Radiosensitive Smooth Muscle Cells Populate Neointimal Lesions Through a Platelet Derived Growth Factor Receptor Beta Dependent Mechanism

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Abstract:

Objective: To provide direct evidence supporting or refuting the dogma that SMC-specific PDGFRB signaling is required for SMC phenotypic switching and neointima formation following vascular injury *in vivo*.

Approach and Results: Utilizing a novel conditional SMC-specific lineage tracing PDGFRB knock out mouse, we demonstrated that PDGFRB signaling is required for SMC phenotypic switching and that loss of PDGFRB in SMCs virtually abolished the capability of SMCs to be recruited into the neointima. Loss of PDGFRB in SMCs, however, did not attenuate neointima formation due to compensation by alternative cell types, including myeloid cells. Lethal irradiation and bone marrow transfer experiments revealed neointimas devoid of SMC-derived cells in both the WT and KO animals with the majority of the neointima in both genotypes being myeloid derived.

Conclusions: Taken together, results demonstrate that there is a radiosensitive subpopulation of SMCs within the vessel wall which normally proliferates and invests the neointima through a PDGFRB dependent mechanism. Further, in the absence of SMC-rich neointimas other cell types including myeloid cells compensate by investing the neointima and activating a subset of SMC marker genes.

Dedication

I dedicate this dissertation to my wife and kids. Without my wife's love, support, and sacrifice I would have not been able to complete my degree. Thank you, Erica, for all of your support, sacrifice, and encouragement! I wouldn't have been able to do this without you.

Acknowledgements

I would like to thank my mentor, Gary Owens, for the guidance and support on a project which kept throwing curveballs at us, despite the dogma and everyone supposedly "knowing" what the results should be. This just goes to show that even "safe projects" can have very surprising and exciting results.

I'd also like to thank the members of the Owens lab past and present for their advice and support on all topics scientific, professional, and personal. In particular I'd like to thank: Dr. Anh Nguyen for teaching me the basics of immunofluorescence staining, a critical component of my dissertation; Dr. Olga Cherepanova for her continued support and encouragement in pursuit of the PhD degree; Rupande Tripathi for cell culture and cell isolation technical expertise; Melissa (Missy) Bevard for tissue processing and histology expertise and advice; Mary McCanna for assistance in tissue sectioning and IHC staining; Alex Young for animal surgery expertise and assistance in all things mouse related; and both Ryan Haskins and Gabe Alencar for moral support, harvest assistance, and various other technical assistance.

I'd also like to thank the members of my thesis committee for their guidance, including Dr. Jeff Smith (chair), Dr. David Wotton, Dr. Hui Li, and Dr. Brant Isakson. This project has had a pretty big evolution from what it initially began as and I very much appreciate the freedom they've allowed me to explore and to develop as a scientist.

Last but not least, I thank the members of my church and my community group for their moral support throughout the difficult pursuit of my degree.

God is Good--All the Time!

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Chapter I: Introduction

Smooth Muscle Cells

Location and Function

The Vascular Smooth Muscle Cell (SMC) is one of the primary contractile components of the vascular system[1]. Located morphologically in circumferential rings around blood vessels, the principle function of the SMC is contraction and regulation of blood vessel tone-diameter, blood flow distribution, and blood pressure[2,3]. SMCs are developmentally derived from multiple independent sources, including the neural crest, secondary heart field, somites, various stem cell sources, mesangioblasts, proepicardium, splanchnic mesoderm, and the mesothelium (reviewed in ref 4). SMCs located in adult blood vessels have an extremely low proliferative rate, exhibit low synthetic activity, and express a unique combination of contractile proteins, ion channels, and signaling molecules that clearly distinguish them from both skeletal muscle and cardiac muscle[5]. Interestingly, recent studies by our lab[6,7] and others[8,9] have begun to demonstrate that SMCs are capable of many more functions than those just related to contraction, dilation, and ECM deposition. The role of SMCs in alternative pathways is thought to be mediated through a process known as SMC phenotypic switching.

In an uninjured vessel, blood flow and pressure exert a force against the endothelial layer as blood circulates throughout the body. It is the SMCs which exert an opposing force and as has been mentioned are responsible for contraction and dilation of blood vessels in the face of this pressure. Large conduit arteries contain a single layer of endothelial cells surrounded by multiple

continuous, circumferential rings of SMCs. This layer of SMCs is termed the media. Located between the media layer and the endothelial cells is a thin, acellular region known as the intima. In response to vascular damage, SMCs undergo phenotypic switching, a process described in the next section, and proliferate and migrate into the intima space, forming what's called the neointima[10]. It's important to note that under most physiological conditions, blood still flows through damaged vessels meaning the media layer must not become SMC depleted, else the vessel may rupture. Aneurysms, for example, are generally caused when blood pressure is increased coupled with a damaged or weakened media layer, leading to rupture. The neointima is an example of inward vessel remodeling in response to vascular damage. Daniel et al[11] has demonstrated that under conditions where blood flow is retained (the femoral wire injury), the neointima actually resolves after a certain time period such that the vessel appears to never have been damaged. The specifics of what happens to the cells in the neointima following injury resolution remain unclear, although several likely possibilities are apoptosis, phagocytosis, and migration away from the site of injury.

Phenotypic Switching

Unlike it's cardiac muscle and skeletal muscle cousins, SMCs, although fully differentiated, are not terminally differentiated and in response to vascular injury [12,13] or disease [6–8] retain the capability to undergo phenotypic switching; a process characterized by coordinate and profound down regulation of SMC marker genes and upregulation of ECM synthesis, pluripotency genes, and migratory genes such as KLF4[6,14,15] and OCT4[7]. Although multiple SMC genes

have been described and used to identify SMCs *in vivo*, including smooth muscle alpha actin (ACTA2)[16], smooth muscle myosin heavy chain (MYH11)[17], h1-calponin[18], and smooth muscle 22-alpha (TAGLN)[19], phenotypic switching of SMCs is a process which is defined by down regulation (or loss) of these genes, making identification of phenotypically modulated SMCs *in vivo* exceedingly difficult using traditional methods which rely on gene or protein expression. Further, multiple genes originally thought to be SMC-specific have now been identified as being expressed in alternative cell types including bone marrow cells[11,20–23], adventitial cells[24–26], endothelial cells[27], and more[28,29], with the only gene currently thought to be specific to SMCs (and pericytes, a perivascular cell similar to SMCs in the microvasculature) being Myh11[17]. Indeed this ambiguity led to several papers[23,30,31] suggesting additional de novo sources of SMCs being derived from cells other than SMCs.

The most controversial of these papers, that by Tang et al.[32], challenged the whole phenomenon of reversible phenotypic switching by suggesting a subpopulation of cells which don't express Myh11 are responsible for complete and total recellularization of the vessel following vascular injury and claimed that SMC phenotypic switching is a complete *in vitro* artifact. In their studies, Tang et al.[32] utilized a non-inducible lineage tracing mouse system, Myh11Cre/eGFP, to derive cultured SMCs using a tissue explant protocol. Surprisingly, the cells obtained were GFP-. However, a key limitation of studies was that they appear to have failed to remove the adventitia from vascular samples such that it is likely the cells they obtained were derived from adventitial fibroblasts and/or Sca1+

adventitial stem cells which are much more highly proliferative than SMCs[24,25]. The authors also used their lineage tracing mouse to analyze cell proliferation at a single time point of 5 days post-wire injury. In their system, they did not observe any Ki67+ GFP+ cells (Ki67 being a marker of cells undergoing proliferation), suggesting that SMC do not proliferate *in vivo* in response to injury, although this was likely an artifact of their inappropriate fixation method since they observed almost no GFP+ cells period. Indeed, the results of Tang et al.[32] are in direct contrast to work previously published by Nemenoff et al.[13] in which they observed the presence of BrdU+ SMCs using a tamoxifen inducible Myh11-B-galactosidase lineage tracing mouse. In addition to the methodological deficiencies in the Tang et al.[32] studies, their studies were also compromised by use of a non-conditionally regulated SMC lineage tracing model which precludes rigorous documentation of the SMC specificity of the model. As such, one has no idea when reporter gene activation occurs, the phenotype of the cell when activated, and most importantly whether some labeled cells might represent a non-SMC which activated the promoter driven reporter during development/maturation, following injury, or disease development. Further, several papers have now been published by our lab[6,7,33] and others[12,34] which have directly and convincingly refuted these conclusions and instead demonstrated through rigorous, conditional, quantitative lineage tracing methods that in response to vascular injury and disease SMCs do undergo phenotypic switching and lose expression of traditional SMC marker genes, including ACTA2 and MYH11.

In addition to being able to down regulate SMC marker genes, we[6] and others[8,9] have shown that under various physiological conditions and disease states, SMCs both lose expression of SMC marker genes and activate genes characteristic of alternative cell types. For example, in the context of atherosclerosis, Shankman et al.[6] used SMC-specific conditional lineage tracing mouse and found that ~80% of SMCs present within atherosclerotic lesions were completely unidentifiable using traditional methods; i.e they were eYFP+ ACTA2-MYH11-. Further analysis by flow cytometry revealed that 13% of these cells were positive for mesenchymal stem cell markers (SCA1 and CD105) and ~30% were positive for macrophage markers (LGALS3, CD11b, F4/80). In a separate study, Long et al.[9] found that a significant fraction of thermogenic UCP1+ cells, specifically beige adipocytes, are derived from pre-existing mature SMCs through the use of inducible SMC-specific lineage tracing. Finally, unpublished data from our lab demonstrates that SMCs are capable of activating at least a subset of genes typically associated with kupffer cells following liver hepatectomy. Taken together, these results highlight the plasticity of SMCs and the absolute requirement of rigorous, conditional, SMC lineage tracing to fully define the SMC contribution, or lack thereof, *in vivo*.

Summary

Transitions in smooth muscle cell (SMC) phenotype have been postulated to play a key role in a variety of disease states including restenosis[35], coronary heart disease[36], and atherosclerosis[37]. Remarkably, in spite of decades of research in this area, only recently have SMC lineage tracing studies been completed indicating that this process occurs *in vivo*[6,34,38]. The reasons for this

lack of information are as follows: **First**, SMC phenotypic switching is a process characterized by profound and coordinate down regulation of SMC marker genes making identification of phenotypically modulated SMCs *in vivo* by traditional markers exceedingly difficult[39] as SMCs lose expression of nearly all SMC marker genes. **Second**, traditional SMC marker genes, such as ACTA2 and TAGLN, have been used for decades to identify SMCs *in vivo*, however we[6,40] and others[12,24–26] have shown that a variety of other cell types are capable of expressing them as well as other SMC marker genes raising the question of whether or not cells previously identified and studied as SMCs are in fact SMCs or an alternative cell type expressing SMC marker genes. **Third**, our recent studies[6,7] and others[8,24,34] have identified potential other sources of SMCs under certain conditions including macrophages, Sca1+ adventitial stem cells, endothelial cells, etc. The contribution of these alternate-origin SMCs to the overall SMC response to injury and disease remains unclear. Taken together, these results highlight the plasticity of SMCs and further underscore the importance of rigorous, conditional SMC lineage tracing in order to fully define the ultimate contribution of SMCs in the context of both vascular injury and vascular disease.

Platelet Derived Growth Factor and Receptors

Brief History

Platelet Derived Growth Factors were identified in a search for chemokines that were able to stimulate SMC proliferation *in vitro*[41–43]. In

response to vascular injury, platelets adhere to the site of injury and release the contents of their granules, which contain platelet derived growth factors (PDGFs)[44] and other platelet constituents. We now know that there are four PDGFs: PDGF-A, PDGF-B, PDGF-C, and PDGF-D which function as homo- and hetero-dimers, and two tyrosine kinase receptors, platelet derived growth factor receptor alpha (PDGFRA) and platelet derived growth factor receptor beta (PDGFRB) which can also function as either a homo- or hetero-dimer (see ref [45] for more details on the specific homodimer/heterodimer binding combinations). Of importance for this dissertation, PDGF-BB[46] and PDGF-DD[47], which both signal through the PDGFRB, are regularly used by multiple labs to induce SMC phenotypic switching *in vitro* whereas PDGF-A and PDGF-C, signaling through the PDGFRA, do not induce SMC phenotypic switching *in vitro*[47]. Further, blockade of PDGFRA (or it's ligands, PDGF-A and PDGF-C) has no effect on SMC migration or proliferation *in vitro*[48] nor on overall disease burden *in vivo*[48].

Interestingly, global knock out studies performed by Hellström et al.[49] found that loss of PDGF-B or PDGFRB resulted in embryonic lethality due in large part to multiple vascular-related defects, including but not limited to reduced SMC coverage, complete lack of pericyte coverage, thrombocytopenia, and microaneuryism formation. Unfortunately we can only conclude from this study that PDGF-B and PDGFRB play an important role during development and we cannot draw any conclusions about the importance of SMC-specific PDGFRB signaling due to this being a global knock out study and subsequent loss of

PDGF-B or PDGFRB in the many cell types that express this receptor throughout development.

Intracellular Signaling

PDGFRB is a receptor tyrosine kinase meaning in response to its ligands, it dimerizes (either with itself or with PDGFRA) and activates multiple phosphorylation events[50]. Among other proteins and kinases, PDGFRB autophosphorylation activates the MEK/ERK MAP kinase signaling pathway, stimulates autocrine secretion of PDGF-BB, and stimulates secretion of bFGF, another factor known to induce SMC phenotypic switching in vitro. This autocrine secretion of bFGF has been shown to be critical for maintained ERK phosphorylation and activation such that loss of FGFR signaling (siRNA) in cultured cells in the presence of PDGF-BB results in early ERK phosphorylation (30-60 minutes) yet loss of ERK phosphorylation at later time points (2-4 hours)[51]. In addition, we have preliminary data demonstrating that in the absence of PDGFRB (siRNA knock down in cultured cells), bFGF alone is unable to induce SMC phenotypic switching (Griffith and Owens, unpublished data). One of the immediate targets of phosphorylated ERK is Elk, a protein directly involved in the down regulation of SMC genes (when phosphorylated, pELK).

One of the other factors upregulated in response to PDGFRB autophosphorylation is Sp1, a protein critical to the activation of KLF4. The Owens lab previously demonstrated that truncation or loss of the three SP1 binding sites in the KLF4 promoter resulted in failed activation/upregulation of KLF4 in response to PDGF-BB[14] stimulation. KLF4 binds to the GC repressor

region in SMC promoters and recruits HDAC2 which deacetylates the surrounding histones promoting epigenetic silencing[52]. In addition, KLF4 recruits pELK which competes against myocardin (the SMC master regulator[53]) for binding to SRF and degenerate CArG boxes, and further the Owens lab has demonstrated that increased KLF4 expression results in an overall decrease in myocardin expression[54]. Under mature, differentiated conditions, the myocardin-SRF complex binds to regions of DNA known as degenerate CArG boxes which the Owens lab has shown are absolutely critical for mature SMC gene expression[55].

In summary, PDGFRB signaling indirectly upregulates KLF4 expression which in turn promotes downregulation of myocardin, and further recruits HDACs to SMC gene promoters. All of these changes work in tandem to repress SMC marker genes in the process known as phenotypic switching.

PDGFRB and Injury

While multiple studies have examined the effects of inhibiting the PDGF signaling pathway in the context of injury, no study to date has been able to examine the specific contributions of SMC-specific PDGFRB in the context of vascular injury[56–59]. For example, one of the earliest studies examining the role of PDGF signaling in vascular injury came from the Russell Ross lab. In this study, Ferns et al[57]. performed intraarterial balloon catheter de-endothelialization on rat carotid arteries and continually administered a polyclonal antibody against PDGF-A/B. The authors found that global inhibition of the PDGFs resulted in a

significant decrease in intimal area and immediately assumed that, since the PDGFs are a SMC chemoattractant *in vitro*, that the decrease in intima area was due to a lack of SMCs in the intima *in vivo*. Several years later, a second study from the Alexander Clowes lab[58] performed balloon injury on the baboon saphenous artery and found that continuous infusion of heparin combined with 5 injections of a neutralizing antibody against the PDGFRB over 28 days resulted in a significant decrease in intima area. The authors then concluded that "...the most likely explanation is inhibition of migration of SMCs from the media to the intima..." and while this is consistent with the dogma at the time and the *in vitro* observations that PDGF signaling through the PDGFRB *in vitro* is capable of inducing SMC migration, there is no direct evidence confirming that it was in fact a deficit of SMCs which led to the observed decrease in intima area *in vivo*.

Indeed, since PDGFRB is expressed by many other cell types, including macrophages[60] and adventitial cells[61] in addition to SMCs[59], the specific role of PDGFRB in SMC phenotypic switching cannot be definitively identified through studies employing antibody-inhibitor or pharmacological agents since those studies involve global inhibition of PDGFRB in all cell types. The focus of the next chapter of this dissertation will be on rigorously examining the role of PDGFRB specifically in SMCs during the response to vascular injury.

Summary

PDGF-BB, signaling through the PDGFRB, has been used by multiple labs including ours to induce SMC phenotypic switching and migration *in vitro*. Although inhibition of PDGFRB signaling has been well documented to reduce

overall neointima area in response to a variety of injuries in multiple animal systems, there is no direct evidence demonstrating that PDGFRB is the critical signaling pathway for SMC phenotypic switching *in vivo*, nor is there evidence supporting that intact SMC-PDGFRB signaling is required for SMC investment of the neointima following vascular injury. Previous studies which have drawn conclusions on the role of SMC-PDGFRB *in vivo* in response to vascular injury are inherently limited in that usage pharmacological inhibitors impact multiple other cell types known to express PDGFRB in addition to SMCs. Further, global knockout studies have a wide array of developmental effects, precluding any definitive determination of the role of PDGFRB in SMCs *in vivo*. Taken together, while it's been definitively proven that ***global*** inhibition of PDGFRB signaling reduces overall neointima formation *in vivo* in response to vascular injury, we do not know what the role of SMC-specific PDGFRB is *in vivo* in response to vascular injury.

Chapter II: Radiosensitive SMCs populate neointimal lesions through a platelet derived growth factor receptor beta dependent mechanism

Introduction

Growth, development, and survival of all multicellular higher organisms is absolutely dependent on the ability to form functional blood vessels and blood vessel networks, and to repair vascular damage[49]. Whereas there have been great advances in our understanding of the role of endothelial cells in this process, much less is known regarding the regulation of vascular smooth muscle cells (SMCs). Indeed, a previous study by Tang et al.[32] challenged the long standing dogma that fully differentiated SMCs were capable of proliferating and postulated that mature SMCs were terminally differentiated, and that newly generated SMCs following vascular injury were derived from a putative "medial stem cell" population negative for SMC markers such as smooth muscle myosin heavy chain (MYH11)[32]. However, as summarized by Nguyen et al.[62] there were a number of major technical limitations with the Tang et al.[32] studies that undermined their major conclusions. In addition, their findings were completely at odds with numerous major studies in the field[63–68] including seminal studies by Clowes et al.[66] who based on continuous 3H-thymidine labeling studies showed that nearly 40% of medial cells underwent at least one cell division in the initial 14 days following balloon injury of the rat carotid artery, and studies by Thomas et al.[68] who used innovative 3H-thymidine labeling studies combined with construction of ancestor tables based on autoradiographic grain count analyses to show that 66-99% of medial SMCs initiate DNA synthesis and undergo cell division during the course of development of porcine atherosclerosis. Even more importantly, there is now irrefutable evidence based on rigorous SMC lineage tracing studies that fully differentiated MYH11+ ACTA2+ medial SMCs are the

primary source of newly generated SMCs following vascular injury. Herring et al[34] utilized an innovative tamoxifen inducible MYH11-CreERT2/CAG flox tdtomato Stop-flox/eGFP mouse model to show that the vast majority of the neointima was derived from preexisting MYH11+ SMCs rather than an alternative source. In addition, SMC lineage tracing studies by our lab demonstrated that SMC-derived cells that are present within advanced atherosclerotic lesions are derived from mature differentiated (MYH11+) medial SMCs. However, of major interest, >80% of these SMC- derived lesion cells have undergone phenotypic switching characterized not only by the lack of expression of typical SMC lineage marker proteins such as ACTA2 and MYH11, but nearly half of these cells have activated markers of other cell lineages including macrophages, mesenchymal stem cells (MSCs), and myofibroblasts[6,33]. As such, studies show that essentially all previous studies in the field have not only grossly under-estimated the number of SMC-derived cells within atherosclerotic lesions, but have also mis-identified them as being other cell types. In addition, of major significance we demonstrated that these SMC phenotypic transitions play a critical role in lesion pathogenesis in that SMC- specific knockout of the stem cell pluripotency genes Oct4 or Klf4 resulted in major changes in lesion size, SMC phenotype, and indices of plaque stability[6,7]. Whereas the preceding studies have clearly established that phenotypic transitions of fully differentiated SMCs play a critical role in vascular injury-repair and atherogenesis, very little is known regarding environmental cues that control SMC phenotypic transitions *in vivo* in response to vascular injury.

Our lab[14,37,47,69,70] and others[45,46,48,58,59,71–73] have previously shown that PDGFBB and DD, major mesenchymal mitogens that activate the PDGFβ receptor (PDGFRB), contribute to SMC dedifferentiation *in vitro* and induce a phenotype characterized by marked increases in proliferation, migration, and gene expression patterns including coordinate down-regulation of SMC marker genes and activation of a number of extracellular matrix (ECM) genes. Moreover, these *in vitro* results, and evidence that vascular injury is associated with platelet activation and release of multiple growth factors including platelet derived growth factors (PDGFs), has led to the hypothesis that PDGFs play a key role in regulating SMC phenotypic transitions *in vivo* following vascular injury. Consistent with this hypothesis, a number of groups[46,56–58,65,72,73] have demonstrated inhibition of neointima formation following vessel injury by administration of anti-PDGF antibodies, pharmacological inhibitors of PDGFRB, and antisense oligonucleotides against members of the PDGF family. In addition, Buetow et al[74] showed marked reductions in the proportion of PDGFRB+/+ to PDGFRB-/- cells in the neointima of chimeric mice produced by fusion of blastocysts from PDGFRB+/+ and PDGFRB-/- ESCs, indicating that PDGFRB+/+ cells had a competitive advantage in outcompeting PDGFRB-/- cells in contributing to formation of the neointima. Whereas the preceding studies have clearly established that PDGFRB signaling plays a key role in neointima formation following vascular injury, it is unclear if this is the result of cell autonomous activation of PDGFRB signaling directly in SMCs as opposed to being a secondary effect due to effects of PDGFRB signaling in non-SMC

including adventitial cells[45], bone marrow derived cells including monocytes/macrophages[75,76], T cells[77], and/or B cells[78] all of which are known to express the PDGFRB. As such, there is no direct evidence that PDGFRB is a critical SMC mitogen that mediates SMC phenotypic switching *in vivo*, in spite of this being the widely held dogma for several decades[79]. To directly test this, we generated Pdgfrb$^{FL/FL}$ Myh11-CreERT2/Rosa flox-Stop/eYFP mice which allow us to simultaneously lineage trace SMCs and study the effects of loss of the Pdgfrb exclusively in SMCs. Consistent with the long term dogma, we showed that investment of eYFP+ SMCs within the neointima was completely dependent on SMC-derived PDGFRB. However, completely unexpectedly we found that: 1) there was complete compensation by non-SMC derived cells such that neointima size was unaltered in SMC-specific Pdgfrb knockout mice; 2) these non-SMC derived cells were predominantly ACTA2+ and in a few cases ACTA2+ and MYH11+ and; 3) differentiated medial SMCs that give rise to neointimal SMCs are radiosensitive such that this is an unexpected and undefined variable in previous bone marrow transplant (BMT) experiments in the field.

Results

Knockout of Pdgfrb within SMCs results in nearly complete loss of SMC-derived neointimal cells but no change in neointima formation.

In order to test if PDGFRB signaling in SMCs is required for SMC phenotypic switching *in vivo*, we generated a tamoxifen inducible SMC-specific PDGFRB knock out lineage tracing mouse, Pdgfrb$^{FL/FL}$ Myh11-CreERT2 Rosa Flox-Stop/eYFP, (Figure 2.1, dubbed PDGFRB$^{SMC\ \Delta/\Delta}$ or PDGFRB$^{SMC\ WT/WT}$

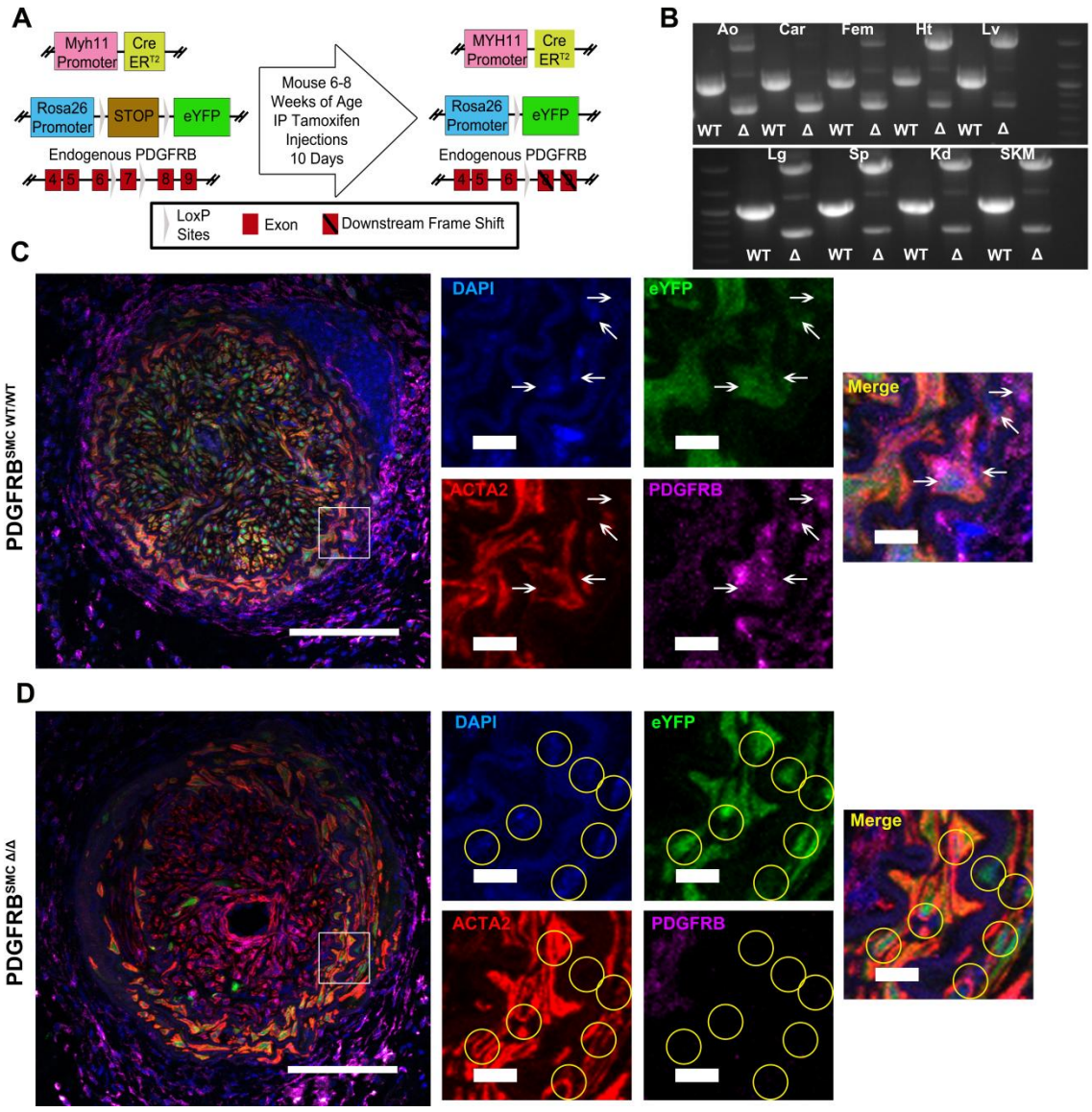

Figure 2.1: Generation and validation of our SMC-specific lineage tracing, conditional PDGFRB knock out mouse.

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(A) Schematic representation of the PDGFRB$^{SMC\ FL/FL}$ mouse pre- and post-tamoxifen injection (IP=Intraperitoneal). (B) Validation of recombination via PCR amplification. FL/FL-1500bp band, WT/WT-1000bp band, and Δ/Δ-800bp excision band. (C,D) Immunofluorescence staining of carotid arteries 21 days post ligation demonstrating loss of PDGFRB protein expression in eYFP+ cells following vascular injury. White arrow indicates eYFP+ PDGFRB+ medial SMCs. Yellow circles indicate eYFP+ PDGFRB- medial SMCs. Small images (right) represent magnified area in white boxes (left). Scale bars are 100μm for left panels and 10μm for magnified images. Ao-Aorta, Car-Carotids, Fem-Femorals, Ht-Heart, Lv-Liver, Lg-Lung, Sp-Spleen, Kd-Kidney, and SKM-Skeletal Muscle. WT-PDGFRB$^{SMC\ WT/WT}$. Δ-PDGFRB$^{SMC\ Δ/Δ}$.

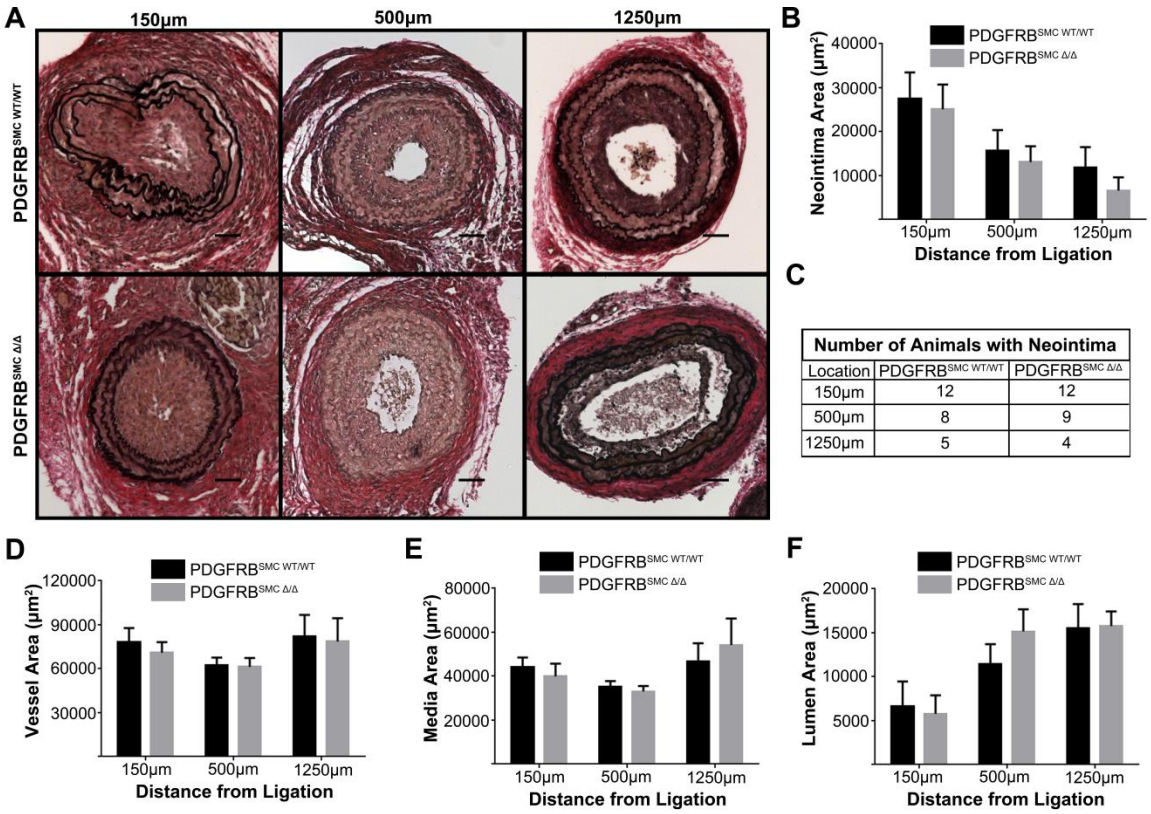

Figure 2.2: SMC specific conditional knock out of PDGFRB did not alter neointima area of carotid arteries 21 days post-ligation.

Figure 2.2: SMC specific conditional knock out of PDGFRB did not alter neointima area of carotid arteries 21 days post-ligation.

(A) Verhoeff's stain of $PDGFRB^{SMC\ WT/WT}$ and $PDGFRB^{SMC\ \Delta/\Delta}$ carotids 21 days post ligation. Micrographs show cross-sections of the ligated carotid artery at different distances from the ligature. Scale bars are 100μm. There was no significant difference in (B) neointima area. (C) Describes the number of animals with neointimas at the various locations. There was no significant difference in (D) vessel area, (E) media area, or (F) lumen area as measured by Two Way ANOVA comparing $PDGFRB^{SMC\ WT/WT}$ versus $PDGFRB^{SMC\ \Delta/\Delta}$ across multiple locations within carotid arteries. Bars for each graph are mean ± s.e.m. n=12 unless otherwise indicated.

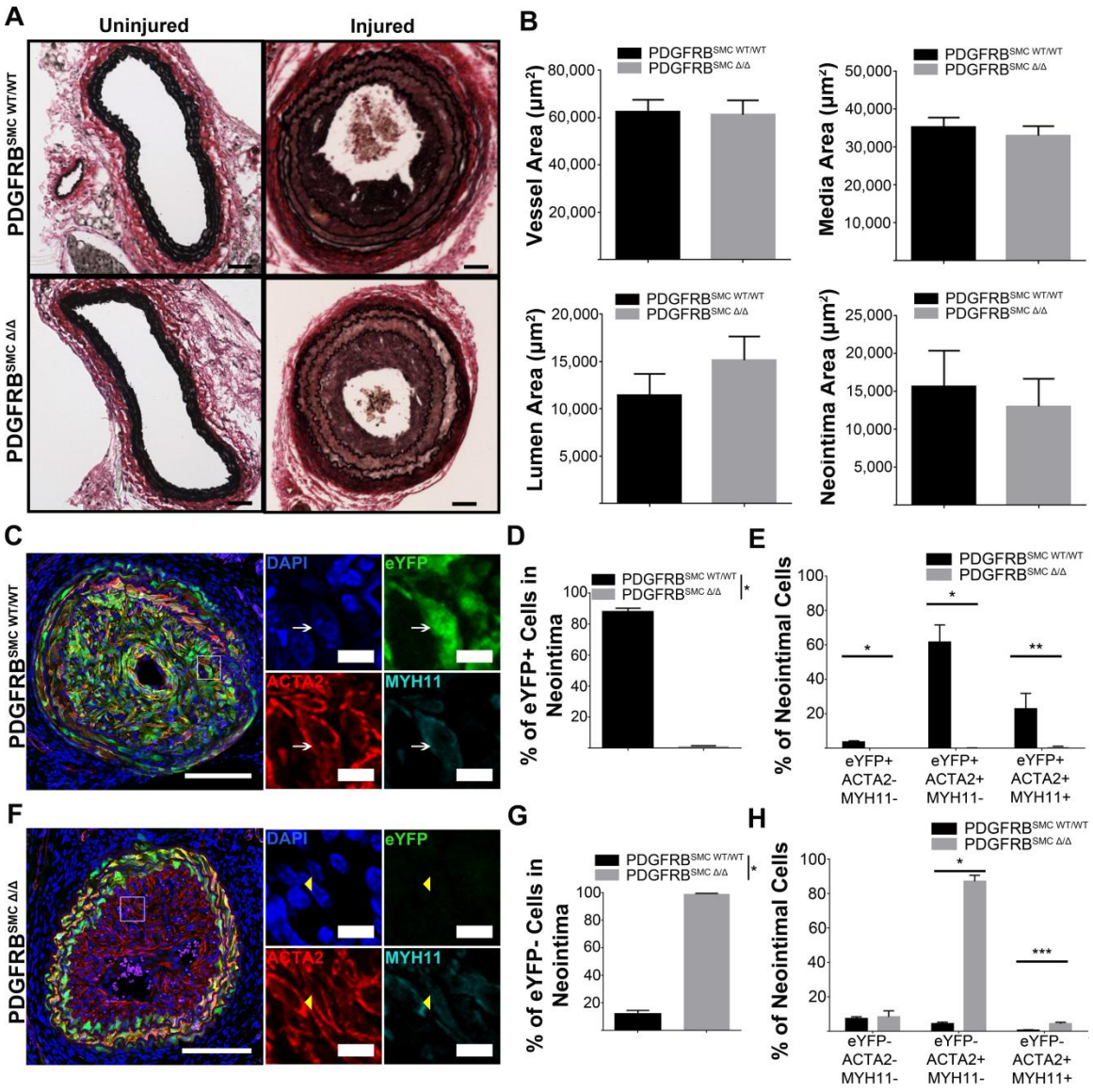

Figure 2.3: SMC specific conditional knock out of PDGFRB resulted in no change in neointima area yet nearly complete loss of SMC-derived cells in the neointima following carotid artery ligation.

Figure 2.3: SMC specific conditional knock out of PDGFRB resulted in no change in neointima area yet nearly complete loss of SMC-derived cells in the neointima following carotid artery ligation.

(A) Verhoeff's stain of representative uninjured and injured $PDGFRB^{SMC\ WT/WT}$ and $PDGFRB^{SMC\ \Delta/\Delta}$ carotids 21 days post ligation. Scale Bars 100μm. (B) Quantitative analysis based on Verhoeff's stain revealed no difference in vessel area, media area, lumen area, or neointima area. (C) Representative immunofluorescence staining of $PDGFRB^{SMC\ WT/WT}$ carotids. Small images (right) represent magnified areas in white boxes (left). Scale bars are 100μm and 10μm, respectively. White arrows indicate eYFP+ ACTA2+ MYH11+ neointimal cells. (D) Quantification of the eYFP+ contribution to the overall neointima. (E) Quantification of dedifferentiated (eYFP+ ACTA2- MYH11-) cells, transition state (eYFP+ ACTA2+ Myh11-) cells, and fully differentiated (eYFP+ ACTA2+ MYH11+) cells to the overall neointima. (F) Representative immunofluorescence staining of $PDGFRB^{SMC\ \Delta/\Delta}$ carotids. Small images (right) represent magnified areas in white boxes (left). Scale bars are 100μm and 10μm, respectively. Yellow arrowheads indicate eYFP- ACTA2+ MYH11+ neointimal cells. (G) Quantification of the eYFP- contribution to the overall neointima. (H) Quantification of the various eYFP- cell expression patterns. *$p<0.0001$, ***$p<0.001$, Unpaired t test. **$p<0.05$ Mann Whitney test.

following tamoxifen injections) and subsequently performed carotid ligation. Despite examining multiple locations (Figure 2.2) at 21 days post-ligation, we found no difference in PDGFRB$^{SMC\ WT/WT}$ and PDGFRB$^{SMC\ \Delta/\Delta}$ neointimal area, lumen area, media area, or vessel area within external elastic lamina (Figure 2.3 A,B). However, results of high resolution confocal microscopic studies of SMC lineage tracing mice showed the nearly complete absence of SMC-derived cells within the neointima of PDGFRB$^{SMC\ \Delta/\Delta}$ mice. Remarkably, ~88% of the neointimal cells within PDGFRB$^{SMC\ WT/WT}$ animals were derived from pre-existing SMCs (eYFP+) as compared to only ~1% of neointimal cells within PDGFRB$^{SMC\ \Delta/\Delta}$ mice (Figure 2.3 C-E). In addition, we found that the majority of the eYFP- cells present in the PDGFRB$^{SMC\ \Delta/\Delta}$ neointima expressed ACTA2 or both ACTA2 and MYH11 (Figure 2.3 F-H) meaning that these cells would likely have been misidentified as being of SMC origin in previous studies in the field. We further confirmed these findings in an alternative model of injury, the femoral wire injury, and found similar results in that the neointima of PDGFRB$^{SMC\ \Delta/\Delta}$ mice were completely devoid of eYFP (Figure 2.4). Consistent with our *in vivo* results and previous *in vitro* studies by multiple labs[28,46,64,80–85], we found that disruption of PDGFRB signaling in cultured SMCs led to a significant reduction in the number of migratory cells in both a scratch wound assay and a transwell migration assay (Figure 2.5). We saw no significant difference in the eYFP+ or eYFP- cells in the media of ligated arteries (Figure 2.6), nor did we see any difference between contralateral controls vessels of PDGFRB$^{SMC\ WT/WT}$ or PDGFRB$^{SMC\ \Delta/\Delta}$ animals (Figure 2.7), suggesting that conditional loss of Pdgfrb

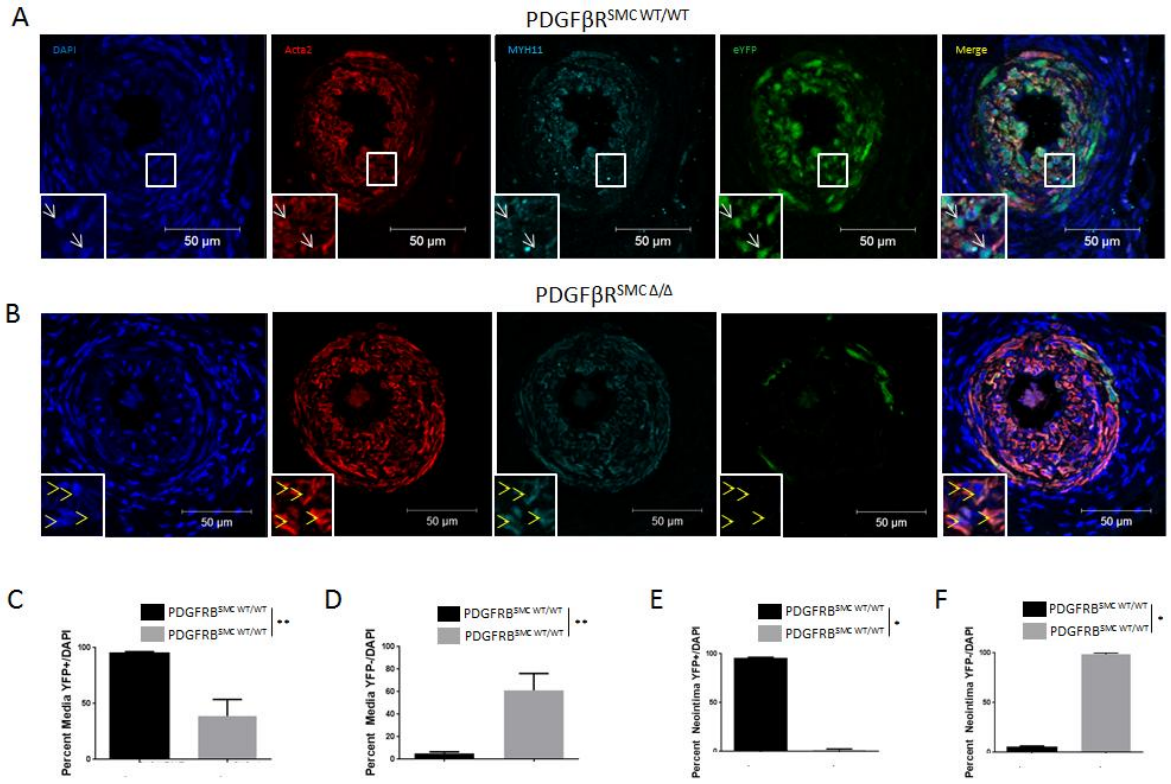

Figure 2.4 SMC specific conditional knock out of PDGFRB resulted in nearly complete loss of SMC-derived cells in the neointima following femoral wire injury.

Figure 2.4 SMC specific conditional knock out of PDGFRB resulted in nearly complete loss of SMC-derived cells in the neointima following femoral wire injury.

Representative IF images of injured femoral arteries from (A) $PDGFRB^{SMC\ WT/WT}$ and (B) $PDGFRB^{SMC\ \Delta/\Delta}$ animals. (C-F) Quantification of the eYFP+ and eYFP- contribution to the media and neointima. Scale bars 50µm. White arrows indicate ACTA2+ MYH11+ eYFP+ cells. Yellow arrowheads indicate ACTA2+ MYH11+ eYFP- cells. *$p<0.0001$, **$p<0.05$ Unpaired t test. $PDGFRB^{SMC\ WT/WT}$ n=5 vs $PDGFRB^{SMC\ \Delta/\Delta}$ n=5

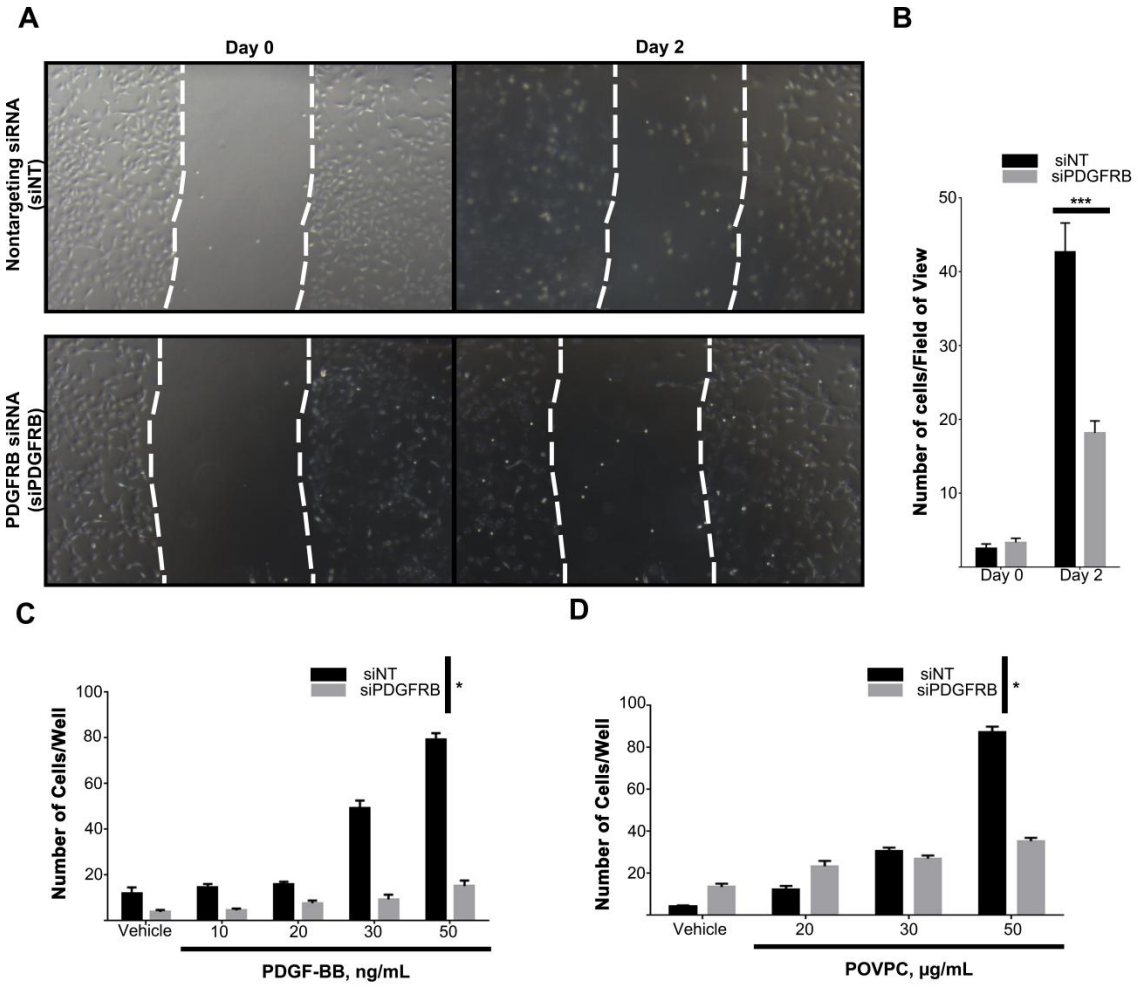

Figure 2.5: Knockdown of PDGFRB inhibited SMC migration *in vitro*.

Figure 2.5: Knockdown of PDGFRB inhibited SMC migration *in vitro*.

Mouse aortic SMC were transfected with blocking PDGFRB siRNA (siPDGFRB) or control non target siRNA (siNT) followed by the Scratch Wound assay (A) or the Boyden Chamber Transwell Migration assay (B). (A) Representative images of the Scratch Wound Assay. (B) Quantification of the Scratch Wound Assay demonstrated that siPDGFRB significantly decreased the number of cells present in the wound 48 hours after scratch. Values represent number of cells from 6 randomly chosen fields of view. Values=mean ±s.e.m. n=4 experiments. (C,D) siRNA knock down of PDGFRB in a Boyden Chamber Transwell Migration Assay resulted in a decrease in migration with both PDGF-BB (C) and POVPC (D) chemoattractants. Graphs are representative experiments. Bars are mean ± s.d. n=4. *$p<0.0001$ Two Way ANOVA, ***$p<0.001$ Unpaired t-test for siNT versus siPDGFRB

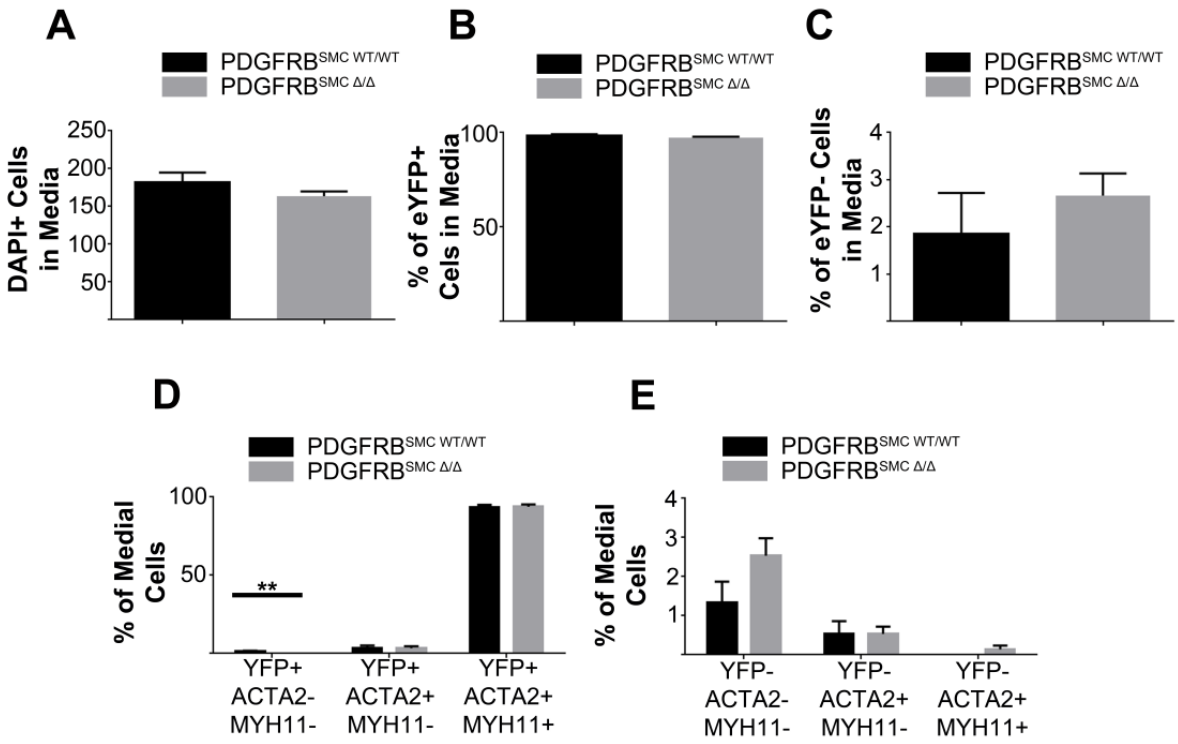

Figure 2.6: SMC specific conditional knock out of PDGFRB did not impact the cell composition of the media after carotid ligation.

Figure 2.6: SMC specific conditional knock out of PDGFRB did not impact the cell composition of the media after carotid ligation.

Quantification of the total number of cells in the media (A), eYFP+ (B), and eYFP- (C) cell contribution to the media. (D) There are no dedifferentiated eYFP+ cells in the media of $PDGFRB^{SMC\ \Delta/\Delta}$ carotids. (E) There is no change in the eYFP- populations in the media of ligated $PDGFRB^{SMC\ WT/WT}$ carotids as compared to $PDGFRB^{SMC\ \Delta/\Delta}$ carotids. Values represent the percent of eYFP+ or eYFP- cells within the total cell population based on DAPI staining. **p<0.05, Mann Whitney test. Error bars are mean +/- s.e.m. $PDGFRB^{SMC\ WT/WT}$ n=8 versus $PDGFRB^{SMC\ \Delta/\Delta}$ n=9.

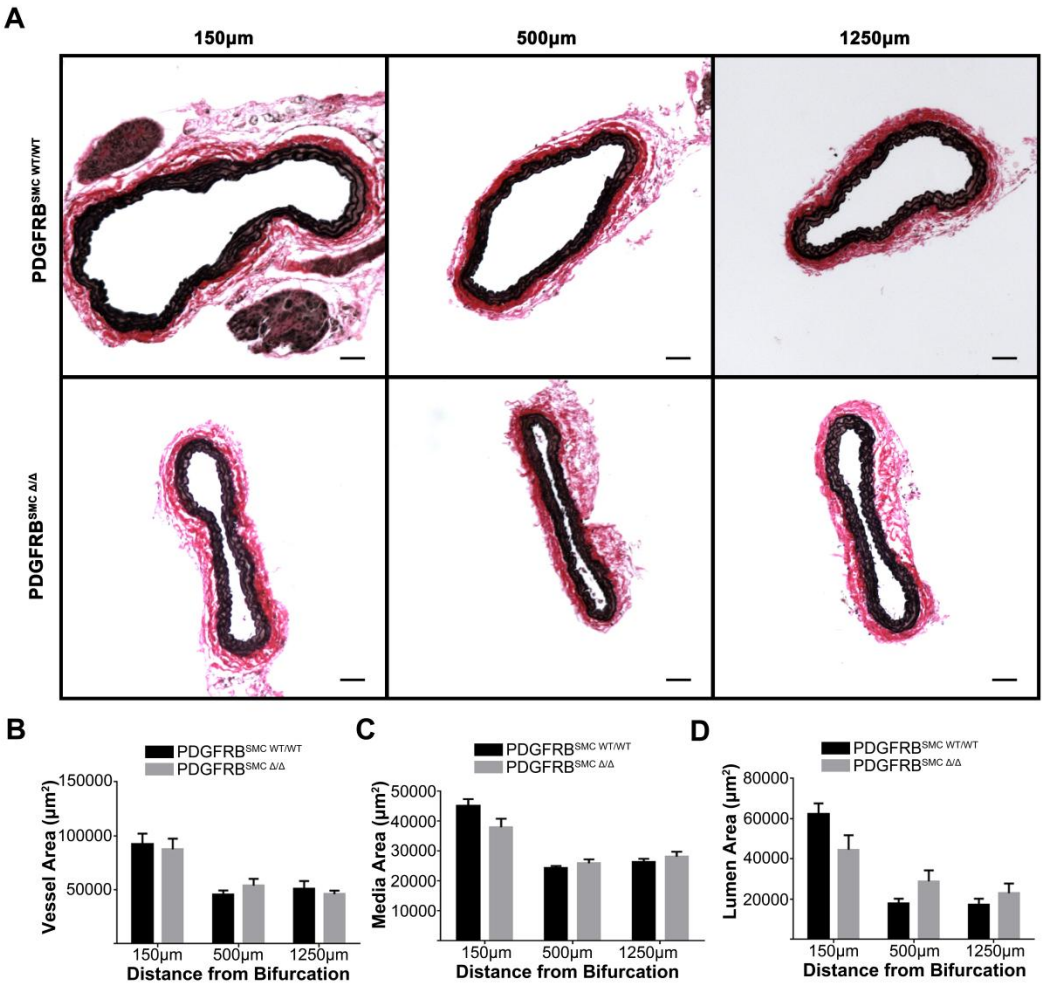

Figure 2.7: SMC specific conditional knockout of PDGFRB had no impact on contralateral control carotid morphology.

Figure 2.7: SMC specific conditional knockout of PDGFRB had no impact on contralateral control carotid morphology.

(A) Verhoeff's stain of representative contralateral, uninjured carotids at varying distances from the internal/external bifurcation. Morphometric analysis of vessel area (B), media area (C), and lumen area (D) revealed no statistically significant differences across multiple locations along the carotid artery as measured by Two Way ANOVA. Scale Bars are 100μm. Error bars are mean ± s.e.m

within SMCs did not have an effect on SMC investment or maintenance of the arterial media in the absence of injury.

Bone Marrow (BM)-Derived Cells compose the majority of the PDGFRB$^{SMC\ \Delta/\Delta}$ neointimas.

We next wanted to determine the origins of the eYFP- cells present in the PDGFRB$^{SMC\ \Delta/\Delta}$ neointima. It's been reported by multiple groups that BM-derived cells are capable of both investing the neointima to varying degrees and expressing a subset of SMC marker genes, including ACTA2[11,12,86–88]. To test the hypothesis that the eYFP- cells within the neointima of PDGFRB$^{SMC\ \Delta/\Delta}$ mice are primarily BM-derived, we performed a BMT experiment utilizing ACTB-tdTomato flox stop flox eGFP (designated as tdTomato) mouse donor BM which constitutively and permanently express tdTomato in all tissues. Importantly, these mice as well as our PDGFRB$^{SMC\ WT/WT}$ and PDGFRB$^{SMC\ \Delta/\Delta}$ SMC eYFP lineage tracing mice are all on a congenic C57BL6 background thus avoiding possible host versus graft disease responses. After confirming ablation of the endogenous hematopoietic cell lineages (Figure 2.8), we then performed immunofluorescence staining and confocal microscopic analyses of ligated arteries to determine if eYFP- cells within the neointima of PDGFRB$^{SMC\ \Delta/\Delta}$ mice were of BM origin, i.e. tdTomato+ (Figure 2.9 A-C) and/or positive for SMC marker genes. These analyses yielded a number of expected as well as completely unexpected results including the following. First, consistent with previous studies[11,21,22,89], we observed that significant numbers of bone marrow derived cells had activated ACTA2 and were eYFP- tdTomato+ ACTA2+ (Figure 2.9 D). Second, 64% of all cells within the neointimas of PDGFRB$^{SMC\ \Delta/\Delta}$ mice

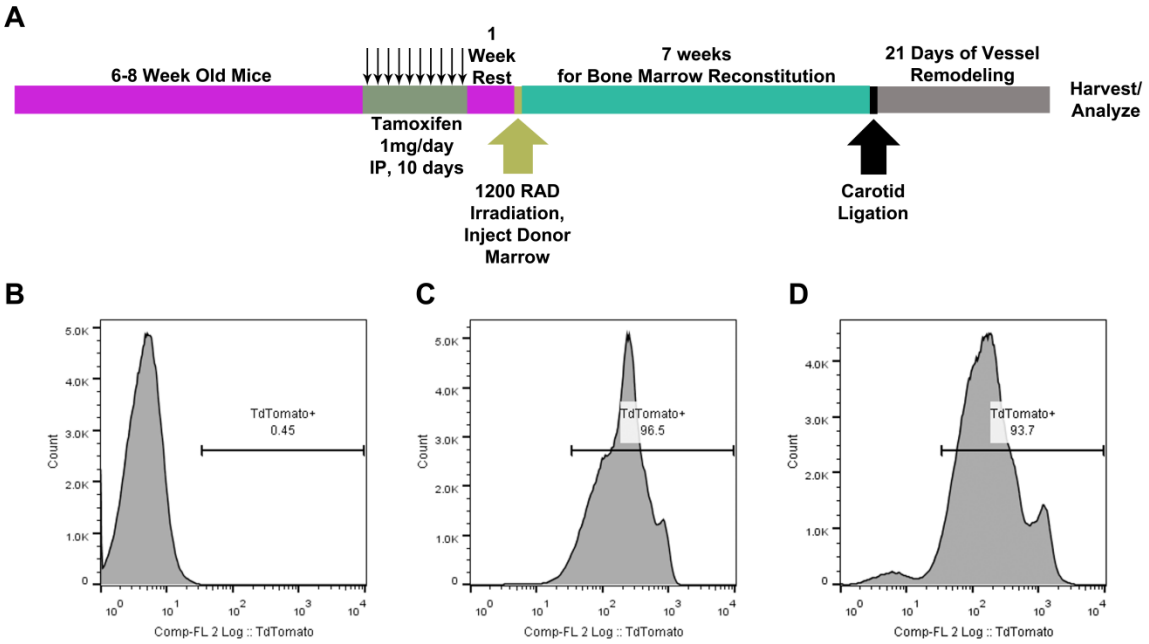

Figure 2.8: Flow cytometry confirmed endogenous bone marrow was ablated and replaced with tdTomato+ donor marrow.

Figure 2.8: Flow cytometry confirmed endogenous bone marrow was ablated and replaced with tdTomato+ donor marrow.

(A) Experimental design of the BMT experiment. (B) Negative tdTomato control. (C) Positive tdTomato control. (D) Representative image of blood taken from experimental mice which had undergone BMT.

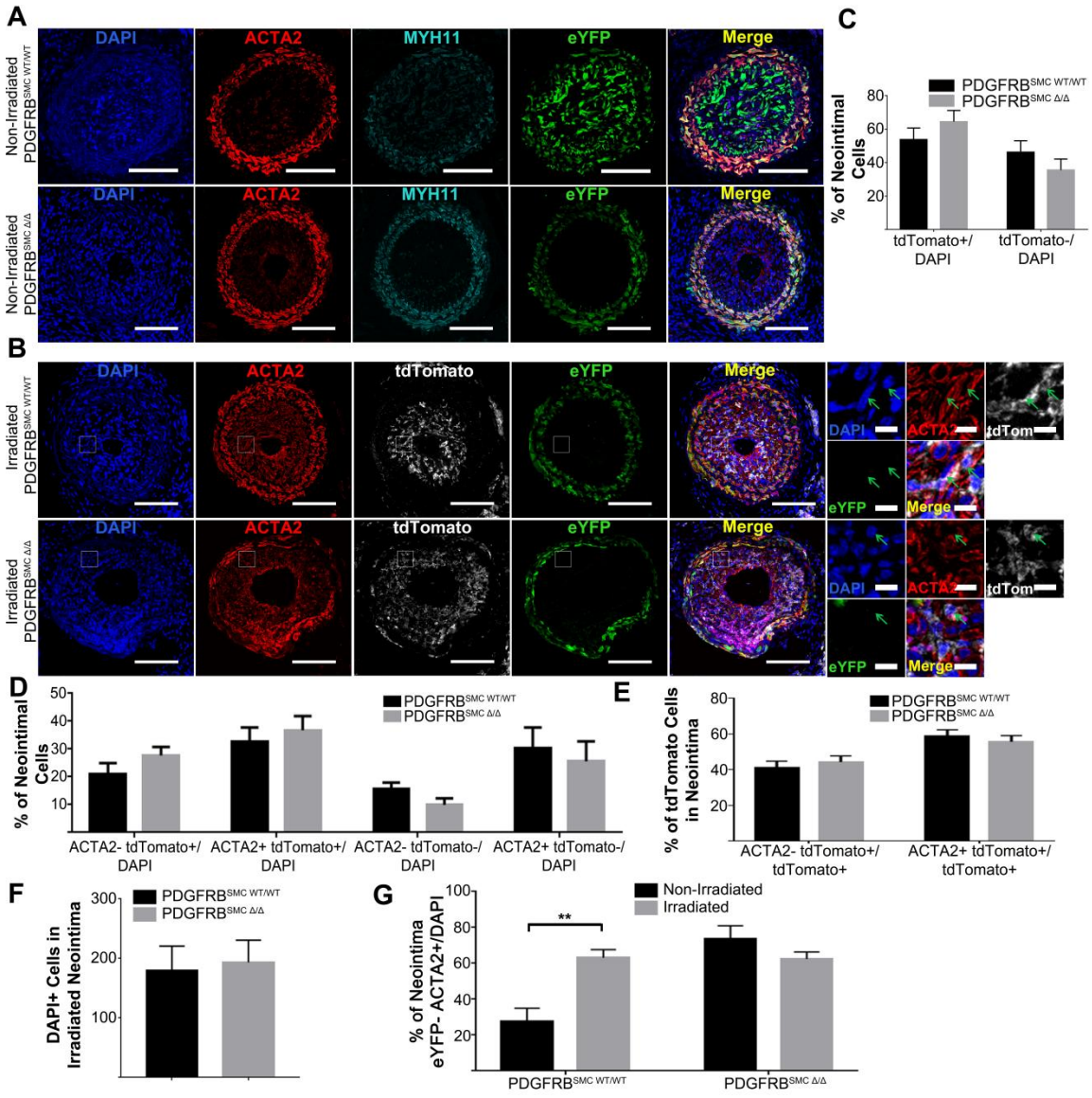

Figure 2.9: BMT irradiation completely abolished the contribution of eYFP+ SMCs to the neointima following carotid ligation.

Figure 2.9: BMT irradiation completely abolished the contribution of eYFP+ SMCs to the neointima following carotid ligation.

(A) Representative immunofluorescence staining of carotids from age-matched, nonirradiated (A) or irradiated (B) PDGFRB$^{SMC\ WT/WT}$ and PDGFRB$^{SMC\ \Delta/\Delta}$ carotids. Small images (B, right) represent magnificed areas in white boxes (B, left). Scale bars are 100μm and 10μm, respectively. Green arrows indicate eYFP-tdTomato+ACTA2+ cells. (C) Quantification of bone marrow-derived (tdTomato+) cells overall contribution to the neointima. Further, irradiation virtually completely abolished the normal SMC (eYFP+) contribution to the PDGFRB$^{SMC\ WT/WT}$ neointima (compare top panels of A and B). (D) Quantification revealed no significant difference in ACTA2+/- tdTomato+/- neointimal cells between genotypes. (E) The majority of tdTomato+ cells express ACTA2+. (F) There was no change in cell density based on DAPI+ cells between irradiated PDGFRB$^{SMC\ WT/WT}$ and PDGFRB$^{SMC\ \Delta/\Delta}$ carotids. (G) There as a significant increase in the eYFP- ACTA2+ cell contribution to the neointima in irradiated PDGFRB$^{SMC\ WT/WT}$ versus non-irradiated PDGFRB$^{SMC\ WT/WT}$ animals. (C-G) Values represent the percentage of tdTomato+ cells within total neointimal cell population basd on DAPi staining. Error bars are mean +/- s.e.m. Scale bars represent 100μm and 10μm, respectively. Green arrows indicate eYFP- tdTomato+ ACTA2+ cells. **$p<0.05$ Mann Whitney test. n=6-9 for all groups.

were tdTomato+ and thus of BM origin; of these 55% were ACTA2+ (Figure 2.9 E). As such, the primary cell type that appears to compensate for PDGFRB dependent loss of SMC contributions to the neointima appear to be of myeloid origin with many of these activating expression of the SMC marker ACTA2 in response to loss of Pdgfrb within SMC. Third, and completely unexpectedly, we found that the neointima of PDGFRB$^{SMC\ WT/WT}$ control mice (which received irradiation) were completely devoid of eYFP+ cells indicating that eYFP+ SMC-derived cells that normally contribute to neointima formation are radiosensitive (Figure 2.9 B). Fourth, there were no differences in the overall frequency of eYFP- tdTomato+ neointimal cells between irradiated PDGFRB$^{SMC\ \Delta/\Delta}$ and PDGFRB$^{SMC\ WT/WT}$ mice, but far more eYFP negative ACTA2+ neointimal cells in irradiated PDGFRB$^{SMC\ WT/WT}$ BMT mice as compared to 17-19 week old age matched controls (Figure 2.9 D-F). These latter observations suggest that similar compensatory mechanisms occurred within non-SMC with loss of the normal contribution of differentiated medial SMCs either by SMC specific conditional knockout of the Pdgfrb or by lethal irradiation. Consistent with this, we observed no differences in total neointimal cells, vessel area, media area, lumen area, or neointima area (Figure 2.9 F, Figure 2.10) between irradiated PDGFRB$^{SMC\ WT/WT}$ and irradiated PDGFRB$^{SMC\ \Delta/\Delta}$ mice. Fifth, we found significantly fewer eYFP+ cells in the neointima of PDGFRB$^{SMC\ WT/WT}$ 17-19 week old mice as compared to 10-12 week old mice used in our initial studies (only 35% eYFP+ cells, Figure 2.11 versus ~88% eYFP+ cells, Figure 2.3). Taken together these findings indicate that: 1) BM-derived cells contribute to the majority of

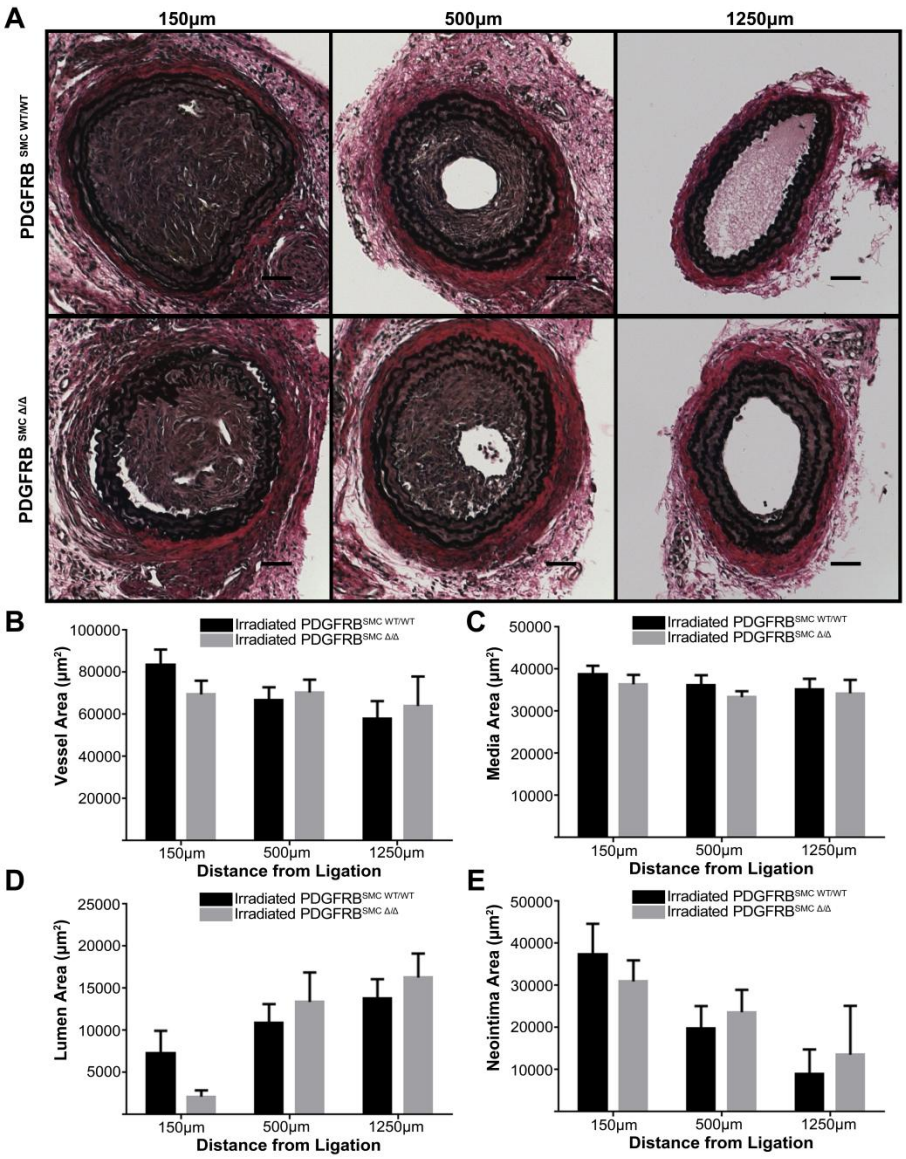

Figure 2.10: There were no morphometric differences detected between carotid arteries from irradiated PDGFRB$^{SMC\ WT/WT}$ and PDGFRB$^{SMC\ \Delta/\Delta}$ mice following carotid ligation.

Figure 2.10: There were no morphometric differences detected between carotid arteries from irradiated PDGFRB$^{SMC\ WT/WT}$ and PDGFRB$^{SMC\ \Delta/\Delta}$ mice following carotid ligation.

(A) Verhoeff's stain of representative PDGFRB$^{SMC\ WT/WT}$ and PDGFRB$^{SMC\ \Delta/\Delta}$ ligated carotids 21 days post injury. There was no statistically significant change in vessel area (B), media area (C), lumen area (D), or neointima area (E) as analyzed by Two Way ANOVA. Error bars are mean ± s.e.m. Scale Bars are 100μm. PDGFRB$^{SMC\ WT/WT}$ n=7 versus PDGFRB$^{SMC\ \Delta/\Delta}$ n=9.

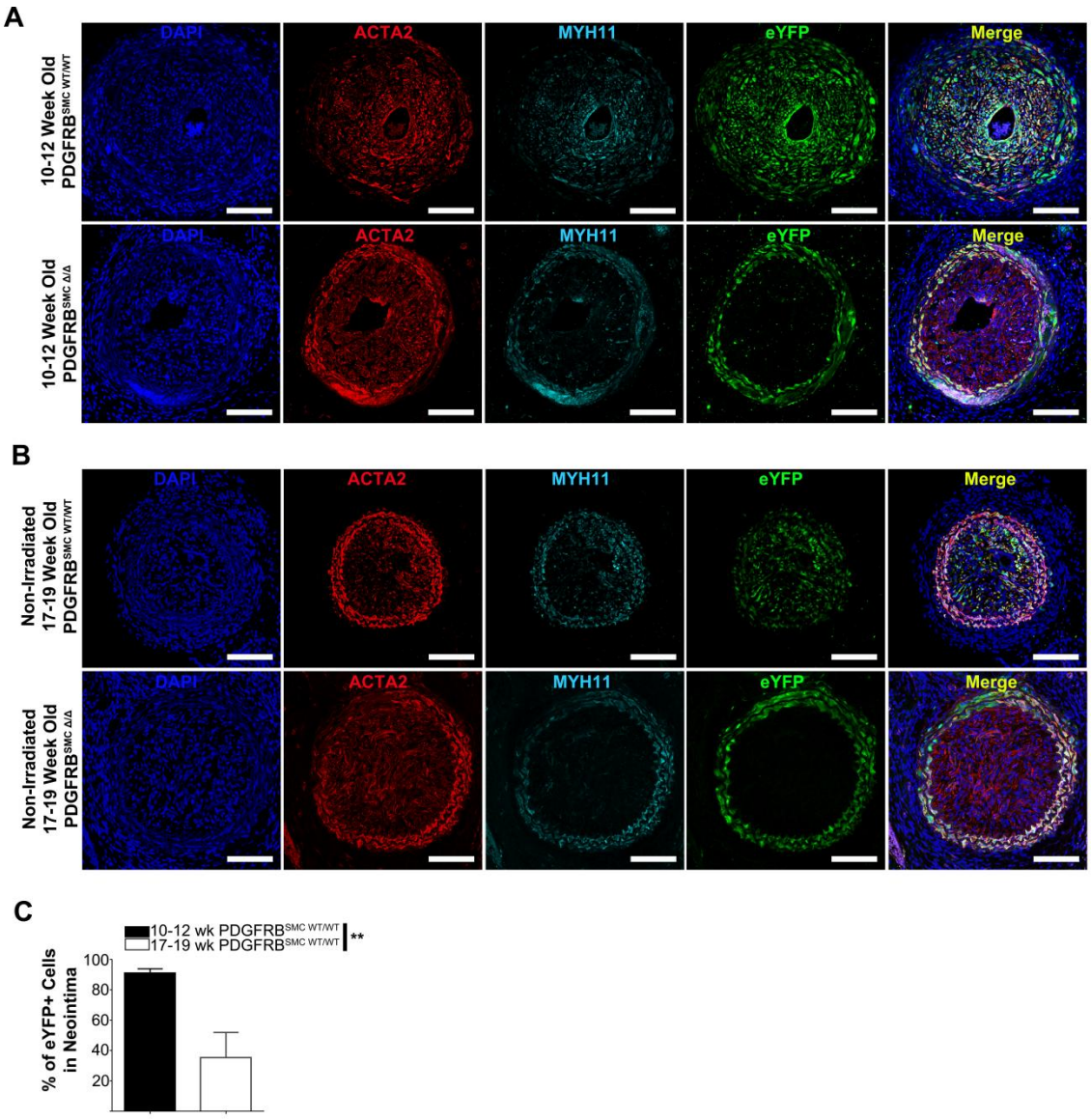

Figure 2.11: Modest increases in age were associated with significant reductions in the overall contribution of medial SMCs to the neointima upon vascular injury.

Figure 2.11: Modest increases in age were associated with significant reductions in the overall contribution of medial SMCs to the neointima upon vascular injury.

(A,B) Representative immunofluorescent staining of ligated non-irradiated10-12 week old carotids (A) or ligated non-irradiated 17-19 week old (B) $PDGFRB^{SMC\ WT/WT}$ and $PDGFRB^{SMC\ \Delta/\Delta}$ mice. (C) Quantification of the eYFP+ cell contribution to the neointima. Error bars are mean ± s.e.m. Scale bars are 100μm. **p<0.05, Mann Whitney test 10-12 week animals (n=8-9) versus 17-19 week animals (n=6). 10-12 week animals n=8-9 per group, 17-19 week animals n=6 per group.

neointimal cells in both PDGFRB$^{SMC\ WT/WT}$ and PDGFRB$^{SMC\ \Delta/\Delta}$ neointimas following BMT; 2) irradiation abolishes the normal response of differentiated medial SMCs to vascular injury; and 3) modest increases in age have a large impact on the overall contribution of differentiated medial SMCs to the neointima upon vascular injury, i.e. the response of these cells declines rapidly with relatively modest increases in age.

There is a relatively abundant subset of SMCs which proliferate in response to ligation injury

A recent study by Greif and co-workers[90] employing multiple clonal SMC lineage tracing mouse models showed that clonal expansion of a PDGFRB+ ACTA2+ MYH11+ arteriolar SMCs is responsible for muscularization of pulmonary capillaries during development of hypoxia-induced pulmonary hypertension. In addition there have been numerous claims that clonal expansion of a rare subset of SMCs or SMC stem cells contribute to neointima formation[31,32,82,91–95], although none of these studies included rigorous SMC specific lineage tracing. Based on these findings, we next sought to determine if there is a rare subset of SMCs which respond to ligation injury, or if a significant subset of differentiated medial SMCs respond to injury. To address this question, we implanted osmotic mini-pumps containing BrdU into our 10-12 week old Myh11 eYFP SMC lineage tracing mice and performed ligations. Since the pumps will deliver a constant infusion of BrdU[96] , we were able to determine whether or not an individual cell has undergone replication at any point during the

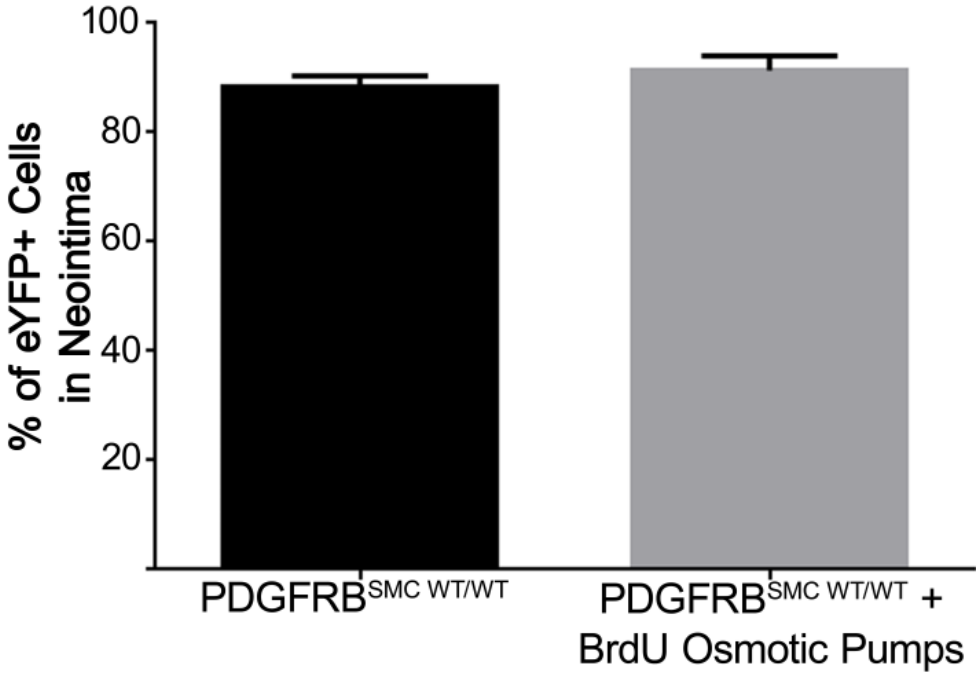

Figure 2.12: The presence of BrdU did not alter the contribution of eYFP+ cells to the neointima.

Figure 2.12: The presence of BrdU did not alter the contribution of eYFP+ cells to the neointima.

Quantification of eYFP+ cells in the neointima of PDGFRB$^{SMC\ WT/WT}$ mice. Error bars are mean +/- s.e.m. No statistical differences were determined by Unpaired *t*-test. PDGFRB$^{SMC\ WT/WT}$ n=8 versus PDGFRB$^{SMC\ WT/WT}$ + BrdU Osmotic Pumps n=5.

remodeling process. After confirming that BrdU did not alter the eYFP composition of neointimal lesions (Figure 2.12), we then stained and quantified the number of cells which had undergone proliferation (BrdU+) in the media and found that ~38% of the medial eYFP+ cells were BrdU+ in the 10-12 week old PDGFRB$^{SMC\ WT/WT}$ mice and ~14% were BrdU+ in the 10-12 week old PDGFRB$^{SMC\ \Delta/\Delta}$ animals (Figure 2.13 A, B). In addition, when examining the BrdU+ cells in the neointima we found that ~74% of the neointimal cells were BrdU+ in the 10-12 week old PDGFRB$^{SMC\ WT/WT}$ mice whereas only 13% of neointimal cells were BrdU+ in the 10-12 week old PDGFRB$^{SMC\ \Delta/\Delta}$ mice (Figure 2.13 C), despite the neointimas having no difference in area or cell density. These results suggest that recruitment, not proliferation, of non-SMC was the predominant mechanism for compensation for PDBFBR-dependent loss of the normal SMC contribution to neointima. In order to confirm that this surprising observation was not a result of failure of the Alzet osmotic mini-pumps to continuously deliver BrdU during the entire post-ligation period, we confirmed nearly complete labeling of the gut epithelium with BrdU (2.14 A, B).

The subset of SMCs that proliferate in response to ligation injury are radiosensitive

We then wanted to further validate our earlier findings that the subpopulation of SMCs that normally respond to injury are radiosensitive. To do this, we first performed a BMT, and after reconstitution with tdTomato BM we implanted BrdU osmotic mini-pumps and then performed ligations. Strikingly, we saw almost complete ablation of eYFP+ BrdU+ cells in the media of irradiated

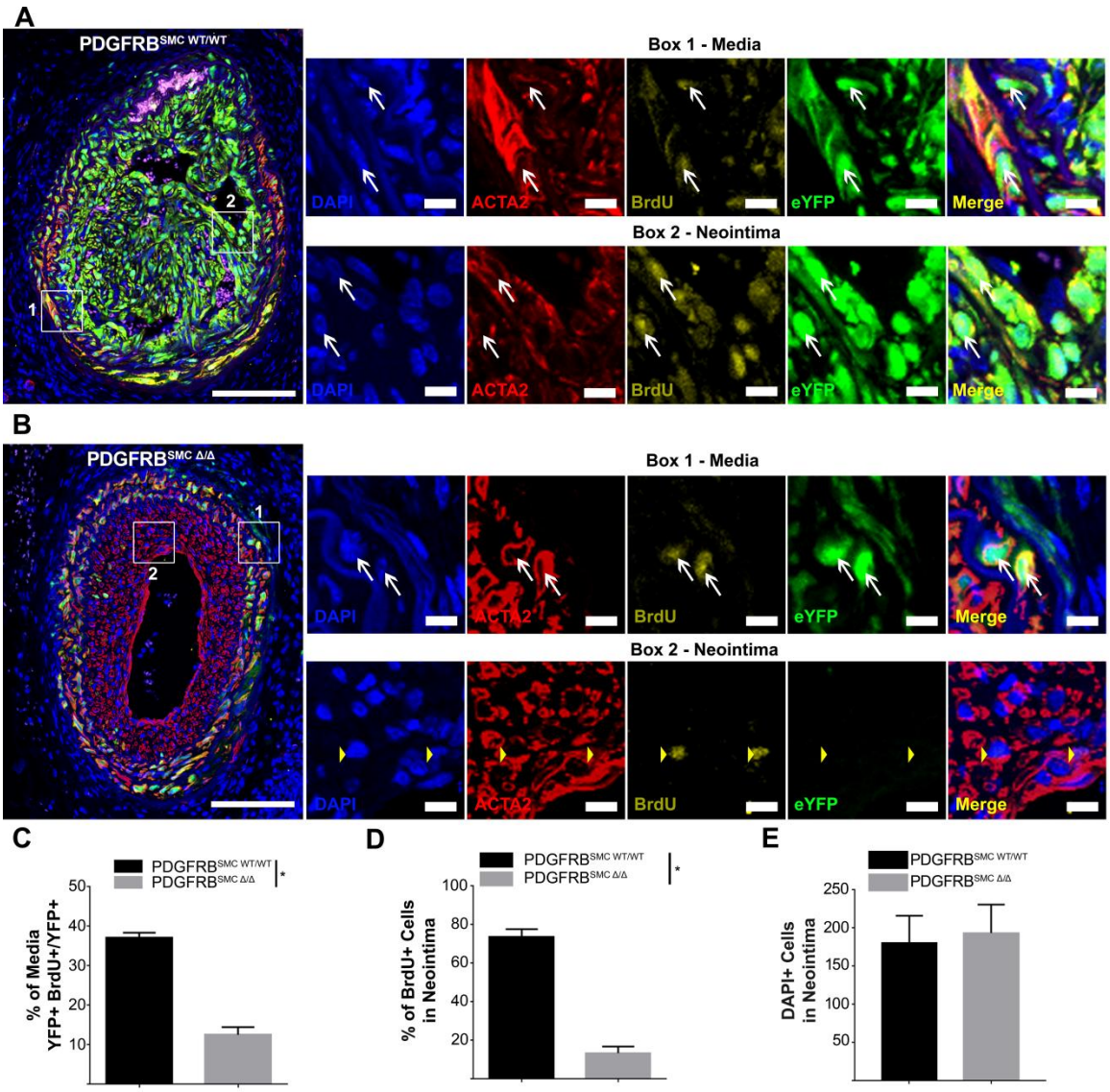

Figure 2.13: 38% of medial cells in young nonirradiated PDGFRB$^{SMC\ WT/WT}$ mice were BrdU labeled following continuous infusion of BrdU during the 21-day post injury period.

Figure 2.13: 38% of medial cells in young nonirradiated PDGFRB$^{SMC\ WT/WT}$ mice were BrdU labeled following continuous infusion of BrdU during the 21-day post injury period.

(A, B) Representative immunofluorescence staining of ligated carotids from 10-12 week old PDGFRB$^{SMC\ WT/WT}$ (A) and PDGFRB$^{SMC\ \Delta/\Delta}$ (B) carotid ligations which received continuous infusion of BrdU. Small images (right) represent magnified areas in white boxes (left). Scale bars are 100μm and 10μm, respectively. White arrows indicate media-derived neointimal SMCs which underwent proliferation during the course of vascular remodeling (YFP+ BrdU+). Yellow arrows indicate YFP- BrdU+ cells. (C) Quantification of YFP+ BrdU+ medial cells. (D) Quantification of total BrdU+ cells in the neointima. (E) Neointimal cell density. Error bars are mean ± s.e.m. *$p<0.0001$ Unpaired t test. PDGFRB$^{SMC\ WT/WT}$ n=5 versus PDGFRB$^{SMC\ \Delta/\Delta}$ n=5.

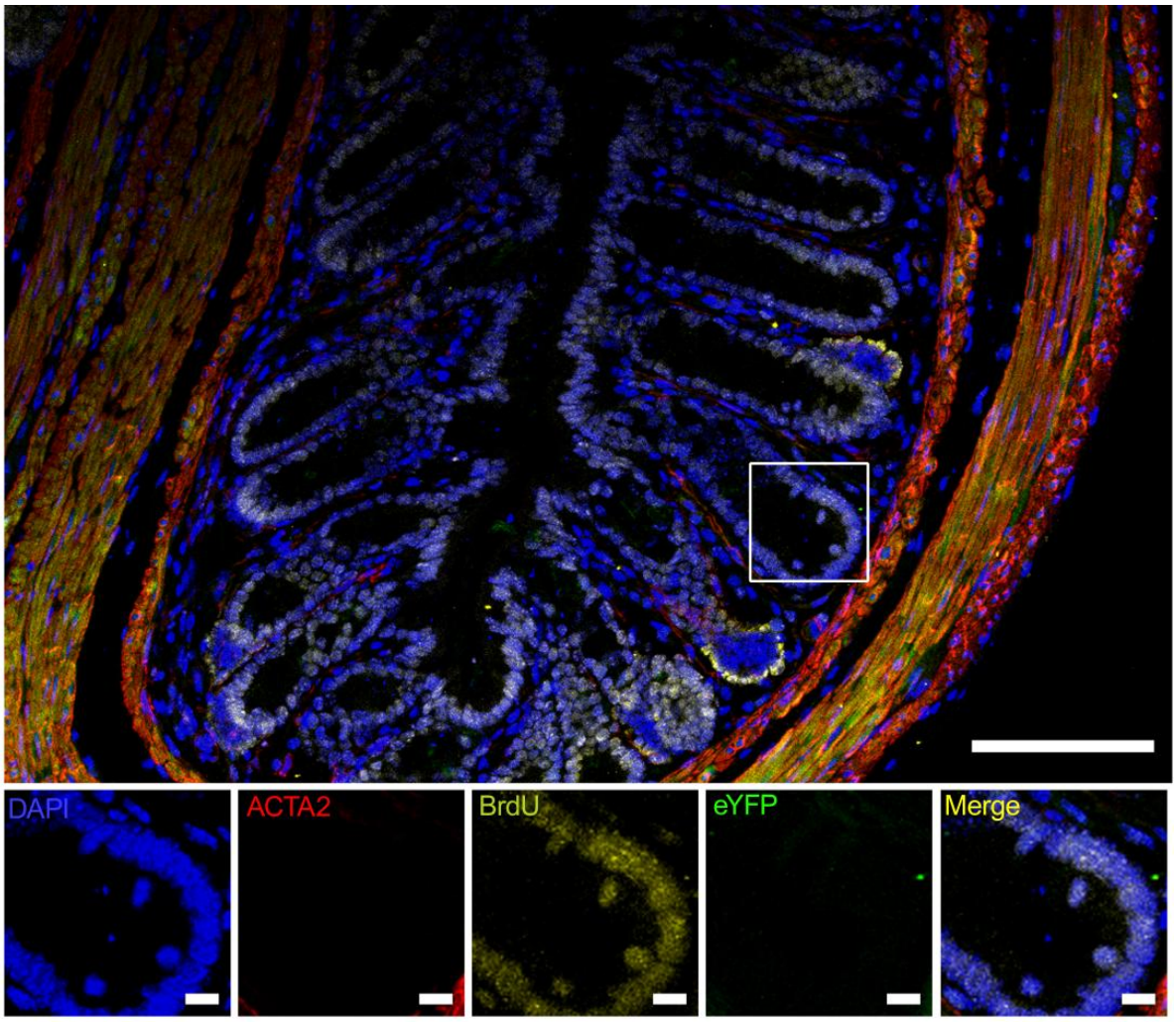

Figure 2.14: BrdU staining of the gut epithelium confirmed that BrdU was present throughout the 21-day post-ligation vessel remodeling period.

Figure 2.14: BrdU staining of the gut epithelium confirmed that BrdU was present throughout the 21-day post-ligation vessel remodeling period.

Representative image from a PDGFRB$^{SMC\ \Delta/\Delta}$ animal. Small images (bottom) represent magnified areas in white boxes (top). Scale bar 100μm and 10μm, respectively.

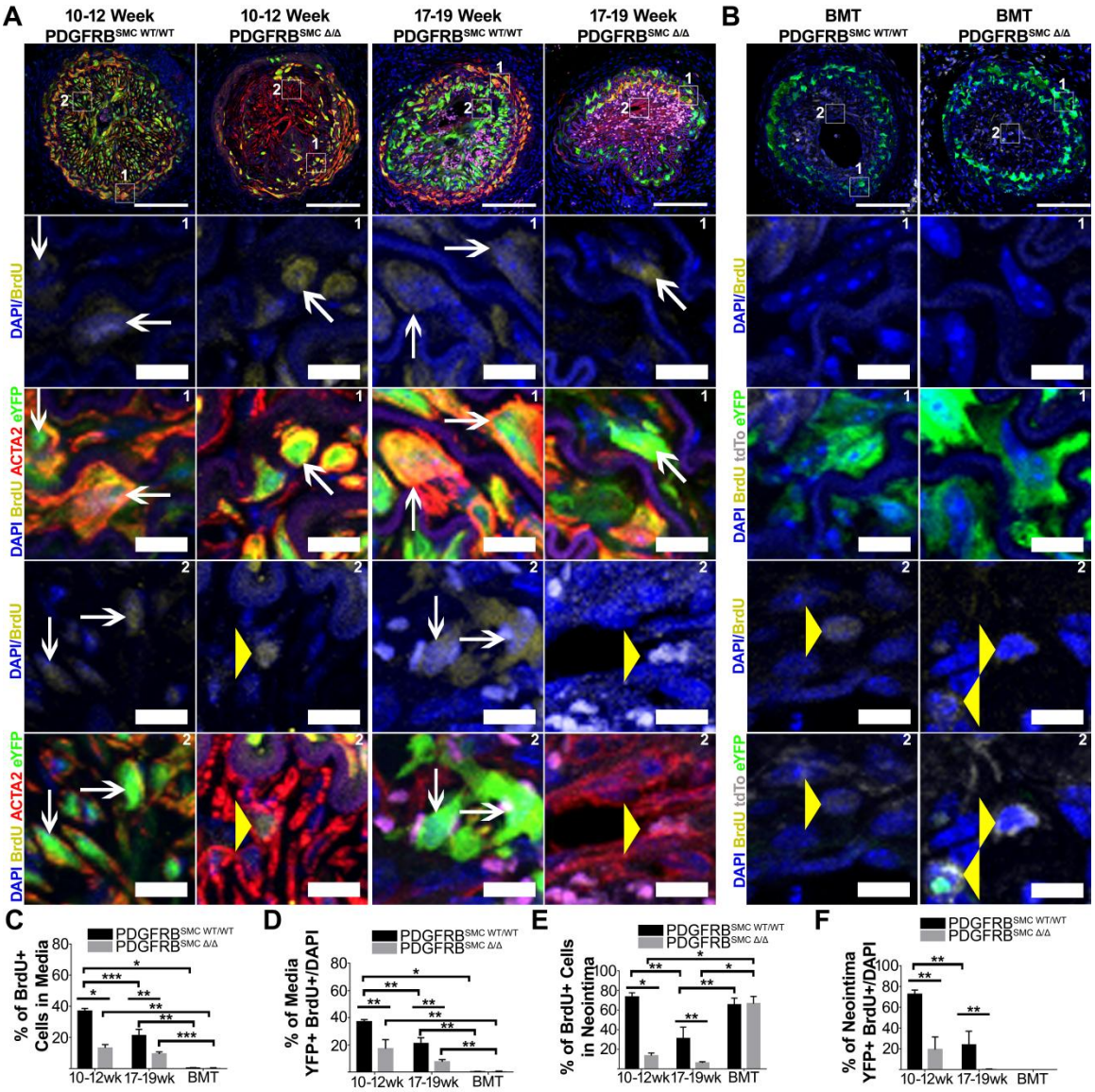

Figure 2.15: Lethal irradiation as part of BMT protocols completely inhibited proliferation of differentiated eYFP+ medial SMC and activated a marked increase in proliferation of a compensatory non-SMC population.

Figure 2.15: Lethal irradiation as part of BMT protocols completely inhibited proliferation of differentiated eYFP+ medial SMC and activated a marked increase in proliferation of a compensatory non-SMC population. (A, B) Representative immunofluorescence staining of carotids from 10-12 week old (A), 17-19 week old non-irradiated (A), and 17-19 week old irradiated PDGFRB$^{SMC\ WT/WT}$ and PDGFRB$^{SMC\ \Delta/\Delta}$ mice (B) which received continuous BrdU infusion throughout vessel remodeling 21 days post ligation injury. Small images (bottom) represent magnified areas in white boxes (top). Scale bars are 100μm and 10μm, respectively. White arrows indicate YFP+ BrdU+ cells. Yellow arrowheads indicate YFP- BrdU+ cells. tdTo=tdTomato. (C) Quantification of total BrdU+ medial cells. (D) Quantification of total YFP+ BrdU+ medial cells. (E) Quantification of total neointimal BrdU+ cells. (F) Quantification of total YFP+ BrdU+ neointimal cells. Error bars are mean ± s.e.m. *p<0.0001 Unpaired t test. **p<0.05 Mann Whitney test. ***p<0.001 Unpaired t test. All groups, n=5-9.

PDGFRB$^{SMC\ WT/WT}$ and irradiated PDGFRB$^{SMC\ \Delta/\Delta}$ vessels (Figure 2.15 A-D). Moreover, knockout of SMC-specific Pdgfrb resulted in a significant reduction of eYFP+ BrdU+ cells in the media of both 10-12 week old and non-irradiated 17-19 week old PDGFRB$^{SMC\ WT/WT}$ and PDGFRB$^{SMC\ \Delta/\Delta}$ animals (Figure 2.15 C, D). We also observed a significant decrease in eYFP+ BrdU+ cells when comparing 10-12 week old and 17-19 week old PDGFRB$^{SMC\ WT/WT}$ medias, but no change when comparing 10-12 week and 17-19 week PDGFRB$^{SMC\ \Delta/\Delta}$ animals (Figure 2.16 and 2.17). These findings help explain the absence of eYFP+ cells in irradiated PDGFRB$^{SMC\ WT/WT}$ neointimas by demonstrating that irradiated, fully differentiated medial SMCs do not enter the cell cycle in response to vascular injury. We also found that: 1) modest increases in age significantly reduced the proportion of medial SMCs that proliferated in response to injury (Figure 2.15 A-D); 2) SMC-specific knockout of Pdgfrb does not impact the proliferative capacity of irradiated neointimas (Figure 2.15 E, F); 3) the majority (~72 and ~78% respectively) of the proliferative cells in irradiated neointimas are derived from the bone marrow (Figure 2.18); 4) SMC-specific knockout of Pdgfrb results in a reduction of SMC proliferation in both 10-12 week old and 17-19 week old mice (Figure 2.15 E, F); and 5) the non-SMC derived neointima cells within irradiated PDGFRB$^{SMC\ \Delta/\Delta}$ mice are more proliferative than those neointimal cells of 10-12 week old or 17-19 week old PDGFRB$^{SMC\ \Delta/\Delta}$ mice (Figure 2.15 E). These latter findings suggest that BMT and irradiation alter the proliferative status/capability of the BM-derived cells which compose the neointima.

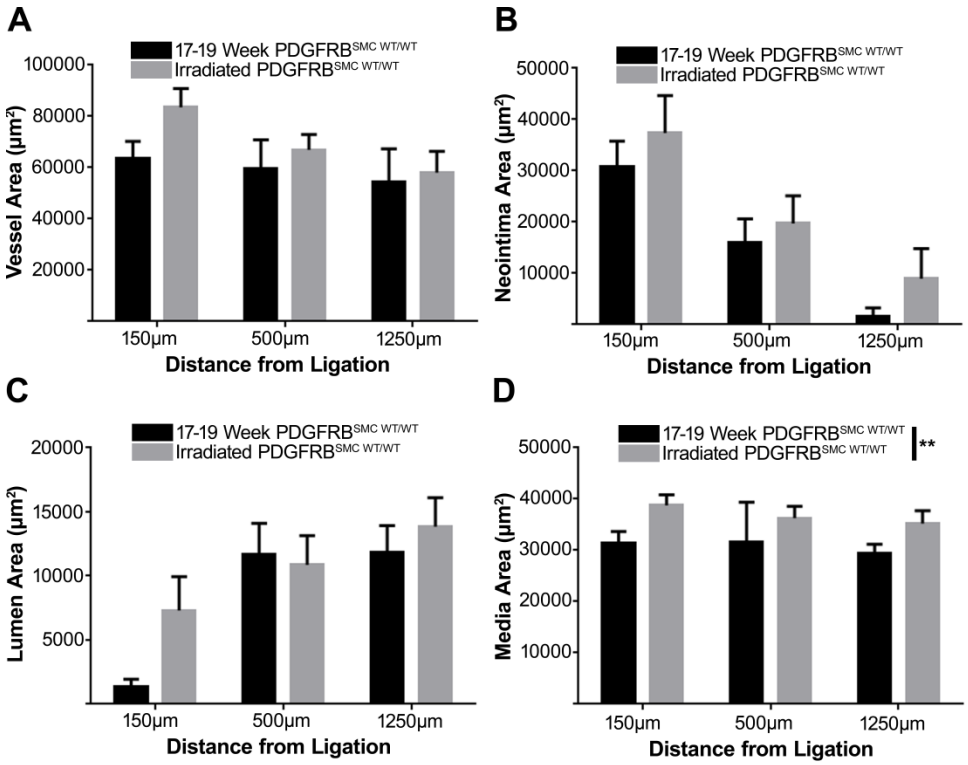

Figure 2.16: Irradiated PDGFRB$^{SMC\ WT/WT}$ carotids had a larger media area than age matched controls.

Figure 2.16: Irradiated PDGFRB$^{SMC\ WT/WT}$ carotids had a larger media area than age matched controls.

Morphometric analysis revealed no differences in vessel area (A), neointima area (B), or (C) lumen area between non-irradiated and irradiated PDGFRB$^{SMC\ WT/WT}$ mice following carotid ligation. (D) Irradiated PDGFRB$^{SMC\ WT/WT}$ medias have a larger area than age matched controls. Error bars are mean ± s.e.m. **p<0.05, Two Way ANOVA. 17-19 week PDGFRB$^{SMC\ WT/WT}$ n=6 versus Irradiated PDGFRB$^{SMC\ WT/WT}$ n=7.

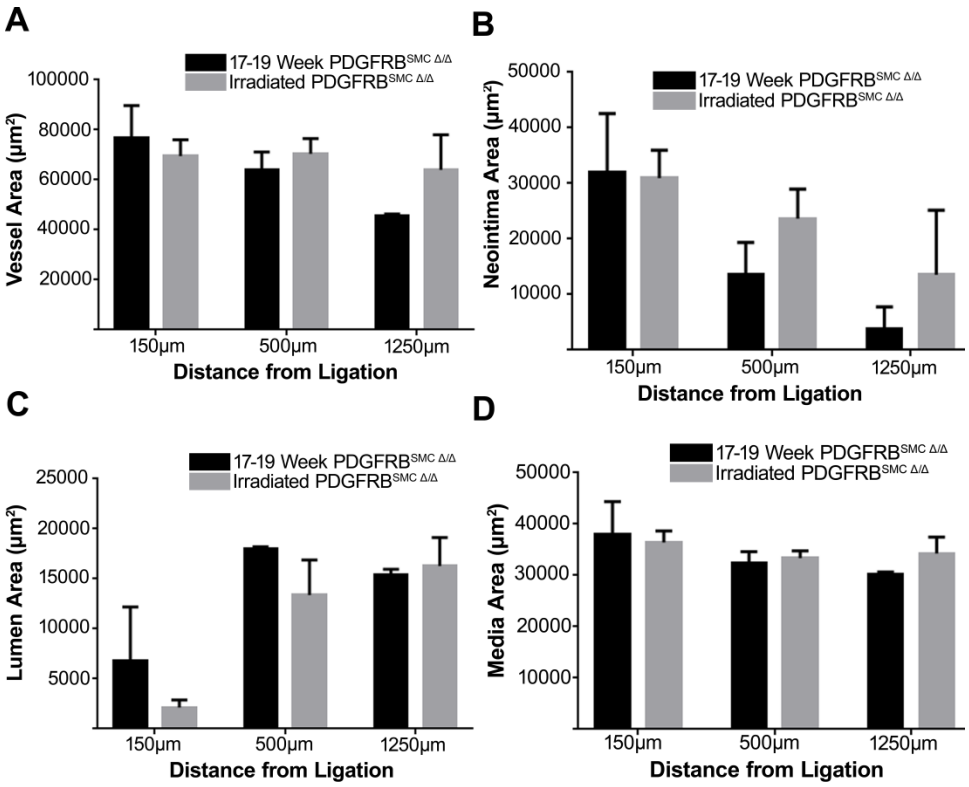

Figure 2.17: There were no morphometric differences detected between age matched non-irradiated and irradiated PDGFRB$^{SMC\ \Delta/\Delta}$ carotids.

Figure 2.17: There were no morphometric differences detected between age matched non-irradiated and irradiated PDGFRB$^{SMC\ \Delta/\Delta}$ carotids.

(A) Vessel area, neointima area (B), lumen area (C), and neointima area (D) were unaffected by irradiation in PDGFRB$^{SMC\ \Delta/\Delta}$ carotids. Error bars are mean ± s.e.m. Two Way ANOVA was used to confirm no statistical difference. 17-19 week PDGFRB$^{SMC\ \Delta/\Delta}$ n=6 versus Irradiated PDGFRB$^{SMC\ \Delta/\Delta}$ n=9.

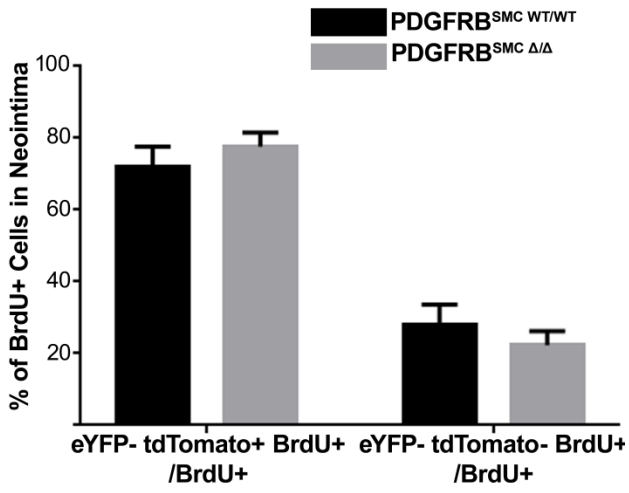

Figure 2.18: The proliferative neointimal cells in BMT mice were predominantly of bone marrow origin.

Figure 2.18: The proliferative neointimal cells in BMT mice were predominantly of bone marrow origin.

There is no statistical significance between PDGFRB$^{SMC\ WT/WT}$ and PDGFRB$^{SMC\ \Delta/\Delta}$ mice in either eYFP- tdTomato+ BrdU+/BrdU+ or eYFP- tdTomato- BrdU+/BrdU+ cells via Unpaired *t*-test. Bars are mean ± s.e.m.

A critical, often overlooked assessment of cell proliferation is to evaluate what fraction of the original cell population enters the cell cycle, a term referred to as the growth fraction[64,66]. In order to determine the SMC growth fraction (i.e. the percentage of initial medial SMCs which proliferated in response to vascular injury) within PDGFRB$^{SMC\ WT/WT}$ and PDGFRB$^{SMC\ \Delta/\Delta}$ mice we used the results of our continuous BrdU labeling studies (Figure 2.19 A-C) to calculate the SMC growth fraction using the method described previously by Alexander Clowes' group[66] (Figure 2.19 D) when they calculated an overall cellular growth fraction in a rat balloon injury model. We first determined the non-growth fraction by subtracting the quotient of the average number of BrdU- YFP+ cells divided by the average number of eYFP+ cells present in sham arteries. We next calculated the growth fraction as 1 minus the non-growth fraction. Using this formula, we then determined that ~23% of the initial medial SMCs in the 10-12 week old PDGFRB$^{SMC\ WT/WT}$ animal (Figure 2.19 E) underwent proliferation in response to ligation injury whereas only ~1% of medial SMCs underwent proliferation in the 10-12 week old PDGFRB$^{SMC\ \Delta/\Delta}$ ligations (Figure 2.19 F). Similarly, we determined the growth fractions to be 18% and 2% in our 17-19 week old PDGFRB$^{SMC\ WT/WT}$ and PDGFRB$^{SMC\ \Delta/\Delta}$ mice used in BMT experiments (Figure 2.19 G-K). Taken together, these results demonstrate that: 1) there exists a significant subset of differentiated medial SMCs which proliferate in response to injury, i.e. the proliferation capable differentiated medial SMC population is not a rare population; and 2) the subset of differentiated medial SMCs which normally respond to injury are radiosensitive.

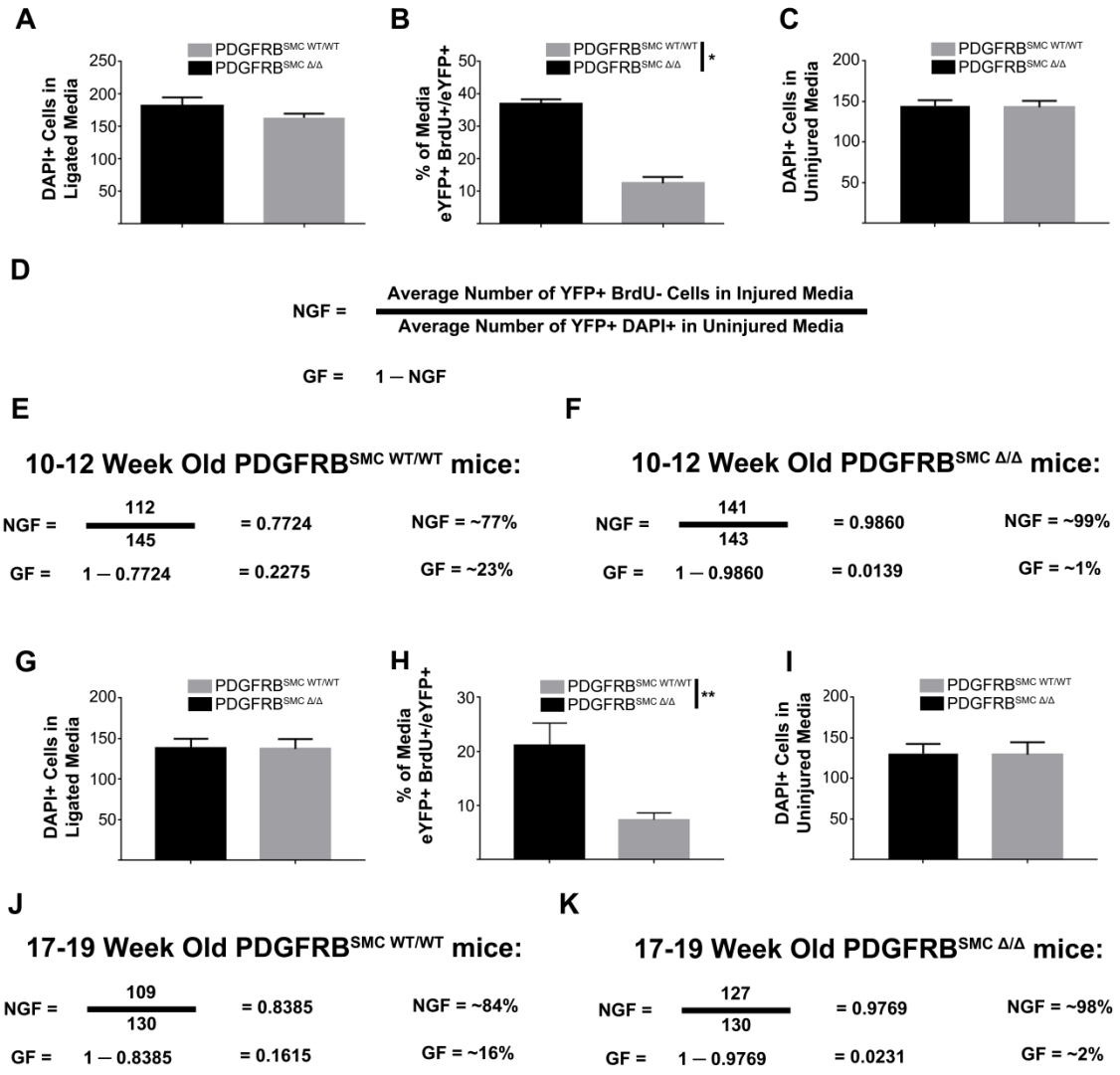

Figure 2.19: Modest increases in the age of mice were associated with a significant decrease in the fraction of medial SMCs that respond to carotid ligation injury.

Figure 2.19: Modest increases in the age of mice were associated with a significant decrease in the fraction of medial SMCs that respond to carotid ligation injury.

(A) The average number of total cells present in the ligated media of 10-12 week old mice at the time of ligation. (B) The average percentage of medial SMC-derived cells in 10-12 week old animals which have incorporated BrdU in response to injury. (C) The average number of cells in 10-12 week old uninjured vessels. (D) Non-growth fraction (NGF) and growth fraction (GF) equation. The non-growth fraction and growth fraction of 10-12 week old $PDGFRB^{SMC\ WT/WT}$ (E) and $PDGFRB^{SMC\ \Delta/\Delta}$ animals (F). (G) The average number of cells present in the ligated media of 17-19 week old mice at the time of ligation. (H) The average percentage of medial SMC-derived cells in 17-19 week old animals which have incorporated BrdU in response to injury. (I) The average number of medial cells in 17-19 week old uninjured vessels. Non-growth fraction and growth fraction of 17-19 week old $PDGFRB^{SMC\ WT/WT}$ (J) and $PDGFRB^{SMC\ \Delta/\Delta}$ animals (K). Error bars are mean ± s.e.m. *$p<0.0001$ Unpaired t test. **$p<0.05$ Mann Whitney test. n=5-9 per group.

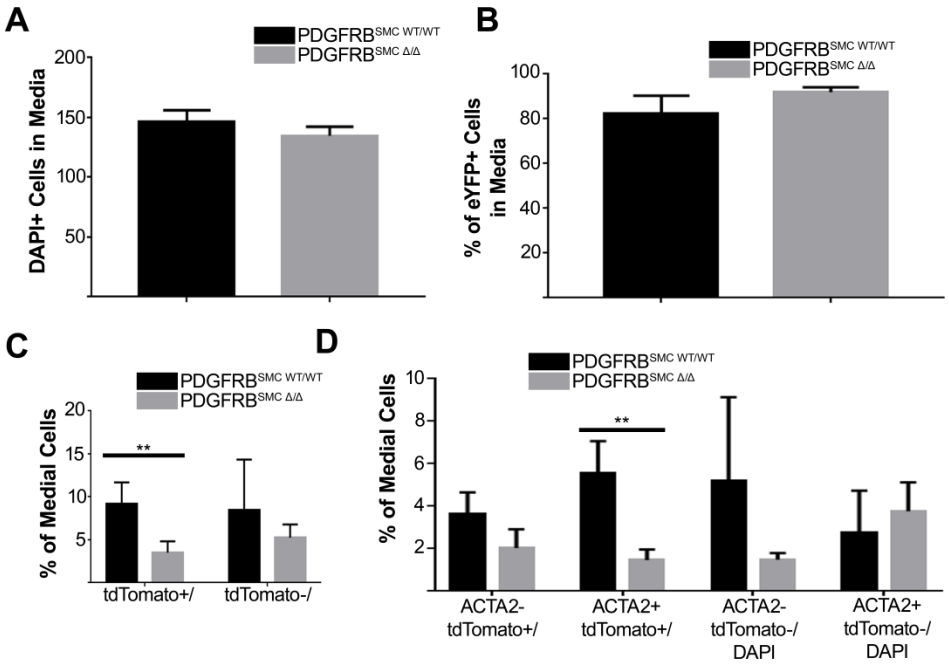

Figure 2.19: There are more bone marrow cells in the media of PDGFRB$^{SMC\ WT/WT}$ carotids than PDGFRB$^{SMC\ \Delta/\Delta}$ carotids following irradiation.

Figure 2.20: There are more bone marrow cells in the media of PDGFRB$^{SMC\ WT/WT}$ carotids than PDGFRB$^{SMC\ \Delta/\Delta}$ carotids following irradiation.

Media cell density (A) and total eYFP+ medial contribution (B) remain unchanged in irradiated PDGFRB$^{SMC\ WT/WT}$ and PDGFRB$^{SMC\ \Delta/\Delta}$ carotids. (C,D) There were less total bone marrow-derived (tdTomato+) cells in the media of ligated PDGFRB$^{SMC\ \Delta/\Delta}$ carotids (C). (D) There are less tdTomato+ ACTA2+ cells in the media of PDGFRB$^{SMC\ \Delta/\Delta}$ ligated vessels. **p<0.05 Mann Whitney test. Error bars are mean ± s.e.m. PDGFRB$^{SMC\ WT/WT}$ n=7 versus PDGFRB$^{SMC\ \Delta/\Delta}$ n=9.

Discussion

The initial goal of this work was to provide direct evidence either supporting or refuting the dogma that SMC-specific PDGFRB signaling is required for SMC phenotypic switching following vascular injury *in vivo*. Utilizing our conditional, SMC-specific Myh11 eYFP lineage tracing Pdgfrb knockout mouse, we demonstrated for the first time that SMC-specific PDGFRB is required for SMC investment into the neointima; indeed, the neointima of PDGFRB$^{SMC\ \Delta/\Delta}$ mice virtually completely lacked eYFP+ SMCs within neointimal lesions (Figure 2.3, Figure 2.9, Figure 2.13, and Figure 2.20). We also confirmed recent studies of Herring et al.[34] showing that proliferative SMCs within the neointima following vascular injury are derived from fully differentiated medial SMCs that have undergone phenotypic switching, and not a rare stem cell population as has been previously postulated[32]. However, we made a number of completely surprising and unexpected observations including the following. First, in the absence of SMC-specific PDGFRB dependent investment of SMCs into the neointima, other cell types including those of myeloid origin migrate into the neointima and give rise to ACTA2+ cells and completely compensate for loss of SMCs in terms of overall lesion size, lumen area, and other morphometric parameters. Second, we show that the subset of differentiated medial SMCs which normally respond to injury are radiosensitive and fail to invest into the neointima following lethal irradiation and BM reconstitution. These observations have a number of important implications as discussed below.

The capability of global blockade of PDGFRB to reduce neointima area has been well established[56–59,80,85,97] and this, combined with the results from

Herring et al[34] which definitively demonstrated that pre-existing differentiated medial SMCs give rise to neointimal cells, have further strengthened the dogma that inhibition of SMC-specific PDGFRB results in a reduction of neointima. Our results directly challenge this paradigm, however, and argue that while inhibition of SMC-specific PDGFRB does profoundly reduce the SMC contribution to the neointima, it does not affect the overall neointima area. Instead, our results suggest that it is inhibition of PDGFRB in all cell types which express PDGFRB that leads to a reduction in neointima and not just SMCs. Indeed, our results from Figure 2.1 demonstrate that at least a subset of the eYFP- cell types composing the neointima of PDGFRB$^{SMC\ \Delta/\Delta}$ animals are distinctly PDGFRB positive meaning the compensation for loss of SMC-PDGFRB is likely dependent on PDGFRB signaling. Furthermore, we have provided evidence supporting the current literature in that defects in PDGFRB in SMCs result in defects in both migration[66,80] and proliferation[64,98,99].

The logical next question, then, is what is the origin/identity of the eYFP-ACTA2+ cells? Based on the literature, we propose that the eYFP- cells are most likely derived from one (or more) of three perivascular cell sources.

First, a recent study by Mike Simon's group[27] has provided *in vivo* evidence of the occurrence of endothelial-to-mesenchymal transition (EndoMT) in the context of vascular injury[12]. Utilizing a conditional Cdh5-CreERT2 endothelial cell (EC) lineage tracing system, they found that approximately 6% of the neointima in the femoral wire injury was derived from ECs. Furthermore, they found that approximately half of these cells (2%-3% of the total neointima)

retained expression of CD31 (eGFP+ CD31+), an common marker of endothelial cells. Staining of our PDGFRB$^{SMC\ WT/WT}$ and PDGFRB$^{SMC\ \Delta/\Delta}$ neointimas for CD31 did not reveal any CD31+ ACTA2+ cells (data not shown) within the neointima suggesting that EndoMT is unlikely the primary mechanism of compensation in PDGFRB$^{SMC\ \Delta/\Delta}$ neointimas. Unfortunately, we are unable to directly test the contribution of EndoMT in our studies since it would require development of a completely novel, non-Cre inducible EC lineage tracing mouse model to replace the tamoxifen inducible Cdh5-CreERT2 mouse since crossing this mouse to ours would induce knockout of the Pdgfrb in both ECs and SMCs and we would not be able to distinguish SMC- versus EC-derived neointimal cells.

Second, as described by multiple labs, there exists a population of SCA1+ resident adventitial cells [24–26,30,100–103] which under culture conditions are capable of activating the full repertoire of SMC marker genes, including myocardin, a master regulator of SMC differentiation[53,104]. One study found that in response to femoral wire injury, a few SCA1+ cells were detectable in the media morphologically perpendicular to the surrounding SMCs, which they concluded to be a SCA1+ adventitia cell migrating through the media to the neointima[11]. However, these results are ambiguous since multiple cell types including SMCs are known to activate SCA1 and other mesenchymal stem cell like genes[6,105]. While lineage tracing of the SCA1+ adventitial cell population would be ideal, there is no definitive, adventitial stem cell-specific marker currently described which to conduct lineage tracing studies of these cells. While we speculate that

a portion of the eYFP- cells are derived from a SCA1+ adventitial stem cell source, there is not currently a way to rigorously test this possibility. Indeed, although these cells are of major potential interest, at present there is no direct evidence that these cells play a critical functional role during the course of neointima formation in response to vascular injury.

Third, our data (Figure 2.8) show that a significant subset of neointimal cells within the neointimas of both $PDGFRB^{SMC\ WT/WT}$ and $PDGFRB^{SMC\ \Delta/\Delta}$ mice are of myeloid origin including many that activate SMC marker genes. These data are consistent with several previous BMT studies in the field showing that [22,106] BM-derived cells contribute to the neointima with varying degrees of ACTA2 expression[20] depending on the model and lab performing the experiments. For example, Tanaka et al.[23] used a mouse model of parabiosis and joined a transgenic eGFP mouse (eGFP in all tissues) with a C57BL/6 mouse and after femoral wire injury found a subset of both neointima and media cells that were eGFP+ ACTA2+ indicating that circulating cells do contribute to neointima formation following vascular injury. Similarly, Iwata et al.[21] transferred bone marrow from an ACTA2-eGFP mouse into irradiated wild type mice and found that ~20% of the neointima formed in response to wire injury expressed eGFP, clearly indicating at least a subpopulation of bone marrow derived cells are capable of investing the neointima and expressing ACTA2. However, these previous BMT experiments did not include SMC lineage tracing and as such were completely unaware that lethal irradiation was also abrogating the normal medial SMC response. That is, previous BMT experiments such as these were

not studying the normal response to vascular injury but rather a compensatory mechanism which is activated when SMCs fail to invest within the neointima.

Indeed, perhaps our most unexpected finding was that differentiated medial SMCs that proliferate following vascular injury are radiosensitive. Whereas it has been well documented that irradiation can reduce neointima formation in the context of an acute vascular injury[107–112], the assumption has been that this is due to effects on a smaller subset of cells induced to proliferate by the vascular injury. Indeed, prior to the advent of drug eluting stents, gamma radiation had been used in the clinic as a way to prevent in-stent restenosis in patients receiving stents or undergoing balloon angioplasty[107]. What was surprising, however, is our finding that irradiation appears to profoundly impact virtually the entire population of differentiated medial SMCs that normally contribute to neointimal formation following vascular injury even when irradiation is done 6 weeks prior to vascular injury. Indeed, our findings indicate that there has likely been a major undefined variable in previous BMT experiments other than the simple absence or presence of a candidate gene of interest within myeloid cells as generally assumed. That is, the BMT protocol also likely involved loss of the normal contribution of medial SMCs to neointimal formation, and the associated compensatory responses to this.

There are literally thousands of previous BMT studies touching most biomedical disciplines. However, we would like to address one seminal study by Robbins et al[113] to illustrate the possible implications of these findings. We want to emphasize that our new findings do not in any way invalidate the findings of

these studies or negate their critical importance for the field. However, they do profoundly impact how the results of these studies can be interpreted. In brief, based on an elegant and complex series of BMT and parabiosis experiments, the authors provided clear evidence that the majority of lesional macrophages in established lesions were derived from local macrophage proliferation rather than by influx of monocytes-macrophages from the circulation. Somewhat paradoxically, the authors then found using BMT that within 5 months, donor derived macrophages became the dominant macrophage population within lesions. In light of our recent findings, however, it's possible that: 1) the absence/loss of a growing SMC population and the subsequent loss of cytokine secretion by these SMC-derived lesional SMCs contributed significantly to the observed responses, i.e. local proliferation of macrophages may not be the normal response but rather occurs only when the normal SMC response is abrogated; and/or 2) irradiation induced death of SMCs within established lesions is permissive for circulating monocyte-macrophage infiltration and subsequent expansion such that they are capable of outcompeting the local macrophages. Given these uncertainties, there needs to be far less dependence on lethal irradiation in BMT experiments, as well as an increased emphasis on myeloid cell specific lineage tracing and conditional gene knockout approaches, in future studies of the putative functions of myeloid cells *in vivo*.

These questions are particularly relevant given several previous studies which we believe have not been as impactful as they should have been, having demonstrated that irradiation had a severe and profound effect on overall lesion

composition[114–116]. Although our study was focused on the carotid ligation model and future studies are needed to define the impact of radiation on SMCs present within atherosclerotic lesions, Linda Curtiss's group has previously reported that irradiation alone in the LDLR-/- mouse induces drastic changes in atherosclerotic lesion composition when compared to non-irradiated controls, including an increase in MOMA-2 staining and a decrease in the cellular cap[116]. In addition, these lesions had large lipid cores and severely reduced collagen staining, indicative of unstable lesions. A second study looking in the ApoE-/- model of atherosclerosis[115] published a similar finding in that lesions of mice who had undergone BMT were highly macrophage rich, had an increased presence of inflammatory cells, and increased intraplaque hemorrhage. We postulate that these effects were due at least in part to radiation-induced loss of the normal contribution of medial SMCs to formation of a protective fibrous cap, findings which are highly consistent with our recent SMC lineage tracing studies +/- SMC conditional knockout of Oct4 or Klf4 within long term Western diet fed ApoE-/- mice[6,7,33].

Perhaps of most interest, is whether lethal irradiation in humans undergoing BMT or stem cell infusion procedures also results in loss or abrogation of the normal response of differentiated medial SMCs during vascular injury and/or remodeling. For example, it is likely this unknowingly impacted results of studies by Caplice et al.[89] in which they attempted to ascertain the contribution of myeloid cells to ACTA2+ cells within advanced atherosclerotic lesions by performing Y-chromosome lineage tracing studies in coronary artery

specimens from subjects who had undergone a cross gender bone marrow transplant. Whereas they observed that >10% of ACTA2+ lesion cells were of myeloid origin, these results may in part reflect a compensatory response to loss of the normal response of medial SMC to lesion pathogenesis. Key questions include: Do BMT subjects show impaired angiogenic responses, perivascular cell investment of newly formed vessels, wound healing, or perhaps most importantly, an increased incidence of plaque rupture and associated major adverse cardiovascular events such as myocardial infarction or stroke due to loss of a SMC-derived protective fibrous cap? Although extensive further studies including validation of our findings in human are required, consistent with this possibility, patients who received irradiation for head and neck (H&N) cancer are documented to have a higher intima to media ratio than non-irradiated patients[117] and biopsies of irradiated medium size arteries from H&N and breast cancer patients displayed an increase in inflammatory cell content and proteoglycan content[118].

Taken together with our study and that of others, we propose that gamma irradiation exacerbates lesion formation via ablation of a radiosensitive subpopulation of SMCs such that over time there is an undesirable increase in the ratio of macrophages to SMC within lesions and plaque destabilization. Future studies are needed to determine the effect of whole body irradiation on both early and late stage lesion development and defining the effect irradiation has on SMCs located in the lesion and the media of atherosclerotic vessels keeping in mind studies cannot rely on use of conventional SMC and

macrophage marker genes to identify each of these respective cell types within lesions since each cell type can activate markers of the other cell type in the context of lesions[6,8].

Conclusions

In summary, the studies described herein have directly validated that loss of SMC-specific PDGFRB significantly attenuates the capability of SMCs to contribute to the neointima following vascular injury. Surprisingly, this lack of SMCs in the neointima did **not** result in a reduced neointima area due to unexpected complete compensation by myeloid and other non-SMC sources. Further, only a subset of differentiated medial SMCs normally proliferate in response to ligation injury and this subset is radiosensitive such that irradiation virtually completely abolished the SMC contribution to the neointima. Finally, we show that modest increases in age have a significant impact on the percentage of SMCs which respond to vascular injury.

Materials and Methods

Mice:

Animal protocols were approved by the University of Virginia Animal Care and Use Committee. PdgfrbFL/FL mice were a generous donation from the labs of Dr Ralf Adams and Dr. Ruediger Kline. Our previously published Myh11-CreERT2 Rosa26 STOP-flox eYFP mice[6,7,33] were crossed to the global PdgfrbFL/FL line to generate PdgfrbWT/WTMyh11-CreERT2 Rosa26 STOP-flox eYFP mice and PdgfrbFL/FL Myh11-CreERT2 Rosa26 STOP-flox eYFP mice. Only homozygous animals were used in this study. Cre recombinase was activated in male mice

with a series of ten 1-mg tamoxifen (Sigma, cat. no. T-5648) intraperitoneal injections from 6 to 8 weeks of age, for a total of 10 mg of tamoxifen per mouse, which averaged 25 g of body weight during this 2-week period. Mice were then allowed 1 week for residual tamoxifen to leave the system before any experiments were performed. The Myh11-CreERT2 transgene is located on the Y chromosome, so only male experimental and male littermate controls were used for all studies. Homozygous congenic B6.129(Cg)-Gt(ROSA)26Sortm4(ACTB-tdTomato,-EGFP)Luo/J (Jackson Lab #007676) male mice age 4-6 weeks were purchased for BMT experiments and BM was harvested when mice were 5-7 weeks of age.

Ligation Injury:

Carotid Ligations were performed as previously described[119]. Briefly, mice were put under anesthesia using the Kent Scientific SomnoSuite Isofluorane delivery system. Once mice were anesthetized, a midline incision was made and the left carotid artery was exposed. A 7-0 suture was placed immediately proximal to the internal/external carotid bifurcation. The right carotid served as an uninjured contralateral control.

Tissue Processing:

Mice were euthanized 21 days after carotid ligation via carbon dioxide asphyxiation. Left ventricles were punctured and mice were the perfused sequentially with 5 mL of PBS, 10 mL of 4% paraformaldehyde, and 5 mL of PBS using a gravity flow system. Left and right carotids were carefully dissected out,

post-fixed in 4% paraformaldehyde for 2 hours, processed, and paraffin embedded. Paraffin embedded carotid arteries were sectioned serially at 10μm from ligation (left carotid) or internal/external bifurcation (right carotid) through the first 2.5mm for analysis.

BrdU Osmotic Minipump Studies:

BrdU (Sigma B5002) was dissolved in 10% DMSO and diluted in PBS to a final working concentration of 9.057 x 10-3 mg/μL. Alzet Osmotic Minipumps (Alzet 2004) were primed 48hrs in advance as directed by the manufacturer with 210 μL of the BrdU solution and implanted subcutaneously along the flank at the same time as carotid ligations.

Bone Marrow Transplant Studies:

Bone Marrow reconstitution was performed as previously described[21]. Briefly, recipient male mice were irradiated 2X with 600RADs (1200RADs total) 3 hours apart. 30 minutes later, unfractionated bone marrow cells (1x106 cells/mouse) extracted from both femurs of tdTomato mice were administered to each recipient mouse via tail vein injections. Bone marrow was allowed to reconstitute for 7 weeks prior to ligation injury.

Morphometric Analysis

Verhoeff Van Gieson and Hematoxylin and Eosin staining were conducted to analyze vessel morphometry at three distinct locations across all carotids. Stains were imaged using a Zeiss Axioscope2 microscope fitted with an AxioCamMR3

camera. Image acquisition was performed with AxioVision40 V4.6.3.0 software (Carl Zeiss Imaging Solution). Digitized images of these stains were all analyzed directly using Image Pro Plus Software 7.0 (MediaCybernetics Inc.). Areas of interest were drawn in the software to delineate external elastic lamina (termed vessel area), inner elastic lamina , and lumen area with medial and neointima areas extrapolated from these measurements.

Immunofluorescence Analysis

Immunofluorescence staining for PDGFRB (Abcam, ab32570) was used to verify loss of PDGFRB in damaged vessels, since PDGFRB is not expressed or lowly expressed in uninjured vessels[59].

To characterize cells within neointimas, the 500 μm location was stained with antibodies specific for eYFP/GFP (Abcam ab6673), ACTA2 (Sigma F3777), CD31 (Abcam ab6327), BrdU (Abcam ab6326), RFP (Rockland Antibodies & Assays, 600-401-379), MYH11 (Kamiya Biomedical Company MC-352) and DAPI (ThermoFisher Scientific D3571). Species matched immunoglobulin staining was used as a negative control. Imaging was conducted using a Zeiss LSM700 confocal microscope or a Zeiss LSM720 multiphoton microscope where a series of 1 μm interval z-stacks were acquired throughout the width of the tissue section for analysis. Careful analysis of each z-stack image using Zen 2009 Light Edition Software (Zeiss) was done to assess colocalization of markers for each cell. All cells within the neointima and media were counted. Researchers were blinded to genotype. An antigen unmasking solution (Vector Laboratories, H-3300) was used for and immunofluorescent staining protocols.

siRNA Transfection

The wild type mouse aortic SMCs were isolated as previously described[7]. All experiments were performed between passages 11-15. The transient transfection of siRNA oligonucleotide was carried out using Oligofectamine (Invitrogen 12252-011) according to the protocol of the manufacturer. Cells were then incubated for 24 hours before being used for experiments.

Scratch Wound Assay

The scratch wound assay was performed as previously described[80,83]. Briefly, three parallel lines were drawn across the bottom of each well of a 6-well plate. Cells were then plated at 1x104 cells per cm2 density and allowed to grow to 60%-70% confluency before siRNA transfection. Following transfection, a sterile 200 µL pipette tip was dragged from one end of the well to the other, perpendicular to the three lines. Cells were gently washed 1X with PBS, and then 2mL/well of SFM no penicillin/streptomyocin added were added to each well. Pictures were taken immediately adjacent to each side of the three lines and the number of cells present in each field of view was counted. Pictures were again taken at each location (6 pictures/well) at 24 hours post scratch, following which the media was again changed. Final pictures were taken at 48 hours. Cells were imaged on an Olympus CKX41 microscope and images were taken with a Olympus QColor 3 camera using the QCapture software.

Boyden Chamber Transwell Migration Assay

Boyden Chamber Transwell Migration Assays were performed as previously described[7]. Briefly, we used a cell suspension of siRNA transfected mouse aortic SMCs (1 × 105 cells/ml, 150 µl) added to the upper well of a HTS Transwell plate containing 8-µm pores (Fisher) in SFM containing 0.1% bovine serum albumin (BSA, Sigma-Aldrich). Concentrations of POVPC from 0 to 10 µg/ml of PDGF-BB (Millipore GF149) from 0 ng/mL-50 ng/mL were added to the bottom chamber in SFM with 0.1% BSA and 5 µg/ml fibronectin (Sigma-Aldrich). Cells were incubated at 37 °C in a CO2 incubator for 18 hours, fixed in 4% formalin and stained with 0.2% Crystal Violet in 7% ethanol. Upon removing cells from the upper surface of each transwell, the remaining cells were counted in 3-4 randomly chosen high-power fields (magnification, ×20). Results are representative sample from 3 independent experiments.

Genotyping and Excision

PDGFRB-Excision

TAGACTTCCCACCGAGCCTAGT

ACGGCCAGTTCCCCTAAACTAC

PDGFRB-Genotyping

GCCATCAACATCTCTGTGATCGG

CTCCAGCAGCCGCACGTAGCCAT

eYFP-Genotyping

GGAGCGGGAGAAATGGATATG

AAGTTCATCTGCACCACCG

TCCTTGAAGAAGATGGTGCG

CGTGATCTGCAACTCCAGTC

Myh11-CreER^{T2} Genotyping

TGACCCCATCTCTTCACTCC

AACTCCACGACCACCTCATC

AGTCCCTCACATCCTCAGGTT

Statistical Analysis:

Statistics were performed using GraphPad Prism Version 6 software. For multiple comparisons across multiple locations, two-way analysis of variance (ANOVA) was performed. For individual comparisons of normally distributed data, unpaired two tailed t tests were performed with Welsh's correction applied only in cases of unequal variance between groups. Mann Whitney tests were conducted if data was non-normally distributed.

Chapter III: Additional Studies – Preliminary Data

Potential Role for PDGFRB in SMC contraction

Observations Leading to Experiments

During the course of the studies mentioned in Chapter II, we made an observation that the vessels of the 10-12 week old PDGFRB$^{SMC\ \Delta/\Delta}$ mice appeared to be “more sticky” and “more fragile” than the 10-12 week old PDGFRB$^{SMC\ WTWT}$ control carotids. This observation was repeated and confirmed by another graduate student and two senior research scientists in the lab, Dr. Olga Cherepanova and Dr. Anh Nguyen. As is mentioned in Chapter I, the primary role of SMCs in the vasculature is contraction and dilation in order to regulate blood vessel tone-diameter, blood flow distribution, and blood pressure. Therefore, if SMC-specific knockout of PDGFRB is causing the vessels to become “more fragile”, then this may indicate a critical unidentified role for PDGFRB in SMC homeostasis and maintenance of the vasculature. If true, this observation would be particularly perplexing as it is well documented that PDGFRB is not expressed or is expressed in very low levels as to be undetectable in mature, uninjured SMCs[59,120,121]. Indeed, many SMC knock out studies focus exclusively on the vessel's response to injury or remodeling and fail to examine whether or not their are alterations in basic SMC function.

Results

In order to try to better define “sticky” and “fragile”, we first attempted to insert telemetry probes into the carotids of PDGFRB$^{SMC\ WT/WT}$ and PDGFRB$^{SMC\ \Delta/\Delta}$ animals to measure blood pressure. While insertion of the probes into the carotids of the PDGFRB$^{SMC\ WT/WT}$ mice went fine, attempting to insert the probes

into the PDGFRB$^{SMC\ \Delta/\Delta}$ animals resulted in shearing of the carotid all the way to the aortic arch in each of the three animals. We next collaborated with the Isakson lab to perform pressure myography on these vessels and found that, while there was a trending increase in contraction in response to KCL stimulation (Figure 3.1 G), there was no significant difference in lumen diameter (Figure 3.1 A, D), wall thickness (Figure 3.1 B, E) or the media to lumen ratio (Figure 3.1 C, F) in response to varying calcium containing flow pressure, meaning that loss of PDGFRB in SMCs for up to 4 weeks did not result in any measurable morphological change in these preliminary studies (similar to Figure 2.3).

We then collaborated with the Somlyo lab to perform wire myography, which allowed us to assess the mechanical properties of PDGFRB$^{SMC\ \Delta/\Delta}$ vessels. Briefly, the wire myography experiment involves loading same diameter, same length aortic strips between two clamps and then recording the force exerted by the muscle on each clamp in response to stretch alone (passive force) and KCL stimulated contraction (active force). When abdominal aortic strips were loaded and then subjected to varying amounts of stretch and KCL stimulation, we found that PDGFRB$^{SMC\ \Delta/\Delta}$ had no change in total force generated, but had a significant leftward shift in the length/force relationship (Figure 3.2), indicating that at lower lengths/stretch and in response to KCL stimulation, PDGFRB$^{SMC\ \Delta/\Delta}$ SMCs exert more force than the PDGFRB$^{SMC\ WT/WT}$ SMCs at the same length yet the aggregate total force generated is not significantly different.

Discussion

Briefly, SMC contraction occurs in response to an increase in intracellular calcium which in turn interacts with calmodulin. This calcium-calmodulin complex

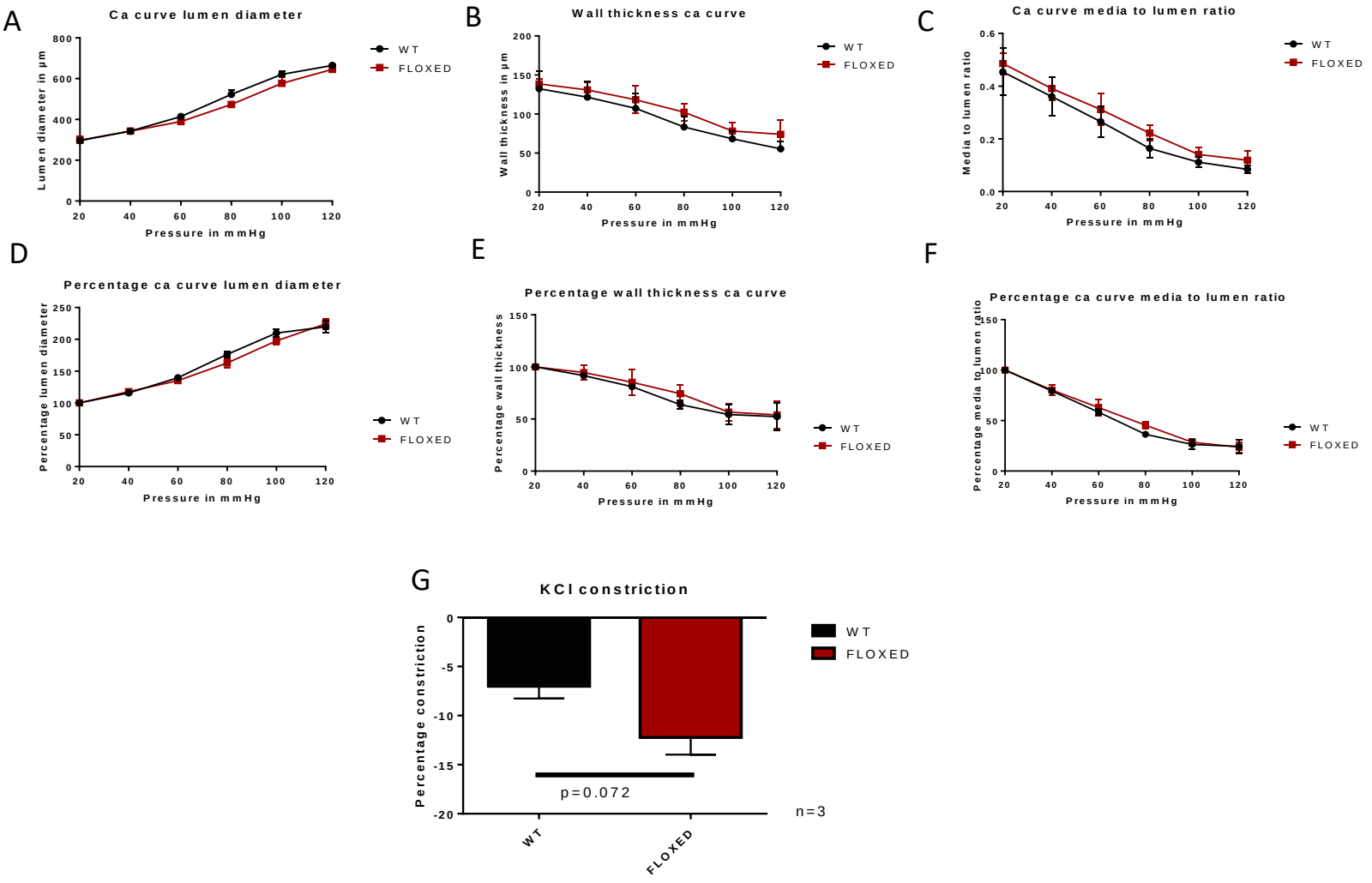

Figure 3.1: There is a trending increase in contraction in PDGFRB$^{SMC\ \Delta/\Delta}$ carotids as measured by pressure myography, but no significant difference.

Figure 3.1: There is a trending increase in contraction in PDGFRB$^{SMC\ \Delta/\Delta}$ carotids as measured by pressure myography, but no significant difference.

Pressure myography revealed no difference in (A) lumen diameter, (B) wall thickness, or (C) the media to lumen ratio in response to increasing flow pressure. (D-F) Further, normalization of those measurements to starting values did not reveal any significant difference. In response to KCL stimulation, PDGFRBSMC Δ/Δ carotids had a trending increase in contractility. n=3 for each group. *=$p<0.05$ as measured by Two tailed Unpaired t-test. Two Way ANOVA was used for al other measurements. Bars are mean ± s.e.m.

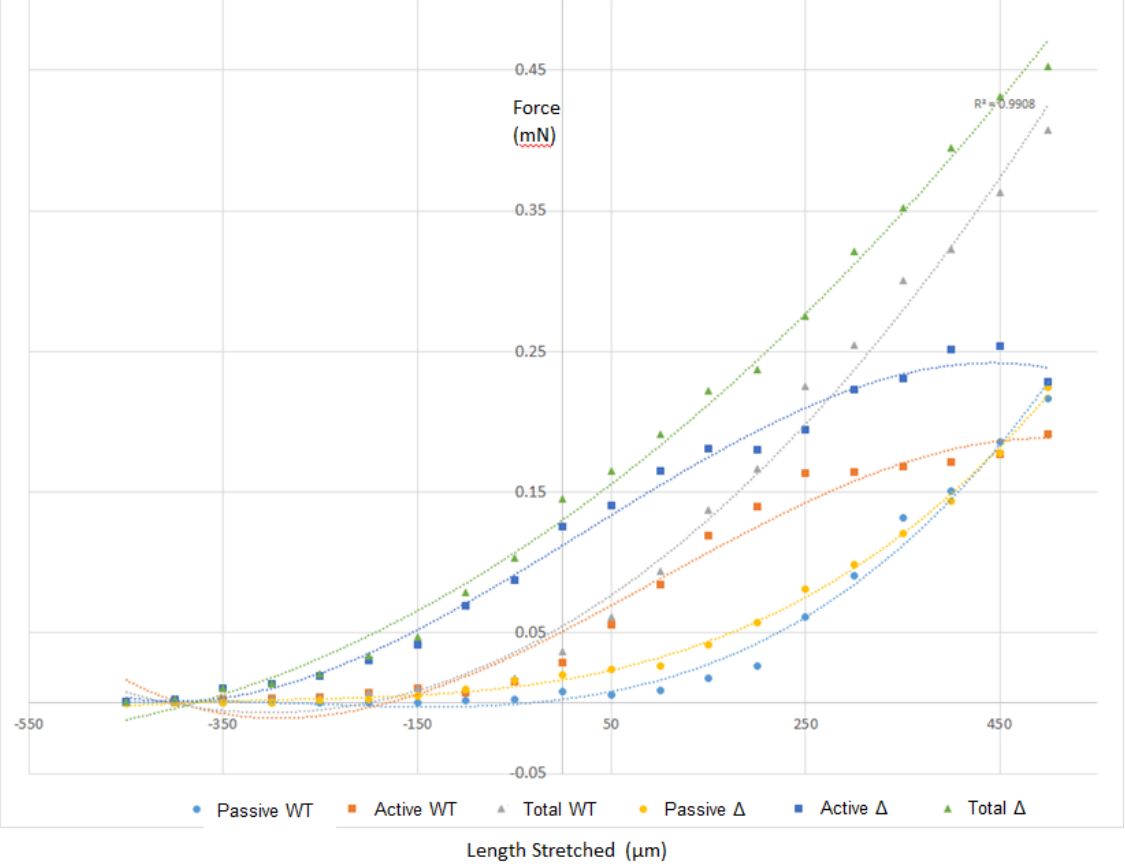

Figure 3.2: Wire Myography of PDGFRB$^{SMC\ WT/WT}$ and PDGFRB$^{SMC\ Δ/Δ}$ revealed a significant leftward shift in the active force generated by the PDGFRB$^{SMC\ Δ/Δ}$ vessels.

Figure 3.2: Wire Myography of PDGFRB$^{SMC\ WT/WT}$ and PDGFRB$^{SMC\ \Delta/\Delta}$ revealed a significant leftward shift in the active force generated by the PDGFRB$^{SMC\ \Delta/\Delta}$ vessels.

Wire Myography results from 2 animals per group (n=2 PDGFRB$^{SMC\ WT/WT}$ and n=2 PDGFRB$^{SMC\ \Delta/\Delta}$ with 3 strips used per animal.

activates myosin light chain kinase (MLCK) which in turn phosphorylates myosin light chain (MLC)[1]. Phosphorylated MLC initiates contraction and myosin phosphatase then removes the phosphate group and the process is then able to repeat[5]. In this situation, with our limited preliminary data we hypothesize that through some unknown mechanism loss of SMC-specific PDGFRB allows for a buildup of phosphorylated MLCK which in turn causes remodeling so that at lower tensions (length), the SMCs are in a more contracted state. In order to test this, however, we would need to isolate protein from both $PDGFRB^{SMC\ WT/WT}$ and $PDGFRB^{SMC\ \Delta/\Delta}$ vessels and then perform a western blot to determine if there is an increase in phosphorylated myosin light chain or its constituents. An alternative hypothesis would be that there is an increase in overall myosin and actin within the SMCs, meaning lower tensions would produce higher force since more myosin heads would be pulling on actin filaments, however if this is the case we would expect to see an overall increase in total force generated, which is not observed.

If the former hypothesis is true, we would expect to see a basal level of PDGFRB, with the assumption that PDGFRB either inhibits phosphorylated MLCK or promotes/increases myosin phosphatase to remove the phosphate group on the active MLC head. In other words, when PDGFRB is absent, a critical intermediary is not activated allowing phosphorylated MLC to build up. This may be through direct phosphorylation or, as is more likely due to the PDGFRB being bound in the plasma membrane and MLC being bound near actin filaments, through an intermediary such as RhoGTPase. Indeed, Mark

Majesky has shown that in uninjured rat carotids, there is PDGFRB mRNA but no detectable expression of PDGFRB protein, which may be due to the inability of antibodies to detect such a low level of protein expression[59]. Future studies may be aimed at elucidating this previously unreported interaction between PDGFRB and basic SMC contraction/function.

PDGFRB$^{SMC\ \Delta/\Delta}$ primary culture SMCs are outcompeted by alternative cell types within 3 passages

Observations Leading to the Experiments

In order to better study the effects of PDGFRB$^{SMC\ \Delta/\Delta}$ on SMC proliferation and migration *in vitro*, we attempted to isolate a primary cell culture of both PDGFRB$^{SMC\ WT/WT}$ and PDGFRB$^{SMC\ \Delta/\Delta}$ cells. We then planned to flow sort based on the presence or absence of endogenous eYFP, since in our model system only SMCs express eYFP and we can confirm we have a pure population of SMC derived culture cells. This rationale was due in part to the fact that it has been well documented that adventitial cells are capable of activating the whole repertoire of SMC marker genes *in vitro*, including both smooth muscle myosin heavy chain (Myh11), the most specific SMC marker known to date and myocardin, the SMC/cardiac muscle master regulator[24,25]. Indeed, in one study the authors found that plating purified Sca1+ adventitial cells resulted in these cells activating early SMC marker genes including ACTA2+ after 2 passages and by passage 6 the entire repertoire of SMC marker genes could be seen through RT-PCR and western blot analysis[24].

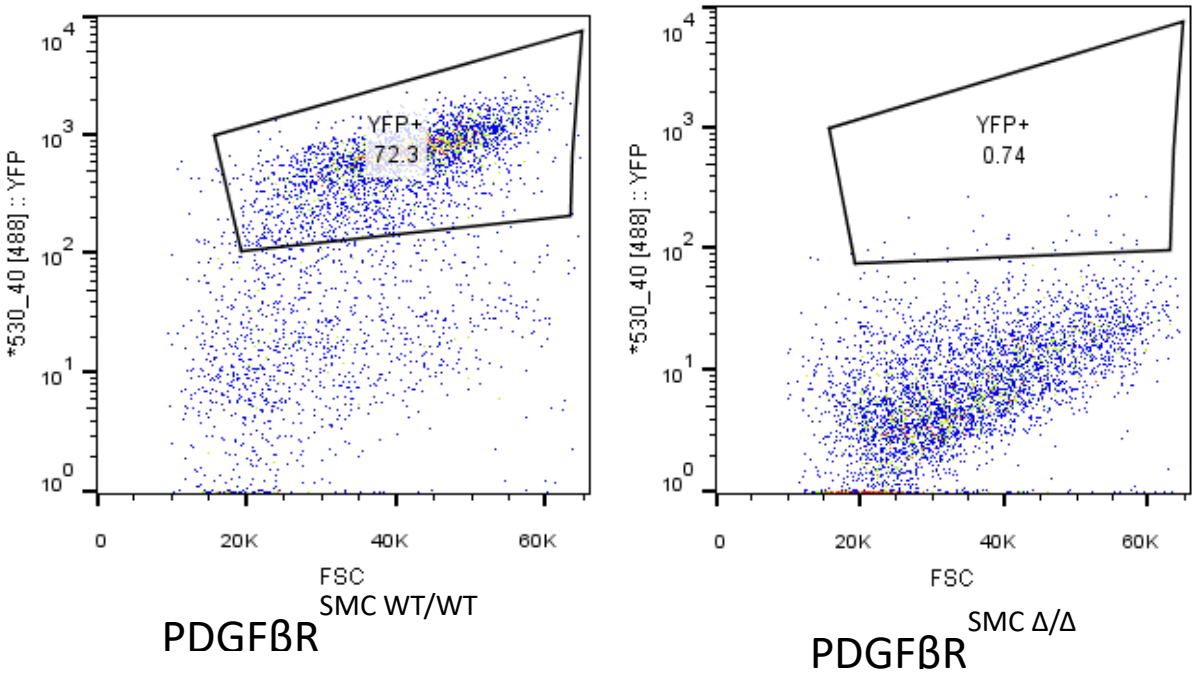

Figure 3.3: PDGFRB$^{SMC\ \Delta/\Delta}$ primary cultures are outcompeted by passage 3 by alternative cell types.

Figure 3.3: PDGFRBSMC Δ/Δ primary cultures are outcompeted by passage 3 by alternative cell types.

PDGFRB$^{SMC\ WT/WT}$ and PDGFRB$^{SMC\ Δ/Δ}$ cell lines were each composed of 10 pooled thoracic aortas and each cell line was sorted based on eYFP.

Results

We independently pooled 10 PDGFRB$^{SMC\ WT/WT}$ and 10 PDGFRB$^{SMC\ \Delta/\Delta}$ thoracic aortas and performed a rigorous enzymatic digestion as previously described on both cultures[40,55,122,123]. Following 3 passages, we found via flow cytometry sorting based on the presence of eYFP that 73% of the PDGFRB$^{SMC\ WT/WT}$ cell line was eYFP+ whereas less than 1% of the PDGFRB$^{SMC\ \Delta/\Delta}$ cell line was eYFP+ (Figure 3.3). This was despite starting the sort with an equivalent number of cells and only allowing 3 passages before sorting.

Discussion

The current method for isolating and culturing primary SMCs rely on either enzymatic digestion or explant culture[124]. Validation of these culture procedures includes RT-PCR analysis of the presence or absence of SMC marker gene transcription and western blot analysis on the presence or absence of a variety of SMC marker proteins including Myh11, Tagln, Smoothelin, and more. Our finding that both PDGFRB$^{SMC\ WT/WT}$ and PDGFRB$^{SMC\ \Delta/\Delta}$ cell cultures had an equivalent number of cells after 3 passages yet a drastically different origin of those cells (i.e ~73% eYFP+ vs ~1% eYFP+) underscores the fact that lineage tracing is absolutely critical to verify/confirm that primary SMC cultures are in fact SMC derived. As Hu et al.[25] has shown and as has been supported by Passman et al.[24], other cell types (including Sca1+ adventitia cells) are capable of activating multiple SMC genes *in vitro* with no special stimulant/media meaning traditional methods of cell characterization are not enough. In other words, isolating and validating SMC-specific KO cultures based on the presence or absence of SMC marker genes is not rigorous enough to ensure that the isolated

cells are in fact SMC-derived versus an alternative cell type activating SMC marker genes. Taken together with our results from Figure 2.3, it appears that loss of the PDGFRB within SMCs allows for alternative cell types to outcompete endogenous SMCs in both an injury setting and in a culture setting. This has profound implications on multiple papers which have isolated primary knock out cultures and subsequently used these cultures for *in vitro* testing. If the primary culture is not SMC-derived, but is expressing SMC marker genes, how can you be sure that any *in* vitro observations accurately recapitulate how a SMC would respond versus a "pseudo-SMC"? For example, one study[125] found that primary cultured SMCs (passage 5-7) from RAGE-null mice failed to proliferate or migrate in response to S100B, a RAGE ligand and further concluded that intact RAGE signaling in SMCs is required for RAGE induced proliferation/migration *in vitro*. The authors validated that their cultures were SMC derived via western blotting for various SMC marker genes. Could the failure to proliferate or migrate in response to S100B be due to the fact that these cells are adventitia-derived and are not true SMCs? And further, could SMCs have a compensatory mechanism in place for loss of RAGE induced proliferation/migration which is not observed in “pseudo-SMCs”?

Our finding that loss of PDGFRB in SMCs can result in primary cultures being completely overgrown by alternative cell population(s) has profound implications in that it demonstrates that current primary SMC isolation validation protocols are inadequate. Further, these results indicate that utilizing SMC cultures derived from WT and KO mice is probably not a valid means with which

to study functional consequences of gene knockout since in most cases the knockout line will impact not only the purity of the cultures, as we've demonstrated above, but their functional properties due to continuous and progressive activation of compensatory gene pathways that likely are highly variable depending on the conditions and reagents (serum, media, antibody, etc) used to culture the cells.

SMC conditional lineage tracing is absolutely vital to ensure that primary cultures are in fact derived from SMCs and not an alternative source which has activated SMC marker genes.

Chapter IV: Future Directions

PDGFRB Related Studies

The experiments presented herein are the first studies to rigorously examine and define the role of SMC-specific PDGFRB in the context of vascular injury. While the dogma has long suggested that inhibition of PDGFRB specifically in SMCs is what leads to a reduction in neointima formation, our results are the first to provide evidence that this is *not* the case. Instead, our studies suggest that the reduction in neointima seen in inhibitor studies is due to the global effect of PDGFRB inhibition rather than a SMC specific effect. Indeed, as seen in figure 2.1, the neointima of the PDGFRB$^{SMC\ \Delta/\Delta}$ mouse is eYFP- but PDGFRB+ suggesting that the PDGFRB pathway may be involved in the compensation process by alternative cell types. Intriguingly, up to 10% of these eYFP- cells expressed Myh11, the most specific SMC marker gene currently known, meaning another cell type appears to have the capability to activate both early and late stage SMC differentiation markers. We also found that PDGFRB$^{SMC\ \Delta/\Delta}$ mice had a significant reduction in the growth fraction, with ~1% of the cells proliferating in response to injury whereas ~23% proliferated in the PDGFRB$^{SMC\ WT/WT}$ mice.

While we attempted to address these questions through the use of BMT, our surprising finding that even the PDGFRB$^{SMC\ WT/WT}$ mice lacked eYFP+ cells in the neointima and further that the neointimas had equivalent contributions of tdTomato+ cells raises the possibility that the origin of the eYFP- neointima may be different in the nonirradiated vs irradiated animals. Further, it was particularly interesting to note that in Figure 2.17 E, there was a significant difference between the BrdU+ neointimal cell population in the 10-12 week old PDGFRB^{SMC}

$^{\Delta/\Delta}$ mice versus the irradiated PDGFRBSMC Δ/Δ mice. Could this be due to different cell populations with different proliferative rates populating the neointima? Or did irradiation somehow ramp up the proliferative rate of circulating cells? In light of these observations, there remain several unanswered questions.

Although we know bone marrow cells contribute to the eYFP- neointima, do endothelial or adventitial cells also contribute to the eYFP- neointimal cell population in the PDGFRB$^{SMC\ \Delta/\Delta}$ vessel?

As is discussed in Chapter II, according to the literature there are 3 most-likely sources of the eYFP- neointima. Unfortunately, there is not a lineage specific marker for bone marrow derived cells or for Sca1+ adventitial cells. Although staining for or crossing a CD45 lineage tracing system to our SMC-PDGFRB KO lineage tracing system is tempting and would classically be used to identify circulating cells, there are several points which would need to be considered prior to this experiment which would confound interpretation of the results: 1) there has recently been identified a Sca1+ CD45+ adventitial stem cell population which would make it unclear whether any eYFP- CD45+ cells are myeloid derived or adventitia derived[26,103]; 2) there is no gene which is specific to the Sca1+ adventitial stem cell population or to bone marrow cells meaning other cell types would be labeled with any lineage tracing system employed and confound interpretation of results[105]; 3) any combinatorial lineage tracing system would need to be an inducible, non-Cre based system or else the PDGFRB would be knocked out in both SMCs and myeloid/adventitial lineage tracing. In other words, generation of a doxycyclin inducible[126] CD45-FLP CAG frt-stop

mOrange and crossing it to our PDGFRB$^{SMC\ FL/FL}$ mouse system would determine if any of the cells were from a CD45 origin, however any eYFP- mOrange+ cells could be derived from either the myeloid lineage[127] or the Sca1+ CD45+ adventitial cell lineage[103].

In order to determine if endothelial cells are contributing to the neointima, the most rigorous approach would be to generate a doxycycline inducible VE-Cadherin-FLP CAG frt-stop mOrange mouse. VE-Cadherin is the most specific endothelial cell marker known to date and is thought to be expressed only in endothelial cells[128]. mOrange is a fluorophore not commonly used which would allow for later crosses to other lineage tracing systems, including tdTomato, dsRed, etc. Crossing this mouse to our PDGFRB$^{fl/fl}$ Myh11-CreER^{T2} Rosa fl-stop eYFP mouse would allow for SMC-specific, conditional loss of PDGFRB and SMC-specific, conditional lineage tracing combined with endothelial specific lineage tracing. Examining the neointima, we could then calculate what percentage of the neointima is eYFP- mOrange+, and thus determine the overall endothelial cell contribution to the neointima in the presence and absence of SMC-specific PDGFRB.

Does the deficit of SMCs also occur in atherosclerosis?

These studies are currently underway by another graduate student in the lab, Alexandra Newman, and will be the focus of her thesis work. Briefly, we do see that SMC-specific loss of PDGFRB results in a significant decrease in the number of SMCs present in the lesion and an expected overall increase in lesion size.

Age Related Studies

As was discussed in Chapter II, when we looked at mice which were slightly older, those around 17-19 weeks of age and compared them to our 10-12 week old mice, we saw a significant reduction in the eYFP+ contribution to the neointima in the PDGFRB$^{SMC\ WT/WT}$ mice. We then found through continuous BrdU infusion that PDGFRB$^{SMC\ WT/WT}$ mice 10-12 weeks of age had a higher growth fraction than those PDGFRB$^{SMC\ WT/WT}$ mice 17-19 weeks of age. This reduction in growth fraction may have significant implications across the vascular injury/disease field, however several unanswered questions remain and are discussed below.

As mice continue to age, does the SMC growth fraction continue to decrease, and if so does it ever reach 0?

Since we found that modest increases in age resulted in a significant reduction in the overall SMC growth fraction, the logical next question would be does this growth fraction reach 0? Indeed, while a search of the literature reveals that the majority of studies rely on mouse experimental groups similar in age[11,129]; there is a not-insignificant fraction of rat and mouse studies which rely on animals of similar body weights rather than similar ages. These studies are usually focused around either the carotid or femoral wire injury where, presumably, mice of similar weight would have similar sized vessels, meaning the injury to the vessel would be more consistent across mice. Indeed, a recent review article[130] providing a step-by-step protocol for rat and mouse vascular injury studies encourages researchers to use mice weighing around 23-25 grams to minimize injury variability between animals.

In order to answer the above question, mice which the literature would consider old (at least 52 weeks of age) would need to be TAM injected at 6-8 weeks of age, and allowed to grow old. Upon reaching the desired age, carotid ligations immediately followed by BrdU osmotic minipump implantation would need to be carried out. At 21 days post injury, vessels should be harvested and the overall SMC contribution to the neointima would need to be calculated (eYFP+) followed by the growth fraction. Varying time points earlier than 52 weeks could be harvested to visualize the decline, if present, in SMC growth fraction in response to age.

Correlating the age of the mouse to its weight at the time of surgery may also help to explain some of the apparent discrepancies in the literature. For example, Tang et al.[32] appear to have used weight as the metric behind when they performed their carotid wire injuries. Could it be that the mice were old enough that the SMC growth fraction was reduced to zero, and that how they concluded SMCs don't undergo phenotypic switching *in vivo*? Yang et al.[12] performed carotid ligations similar to Herring et al.[34], yet found that ~30% versus the Herring et al 80% of the neointima was derived from pre-existing SMCs. Could this be because Yang et al.[12] used older mice than Herring et al.[34] (10-12 week Herring et al versus 18-20 week Yang et al)?

Does the decrease in SMC growth fraction via modest increases in age also occur in the resolvable injury setting?

The carotid ligation injury model is a non-resolvable injury setting in that the injury does not go away. Although we confirmed our observations on the role of loss of SMC specific PDGFB in the femoral wire injury, we did not perform

growth fraction analysis on 10-12 week animals or 17-19 week femoral wire injured animals. Could the reduction in SMC growth fraction with modest increases in age be due to the injury model used (including altered environmental cues, lack of blood flow, etc) and not age as we believe?

In order to test this, we would perform femoral wire injury on 10-12 week, 17-19 week, and 52+ week $PDGFRB^{SMC\ WT/WT}$ and $PDGFRB^{SMC\ \Delta/\Delta}$ mice and immediately implant BrdU osmotic minipumps. Following 28 days of injury, we would then harvest the animals and calculate the growth fraction of the medial SMCs. While it would be interesting to compare the carotid ligation growth fraction to the femoral wire injury growth fraction, it's important to note that they are two distinct types of injury[131]. The carotid ligation causes a cessation of blood flow which induces vessel remodeling whereas the femoral wire injury induces mechanical injury to the endothelial layer and the medial SMC layer to which the vessel responds[129]. I would expect the femoral wire injury to have a higher growth fraction due to the directly induced medial SMC death, however this experiment would be telling. Regardless, the eYFP+ contribution to the media and neointima will allow for growth fraction calculations and further define if SMCs growth fraction decreases in a different injury setting with age.

If these observations were proven true and there was a decrease in SMC growth fraction with varying increases in age, it would be particularly interesting to speculate why. Do SMCs have a "Use it or Lose it" philosophy in regards to their capability to undergo phenotypic switching? In other words, as SMCs get older, do they lose the capability to undergo phenotypic switching? Alternatively,

is there a factor or factors secreted which decreases over time that keeps SMCs "primed" for phenotypic switching and proliferation/migration? Interestingly, the Owens lab has long known that in order to isolate a primary SMC culture line, the younger the mice at the time of harvest, the better (Owens lab, unpublished observations). Could this be due to a higher growth fraction of the medial SMCs at earlier time points? Further, as the mice age, does the SMC growth fraction decrease meaning the original population capable of contributing to the primary cell culture also decreases?

Wilbur Thomas's studies[68,132–134] would seem to argue against this as he found that 66-99% of the medial SMCs had undergone at least one round of proliferation during the development of juvenile porcine atherosclerosis. However, it's also possible that: 1) in the context of a chronic disease such a atherosclerosis, there is/are some factor or factors being secreted, environmental cues, etc which keep SMCs "primed" for phenotypic switching and thus in that setting SMCs do not lose the capability to undergo phenotypic switching; or 2) since he was studying young pigs for 30-60 days, perhaps the medial growth fraction was still high and would have decreased with age. Regardless, whether or not there is a subpopulation of SMCs which normally respond to vascular disease (such as atherosclerosis) and does this subpopulation decreases with age are both interesting questions which may be addressed through BrdU osmotic minipump and SMC growth fraction studies.

Irradiation Related Studies

Our finding that irradiation completely abolished the SMC contribution to the neointima are extremely provocative. Considering that the BMT experiment

is one of the most commonly employed techniques in the vascular and myeloid field[135], our finding that lethal irradiation fundamentally alters the composition of the neointima necessitate re-examining the conclusions drawn from thousands of papers and while this most certainly does not invalidate those conclusions, they must now be reconsidered in the light that irradiated neointimas do not contain SMCs, the primary component of nonirradiated neointimas. Although the BMT is used by thousands of labs across the globe, the exact amount of whole body irradiation and type of injury induced differs, with the lethal range generally being from about 900RADs to 1300RADs and multiple sub-lethal studies using between 300RADs-800RADs. This raises several unanswered questions addressed below.

At what radiation dose are SMCs no longer impacted by irradiation?

Since the literature generally agrees that lethal irradiation of the bone marrow takes place at radiation doses above 900RADs[135], and it takes between 2000RADs to 3000RADs to induced cultured SMC death in response to irradiation[136,137], how much irradiation can *in vivo* SMCs take and still respond normally to injury? Our lab is particularly primed to answer this question, given our SMC specific, conditional lineage tracing, and depending on the results, it may requirere-evaluating the conclusions of papers which used lower doses of radiation when compared to our radiation dose (1200RADs).

In order to address this question, multiple different experimental groups with differing doses of irradiation would be needed followed by bone marrow reconstitution and carotid ligation. Following the carotid ligations, immediate implantation of BrdU osmotic minipumps would allow for determination of the

SMC growth fraction. Knowing the SMC growth fraction at decreasing doses of irradiation and comparing those growth fractions to age matched nonirradiated controls would allow for determination of which radiation doses SMCs are able to tolerate without inhibiting proliferation and response to injury. Further analyzing the SMC contribution to the neointima and again comparing to controls would allow for better definition of the impact smaller doses of irradiation have on neointima formation.

Does irradiation also inhibit the SMC growth fraction and contribution of SMCs to the neointima following other modes of vascular injury and disease?

Next, although we know irradiation significantly alters the SMC growth fraction and contribution to the neointima in the carotid ligation, we do not know if this is also the case in the femoral wire injury. The femoral wire injury is distinct from the carotid ligation in many ways, including: 1) a different developmental origin of femoral SMCs and carotid SMCs[4] and; 2) the femoral wire injury is a resolvable injury whereas the carotid is a permanent injury. Further, several studies have performed lethal irradiation and found increases in plaque instability, something we would expect to see if there were more macrophages present and less SMCs[116,138].

In order to test whether or not our observations in the carotid ligation also occur in the femoral wire injury, we would need to repeat our BMT experiment in the femoral wire injury. Following BM reconstitution and femoral wire injury, we could then immediately implant BrdU osmotic minipumps to determine the

percentage of cells which had undergone proliferation in response to femoral wire injury, growth fraction in response to irradiation, etc.

Similarly, we would next want to determine whether or not radiation abolishes the SMC contribution to the lesion, as has been suggested by other groups which did not have SMC specific lineage tracing and have performed BMTs. We would first irradiate, reconstitute, and then allow ApoE-/- PDGFRB$^{SMC\ WT/WT}$ mice to be on western diet for 18 weeks. Following this, we would then harvest the BCA and/or aortic root and examine whether or not SMCs are present in the atherosclerotic lesion. If there are no SMCs present in the lesion as we hypothesize, then this necessitates reexamining any paper which has performed a BMT in the context of atherosclerosis. Similar to studies suggested earlier, it may be beneficial to understand whether or not SMCs are capable of contributing to the lesion at lower doses of radiation.

Regardless, understanding whether or not lethal irradiation abolishes the SMC contribution to atherosclerotic plaques is vital to our interpretation of atherosclerotic BMT studies moving forward, as is discussed in Chapter II. Since BMTs are usually performed well before any lesion is developed in mice (young mice, pre-western diet), it's extremely important to understand if irradiation from a BMT alters the basic composition and content of an atherosclerotic plaque as this will impact our interpretation of the results.

Does whole body irradiation abrogate/attenuate the SMC response to atherosclerosis in humans?

As is discussed in Chapter II, ApoE-/- or LDLR-/- mice which have undergone a BMT experiment have an increase in multiple indices of plaque

instability[115,116]. Looking at humans, patients who had received irradiation for H&N cancer have a documented higher intima-to-media ratio (meaning larger lesions or smaller vessels) than nonirradiated patients, and biopsies of irradiated medium size arteries from H&N and breast cancer patients displayed increases in several indices of plaque stability. Further, studies examining long term survival of adults (10+ years) post-irradiation have found that approximately 1/3 of the patients examined had some sort of cardiovascular disease/event, included but not limited to congestive heart failure, heart attack, or stroke[139]. Even more striking, two independent studies found that children who received whole body irradiation were ~10 times as likely to develop cardiovascular disease by age 40[140] and were 5 times as likely to have 3 or more significantly increased cardiovascular risk factors[141]. In summary, we now know that: 1) whole body lethal irradiation completely abolishes the SMC contribution to the remodeling vessel *in vivo* in mice; 2) mice which underwent BMT have a significant increase in multiple indices of plaque instability including thin fibrous caps and an increase in the macrophage-to-SMC ratio[115,116]; 3) childhood cancer survivors which received whole body irradiation are 10 times more likely to have cardiovascular disease (including a heart attack) than siblings[140]; and 4) lesions studied from H&N cancer patient biopsies are documented to be larger and less stable[117,118]. Taken together, we hypothesize that patients who receive radiation therapy have an increase in cardiovascular disease and a decrease in atherosclerotic plaque stability due to a defect in the SMCs response to cardiovascular disease. Specifically, that SMCs in patients who receive radiation therapy either fail to

invest atherosclerotic lesions or invest at a reduced frequency, causing unstable lesions and an increase in cardiovascular events including but not limited to stroke and heart attacks.

Unfortunately, to rigorously test this hypothesis we would need a way to quantitatively lineage trace SMCs in humans as, given the process of SMC phenotypic switching, we cannot rely on SMC protein expression to definitively identify a SMCs. Interestingly, a former graduate student in the lab found that, although SMCs lose expression of SMC marker genes in response to phenotypic switching and undergo drastic epigenetic changes, they actually retain H3K4me2 on SMC gene promoter loci[142]. Although the H3K4me2 mark itself is not unique to SMCs, the presence of this mark on SMC gene promoters is unique and allows for identification of phenotypically modulated SMCs in the absence of a SMC lineage tracing system. Utilizing a novel method developed here in the Owens lab which allows us to visualize specific epigenetic marks at individual gene loci in tissue sections (called ISH-PLA), the Owens lab has successfully demonstrated the capability to identify phenotypically modulated SMCs by the presence or absence of H3K4me2 in both our mouse atherosclerosis SMC lineage tracing system and in human atherosclerotic tissue[33]. Further, we've confirmed that cells not of SMC origin which are capable of activating ACTA2+ expression do NOT acquire this mark on the Myh11 promoter *in vivo*[6,7,33]. The drawback to this technique, however, is that it is semi-quantitative; that is, it labels around 70% of the SMCs in our SMC lineage tracing mouse.

Utilizing this unique method, we should be able to test our earlier hypothesis that SMCs in patients which received radiation therapy fail to invest the lesion formed during the development of atherosclerosis leading to more unstable plaques and a higher risk of a cardiovascular event. To test this hypothesis, we should be able to perform ISH-PLA on tissue sections from patients which received radiation therapy and determine whether or not there are cells which have H3K4me2 on the Myh11 promoter (ISH-PLA+) present within the lesions. If there are no cells or a significant reduction of cells which are H3K4me2+ (on the Myh11 promoter) in the lesion, then we can conclude that radiation therapy inhibits the SMCs participation in the development of atherosclerosis leading to less stable plaques and an increase in the risk of a cardiovascular event.

Summary

In summary, our findings demonstrate that SMC-specific loss of PDGFRB does NOT reduce neointima area. These results would seem to indicate that all previous studies which demonstrated a reduction of neointima area in response to PDGRRB inhibition were achieved through a global mechanism rather than a SMC-specific mechanism, as had been suggested. Indeed, our results from Figure 2.1 demonstrate that at least a subset of the eYFP- cell types composing the neointima of PDGFRB$^{SMC\ \Delta/\Delta}$ animals are distinctly PDGFRB positive meaning the compensation for loss of SMC-PDGFRB is likely dependent on PDGFRB signaling. Furthermore, we have provided evidence supporting the current literature in that defects in PDGFRB in SMCs result in defects in both migration[66,80] and proliferation[64,98,99] . While we attempted to use a BMT to

determine if myeloid cells formed the majority of the neointima in the absence of SMCs, we unexpectedly found that irradiation alone significantly altered the origin of the cells in the neointima of wild type animals such that the neointima was virtually completely devoid of SMC-derived cells in response to irradiation alone.

So what is the link between loss of SMC-specific PDGFRB and irradiation in the context of vascular injury? While both instances result in a neointima devoid of SMC-derived cells, do the neointimas form in a similar manner? Surprisingly, when comparing the number of cells in the neointima which had undergone proliferation (BrdU+), we found that there was a significant increase in the BrdU+ cells in the irradiated PDGFRB$^{SMC\ \Delta/\Delta}$ neointima as compared to the nonirradiated. In fact, the nonirradiated neointima of the PDGFRB$^{SMC\ \Delta/\Delta}$ animal (predominantly eYFP-) appeared to be formed primarily through recruitment of cells rather than proliferation (as evidenced by the low overall BrdU+ cells) whereas the neointima of the irradiated PDGFRB$^{SMC\ \Delta/\Delta}$ animal appeared to be formed primarily through proliferation rather than recruitment (Figure 2.15 E, F). The specific mechanisms behind the neointima formation in each case, however, remain unclear. Are there cytokines released in the nonirradiated animal which favor a migratory response versus a proliferative one? When SMCs lose the PDGFRB, do they secrete factors which increase migration of other cell types? Are there specific chemokines secreted in the irradiated animal which promote a proliferative response versus a migratory one? What are those chemokines? Is the neointima of the irradiated animal devoid of SMCs due to a SMC-specific

effect (DNA breaks, increased senescence, SMC death, etc), or is it due to a secondary effect caused by endothelial cell dysfunction? These and other questions need to be answered in order to fully understand the mechanism of formation of these two SMC-devoid neointimas and further to understand the mechanism by which irradiation impacts SMCs.

Perhaps of most interest, is whether lethal irradiation in humans undergoing BMT or stem cell infusion procedures also results in loss or attenuation of the normal response of differentiated medial SMCs during vascular disease. Key questions include: Do BMT subjects show impaired angiogenic responses, perivascular cell coverage of newly formed vessels, wound healing, or most importantly, an increased incidence of plaque rupture and associated major adverse cardiovascular events such as myocardial infarction or stroke due to loss of a SMC-derived protective fibrous cap? Although extensive further studies including validation of our findings in human are required, consistent with this possibility patients who received irradiation for head and neck (H&N) cancer are documented to have a higher intima to media ratio than non-irradiated patients[117] and biopsies of irradiated medium size arteries from H&N and breast cancer patients displayed an increase in inflammatory cell content and proteoglycan content[118]. Further, another study found that children which received irradiation were ~10 times more likely to develop cardiovascular disease or have a cardiovascular event (including myocardial infarction and stroke) by age 40 than their siblings[140,141]. Is this due to poor SMC investment of lesions and a subsequent increase in lesion instability?

Taken together with our study and that of others, we propose that gamma irradiation exacerbates lesion formation via ablation of a radiosensitive subpopulation of SMCs such that over time there is an increase in the ratio of macrophages to SMC within lesions and an increase in plaque destabilization. Future studies are needed to determine the effect of whole body irradiation on both early and late stage lesion development and defining the effect irradiation has on SMCs located in the lesion and the media of atherosclerotic vessels. In other words, does irradiation in children result in SMC-devoid lesions later in life, and a concomitant increase in plaque instability? How does irradiation on pre-existing lesions impact the plaque? These are just a few of the questions which need to be answered in order for us to better understand the overall impact irradiation has on SMCs and the SMC response to cardiovascular injury and disease in humans.

Chapter V: Citations

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