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Pre-existing smooth muscle cells contribute to neointimal cell repopulation at an incidence varying widely among individual lesions

Pu Yang, MD, PhD^{1,*}, Michael S. Hong, MD¹, Chunhua Fu, PhD¹, Bradley M. Schmit, MD¹, Yunchao Su, MD, PhD², Scott A. Berceli, MD, PhD^{1,3}, and Zhihua Jiang, PhD¹

¹Division of Vascular Surgery and Endovascular Therapy, University of Florida College of Medicine

²Department of Pharmacology and Toxicology, Georgia Regents University

³The Malcom Randall VAMC, United States

Abstract

Background—With the diverse origin of neointimal cells, previous studies have documented differences of neointimal cell-lineage composition across models, but the animal-to-animal difference has not attracted much attention though the cellular heterogeneity may impact neointimal growth and its response to therapeutic interventions.

Methods—The *R26R⁺;Myh11-CreER⁺* and *R26R⁺;Scl-CreER⁺* mice were utilized to attach LacZ tags to the pre-existing smooth muscle cells (SMCs) and endothelial cells (ECs), respectively. Neointimal lesions were created via complete ligation of the common carotid artery (CCA) and transluminal injury to the femoral artery (FA).

Results—LacZ-tagged SMCs were physically relocated from media to neointima and changed to a de-differentiated phenotype in both CCA and FA lesions. The content of SMCs in the neointimal tissue, however, varied widely among specimens, ranging from 5–70% and 0–85%, with an average at low levels of 27% and 29% in CCA ($n=15$) and FA ($n=15$) lesions, respectively. Bone marrow cells, while able to home to the injured arteries, did not differentiate fully into SMCs after either type of injury. Pre-existing ECs were located in the sub-endothelial region and produced mesenchymal marker α -actin, indicating endothelial-mesenchymal-transition (EndoMT), however, EC-derived cells represented only 7% and 3% of the total neointimal cell pool of CCA ($n=7$) and FA ($n=7$) lesions, respectively. ECs located on the luminal surface exhibited little evidence for EndoMT.

Address correspondence to: Zhihua Jiang, PhD, PO Box 100128, Gainesville, FL 32610-0128, Phone: (352) 294-5688, Fax: (352) 273-9644, jiangzh@surgery.ufl.edu.

*Currently Xiangya Hospital, Central-South University, China.

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Conclusion—Neointimal hyperplasia proceeds with a wide range of variation in its cellular composition between individual lesions. Relative to ECs, SMCs are major contributors to the lesion-to-lesion heterogeneity in neointimal cell-lineage composition.

Neointimal hyperplasia is the primary pathology that leads to early failure of vascular procedures aimed at treating occlusive arterial diseases. Animal models, particularly those created with transgenic mice, have been utilized widely in studies to obtain mechanistic insights into the hyperplastic response of injured vessels. Of these models, common carotid artery ligations, transluminal injury to femoral arteries, and interpositional vein grafting have been used widely for these studies. Using these models, studies have identified numerous potential therapeutic molecular targets, but treatments developing from preclinical studies have, so far, failed to prove to be translational in clinical practice. While an easy explanation for the failure is that “animals are not humans”, the potential for true translational application may require a better understanding of the cellular events occurring in animal models.

In humans, work in early 1970s suggested that neointimal hyperplasia is an adaptive response of the vessel wall to injuries, with smooth muscle cells (SMCs) being the key player in this process^{1–3}. Later experimental studies, however, suggested that the source of neointimal cells is not limited to SMCs. Over 40% of neointimal cells were of bone marrow origin^{4, 5}. Other sources, including both local vessel wall^{6, 7} and remote perivascular tissues⁸, have also been documented as donating progenies to the neointimal cell population. Such a diverse origin of neointimal cells has sparked a hot debate on the contribution of SMCs to neointimal cell population^{7, 9, 10}. Although genes in SMCs may be manipulated to alter the progression of neointimal thickening¹¹, uncertainty has remained about whether SMCs make up the majority of neointimal cells. A study tracing the lineage fate of these cells demonstrated that over 80% of neointimal cells originated from the pre-existing SMCs¹², however, juvenile mice (<6 weeks of age) were chosen in this study, and given the significant disparity of the *in vivo* SMC biology between young and adult or aged animals¹³, the contribution of SMCs to the neointimal cell repopulation remains to be evaluated with models that are more relevant to patients who often develop this pathology after adult age. More recently, endothelial cells (ECs) were identified as sources for neointimal cell population^{14, 15}. In a model of vein bypass grafting, EC-derived cells donated more than 50% of total neointimal cell population via a process termed endothelial-mesenchymal transition (EndoMT)¹⁵. The discovery of non-SMC origin of neointimal cells has challenged the long-standing paradigm that assumes SMCs to be the predominant source for neointimal cell population.

The current study sought to tackle this fundamental issue with a focus on the fate of SMCs and ECs. Using inducible Cre-loxP systems driven by a SMC- or EC- specific promoter, we mapped the fate of these cell groups in neointimal lesions induced by different types of arterial injury. The results show that medially derived SMCs lose their markers of differentiation and assume a more dedifferentiated state on relocating to neointimal lesions. To our knowledge, it is the first direct evidence that reaffirms the concept of “SMC dedifferentiation” suggested by Regan et al¹⁶. In addition, we show that, in adult animals, SMCs donate to the total neointimal cell pool with a wide range of animal-to-animal

variation in models that have been used widely in the past in both mechanistic and pre-clinical studies. EndoMT, while serving as a major mechanism for neointimal cell repopulation in selected vascular settings¹⁵, appears to occur infrequently in neointimal lesions induced by complete cessation of blood flow or intraluminal injury. Our findings suggest that experimental neointimal lesions are highly heterogeneous pathologically, which raises a question as to the pathologic property, and thus the response to therapeutic intervention in humans.

METHODS

Animal models

This study conforms to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by Institutional Animal Care and Use Committee of the University of Florida. The *Gt(ROSA)26Sor (R26R)*, *CAG-EGFP*, and C57BL/6-Ly5.1 mice were purchased from Jackson Laboratory. The *Myh11-CreER* and *Scl-CreER* strains were kindly provided by Drs. Offermanns and Gothert, respectively. All animals were on C57BL/6 background. To avoid the potential issue of sexually dimorphic response to vascular injury, only male mice were used in this study, and the responses in female mice were not studied. The Cre lines were bred with *R26R*, and offspring littermates were screened for *R26R⁺;Myh11-CreER⁺* and *R26R⁺;Scl-CreER⁺* animals with PCR-based genotyping. The *R26R⁺;Myh11-CreER⁺* mice received 10 consecutive doses of tamoxifen (2.5 mg/mouse×day, I.P.), while the *R26R⁺;Scl-CreER⁺* mice were dosed with five daily injections (2.5 mg per mouse×day, I.P.) followed by additional five doses administered every other day. These protocols were selected from our pilot experiments, where various dosing schedules were executed to achieve maximal efficiency of the Cre mediated activation of the reporter gene. Neointimal lesions were induced in the right common carotid artery (CCA) and right femoral artery (FA) in the same animal via complete ligation and a metal wire injury, respectively. For the carotid ligation, an 8-0 ligature was placed immediately proximal to the bifurcation. Mechanical injury to femoral artery was produced via a one-minute distention by a 0.015" guidewire inserted through the profunda femoris artery⁴. Animals received tamoxifen induction at 11 weeks of age, and operative procedures to create vascular injuries were performed on the day following the completion of tamoxifen induction. Surgical samples were perfusion-fixed with 4% paraformaldehyde in PBS and collected at various time points as specified in Table 1. At the time of sample collection, uninjured CCAs and FAs from the contralateral side were also harvested to examine the LacZ-labeling of SMCs and ECs.

Experimental Design

The *R26R;Myh11-CreER* system, in conjunction with a timely and controlled tamoxifen induction, selectively labels cells carrying the active *Myh11* promoter with LacZ tags. This system allows tracking of SMCs and detecting those with acquired ability to express *Myh11* in neointimal lesions. As detailed in table 1, the SMC group received tamoxifen only pre-operatively. Under this scenario, any LacZ-tagged cells in neointimal lesions would be of SMC origin. The bone marrow transplant experiment (e.g. the bone marrow-derived cell (BMC) group) was performed to examine the SMC lineage fate of BMCs in neointimal

lesions. Chimeras were created with $R26R^{+};Myh11-CreER^{+}$ bone marrow, and tamoxifen induction was completed prior to histologic evaluation at time points of d24, d38, or d59. Selective labeling of mature ECs was completed with the $R26R;Scl-CreER$ system. Two $Scl-CreER$ mouse lines with different specificities of EC labeling were created, and the strain utilized in this study was the one with minimal off-target Cre production in hematopoietic cells¹⁷.

Cell tracing

X-gal staining (MIR 2600, Mirus, Madison, WI) was performed on frozen sections (5 μ m in thickness, one section per sample) that were collected at a site 500 μ m proximal to the ligature or site of wire insertion. Sections were counterstained with nuclear fast red. Unmanipulated CCAs and FAs collected from $R26R^{+};Myh11-CreER^{+}$ ($n=6$) and $R26R^{+};Scl-CreER^{+}$ ($n=5$) animals were assayed to estimate the efficiency of LacZ labeling in SMCs and ECs, respectively. To quantify LacZ-tagged SMCs, x-gal positive area was measured using Axiovision as we have detailed previously¹⁸. The percent of the x-gal positive area in the medial layer of unmanipulated arteries was considered as the baseline for the efficiency of LacZ labeling of SMCs. Because of the fusion of the neointima with the media, especially in the FA lesions, determination of the border between these layers was technically challenging. Therefore, the neointima and media were viewed as one layer during morphometric analysis. The x-gal positive area was normalized to the total medial and neointimal (M+I) area of the corresponding section, and normalized data were used for subsequent analyses. The phenotype of the LacZ-tagged SMCs was characterized with immuno-fluorescent assays for SM α -actin and myosin heavy chain (SMMHC).

To quantify LacZ-labeled ECs, the length of the x-gal positive surface on cross sections of unmanipulated arteries was measured and indexed to the circumference of the corresponding section. The percent of the x-gal positive surface was considered as the baseline efficiency of LacZ labeling in ECs. All relocated LacZ-tagged (e.g. EC-derived) cells in the sub-EC neointimal/medial region were taken as events of EndoMT and counted on the x-gal staining sections. The percent of EC-derived cells was calculated to estimate the contribution of ECs to the total neointimal cell pool. The phenotype of the luminal ECs and the EC-derived cells (e.g. those located in the sub-EC region) was further characterized with immuno-fluorescent assays against LacZ tags, the EC lineage marker CD31, and mesenchymal makers of α -actin and collagen 1.

SMC and bone marrow cell culture

Primary aortic SMCs were explanted from tamoxifen-treated $R26R^{+};Myh11-CreER^{+}$ mice using the method we reported previously¹⁹. Cells were expanded in DMEM plus 10% FBS, and the SMC lineage was confirmed with flow cytometry against α -actin (F3777, Sigma, St. Louis, MO). To examine the durability of LacZ tags during de-differentiation and proliferation, SMCs were treated with 10 ng/ml of PDGF-BB (SRP3138, Sigma, St. Louis, MO) for 24 h¹⁶ and assayed with x-gal staining. SMCs separated from non-treated $R26R^{+};Myh11-CreER^{+}$ mice were included as negative controls. Acquisition of the LacZ tags by bone marrow cells was evaluated via PDGF-promoted *Myh11* activation⁵. Whole bone marrow (1×10^6 cells/mL) collected from non-treated $R26R^{+};Myh11-CreER^{+}$ mice

were incubated in RPMI 1640 plus 10% FBS for 3 h. After three gentle washes with serum-free RPMI 1640 to remove non-adherent cells, the remaining cells were treated with 50 ng/ml PDGF-BB for 72 h, followed by induction with the active form of tamoxifen (4-hydroxytamoxifen, H-7904, Sigma, St. Louis, MO) at a concentration of zero or 0.5 μ M for 48 h²⁰. Activity of the *Myh11* promoter in these cells was then examined with x-gal staining assays. All assays were done in triplicates.

Immuno-fluorescent assays

Immuno-fluorescent assays were performed on frozen sections (5 μ m) with specimens omitting primary antibodies included as negative controls. Primary antibodies applied to the assays were cy3 conjugated anti α -actin (c6198, Sigma, St. Louis, MO), chicken anti β -galactosidase (ab9361, abcam, Cambridge, MA), rabbit anti CD31 (ab28364, abcam, Cambridge, MA), rat anti CD31 (NB100-1642, Novus, Littleton, CO), rabbit anti SMMHC (ab53219, abcam, Cambridge, MA), goat anti vimentin (sc-7557, Santa Cruz, Dallas, Texas), and rabbit anti collagen 1 (NBP1-30054, Novus, Littleton, CO). Secondary antibodies were Alexa 488 goat anti-chicken, Alexa 546 goat anti-rabbit, and Alex 546 goat anti-rat, all purchased from Life Technologies (Grand Island, NY). Antigen retrieval was required for CD31 and β -galactosidase staining and achieved by incubating sections with citrate acid (H-3300, Vector Labs, Burlingame, CA) in a pressure cooker for 10 min. All assays were evaluated with confocal microscopy to determine co-localization of the examined markers.

Bone marrow transplantation

The *R26R⁺;Myh11-CreER⁺* or EGFP⁺ marrow cells (on a Ly5.2 background) were harvested from femurs by flushing with buffer-containing PBS plus 2%FBS. Cell clumps were broken by gentle passing through a 27G needle and debris was removed with a 100 μ m cell strainer. After lyses of red blood cells with ammonium chloride buffer (0.8% in 0.01 M Tris-HCl), the remaining cells were re-suspended in flush buffer and evaluated for cell concentration with a hemocytometer. Six-week-old C57BL/6-Ly5.1 male recipients were lethally irradiated with two doses of 500 Rads separated by a period of 4 h and then received 2×10^6 donor cells for bone marrow transplantation (BMT) via retro-orbital injection. Four weeks after BMT, the presence of ly5.2 positive cells in peripheral blood was evaluated with flow cytometry analysis. All chimeras demonstrated engraftment efficiency greater than 90%.

Statistical analyses

All data are expressed as mean $\pm$ SEM. Comparisons were done using an unpaired t-test and linear regression as appropriate. $P < 0.05$ was considered significant.

RESULTS

Medial SMCs migrate to the neointimal layer and become de-differentiated in neointimal lesions

In the tamoxifen-treated *R26R⁺;Myh11-CreER⁺* mice, LacZ tags (appearing in blue) were attached exclusively to SMCs in the media (Fig S1, A–C), with a labeling efficiency of 92%

and 94% for un-manipulated CCAs and FAs, respectively (Fig S1, D). Aortic outgrowths were nearly homogeneously α -actin positive and had LacZ tags (Fig. S1, E–G), indicating that these cells were SMCs. During neointimal hyperplasia, LacZ-tagged cells accumulated in the neointimal layer of both CCA (Fig 1, A–B) and FA (Fig 1, C–D) lesions on d38. Colocalization analyses revealed that some SMC-derived neointimal cells (Fig 1, E–H, green) lost their contractile protein α -actin (Fig 1, E and G, red). Analyses of the neighboring sections uncovered more severe loss of SM myosin heavy chain (SMMHC, Fig 1, F and H, red) than α -actin in SMC-derived neointimal cells in both the CCA and FA lesions. In contrast, SMCs in the media, particularly those in CCA lesions where the tunica media was well-preserved, displayed robust α -actin and SMMHC staining (Fig 1, E and F). Such a pattern was observed consistently in all 15 CCA and FA lesions. Evidently, SMCs physically relocate (e.g. migration) from the tunica media to the developing neointima and de-differentiate with loss of mature SMC markers.

SMCs populate the neointima with significant animal to animal variation

The content of LacZ-tagged SMCs in the neointimal layer varied widely, from a majority (Fig 2, column A) to nearly complete absence (Fig 2, column C) in both CCA and FA lesions. In some extreme cases, although the neointimal lesions were highly cellular and the underlying SMCs were well-preserved as evidenced by robust x-gal staining in the media, LacZ-tagged cells were hardly detected in the neointimal layer (Fig 2, column C). Such a striking phenomenon was noted in both CCA and FA lesions, suggesting that SMCs do not always donate progenies to the neointimal cell population. The paucity of LacZ-tagged cells seems not to be a result of random loss of the LacZ tags since SMCs remained homogeneously on LacZ tags even after several generations of continuous passaging or PDGF stimulation (Fig S1, F–G). Measurements of LacZ-tagged cells in individual CCA and FA lesions are plotted in Fig 2D (top panel); those having more LacZ-tagged cells in CCA lesions displayed more LacZ-tagged cells in their FA lesions as well, and such a correlation was statistically significant (Fig 2, D), indicating an individual dependency of the contribution of SMCs to the neointimal cell population. Overall, the content of LacZ-tagged cells was surprisingly low and averaged 27% and 29% in CCA and FA lesions, respectively, $P>0.05$ (Fig 2, D).

Bone marrow-derived cells cannot fully differentiate into SMC in the developing neointima

In order to capture the activation of *Myh11* promoter in bone marrow-derived cells during neointimal hyperplasia, tamoxifen induction was performed at various time points (Table 1) during neointimal formation. The injured CCAs and FAs produced a robust neointima hyperplasia by d38, with a combined neointimal/medial area measured as $37,293 \pm 10,983$ and $32,926 \pm 6,221 \mu\text{m}^2$ for the CCA and FA lesions, respectively, which were not significantly different from the amount of neointimal formation in non-irradiated mice ($34,748 \pm 5,596$ and $41,630 \pm 7,107 \mu\text{m}^2$ in CCA ($P=0.86$) and FA ($P=0.60$) lesions, respectively); however, microscopic examination did not detect any LacZ-tagged cells in CCA or FA lesions at all indicated time points (Fig 3, A). To confirm the recruitment of the bone marrow cells to neointimal lesions created with our experimental protocol, we generated EGFP bone marrow chimeras and evaluated the recruitment of EGFP cells in FA lesions ($n=3$). The FA lesions displayed abundant bone marrow-derived cells on

postoperative d38 (Fig 3, B). We further validated the negative finding with directed marrow cell differentiation experiments. The $R26R^{+};Myh11-CreER^{+}$ bone marrow cells were stimulated with PDGF, a growth factor known to induce *Myh11* expression in bone marrow progenitor cells⁵. After induction with the active form of tamoxifen, a subset of marrow cells were labeled successfully by LacZ tags (Fig 3, C), while the untreated bone marrow cells were completely LacZ-negative (Fig 3, D). These results suggest that the absence of the *Myh11*-positive cells in the CCA and FA lesions is the result of the inability of bone marrow cells to acquire the *Myh11* promoter activity rather than a labeling artifact or failed homing of bone marrow cells to the neointimal lesions.

Mature ECs undergo EndoMT infrequently to donate progenies to neointimal cell population

Neointimal lesions were created in CCAs and FAs of tamoxifen-treated $R26R^{+};Scl-CreER^{+}$ mice. In un-manipulated arteries, LacZ tags were attached exclusively to cells lining the luminal surface (Fig S2, A). Confocal Z-stack scanning showed a precise co-localization of the LacZ tags with the EC lineage marker CD31 (Fig S2, B), indicating that the LacZ labeling is restricted to mature ECs. The labeling efficiency was calculated as 62% and 82% for CCAs and FAs, respectively (Fig S2, C; $P=NS$). In the CCA and FA lesions, a few LacZ-tagged cells were detected in the sub-endothelial region (Fig 4, column A). In addition to the compartmental relocation, these cells also acquired the ability to produce α -actin (Fig 4, column B), the definitive mesenchymal marker usually not expressed in ECs^{14, 21}, indicating the occurrence of EndoMT in neointimal lesions. This cellular event, however, occurred at a very low frequency and represented only 7% and 3% of the total neointimal cell pool of CCA and FA lesions, respectively (Fig 4, B, $P=0.061$).

ECs may also acquire a mesenchymal phenotype without completely losing their lineage markers²². We examined the “*in situ* EndoMT” in both CCA and FA lesions. Several markers have been utilized to determine EndoMT in injured or diseased organs. Of them, α -actin and collagen 1 are accepted widely as a definitive marker for this process^{14, 15, 23}. Therefore, we examined both markers in luminal ECs. As shown in the right column of Fig 4 A, the LacZ-tagged ECs did not produce α -actin on luminal surface of both CCA and FA lesions. Collagen 1 was detected primarily in the adventitia and deep neointima of both CCA (Fig 4, C) and FA lesions (Fig 4, D). Cells lining the luminal surface maintained their ability to produce CD31, and we were unable to detect collagen 1 in these cells (Fig 4, C–D).

DISCUSSION

Using a strategy of cell tracing, the current study has provided *in vivo* evidence suggesting that neointimal lesions pathologically are highly heterogeneous among individual lesions. We showed that SMCs relocate from the tunica media to neointimal lesions, donating progenies to the neointimal cell population. During neointimal hyperplasia, SMCs become de-differentiated and lose the contractile proteins α -actin and SMMHC when populating the neointimal cell response. These cells, however, do not always comprise the major group in the neointimal cell population, which underscores the importance of individual differences and the importance of individualized therapy that might need to be targeted to control

neointimal hyperplasia. EndoMT, though serving as a major mechanism for neointimal cell repopulation in some vascular settings, occurred infrequently in the models examined in the current study.

The *Myh11*-CreER mouse line was chosen in this study for two reasons: 1) *Myh11* is the most definitive marker identified to date for SMCs; and 2) the Cre activity is inducible, which enables attachment of LacZ tags to SMCs from the media in a timely, controlled fashion. Other systems, such as the *SM22-LacZ*⁵ and *Myh11-LacZ*¹⁶, were utilized in previous studies to map the fate of SMCs. Because of the mosaic and episodic expression of the reporter cassettes, a binary Cre-loxP system was developed later and achieved a more homogenous labeling of SMCs^{7, 24, 25}, however, both *SM22* and *Myh11* promoters are expressed transiently in non-SMCs during embryonic development²⁴. At the adult stage, the *SM22* promoter remains active in myeloid cells²⁶. To avoid off-target Cre activity, several inducible Cre lines have been created, and the *Myh11-CreER* strain²⁷ has gained popularity in studies to fate-map SMCs during pathogenesis of vascular diseases^{9, 11, 12}. Consistent with these previous reports, we showed that about 95% of the SMCs in the tunica media in CCAs and FAs were labeled successfully with LacZ tags. Once attached, these tags become permanent, as evidenced by the results obtained from continuous SMC passaging and PDGF-induced dedifferentiation (Fig S1).

Recent work has raised a fundamental question as to the role of SMCs during neointimal hyperplasia^{9, 10}. Early evidence supporting SMCs as a source for the neointimal cell response comes from observations that SMCs underwent active proliferation during early neointimal hyperplasia, and cells expressing active *SM22* promoter prior to vascular injury accumulated in neointimal lesions^{1, 16}. This concept has now gained a solid foundation from two recent studies^{11, 12}. Consistent with these reports, our results showed that SMCs served as a source of progenies to the neointimal cell pool in lesions induced by different types of vascular injury. The incidence of this event appears, however, to be age-dependent. In juvenile mice, Herring *et al* observed that ~80% of neointimal cells were of SMC origin¹², while in adult mice, we found cells of SMC origin represented less than 30% of the total neointimal cell pool. Another widely accepted concept is that SMCs undergo “phenotypic switching” during neointimal hyperplasia²⁸. Using an *SM22* promoter assay, Regan *et al* demonstrated loss of *SM22* expression in SMCs derived from the tunica media of the vessel wall¹⁶. In the current study, we provided *in vivo* evidence showing that the expression of α -actin and SMMHC proteins are also severely inhibited or completely lost in neointimal SMCs, which reaffirms the concept of “SMC de-differentiation”. Additionally, there was an uncertainty on the lineage identity of the cells collected from aortic outgrowth^{7, 9, 10}. In this study, we provided direct evidence demonstrating that cells explanted from aortic media are truly SMCs.

A surprising finding in our study was the wide-range of mouse-to-mouse variation in the contribution of SMCs to the neointimal cell population in both CCA and FA lesions. Previous studies suggested that the number of living SMCs is the major determinant for the fraction of SMCs in the total neointimal cell pool⁴. While this theory is applicable to FA lesions where the structure of the tunica media is usually severely injured during creation of the model we employed, it is difficult to reconcile the variation among CCA lesions where

the media layer is well preserved and contains LacZ-tagged SMCs (Fig 2). Another explanation would be a random loss of the LacZ tags in the de-differentiated SMCs. Using total RNA extracted from whole tissue block, Cuttler *et al* detected a decrease in LacZ expression in neointimal lesions²⁹. Because of the small fraction of SMCs in the total neointimal cell pool, it is not surprising that the mean LacZ expression is low in neointimal lesions compared to normal arteries where the major cell group is LacZ-expressing SMCs. Our results show that SMCs remained homogeneously on LacZ tags after continuous passaging or PDGF-induced de-differentiation, supporting the concept that the variation seen in our experiments is not the result from random loss of LacZ tags. Complete carotid artery ligation and mechanical injury to the femoral artery are popular models utilized in both mechanistic studies and preclinical evaluations³⁰. Given the extremely high disparity of the dominant cell group between individual neointimal lesions forming in the same vascular setting, a critical but still unanswered question is whether neointimal cells of different origin would respond to molecular interventions and/or pharmaceutical therapies in a similar fashion. The answer to this question will guide the future development of therapeutic strategies to control neointimal growth.

Previous studies have led to confusion about the potential of bone marrow cells to differentiate into SMCs. Some investigators demonstrated that bone marrow cells were transient visitors and unable to acquire the ability to express SM α -actin in neointimal lesions^{31–33}, while other investigators provided evidence that supports the opposite conclusion^{34,35}. These studies used an EGFP reporter driven by various promoters, and the labeling efficiency of the EGFP reporter varies among different strains and cell lineages³⁶. In the current study, we evaluated the SMC-lineage potential of bone marrow cells with a LacZ reporter driven by the *Myh11* promoter. Examination at various time points could not detect any LacZ-tagged cells in either the CCA or the FA lesions. With our observations, it seems unlikely that there was a refractory response of the bone marrow cells to the labeling system, because LacZ tags were activated successfully in bone marrow cells after PDGF-stimulation that is known to induce *Myh11* promoter activity in marrow progenitor cells⁵. Our results, therefore, support the concept that bone marrow cells cannot acquire an SMC lineage fate in neointimal lesions.

Emerging evidence suggests that ECs can differentiate into SMC-like mesenchymal cells via EndoMT. These cells migrate to the sub-endothelial region and participate in the pathogenesis of cardiovascular diseases, such as pulmonary and cardiac fibrosis^{21,37}. We evaluated the importance of EndoMT as a mechanism for repopulating neointimal lesions. In the neointima forming in injured CCAs and FAs, we could only detect a small number of EC-derived cells in the sub-endothelial region. This observation is consistent with the report from Chen *et al* who utilized a Cre line different from ours and showed that EC-derived cells represented only 5% of the total neointimal cell population¹⁴. Interestingly, in neointimal responses forming in bypass vein grafts, Cooley et al observed that over 50% of the neointimal cells were of EC origin¹⁵. In contrast, Chen *et al* reported that EC-derived cells constituted only 6.7% of the neointimal cells in a similar vein bypass model¹⁴. All these studies, including ours, have utilized different lineage-tracing systems to investigate ECs as a source for neointimal cell population in different settings of vascular injuries. It

appears that both the lineage-tracking system and the vascular setting are important factors for the inconsistency between these studies.

In addition to relocation to pathologic sites, another feature of EndoMT is the alteration of their molecular signature, including loss of the markers of EC lineage CD31 and VE-cadherin and the gain of the mesenchymal markers α -actin, collagen 1, and vimentin^{14, 21}. We evaluated these markers in ECs lining the luminal surface of developing neointimal lesions and could not detect any convincing evidence for “*in situ* EndoMT” in both CCA and FA lesions. This finding appears inconsistent with the report from Chen *et al* who observed EndoMT in 46% of luminal ECs in the same model and at the same time point as we chose in the current study¹⁴. Major differences between the study of Chen *et al*¹⁴ and our studies was the age (6 vs. 11 weeks) of animals used in these experiments; indeed an age-dependent phenotypic expression has been documented recently for vascular remodeling in maturing mice¹³.

A few limitations exist in our study. LacZ-labeling of the mature ECs was achieved with the Cre-mediated recombination driven by the *Scl* promoter. Although the Cre activity of the *Scl-CreER* mouse line used in our study is highly restricted to mature ECs with little leakage to the hematopoietic cells, the recombination driven by this mouse line is incomplete in ECs lining the luminal surface of CCAs and FAs. Given the heterogeneity of the EC population³⁸, our system might have labeled selectively a subset of ECs, and, therefore, the fate map obtained with this mouse line may not be extrapolated to the entire EC population. In addition, the expression of SMMHC is a marker of differentiation gained by SMCs at the late stage of lineage maturation³⁹. In the BMT experiment, the SMC lineage potential of bone marrow cells was judged by the ability to acquire the *Myh11* promoter activity, which could not exclude the possibility that some of these cells may be able to differentiate into immature SMCs in neointimal lesions. Also, we only studied male mice, and therefore, we cannot comment on whether a similar response occurs in female mice.

In summary, SMCs are important players that physically relocate/migrate from the tunica media to the neointima, contributing to neointimal hyperplasia. The consistency of this cellular event varies widely between models and between individual animals, causing considerable disparity of the neointimal cell composition between different models and among lesions forming in the same animal model. Although ECs are important contributors to the hyperplastic neointimal thickening, the importance of EndoMT in this process appears limited to certain vascular settings. The extremely high pathologic heterogeneity of neointimal lesions seen in experimental models calls for future studies to characterize the cellular composition of human neointimal lesions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1.

SMCs from the tunica media repopulate neointimal lesions via a process of physical relocation and de-differentiation. A–D: x-gal staining of CCA (A–B) and FA (C–D) lesions. B and D, magnified view of the boxed area in A and C; Blue, LacZ-tagged SMCs; Purple, nuclear counterstain. Arrows point to internal elastic lamina (IEL). The residual media of FA lesions was torn apart during frozen sectioning due to the weak mechanical strength of this layer. Note the accumulation of LacZ-tagged SMCs in the neointimal layer of both CCA and FA lesions. E–H: immunofluorescent images representative of 15 CCA and FA lesions. Green, β -gal (LacZ gene product); Red, α -actin or SMMHC. White dash lines delineate IEL. Note the loss of α -actin (E and G) and SMMHC (F and H, arrows) in the majority of the SMC-derived neointimal cells (arrows). Scale bars, 50 μ m.

Fig 2. SMCs from the tunica media repopulate neointimal lesions with a degree varying widely between animals. A–C: x-gal staining of CCA (top row) and FA (bottom row) lesions (d38). Blue, LacZ-tagged SMCs; Purple, nuclear counterstain. Red dash lines delineate IEL. Note the remarkable variation in the number of LacZ-tagged SMCs in both CCA and FA lesions. Scale bars, 100 μm. D: accumulation of LacZ-tagged SMCs in FA and CCA lesions of individual animals. Regression analysis detected a significant individual dependency (top panel) though the averaged content of LacZ-tagged SMCs was not significantly different between CCA and FA lesions (lower panel).

Fig 3.

Bone marrow cells home to neointimal lesions but cannot acquire *Myh11* promoter activity.

A: x-gal staining of the CCA and FA neointimal lesions created in $R26R^{+};Myh11-CreER^{+}$ bone marrow chimeras. Samples were assayed at time points specified on the left of the panel. Purple, nuclear counterstain. Note the absence of β-gal positive cells in both the CCA and FA lesions at various time points. B: fluorescent imaging of an FA lesion (d38) created in an EGFP bone marrow chimera. Green, bone marrow derived EGFP positive cells; Blue, nuclear counterstain; L, lumen; M, media. C–D: x-gal staining of the $R26R^{+};Myh11-CreER^{+}$ bone marrow cells treated with (C) and without (D) PDGF. Note that LacZ tags (blue) are attached to a subset of bone marrow cells. Scale bars, 50 μm.

Fig 4.

ECs repopulate the neointima with a very low frequency. A: x-gal (left panels) and immunofluorescent (right panels) staining images representing CCA ($n=6$) and FA ($n=6$) lesions on d38. Dash lines, IEL. L, lumen; Blue, LacZ tagged ECs; Purple, nuclear counterstain; Green, β-gal; Red, α-actin. Red arrows point to EC-derived neointimal cells, white arrows denote cells stained positive for both β-gal and α-actin, and arrow heads point to LacZ-tagged luminal ECs. B: Fraction of EC-derived cells in the sub-endothelial region of CCA ($7\pm1\%$, $n=6$) and FA ($3\pm1\%$, $n=6$) lesions. $P=0.061$, by unpaired t-test.; Scale bars, 50 μm.

Table 1

Experimental design

Target	BMT	Pre-op TM	Surgery	Post-op TM	Evaluation
SMCs (n=15)	No	-d10	d0	No	d38
BMCs (n=5-7)	-d38	No	d0	d14, d28, or d49	d24, d38, or d59
ECs (n=6)	No	-d15	d0	No	d38

Note: BMT, bone marrow transplantation; TM, tamoxifen; BMCs, bone marrow-derived cells; d, days pre or post –operatively; and “-”, preoperatively.