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

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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, although the cellular heterogeneity may impact neointimal growth and its response to therapeutic interventions.*

Methods. R26R$^+$;Myh11-CreER$^+$, *and* R26R$^+$;Scl-CreER$^+$ *mice were used to attach LacZ tags to the preexisting 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 dedifferentiated phenotype in both CCA and FA lesions. The content of SMCs in the neointimal tissue, however, varied widely among specimens, ranging from 5 to 70% and 0 to 85%, with an average at low levels of 27% and 29% in CCA (*n = *15) and FA (*n = *15) lesions, respectively. Bone marrow cells, although able to home to the injured arteries, did not differentiate fully into SMCs after either type of injury. Preexisting ECs were located in the subendothelial 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 of EndoMT.*

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. (Surgery 2016;159:602-12.)*

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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 used widely in studies to obtain mechanistic insights into the hyperplastic response of injured vessels. Of these models, common carotid artery (CCA) ligations, transluminal injury to femoral arteries (FA), 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. Although an simple 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

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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. More than 40% of neointimal cells were of bone marrow origin.[4,5] Other sources, including both local vessel wall[6,7] and remote perivascular tissues,[8] 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 thickening,[11] uncertainty has remained about whether SMCs make up the majority of neointimal cells. A study tracing the lineage fate of these cells demonstrated that >80% of neointimal cells originated from the preexisting SMCs[12]; 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 animals,[13] 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 >50% of total neointimal cell population via a process termed endothelial–mesenchymal transition (EndoMT).[15] The discovery of a 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 al.[16] 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 preclinical studies. EndoMT, although serving as a major mechanism for neointimal cell repopulation in selected vascular settings,[15] seems 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 (Bar Harbor, ME). 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 $R26R^{+}$;$Myh11\text{-}CreER^{+}$ and $R26R^{+}$;$Scl\text{-}CreER^{+}$ animals with polymerase chain reaction–based genotyping. The $R26R^{+}$;$Myh11\text{-}CreER^{+}$ mice received 10 consecutive doses of tamoxifen (2.5 mg/mouse per day IP), whereas the $R26R^{+}$;$Scl\text{-}CreER^{+}$ mice were dosed with 5 daily injections (2.5 mg/mouse per day IP) followed by an 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 CCA and right 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 FA was produced via a one-minute distention by a 0.015" guidewire inserted through the profunda femoris artery.[4] Animals received tamoxifen induction at 11 weeks of age, and operative procedures to create vascular injuries were performed on the day after the completion of tamoxifen induction. Surgical samples were perfusion-fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and collected at various time points as specified in the Table. 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