (AR), the femoral artery will be injured with the 0.014" guide wire as described in Aim 1, and either Sca1+, CD34+, or flk-1+ cells ($1x10^5$) mixed in Matrigel (5 mg/ml) will be placed on the periadventitial surface of the artery, as described by [redacted] et al,[37] followed by application of PROLI/NO. These cells will be harvested, isolated, confirmed, and maintained in cell culture as described in Aim 2. Treatment groups will include: 1) control, 2) injury, 3) injury + AR, 4) injury + AR + Sca1+ cells, 5) injury + AR + CD34+ cells, 6) injury + AR + flk-1+ cells. n=6/group. Arteries will be harvested at 2 weeks following arterial injury. Prior to sacrifice, mice will receive BrdU (100 mg/kg IP) administration. Neointimal hyperplasia will be assessed morphometrically as we have previously published.[56, 66, 68] Arteries will also undergo immunohistochemical assessment for the following proteins: Sca1, CD34, flk-1, αSMA, desmin, NM-MHC, SM-MHC, CD31, von Willebrand factor, and BrdU. These experiments should identify if NO-mediated inhibition of neointimal hyperplasia is dependent on adventitial Sca1+, CD34+, or flk-1+ stem and progenitor cells. Furthermore, these experiments will determine which of these three stem and progenitor cells types is most important to mediating the effects of NO.

To determine the effect of increasing the population and possible recruitment of circulating bone marrow-derived stem cells to NO mediated inhibition of neointimal hyperplasia following arterial injury, we will **administer G-CSF** to C57BL/6 wild-type mice at various time points before and after arterial injury. Briefly, mice will undergo the femoral artery wire injury model as described in Aim 1 with and without exposure to periadventitial application of PROLI/NO. Mice will receive G-CSF (300 μg/kg) or saline subcutaneously daily for 5 days prior to arterial injury (days -5 to 0). Treatment groups will include: 1) injury and 2) injury + PROLI/NO. n=6/treatment group. Arteries will be harvested at 1 day, 3 days, 1 wk, 2 wks, and 8 wks following arterial injury. Prior to sacrifice, mice will receive BrdU (100 mg/kg IP) administration. Neointimal hyperplasia will be assessed morphometrically. Arteries will also undergo immunohistochemical assessment for the following proteins: Sca1, CD34, flk-1, αSMA, desmin, NM-MHC, SM-MHC, CD31, von Willebrand factor, and BrdU. This experiment should identify if the number of recruited stem cells has an impact on the ability of NO to inhibit neointimal hyperplasia. Furthermore, if NO supports differentiation of these stem cells toward endothelial-like cells and stimulates growth of the microvasculature, we would expect to observe a more prominent vasa vasorum in the groups treated with G-CSF.

Lastly, we will **determine the effect of endogenous NO production** from iNOS or eNOS on the presence and recruitment of adventitial stem cell populations. It has already been reported in the literature that eNOS deficiency results in greater neointimal hyperplasia using the mouse carotid ligation model.[70] iNOS deficiency results in greater vein graft neointimal hyperplasia, possibly through deficient recruitment of EPC.[71] Thus, we will perform the femoral artery wire injury model in iNOS-/- and eNOS-/- mice and their wild-type controls ([redacted]) with and without periadventitial application of PROLI/NO (1mg). n=6/treatment group. Arteries will be harvested at 2h, 6h, 1d, 3d, 7d, 1wk, 2wk, and 8wk. Prior to sacrifice, mice will receive BrdU (100 mg/kg IP) administration. Neointimal hyperplasia will be assessed morphometrically. Arteries will also undergo immunohistochemical assessment for the following proteins: Sca1, CD34, flk-1, αSMA, desmin, NM-MHC, SM-MHC, CD31, von Willebrand factor, and BrdU. These experiments will elucidate the role of

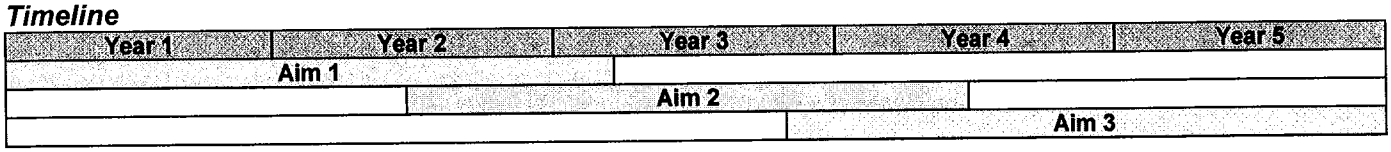
endogenous NO production on resident stem and progenitor cells, as well as recruitment of circulating stem and progenitor cells. We hypothesize that endogenous NO production is necessary to maintain resident adventitial stem cells, and recruit circulating stem cells, in order to inhibit neointimal hyperplasia.

Potential Problems and Alternative Strategies. With respect to potential technical problems, for the experiments in which we reintroduce cells to the outer wall of the artery, it is possible that some or all of the cells will not engraft. If we observe this, it may be that we need to use more cells, or that we need to include the cells in a different matrix or a cuff that we place on the outside of the artery. Another approach we may utilize if this experiment proves challenging would be to study the effect of NO on neointimal hyperplasia in both SCF and c-Kit deficient mice given the importance for this ligand-receptor pair in stem cell signaling. For the stem cell stimulation study with G-CSF, we may need to evaluate a range of different doses of G-CSF. While studies have demonstrated the beneficial effects of G-CSF administration on neointimal hyperplasia, too high of a dose can result in increased inflammation and hence more neointimal hyperplasia.[72, 73] We may also need to consider administering G-CSF during different time periods. Currently, we will administer it pre-operatively; however, we may need to evaluate post-operative administration, or a range of time frames. Alternatively, we may need to administer a different agent, either intravenous SDF-1α or AMD-3100, the CXCR4 inhibitor, to stimulate recruitment of circulating stem cells. With respect to our hypothesis, it is possible that NO inhibits neointimal hyperplasia independent of the stem and progenitor cell populations. If so, we will focus our attention on other cell types in the adventitia, including the fibroblasts and macrophages, as these cell types have been shown to contribute to the neointima through phenotypic differentiation. While our preliminary data demonstrate that NO significantly increases Sca1+ expression following arterial injury, it is possible that this increase is due to stimuli released from NO-stimulated fibroblasts and macrophages and is not directly due to the actions of NO. Thus, to examine this possibility, we will perform the adventitial removal experiment and individually introduce back either adventitial fibroblasts or adventitial tissue macrophages. If our hypothesis is disproved, another avenue we will pursue is the role of ROS in this system, as it is possible that NO differentially affects how cells respond to exogenous NO with respect to pro- and anti-oxidant protein expression. It is also possible that NO exerts its beneficial effects through regulation of cell adhesion molecules, such as VCAM-1, P-selectin, E-selectin, VE-cadherin, CEACAM1, and TIE2. These molecules may attract certain cell types to the site of injury that mediate the actions of NO. To examine this possibility, we will evaluate the expression of these cell adhesion molecules and receptors using immunohistochemical analysis. To determine if the effects of NO are mediated through manipulation of growth factor and cytokine release, we will assess the pattern of cytokine and growth factor release as described in the potential problems section for Aim 1. After determining which cytokines and/or growth factors are affected by NO, we will perform studies to administer inhibitors to these growth factors and cytokines systemically to the animals. For example, if PDGF is shown to be affected by NO, we will administer a compound such as GFB-111, which is a small molecule that binds to PDGF, or AG 1296, a PDGF receptor inhibitor.

Benchmarks for Success. We have several benchmarks for success with this grant proposal, including: 1) demonstrating that NO regulates phenotypic differentiation of Sca1+, CD34+, and flk-1+ cells following arterial injury *in vivo; 2)* demonstrating that NO prevents phenotypic differentiation of Sca1+, CD34+, and flk-1+ cells into αSMC+ cells; 3) demonstrating that NO supports differentiation of the Sca1+, CD34+, and flk-1+ cells into endothelial like cells that contribute to and increase the microvascularity following injury; 4) demonstrating that NO prevents proliferation and migration of the Sca1+, CD34+, and flk-1+ cells under various stimuli and growth conditions; and 5) demonstrating that NO-mediated inhibition of neointimal hyperplasia is dependent on Sca1, CD34, or flk-1 stem and progenitor cells.

Statistical Analysis – For all of the experiments described in Aims 1-3, differences between two groups will be evaluated with the Student's t-test. Differences between multiple groups will be analyzed using one-way analysis of variance (ANOVA) with the Student-Newman-Keuls *post hoc* test for all pair wise comparisons (SigmaStat; SPSS). Correlation will be determined by calculating the Spearman Rank Order Correlation coefficient (SigmaStat, SPSS). Statistical significance will be assumed when $p<.05$.

Timeline



REFERENCES

1. Lloyd-Jones D, Adams R, Carnethon M et al. Heart Disease and Stroke Statistics--2009 Update. A Report From the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Circulation. 2009; 119(3):e21-181.
2. Stone GW, Ellis SG, Cox DA et al. A polymer-based, paclitaxel-eluting stent in patients with coronary artery disease. N Engl J Med 2004;350(3):221-231.
3. Weintraub WS, Clements SD, Jr., Crisco LV et al. Twenty-year survival after coronary artery surgery: an institutional perspective from Emory University. Circulation 2003;107(9):1271-1277.
4. Eagleton MJ, Ouriel K, Shortell C, Green RM. Femoral-infrapopliteal bypass with prosthetic grafts. Surgery 1999;126(4):759-764.
5. Clowes AW, Reidy MA, Clowes MM. Kinetics of cellular proliferation after arterial injury. I. Smooth muscle growth in the absence of endothelium. Lab Invest 1983;49(3):327-333.
6. Clowes AW, Reidy MA, Clowes MM. Mechanisms of stenosis after arterial injury. Lab Invest 1983;49(2):208-215.
7. Clowes AW, Clowes MM, Reidy MA. Kinetics of cellular proliferation after arterial injury. III. Endothelial and smooth muscle growth in chronically denuded vessels. Lab Invest 1986;54(3):295-303.
8. Ross R, Glomset J, Kariya B, Harker L. Platelet-Dependent Serum Factor That Stimulates Proliferation of Arterial Smooth-Muscle Cells Invitro. P Natl Acad Sci USA 1974;71(4):1207-1210.
9. Ross R, Bowenpope DF, Raines EW. Platelets, Macrophages, Endothelium, and Growth-Factors - Their Effects Upon Cells and Their Possible Roles in Atherogenesis. Annals of the New York Academy of Sciences 1985;454:254-260.
10. Fingerle J, Johnson R, Clowes AW, Majesky MW, Reidy MA. Role of platelets in smooth muscle cell proliferation and migration after vascular injury in rat carotid artery. Proc Natl Acad Sci U S A 1989;86(21):8412-8416.
11. Davies MG, Hagen PO. Pathobiology of intimal hyperplasia. Br J Surg 1994;81(9):1254-1269.
12. Libby P, Schwartz D, Brogi E, Tanaka H, Clinton SK. A cascade model for restenosis. A special case of atherosclerosis progression. Circulation 1992;86(6 Suppl):III47-III52.
13. Lindner V, Lappi DA, Baird A, Majack RA, Reidy MA. Role of basic fibroblast growth factor in vascular lesion formation. Circ Res 1991;68(1):106-113.
14. Bendeck MP, Zempo N, Clowes AW, Galardy RE, Reidy MA. Smooth muscle cell migration and matrix metalloproteinase expression after arterial injury in the rat. Circ Res 1994;75(3):539-545.
15. Hasenstab D, Forough R, Clowes AW. Plasminogen activator inhibitor type 1 and tissue inhibitor of metalloproteinases-2 increase after arterial injury in rats. Circ Res 1997;80(4):490-496.
16. Lindner V, Majack RA, Reidy MA. Basic fibroblast growth factor stimulates endothelial regrowth and proliferation in denuded arteries. J Clin Invest 1990;85(6):2004-2008.
17. Nabel EG, Shum L, Pompili VJ et al. Direct transfer of transforming growth factor beta 1 gene into arteries stimulates fibrocellular hyperplasia. Proc Natl Acad Sci U S A 1993;90(22):10759-10763.
18. Majesky MW, Lindner V, Twardzik DR, Schwartz SM, Reidy MA. Production of transforming growth factor beta 1 during repair of arterial injury. J Clin Invest 1991;88(3):904-910.
19. Couffinhal T, Dufourcq P, Jaspard B et al. Kinetics of adventitial repair in the rat carotid model. Coron Artery Dis 2001;12(8):635-648.
20. Wilcox JN, Okamoto EI, Nakahara KI, Vinten-Johansen J. Perivascular responses after angioplasty which may contribute to postangioplasty restenosis: a role for circulating myofibroblast precursors? Ann N Y Acad Sci 2001;947:68-90.
21. Maiellaro K, Taylor WR. The role of the adventitia in vascular inflammation. Cardiovasc Res 2007;75(4):640-648.
22. Okamoto E, Couse T, De LH et al. Perivascular inflammation after balloon angioplasty of porcine coronary arteries. Circulation 2001;104(18):2228-2235.
23. Bayes-Genis A, Campbell JH, Carlson PJ, Holmes DR, Jr., Schwartz RS. Macrophages, myofibroblasts and neointimal hyperplasia after coronary artery injury and repair. Atherosclerosis 2002;163(1):89-98.
24. Shi Y, Obrien JE, Fard A, Mannion JD, Wang D, Zalewski A. Adventitial myofibroblasts contribute to neointimal formation in injured porcine coronary arteries. Circulation 1996;94(7):1655-1664.
25. Scott NA, Cipolla GD, Ross CE et al. Identification of a potential role for the adventitia in vascular lesion formation after balloon overstretch injury of porcine coronary arteries. Circulation 1996;93(12):2178-2187.

26. Sata M, Saiura A, Kunisato A et al. Hematopoietic stem cells differentiate into vascular cells that participate in the pathogenesis of atherosclerosis. Nat Med 2002;8(4):403-409.
27. Werner N, Junk S, Laufs U et al. Intravenous transfusion of endothelial progenitor cells reduces neointima formation after vascular injury. Circ Res 2003;93(2):e17-e24.
28. Werner N, Priller J, Laufs U et al. Bone marrow-derived progenitor cells modulate vascular reendothelialization and neointimal formation: effect of 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibition. Arterioscler Thromb Vasc Biol 2002;22(10):1567-1572.
29. Wang CH, Cherng WJ, Yang NI et al. Late-outgrowth endothelial cells attenuate intimal hyperplasia contributed by mesenchymal stem cells after vascular injury. Arterioscler Thromb Vasc Biol 2008;28(1):54-60.
30. Han CI, Campbell GR, Campbell JH. Circulating bone marrow cells can contribute to neointimal formation. J Vasc Res 2001;38(2):113-119.
31. Wang CH, Anderson N, Li SH et al. Stem cell factor deficiency is vasculoprotective: unraveling a new therapeutic potential of imatinib mesylate. Circ Res 2006;99(6):617-625.
32. Tanaka K, Sata M, Hirata Y, Nagai R. Diverse contribution of bone marrow cells to neointimal hyperplasia after mechanical vascular injuries. Circ Res 2003;93(8):783-790.
33. Passman JN, Dong XR, Wu SP et al. A sonic hedgehog signaling domain in the arterial adventitia supports resident Sca1+ smooth muscle progenitor cells. Proc Natl Acad Sci U S A 2008;105(27):9349-9354.
34. Adolphe C, Narang M, Ellis T, Wicking C, Kaur P, Wainwright B. An in vivo comparative study of sonic, desert and Indian hedgehog reveals that hedgehog pathway activity regulates epidermal stem cell homeostasis. Development 2004;131(20):5009-5019.
35. Ahn S, Joyner AL. In vivo analysis of quiescent adult neural stem cells responding to Sonic hedgehog. Nature 2005;437(7060):894-897.
36. Uhmann A, Dittmann K, Nitzki F et al. The Hedgehog receptor Patched controls lymphoid lineage commitment. Blood 2007;110(6):1814-1823.
37. Hu Y, Zhang Z, Torsney E et al. Abundant progenitor cells in the adventitia contribute to atherosclerosis of vein grafts in ApoE-deficient mice. J Clin Invest 2004;113(9):1258-1265.
38. Farrington-Rock C, Crofts NJ, Doherty MJ, Ashton BA, Griffin-Jones C, Canfield AE. Chondrogenic and adipogenic potential of microvascular pericytes. Circulation 2004;110(15):2226-2232.
39. Doherty MJ, Ashton BA, Walsh S, Beresford JN, Grant ME, Canfield AE. Vascular pericytes express osteogenic potential in vitro and in vivo. J Bone Miner Res 1998;13(5):828-838.
40. Diaz-Flores L, Gutierrez R, Varela H, Rancel N, Valladares F. Microvascular pericytes: a review of their morphological and functional characteristics. Histol Histopathol 1991;6(2):269-286.
41. Doherty MJ, Canfield AE. Gene expression during vascular pericyte differentiation. Crit Rev Eukaryot Gene Expr 1999;9(1):1-17.
42. Zengin E, Chalajour F, Gehling UM et al. Vascular wall resident progenitor cells: a source for postnatal vasculogenesis. Development 2006;133(8):1543-1551.
43. Tigerstedt NM, Savolainen-Peltonen H, Lehti S, Hayry P. Vascular cell kinetics in response to intimal injury ex vivo. J Vasc Res 2010;47(1):35-44.
44. Aicher A, Heeschen C, Mildner-Rihm C et al. Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells. Nat Med 2003;9(11):1370-1376.
45. Iwakura A, Luedemann C, Shastry S et al. Estrogen-mediated, endothelial nitric oxide synthase-dependent mobilization of bone marrow-derived endothelial progenitor cells contributes to reendothelialization after arterial injury. Circulation 2003;108(25):3115-3121.
46. Wegiel B, Gallo DJ, Raman KG et al. Nitric oxide-dependent bone marrow progenitor mobilization by carbon monoxide enhances endothelial repair after vascular injury. Circulation 2010;121(4):537-548.
47. Zhang LN, Wilson DW, da Cunha V et al. Endothelial NO synthase deficiency promotes smooth muscle progenitor cells in association with upregulation of stromal cell-derived factor-1 alpha in a mouse model of carotid artery ligation. Arterioscl Throm Vas Arterioscl Throm Vas 2006;26(4):765-772.
48. Kwon HM, Sangiorgi G, Ritman EL et al. Adventitial vasa vasorum in balloon-injured coronary arteries: visualization and quantitation by a microscopic three-dimensional computed tomography technique. J Am Coll Cardiol 1998;32(7):2072-2079.
49. Barker SG, Tilling LC, Miller GC et al. The adventitia and atherogenesis: removal initiates intimal proliferation in the rabbit which regresses on generation of a 'neoadventitia'. Atherosclerosis 1994;105(2):131-144.

50. Short M, Nemenoff RA, Zawada WM, Stenmark KR, Das M. Hypoxia induces differentiation of pulmonary artery adventitial fibroblasts into myofibroblasts. Am J Physiol Cell Physiol 2004;286(2):C416-C425.
51. Stenmark KR, Gerasimovskaya E, Nemenoff RA, Das M. Hypoxic activation of adventitial fibroblasts: role in vascular remodeling. Chest 2002;122(6 Suppl):326S-334S.
52. Stenmark KR, Bouchey D, Nemenoff R, Dempsey EC, Das M. Hypoxia-induced pulmonary vascular remodeling: contribution of the adventitial fibroblasts. Physiol Res 2000;49(5):503-517.
53. Stenmark KR, Frid M, Nemenoff R, Dempsey EC, Das M. Hypoxia induces cell-specific changes in gene expression in vascular wall cells: implications for pulmonary hypertension. Adv Exp Med Biol 1999;474:231-258.
54. Pearce CG, Najjar SF, Kapadia MR et al. Beneficial effect of a short-acting NO donor for the prevention of neointimal hyperplasia. Free Radic Biol Med 2008;44(1):73-81.
55. Ahanchi SS, Varu VN, Tsihlis ND et al. Heightened efficacy of nitric oxide-based therapies in type II diabetes mellitus and metabolic syndrome. Am J Physiol Heart Circ Physiol 2008;295(6):H2388-H2398.
56. Kapadia MR, Chow LW, Tsihlis ND et al. Nitric oxide and nanotechnology: a novel approach to inhibit neointimal hyperplasia. J Vasc Surg 2008;47(1):173-182.
57. Lee PC, Kibbe MR, Schuchert MJ et al. Nitric oxide induces angiogenesis and upregulates alpha(v)beta(3) integrin expression on endothelial cells. Microvasc Res 2000;60(3):269-280.
58. Murohara T, Asahara T, Silver M et al. Nitric oxide synthase modulates angiogenesis in response to tissue ischemia. Journal of Clinical Investigation 1998;101(11):2567-2578.
59. Ziche M, Morbidelli L, Masini E et al. Nitric oxide mediates angiogenesis in vivo and endothelial cell growth and migration in vitro promoted by substance P. J Clin Invest 1994;94(5):2036-2044.
60. Ii M, Takeshita K, Ibusuki K et al. Notch signaling regulates endothelial progenitor cell activity during recovery from arterial injury in hypercholesterolemic mice. Circulation 2010;121(9):1104-1112.
61. Schlaeger TM, Qin Y, Fujiwara Y, Magram J, Sato TN. Vascular endothelial cell lineage-specific promoter in transgenic mice. Development 1995;121(4):1089-1098.
62. Zambrowicz BP, Imamoto A, Fiering S, Herzenberg LA, Kerr WG, Soriano P. Disruption of overlapping transcripts in the ROSA beta geo 26 gene trap strain leads to widespread expression of beta-galactosidase in mouse embryos and hematopoietic cells. Proc Natl Acad Sci U S A 1997;94(8):3789-3794.
63. Trotman W, Beckett T, Goncz KK, Beatty BG, Weiss DJ. Dual Y chromosome painting and in situ cell-specific immunofluorescence staining in lung tissue: an improved method of identifying donor marrow cells in lung following bone marrow transplantation. Histochem Cell Biol 2004;121(1):73-79.
64. Kapadia MR, Eng JW, Murar J, Jiang Q, Kibbe MR. 26S proteasome: Intracellular localization and regulation by nitric oxide. Circulation 2007;116(16):210.
65. Kibbe MR, Li J, Nie S et al. Inducible nitric oxide synthase (iNOS) expression upregulates p21 and inhibits vascular smooth muscle cell proliferation through p42/44 mitogen-activated protein kinase activation and independent of p53 and cyclic guanosine monophosphate. J Vasc Surg 2000;31(6):1214-1228.
66. Tsihlis ND, Murar J, Kapadia MR et al. Isopropylamine NONOate (IPA/NO) moderates neointimal hyperplasia following vascular injury. J Vasc Surg 2010;51(5):1248-1259.
67. Popowich DA, Vavra AK, Walsh CP et al. Regulation of Reactive Oxygen Species by p53: Implications for Nitric Oxide-Mediated Apoptosis. Am J Physiol Heart Circ Physiol 2010.
68. Ahanchi SS, Tsihlis ND, Kibbe MR. The role of nitric oxide in the pathophysiology of intimal hyperplasia. J Vasc Surg 2007;45 Suppl A:A64-73.:A64-A73.
69. Chignier E, Eloy R. Adventitial resection of small artery provokes endothelial loss and intimal hyperplasia. Surg Gynecol Obstet 1986;163(4):327-334.
70. Rudic RD, Shesely EG, Maeda N, Smithies O, Segal SS, Sessa WC. Direct evidence for the importance of endothelium-derived nitric oxide in vascular remodeling. J Clin Invest 1998;101(4):731-736.
71. Mayr U, Zou Y, Zhang Z, Dietrich H, Hu Y, Xu Q. Accelerated arteriosclerosis of vein grafts in inducible NO synthase(-/-) mice is related to decreased endothelial progenitor cell repair. Circ Res 2006;98(3):412-420.
72. Shoji M, Iso Y, Kusuyama T et al. High-dose granulocyte-colony stimulating factor promotes neointimal hyperplasia in the early phase and inhibits neointimal hyperplasia in the late phase after vascular injury. Circ J 2008;72(11):1885-1893.

73. Wu X, Wang K, Cui L et al. Effects of granulocyte-colony stimulating factor on the repair of balloon-injured arteries. Pathology 2008;40(5):513-519.

VERTEBRATE ANIMAL

1. Species and Detailed Procedure

A total of 964 12-week old male mice ([redacted]) will be used for the entire protocol. Use of mice by strain, and for each Aim is listed in Table 1 below.

Table 1. Number of mice used by Aim and by Strain.

Strain	Aim 1	Aim 2	Aim 3	Subtotal by Strain
C57BL/6	96 + 72 = 168	20	20 + 60 + 120 +96 = 296	**484**
C57BL/6-Tg(CAG-EGFP)	96			**96**
FVB/N-Tgn(TIE2-lacZ)	96			**96**
FVB/N	96			**96**
iNOS KO			96	**96**
eNOS KO			96	**96**
Subtotal by Aim	**456**	**20**	**488**	**964**

The following procedures will be performed:

Preoperative preparation and anesthesia

Anesthetic induction and maintenance will be conducted using isoflurane gas anesthesia (2-5%). The surgical site of the mice will be shaved and prepped with ethanol and betadine. The surgeon will use sterile instruments for all survival procedures. The surgeon will wear a sterile hat, mask, and gloves for all survival procedures.

Bone marrow transplantation

The bone marrow of either C57BL/6 or FVB/N recipient mice will undergo lethal irradiation (12 Gy). $1x10^6$ donor bone marrow cells from either C57BL/6-Tg(CAG-EGFP) or FVB/N-Tgn(TIE2-lacZ) age and strain-matched mice will be injected into the tail vein of the recipient mice. Four weeks after bone marrow transplantation (BMT), a time sufficient for bone marrow regeneration, the standard femoral artery wire injury model will be performed as described below.

Femoral artery wire injury model

The right common femoral artery of anesthetized mice will be dissected and controlled. A 0.014" guidewire will be advanced into the right common femoral artery via a side branch and passed three times. After removal of the wire, the branch will be ligated and flow will be restored. PROLI/NO (1mg) will be administered to the periadventitial surface of the injured femoral artery in some mice. The wound will be closed. At the designated time points, the animals will be anesthetized then euthanized with bilateral thoracotomies and the common femoral arteries will be removed following *in situ* perfusion-fixation. Contralateral uninjured arteries will serve as controls and be harvested.

BrdU injection

Some animals will undergo intraperitoneal (IP) injection of BrdU (100 mg/kg) at designated time points, according to each experiment.

Adventitial removal with periadventitial cell transplantation

To remove the adventitia of the femoral artery *in vivo*, we will follow the method described by [redacted] and [redacted] et al. Briefly, the adventitia and periadventitial fat will be removed by gentle dissection using the dissecting microscope and high power magnification. Cell transplantation will be performed by transplanting $1x10^5$ cells to the periadventitial surface of the artery after the femoral artery wire injury model is performed. The wound will be closed in standard fashion.

G-CSF administration

G-CSF administration will be performed by injecting 300 μg/kg G-CSF subcutaneously daily for 5 days.

Harvest carotid artery for cell isolation

For some experiments, the bilateral common femoral arteries will be harvested in order to isolate adventitial stem and progenitor cells. To do this, mice will undergo general anesthesia as described above, the femoral arteries will be isolated and ligated proximally and distally and resected. After resection, the animals will undergo euthanasia by performing bilateral thoracotomies.

Detailed numbers calculations by Aim:

Aim 1

4 strains (C57BL/6-Tg(CAG-EGFP), C57BL/6, FVB/N-Tgn(TIE2-lacZ), FVB/N)
2 treatment groups (Injury and Injury+NO)
n=6/group
8 time points
2 x 6 x 8 = **96 of each of the four strains**

BrdU pulse chase experiments:
1 strain
6 time points
2 treatment groups (Injury and Injury+NO)
n=6/group
1 x 6 x 2 x 6 = **72 C57BL/6**

Aim 2

Harvest of cells from C57BL/6 mice = **20 C57BL/6**

Aim 3

Harvest of cells from C57BL/6 mice = **20 C57BL/6**

10 treatment groups: (injury, I+AR, I+AR+Sca1, I+AR+CD34, I+AR+flk-1) each with injury vs injury+NO
n=6/group
1 time point
10x6x1 = **60 C57BL/6**

4 treatment groups (G-CSF vs saline; injury vs injury+NO)
n=6/group
5 time points
4 x 6 x 5 = **120 C57BL/6**

3 strains (iNOS KO, eNOS KO, WT)
2 treatment groups: injury and injury+NO
n=6/group
8 time points
2 x 6 x 8 = **96 iNOS KO, 96 eNOS KO, 96 WT**

2. Justification

Mice have been used extensively in the literature to evaluate the arterial injury response *in vivo*. They are the smallest mammal that can provide useful information prior to moving to larger animals. Mice also provide a useful research tool because of the ability to create knockout and transgenic mice. No non-animal model exists that can mimic this arterial injury response and a similar model does not exist in a phylogenetically lower species.

To determine the number of animals required for each experimental group, a power analysis was performed. The power of the study is defined as the probability that an experiment will have a significant (positive) result, that is, have a p-value of less than the specified significance level. This probability is calculated under the assumption that the treatment difference or strength of association equals the minimal detectable difference. From previous experience with these models of neointimal hyperplasia, we know that there is a standard deviation of approximately 10%. We want to detect a minimal difference of 20% within any measurement with

a 5% error (i.e., $p<0.05$). Therefore, in order to achieve a power of 0.8 (i.e., an 80% chance to correctly determine a 10% difference in the data), the n (number of animals in each treatment group) is determined based on the number of treatment groups to be compared.

An example of the power analysis conducted for the mouse femoral artery injury model experiments that includes three groups (control, injury, injury+NO) is as follows (note: the group size [n] is varied to determine the size at which sufficient power is reached):

Power - 0.800
Difference in Means - 0.200
Standard Deviation - 0.100
Number of Groups - 3
P-value - 0.05
Group size (n) = 6

Therefore, in order to achieve sufficient power (>80%) given a $p<0.05$, with 3 groups, we need to include 6 animals per group.

3. Veterinary Care

All animals are housed and cared for by the veterinary staff at the Center for Comparative Medicine (CCM) facility at [redacted]. All animals housed in the CCM facilities are monitored by a team of 8 veterinarians, 8 animal health care technicians, and their expansive support staff. CCM is the centrally administered animal resource that acts as a service and teaching unit for all of the animals used in research, testing, and education at [redacted]. CCM has been AAALAC Accredited since 1985 and a member of AALAS since 1962.

4. Limitation of Pain and Discomfort

The proposed surgeries will cause no more discomfort than is absolutely necessary. Animals will be anesthetized during all surgeries and will receive analgesics post-surgery as indicated. The mouse femoral artery injury model is considered a minor procedure, since it does not involve a major body cavity. This procedure is well-tolerated by the animals. The mice will be administered buprenex following surgery once they are sternal and then as needed. The duration of the discomfort will be as short as possible. The animals will be regularly monitored for signs of discomfort and treated accordingly. Should any of the animals experience disabling complications from the surgery the animal will undergo immediate euthanasia.

5. Euthanasia

Mice will undergo anesthesia with isoflurane (2-5%) and euthanasia by bilateral thoracotomy. This method has been approved by the [redacted] Office for the Protection of Research Subjects and the Animal Care and Use Committee (ACUC), and is consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

DATA SHARING PLAN

All research data generated from this proposal, should it be funded by the NIH, will be made readily available for research purposes to qualified individuals within the scientific community upon publication according to the NIH policy on Sharing Research Resources (SF424, Part III, 1.5).