c-Myb Regulates Proliferation and Differentiation of Adventitial Sca1+ Vascular Smooth Muscle Cell Progenitors by Transactivation of Myocardin

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Objective—Vascular smooth muscle cells (VSMCs) are believed to dedifferentiate and proliferate in response to vessel injury. Recently, adventitial progenitor cells were implicated as a source of VSMCs involved in vessel remodeling. c-Myb is a transcription factor known to regulate VSMC proliferation in vivo and differentiation of VSMCs from mouse embryonic stem cell–derived progenitors in vitro. However, the role of c-Myb in regulating specific adult vascular progenitor cell populations was not known. Our objective was to examine the role of c-Myb in the proliferation and differentiation of Sca1+ adventitial VSMC progenitor cells.

Approach and Results—Using mice with wild-type or hypomorphic c-myb (c-myb<sup>h/h</sup>), BrdU (bromodeoxyuridine) uptake and flow cytometry revealed defective proliferation of Sca1<sup>+</sup> adventitial VSMC progenitor cells at 8, 14, and 28 days post carotid artery denudation injury in c-myb<sup>h/h</sup> arteries. c-myb<sup>h/h</sup> cKit<sup>+</sup>CD34<sup>-</sup>Flk1<sup>+</sup>Sca1<sup>+</sup>CD45<sup>-</sup> cells failed to proliferate, suggesting that c-myb regulates the activation of specific Sca1<sup>+</sup> progenitor cells in vivo and in vitro. Although expression levels of transforming growth factor-β1 did not vary between wild-type and c-myb<sup>h/h</sup> carotid arteries, in vitro differentiation of c-myb<sup>h/h</sup> Sca1<sup>+</sup> cells manifested defective transforming growth factor-β1–induced VSMC differentiation. This is mediated by reduced transcriptional activation of myocardin because chromatin immunoprecipitation revealed c-Myb binding to the myocardin promoter only during differentiation of Sca1<sup>+</sup> cells, myocardin promoter mutagenesis identified 2 specific c-Myb–responsive binding sites, and adenovirus-mediated expression of myocardin rescued the phenotype of c-myb<sup>h/h</sup> progenitors.

Conclusions—These data support a role for c-Myb in the regulation of VSMC progenitor cells and provide novel insight into how c-myb regulates VSMC differentiation through myocardin. (Arterioscler Thromb Vasc Biol. 2016;36:1367-1376. DOI: 10.1161/ATVBHA.115.307116.)

Key Words: c-myb genes ■ cell differentiation ■ myocardin ■ progenitor cells ■ smooth muscle cells

The vessel wall is known to host tissue-resident stem and progenitor cells capable of forming the various cells that constitute its layers. Although the adventitia was long thought to be inert, recent studies have shown the adventitia to be a dynamic tissue playing an active role in the regulation of the vasculature. Various progenitor cell populations in the adventitia can give rise to not only vascular smooth muscle cells (VSMCs) but also endothelial cells, pericytes, and small populations of hematopoietic/myelopoietic cells. Indeed, it has been suggested that the adventitia may be a contributing source of long-lasting neointimal VSMCs after injury. Several potential stem and progenitor cell populations have been identified as residing in the tunica media and/or adventitia, including stem cell antigen-1<sup>+</sup> (Sca1<sup>+</sup>) CD45<sup>-</sup> adventitial macrophage progenitor cells and Sca1<sup>+</sup>CD45<sup>-</sup> progenitor cells that give rise to endothelial and mesenchymal cell lineages. However, few studies have identified factors that regulate the proliferation and differentiation of these vessel-resident adventitial VSMC progenitor cells.

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Hu et al<sup>7</sup> first identified a Sca1<sup>+</sup> cell residing in the adventitia as a VSMC progenitor cell. When transferred to the adventitial side of irradiated vein grafts, ≤30% of neointimal cells in the graft were shown to be derived from transplanted adventitial cells. When isolated and expanded in vitro, Sca1<sup>+</sup> cells were found to be largely cKit<sup>+</sup> and negative for hematopoietic lineage markers (CD45<sup>-</sup>Lin<sup>-</sup>), suggesting that these cells were not derived from the bone marrow (BM) but were vessel-resident vascular progenitor cells. Adventitial Sca1<sup>+</sup> progenitor cells differentiated into smooth muscle cells expressing the mature...
VSMA markers α-smooth muscle actin (ACTA2), calponin (CNN1), and smooth muscle myosin heavy chain (MYH11).3 Passman et al18 later identified Sonic hedgehog (Shh) signaling as critically important to the development of adventitial Sca1+ progenitor cells. Shh−/− mice had greatly reduced numbers of adventitial Sca1+ progenitor cells, suggesting that Shh signaling was critical to the development or homing of adventitial Sca1+ progenitor cells to the adventitia.4 The differentiation of adventitial Sca1+ VSMC progenitors has been shown to be regulated by integrin/collagen-IV interactions2 and through extracellular signal-regulated kinase/β-catenin signaling1 after sirolimus exposure in vitro. Matrix metalloproteinase-811 and Stromal-cell derived factor-12 also seem to regulate adventitial Sca1+ VSMC progenitor cell recruitment during atherosclerosis or neointimal formation in vein grafts. However, in none of these studies were subsets of Sca1+ cells identified as being relevant to vessel remodeling in vivo, nor were their endogenous spatiotemporal patterns of abundance examined.

The hematopoietic transcription factor c-myb is known as a stem/progenitor cell regulator in multiple tissue compartments12–17 and has also been shown to regulate the proliferation of adult VSMCs. We have shown that c-myb regulates the differentiation of VSMCs from embryonic stem cell (ESC)–derived embryoid bodies, specifically by promoting the expansion of a Flk1+PDGFRα− progenitor cell population in vitro.18,19 However, it remains unknown if such a VSMC progenitor cell exists in vivo, and if it persists into adulthood where it might contribute to vascular pathophysiology. In addition, although c-myb was shown to regulate the number of Flk1+PDGFRα− progenitor cells, the mechanism by which it regulates VSMC differentiation from progenitor cells remains unknown.

An in vivo model of c-Myb deficiency was reported by Sandberg et al,17 where an N-ethyl-N-nitrosourea mutagenesis screen identified a mouse line with a nonlethal point mutation in c-myb. This point mutation mapped to the transactivating domain of c-Myb, resulting in defective recruitment of transcriptional coactivators (such as p300), leading to decreased activity of c-Myb. This hypomorphic (c-myb−/−) mouse has several hematopoietic abnormalities caused by dysregulation of hematopoietic stem/progenitor cell maintenance, as well as stage- and lineage-specific blocks on differentiation. Several studies have shown that inhibiting c-myb activity reduces vessel remodeling after injury26–29; however, these studies have not been able to address if c-myb also has effects on vessel-resident VSMC progenitor cells. Given the role of c-myb in other stem and progenitor cell compartments,12–17 as well as evidence for the role of c-myb in the differentiation of VSMCs from embryoid bodies,18,19 we sought to more specifically test the role of c-myb in the proliferation and differentiation of adventitial Sca1+ VSMC progenitor cells. Further defining the role of vessel-resident VSMC progenitor cells, as well as identifying specific factors involved in the proliferation, differentiation, and activity of cells recruited during the injury response may represent a novel approach to modulating vessel injury responses as diverse as neointima formation, atherosclerosis, hypertrophy, and neeurysm formation.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

c-myb−/− Mice Have Defective Vessel Remodeling After Wire Denudation Injury

To examine the effect of c-myb hypomorphism on injury-induced vessel remodeling, groups of 10- to 12-week-old male mice homozygous for the hypomorphic c-myb allele (c-myb−/−) and their wild-type (WT) littermate controls on a C57BL/6 background were subjected to wire denudation injury of the left common carotid artery (CCA) and allowed to recover for 8, 14, or 28 days. The right CCA served as the uninjured control artery. No differences in medial or intimal area were found between WT and c-myb−/− mice at baseline (Figure 1A). Mice were examined 8 days post injury, a time point previously characterized as that of maximal medial proliferation.26 No differences were found in intimal or medial remodeling between injured WT and c-myb−/− mice, suggesting that medial proliferation may not be affected in c-myb−/− mice (Figure 1B–1D). Given that neointimal remodeling is known to be greater at later time points, mice were also examined at 14 and 28 days post injury. At these later time points, c-myb−/− mice had decreased neointima formation at 14 days (16.7±0.3 versus 31.4±5.0×103 μm2; N=6–7 mice per group; P<0.05) and continued to have decreased neointima area at 28 days post injury compared with WT (17.3±3.8 versus 39.6±8.5×103 μm2; N=5–8 mice per group; P<0.001; Figure 1E–1J). c-myb−/− mice on a FVB/N background also manifested a similar phenotype, demonstrating that the phenotype is strain independent (Figure I in the online-only Data Supplement). Medial remodeling in c-myb−/− mice remained no different than that of WT mice (Figure II in the online-only Data Supplement), indicating a preferential defect in cells involved in neointimal, but not medial, remodeling.

c-Myb Regulates Vessel-Intrinsic Remodeling Responses

Because c-Myb has pleiotropic roles in circulating BM-derived leukocytes and vessel-resident cells involved in the injury response, reciprocal bone marrow transplantation experiments were performed. To determine if the diminished injury response of c-myb−/− mice was vessel intrinsic or mediated by circulating BM-derived factors or cells, BM from either WT or c-myb−/− donors were transplanted into c-myb−/− recipient mice. Importantly, BM from WT donors failed to restore the injury response of c-myb−/− recipients, suggesting that the decreased neointimal response was vessel intrinsic (Figure 2).
In agreement with these data, there are no differences in leucocyte recruitment between WT and c-myb h/h injured vessels (Figure III in the online-only Data Supplement). Illustrating the complexity of the biology of neointimal remodeling, when reciprocal experiments were performed using WT recipients, c-mybh/h BM conferred protection from neointimal remodeling as compared with WT BM (Figure IV in the online-only Data Supplement).

**c-Myb Regulates Sca1+ VSMC Progenitor Cell Proliferation in Response to Vessel Injury**

Because Sca1+ cells have been shown to participate in vessel remodeling, we next examined whether c-myb has a role in regulating the formation of these adventitial VSMC progenitor cells. The CCA of WT and c-myb h/h mice were found not to differ in the number of adventitial Sca1+ cells as assessed by immunohistology (Figure 3A and 3B; Figure V in the online-only Data Supplement), suggesting that c-myb may not regulate the development of Sca1+ adventitial VSMC progenitor cells. Although previous studies have relied heavily on histological identification of Sca1+ cells, this method has technical limitations for the identification and quantification of specific subpopulations of Sca1+ cells. Accordingly, we also used flow cytometry to refine the analysis of adventitial VSMC progenitor cell populations, allowing for the exclusion of hematopoietic cells and endothelial cells from the analyzed cell populations. After CCA injury, adventitial Sca1+ cells were increased at 14 days (5629±327 [54.7±1.7%]) and 28 days post injury (6725±321 [55.8±1.4%]) in WT animals compared with baseline (3796±360 [39.8±0.9%]; N=3–10 mice per group; P<0.01 for both comparisons; Figure 3D and 3E). However, there were significantly fewer Sca1+ cells in c-myb h/h mice at 14 days (4371±436 [49.1±2.1%]) and 28 days post injury (5243±218 [48.6±1.2%]; N=3–10 mice per group; P<0.05 for both comparisons versus WT), suggesting that c-myb plays a role in the expansion of Sca1+ cells that occurs after injury. To assess the proliferation of Sca1+ progenitor cells in response to vessel injury, we quantified the incorporation of bromodeoxyuridine (BrdU) post injury.
c-mybh/h CD45−Lin−Sca1+ cells had decreased BrdU incorporation compared with WT cells at 8 days (5.4±0.8% versus 9.1±0.6%), 14 days (2.4±0.4% versus 4.9±0.4%), and 28 days post injury (2.0±0.6% versus 4.1±0.6%; N=3–10 mice per group; 8 days: \( P < 0.001 \), 14 days and 28 days: \( P < 0.05 \)), indicating that c-myb promotes the proliferation of CD45−Lin−Sca1+ adventitial progenitor cells (Figure 3F and 3G).

**Expansion of Sca1+ Adventitial Cells After Injury Is Derived From Vessel-Resident Cells and Not Circulating Cells**

Because Sca1+ adventitial VSMC progenitor cells could potentially arise locally or be recruited from the circulation in response to injury, we injured WT and c-mybh/h mice transplanted with eGFP+c-mybWT BM. This analysis revealed that both at baseline and at 28 days post injury, >99% of all carotid adventitial CD45−Lin−Sca1+ cells remain host vessel derived (ie, eGFP−; Figure 4A), which was consistent in the aortic arch (Figure 4B). Excluding the possibility that incomplete or unsuccessful BM reconstitution might explain the above result, >99% of blood cells were eGFP+ (Figure 4C).

**c-Myb Specifically Regulates Sca1+cKit+CD34−Flk1− Cells in Response to Vessel Injury**

cKit, CD34, and Flk1 have been identified as potential additional markers of adventitial Sca1+ progenitor cells that can differentiate into VSMCs, yet it is not known if one or a combination of these markers identifies VSMC progenitor cell populations that may be involved in the injury response in vivo. Thus, we next examined the expression of cKit, CD34, and Flk1 within CD45−Lin−Sca1+ cells in the uninjured carotid as well as after vessel injury and found that few CD45−Lin−Sca1+ cells in the uninjured carotid artery are cKit+CD34+ (0.15±0.04%) or cKit−CD34+ (0.38±0.09%) and that these proportions were not different between WT and c-mybh/h mice (Figure 5A and 5B). Of interest, the relative abundance of CD45−Lin−Sca1+ Flk1+ cells in carotid arteries was decreased at 14 and 28 days post injury as compared with baseline and did not differ between WT and c-mybh/h mice (Figure VII in the online-only Data Supplement). By contrast, we found that at 8 and 14 days post injury, there is an expansion specifically of the cKit+CD34− population, whereas cKit−CD34+ and cKit+CD34+ cells remained unchanged (Figure 5B) in WT mice injured with supraphysiological doses of c-myb compared with WT mice injured with control BM (Figure VIII)
mice. However, in c-myb<sup>−/−</sup> mice, the cKit<sup>+</sup>CD34<sup>−</sup> population failed to expand to the same extent as in WT mice (10.4±0.8% versus 14.8±1.1%; N=5 mice per group; *P<0.05; Figure 5A and 5B). When adventitial Sca1<sup>+</sup> cells isolated from carotid arteries were cultured in vitro, there was decreased proliferation of c-myb<sup>−/−</sup> Sca1<sup>+</sup> cells (6.1±1.5% versus 21.1±1.1% WT Sca1<sup>+</sup>BrdU<sup>+</sup> cells; Figure 5C), whereas the relative abundance of Sca1<sup>+</sup> cells did not differ between genotypes (Figure VIIA in the online-only Data Supplement). It was also found that there were fewer Sca1<sup>+</sup>cKit<sup>+</sup> cells in cultures isolated from c-myb<sup>−/−</sup> carotid arteries versus WT carotid arteries (3.1±0.7% versus 16.2±0.5%; N=3 mice per group; *P<0.05; Figure 5D).

When the expression of cKit and CD34 was examined on proliferating (BrdU<sup>+</sup>) Sca1<sup>+</sup> cells in vitro, it was found that in both genotypes, proliferating cells were predominantly cKit<sup>+</sup> (Figure 5E).

c-Myb Regulates the Differentiation of Adventitial Sca1<sup>+</sup> Cells Into VSMCs

To examine whether c-myb is involved in the differentiation of Sca1<sup>+</sup> adventitial VSMC progenitor cells, cells were isolated from WT and c-myb<sup>−/−</sup> mice and differentiated in the presence of 0.2% fetal bovine serum and 10 ng/mL transforming growth factor-β1 (TGF-β1) for 7 days. Unlike WT cells, c-myb<sup>−/−</sup> adventitial Sca1<sup>+</sup> VSMC progenitor cells did not effectively upregulate markers of differentiated VSMCs, such as CNN1, MYH11, ACTA2, and myocardin (Figure 6A–6C), suggesting defective VSMC differentiation in c-myb<sup>−/−</sup> adventitial VSMC progenitor cells. Interestingly, endogenous gene expression levels of several VSMC-specific and extracellular matrix genes also differed in the injured carotid arteries of WT versus c-myb<sup>−/−</sup> mice in vivo (Figure IX in the online-only Data Supplement), in a pattern mirroring the defects observed in differentiated c-myb<sup>−/−</sup> adventitial Sca1<sup>+</sup> VSMC progenitor cells in vitro. c-Myb deficiency did not have an effect on adventitial smooth muscle progenitor cell apoptosis at baseline or after induction of differentiation by TGF-β1 (Figure VIIIC in the online-only Data Supplement).

The Myocardin Gene Promoter Is Bound and Transcriptionally Activated by c-Myb During VSMC Differentiation

Although c-myb has been previously demonstrated to regulate ESC differentiation to VSMCs, the mechanism by which c-myb promotes VSMC differentiation remains unclear. When Sca1<sup>+</sup> VSMC progenitor cells were differentiated, c-myb<sup>−/−</sup> Sca1<sup>+</sup> VSMC progenitor cells failed to upregulate myocardin expression (Figure 6A). Similarly, c-myb<sup>−/−</sup> CCA have lower myocardin expression than that of WT CCA (Figure 6D). Adenoviral overexpression of myocardin before differentiation restored SMC gene expression in differentiated c-myb<sup>−/−</sup> adventitial Sca1<sup>+</sup> VSMC progenitor cells (Figure 6E). c-Myb–dependent activation of myocardin was determined using a heterologous system by cotransfection of a myocardin promoter–luciferase construct and either a c-Myb expression or empty control vector into human embryonic kidney 293 cells. Overexpression of c-Myb resulted in activation of the myocardin promoter (Figure 7A). Cotransfection of hypomorphic c-Myb resulted in decreased myocardin–luciferase reporter activity (Figure 7A), as did the cotransfection of a distinct mutant c-Myb (mR3-3),
previously shown to have defective c-Myb target gene activation27 (Figure 7B).

In silico analysis of the myocardin core promoter 28 revealed 8 putative c-Myb–binding sites (MBS) in the core myocardin promoter (Figure 7C). To determine if c-Myb transcriptionally regulates myocardin expression during VSMC differentiation, chromatin immunoprecipitation was performed using an anti–c-Myb antibody. Maximal induction of c-myb was observed 1 day post differentiation (Figure VIIID in the online-only Data Supplement); thus, c-Myb binding to the myocardin promoter was examined 1 day post induction of differentiation (Figure VIIID in the online-only Data Supplement); thus, c-Myb binding to the myocardin promoter was examined 1 day post induction of differentiation. Although c-Myb was not found to bind to any region of the core myocardin promoter in undifferentiated Sca1+ VSMC progenitor cells, c-Myb bound 2 regions of the core promoter on induction of VSMC differentiation with TGF-β1 (Figure 7D). To examine the functional importance of both predicted and chromatin immunoprecipitation–confirmed MBS in the myocardin promoter, WT and point-mutated promoter assays in human embryonic kidney cells cotransfected with c-Myb were performed. These experiments revealed that c-Myb–dependent myocardin promoter activity was critically dependent on MBS1 and MBS8 (Figure 7E). Unexpectedly, mutation of MBS3 resulted in a constitutively overactive myocardin reporter (Figure X in the online-only Data Supplement). These results demonstrate direct association of c-Myb with the myocardin promoter during differentiation of Sca1+ VSMC progenitor cells to VSMCs and its transcriptional activation via specific MBS.

Discussion

It has been established that c-myb regulates the proliferation of VSMCs and the differentiation of VSMCs from ESC-derived progenitors in vitro18–20,29,30; however, it remained unknown if c-myb has a similar role in adult vessel-resident VSMC progenitor cells in vivo. Here, we show that a mouse harboring a point-mutated c-myb has defects in vessel remodeling in response to wire denudation injury and that this is a vessel-intrinsic defect associated with defective proliferation of Sca1+ adventitial VSMC progenitor cells. Although c-myb does not seem to have a developmental role in the formation of Sca1+ adventitial VSMC progenitor cells, our data demonstrate that c-myb regulates the proliferation of CD45-Lin−Sca1+ cells in response to wire denudation injury. Moreover, we have identified that cKit+Sca1+ progenitor cells expand in response to wire denudation injury, whereas CD34+ and Flk1+ cells do not. In vitro differentiation of c-myb−/−–derived adventitial VSMC progenitor cells failed to effectively generate mature VSMCs, demonstrating that c-myb also promotes the differentiation of adventitial progenitor cells. Finally, our data demonstrate that c-myb regulates VSMC differentiation through the binding and transcriptional regulation of the myocardin gene. These results together demonstrate a role for c-myb in
regulating the proliferation and differentiation of an adult vessel-resident VSMC progenitor cell.

c-Myb Regulates Sca1+ Progenitor Cells in Response to Injury

In this study, we have established that adventitial Sca1+ progenitor cell development does not seem to be regulated by c-myb, because no differences in the number of adventitial Sca1+ cells were found by histology or flow cytometry of uninjured vessels in WT and c-mybh/h mice. However, on injury, there is a significant impairment in the ability of c-mybh/h Sca1+ progenitor cells to proliferate as compared with WT Sca1+ cells. This role for c-myb in the proliferation of Sca1+ cells is similar to its already established role in VSMC proliferation, providing evidence that c-myb regulates not only mature VSMC proliferation but also the proliferation of adult vessel-resident VSMC progenitor cells.

Sca1+ Progenitors Are Vessel Resident and Expand After Vessel Injury

Using WT and c-mybh/h mice lethally irradiated and reconstituted with eGFP+ BM, we have shown that CD45−Lin−Sca1+ cells in the carotid artery and aorta are not BM derived. In response to wire denudation injury, the Sca1+ cell population proliferates; when mice reconstituted with eGFP+ BM were injured, the CD45−Lin−Sca1+ cells remained eGFP−, confirming that Sca1+ adventitial progenitor cells are not BM-derived cells at baseline or in response to injury but are in fact adult vessel-resident progenitor cells. In addition, leukocyte recruitment is not impaired in c-mybh/h injured vessels, suggesting that c-myb does not regulate inflammatory cell recruitment in response to vessel injury. Although reconstitution of c-mybh/h mice with WT BM was not sufficient to restore their injury response, the reciprocal experiment in which WT mice were reconstituted with c-mybh/h BM revealed that c-mybh/h BM had a protective effect on neointimal remodeling, demonstrating the complexity of neointimal remodeling. Given the various hematopoietic abnormalities caused by c-myb hypomorphism, precise elucidation of which BM-derived cell populations mediate the latter phenotype would require complex stage- and lineage-specific transplantation or genetic ablation models beyond the scope of this study. Moreover, the contribution of BM-derived protection to the decreased vessel remodeling of c-mybh/h mice, although noteworthy, is overshadowed in the context of wire denudation injury, given that WT BM is insufficient to restore a normal injury response to c-mybh/h mice. Taken together, these results show that although c-mybh/h BM-derived cells also confer some level of protection from neointimal remodeling, the defect in vessel remodeling is intrinsic to the c-mybh/h vessel-resident cells. Consistent with this observation, when TGF-β1 expression was examined in WT and c-mybh/h arteries post injury, there was no difference in TGF-β1 mRNA levels. Rather, Sca1+ cells isolated from c-mybh/h mice manifest defective responses to TGF-β1 treatment, suggesting that the observed phenotype was independent of circulating or secreted factors. A limitation of this study is that other cell compartments such as endothelial cells, pericytes, and BM-derived cells from c-mybh/h mice may also harbor uncharacterized defects; however, our bone marrow transplantation and in vitro experiments suggest that the effects observed in Sca1+ cells are cell intrinsic and not dependent on other cell types.

c-Myb Specifically Regulates the Expansion of cKit+Sca1+ Cells

We have shown previously that c-myb has a role in regulating ESC differentiation into VSMCs through the expansion of a subset of hemogenic VSMC progenitor cells that are Flk1+. However, it was not known if c-myb has such a role in adult vessel-resident progenitor cells in vivo. On the basis of our earlier work, we hypothesized that an Flk1+ progenitor might persist within the Sca1+ adventitial progenitor cell population, and this adult Sca1+Flk1+ cell population would be regulated by c-myb in vivo. Histological examination of mouse adventitia in a previous report suggested that adventitial Sca1+ progenitor cells are a heterogeneous population expressing CD34 or Flk1; however, flow cytometric analysis identified adventitial Sca1+ cells as Lin−cKit+. A separate study found that freshly isolated adventitial Sca1+ progenitor cells were cKit CD34+CD140b−CD45+CD68−; however,
these expression data came from reverse transcription-PCR of magnetically isolated Sca1+ cells without lineage exclusion and were not validated by flow cytometry or immunostaining. In this study, a flow cytometry approach to analyze adventitial Sca1+ progenitor cell populations was used to quantitatively determine markers to identify progenitor cells relevant to vessel remodeling after injury. No baseline differences between WT and c-myb<sup>h/h</sup> Sca1+ cell population expression of cKit, CD34, and Flk1 were found. After vessel injury, although CD34+ and Flk1+ cells declined, cKit+ cells expanded in response to injury in WT mice. However, in c-myb<sup>h/h</sup> mice post injury, a defect in the expansion of Sca1+cKit+ cells was identified. Consistent with these results, when adventitial Sca1+ cells were isolated from WT and c-myb<sup>h/h</sup> carotid arteries, a proliferative defect was observed specifically in c-myb<sup>h/h</sup> cKit+CD34+ Sca1+ cells. It is tempting to speculate that CD34 and Flk1 represent markers of quiescent versus activated Sca1+ VSMC progenitors and that expression of CD34 and Flk1 are downregulated by activation of Sca1+ progenitor cells. Although the baseline number of Sca1+ progenitors and CD34+ and Flk1+ subpopulations did not differ between WT and c-myb<sup>h/h</sup> mice, we have not excluded the possibility that the function of these progenitors remains disturbed and may also contribute to the overall vascular phenotype of this mutant mouse. Additionally, it remains unknown if there are differential responses of adventitial progenitor cells based on embryological origins or vessel size, because previous reports examined aortic arch adventitial VSMC progenitor cells, which may be of embryological origins distinct from that of carotid artery adventitial cells.

c-Myb Regulates the Differentiation of Adventitial Sca1+ VSMC Progenitor Cells

To date, there have been few reports of transcription factors that regulate the differentiation capacity of adventitial Sca1+ VSMC progenitor cells. WT adventitial Sca1+ VSMC progenitor cells differentiated into VSMCs as evidenced by expression of VSMC markers Cnn1, Acta2, Myh11, and myocardin. By contrast, c-myb<sup>h/h</sup> adventitial Sca1+ VSMC progenitor cells incompletely upregulated expression of these genes, suggesting that they are defective in their ability to differentiate into VSMCs, demonstrating the requirement for functional c-myb in the differentiation of this VSMC progenitor cell population.

c-Myb Binds the Myocardin Promoter During VSMC Differentiation

Undifferentiated Sca1+ adventitial VSMC progenitor cells were shown to be primed for VSMC differentiation through the coexpression of serum response factor and myocardin, while also having high expression of transcriptional corepressors of VSMC differentiation Msh homeobox 1, Kruppel-like factor 4, and Forkhead box protein O4. In WT adventitial Sca1+ cells, the expression of Msh homeobox 1, Kruppel-like factor 4, and Forkhead box protein O4 decreased over time because cells differentiated, whereas serum response factor and myocardin were upregulated. Because myocardin expression was lower in c-myb<sup>h/h</sup> carotid arteries at baseline and after injury and incompletely upregulated in response to TGF-β1 in vitro in c-myb<sup>h/h</sup> adventitial Sca1+ VSMC progenitor cells, we hypothesized that c-myb regulates VSMC differentiation of Sca1+ smooth muscle progenitor cells via transcriptional control over myocardin. Adenoviral overexpression of myocardin rescued the expression of SMC genes in differentiated c-myb<sup>h/h</sup> Sca1+ VSMC progenitor cells. Although these data demonstrate that c-Myb regulates VSMC differentiation at least partly through myocardin, we have not excluded the possibility that additional mechanisms may also be regulated by c-myb.

In a heterologous system, c-Myb overexpression was sufficient to activate a myocardin promoter–luciferase construct, further demonstrating that c-Myb activates myocardin expression. Consistent with our observations from c-myb<sup>h/h</sup> Sca1+ VSMC progenitor cells, transfection of our system with hypomorphic c-Myb did not activate the myocardin promoter reporter construct as effectively as WT c-Myb. Importantly, a second c-Myb mutant (mR3-3), previously shown to have defective transactivation of c-Myb target genes, also failed to activate a myocardin reporter. Taken together, these data demonstrate that c-Myb is critical for the activation of myocardin expression. Interestingly, the promyogenic activity of myocardin is enhanced by p300, a histone acetyltransferase known to have defective interaction with the mutant-c-Myb expressed in c-myb<sup>h/h</sup> mice. Given the specific defect in the transactivation domain of the c-Myb hyporphom studied, it is also likely that interaction of c-myb with other transcriptional coactivators is necessary for efficient activation of the myocardin promoter and subsequent differentiation of adventitial Sca1+ VSMC progenitors into VSMCs.

Chromatin immunoprecipitation with anti–c-Myb in Sca1+ VSMC progenitor cells demonstrated binding of c-Myb to the myocardin promoter in 2 regions of the core promoter. Importantly, c-Myb binding to the myocardin promoter could not be detected in undifferentiated cells, demonstrating that there is dynamic and direct regulation of myocardin transcription by c-Myb during differentiation of Sca1+ adventitial VSMC progenitor cells into VSMCs. Through site-directed mutagenesis, it was determined that MBS2 was not as critical for c-Myb–induced promoter activity, whereas mutagenesis of MBS1 and MBS8 significantly decreased c-Myb–induced myocardin promoter activity. Interestingly, the mutation created in MBS3 (CAGCGGTC changed to CccgGGTC) generated a myocardin–luciferase construct with 8.0×10<sup>5</sup>-fold higher activity than the WT myocardin promoter, suggesting either inadvertent creation of a binding site for another potent transcriptional activator or, more likely, loss of an inhibitory element in the myocardin promoter. This is the direct mechanistic demonstration of how c-myb regulates the differentiation of progenitor cells into VSMCs and provides insight into how c-myb regulates the expression of multiple VSMC genes simultaneously.

It had been widely accepted that VSMC-like cells involved in the neointimal injury response are derived from the phenotypic modulation of contractile medial VSMCs into proliferative synthetic VSMCs. However, emerging evidence now suggests that local progenitor populations residing in various compartments of the vessel wall may in fact be a contributing source of synthetic VSMCs in response to injury. Through lineage-tagging experiments, it has been suggested...
that neointimal VSMCs may also be generated in part by the proliferation and differentiation of multipotent vessel-resident stem and progenitor cells. We now demonstrate that c-myb regulates the proliferation and differentiation of an adventitial Sca1+ VSMC progenitor cell. A limitation of this study is that neointimal VSMCs may also be generated in part by the proliferation and differentiation of an adventitial Sca1+ VSMC progenitor cell. A limitation of this study is that we have yet to directly examine the relative contribution of the adventitial Sca1+ VSMC progenitor cell to neointimal remodeling. In future studies, progenitor cell recruitment will need to be demonstrated with coordinated spatiotemporal expression of several inducible lineage markers to conclusively delineate the contribution from heterogeneous populations of adventitial progenitor cells to vessel remodeling. Although this study cannot clarify the relative contribution of adventitial progenitor cells to vessel remodeling, the results do identify the expansion of a CD45−Lin−Sca1+cKit+ progenitor cell population in response to vessel injury, informing specific markers necessary to define a relevant cell population in future studies. Although c-myb has been shown to regulate VSMC differentiation from ESCs in vitro, we have now demonstrated that c-myb also plays a pathophysiological role in the regulation of adult vessel progenitor cells that participate in neointimal remodeling. By regulating both the proliferation and myocardin-dependent differentiation of adventitial VSMC progenitor cells, c-myb has a profound role in the functional capacity of adventitial VSMC progenitor cells and thus may represent a novel approach to modulating injury responses of vessels. Although current therapies for many vascular diseases include systemic administration of therapeutic factors, understanding the functional role of local vessel-resident stem and progenitor cells and the factors that regulate their activity may represent novel refinements to the therapeutic success of vascular interventions.

**Acknowledgments**

We wish to thank Dr Joseph Lipsick for kindly providing the mR3-3 c-Myb activation domain mutant and WT control plasmid. Some equipments used in this study were provided by The 3D (Diet, Digestive Tract, and Disease) Centre funded by the Canadian Foundation for Innovation and Ontario Research Fund, project number 19442 and 30961.

**Sources of Funding**

This work was supported in part by Canadian Institutes of Health Research (CIHR) operating grant MOP-136850 (to M. Husain). M. Husain is a Career Investigator of the Heart and Stroke Foundation of Canada (CI5503). E.A. Shikatani was supported by a CIHR Small Health Organization Partnership Program Priority Area: Hypertension Doctoral Research Award, Ontario Graduate Scholarship, and a Meredith & Malcolm Silver Scholarship in Cardiovascular Studies.

**Disclosures**

None.

**References**


20. You XM, Munguea IN, Kalair W, Afroz F, Ravi B, Sadi AM, Gros R, Husain M. Conditional expression of a dominant-negative c-Myb in...

Highlights

- It has long been thought that mature vascular smooth muscle cells (VSMCs) dedifferentiate, proliferate, and provide the bulk of cells in vascular pathologies as diverse as neointimal remodeling, atherosclerosis, and aneurysm formation. More recently, a role for vessel-resident stem and progenitor cells is being appreciated.
- c-myb regulates the proliferation of CD45-Lin-Sca1-cKit+ vessel-resident adventitial VSMC progenitor cells in vivo in response to vessel injury.
- c-myb regulates the differentiation of adventitial VSMC progenitor cells into VSMCs in vitro.
- VSMC differentiation of adventitial VSMC progenitor cells is mediated by reduced transcriptional activation of myocardin because chromatin immunoprecipitation revealed c-Myb binding to the myocardin promoter only during differentiation of Sca1+ cells. Adenoviral overexpression of myocardin rescued the phenotype of c-myboh progenitors.
c-Myb Regulates Proliferation and Differentiation of Adventitial Sca1+ Vascular Smooth Muscle Cell Progenitors by Transactivation of Myocardin

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Arterioscler Thromb Vasc Biol. 2016;36:1367-1376; originally published online May 12, 2016; doi: 10.1161/ATVBAHA.115.307116

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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**Supplemental Information: Materials and Methods**

**Animals used**
c-myb<sup>h/h</sup> animals on a C57BL/6 genetic background were derived as previously described<sup>1</sup>. To test the strain independence of injury phenotype, C57BL/6/c-myb<sup>h/h</sup> mice were crossbred with eGFP<sup>+</sup> mice (C57BL/6-Tg(UBC-GFP)30Scha/J; Jackson Laboratories #004353) to generate eGFP<sup>+</sup> WT and c-myb<sup>h/h</sup> mice. For bone marrow transplantation, 5 wk old recipient mice were lethally irradiated with a single 10Gy dose of radiation (Gammacell 40 Exactor, Best Theratronics). Bone marrow was harvested from the femur and tibias of donors; 2.0 x10<sup>6</sup> cells were injected intravenously via tail vein injection per recipient. Mice were allowed to recover for 8 weeks. All mice used in the study were male. Mice were housed in a SPF facility with access to water and standard chow *ad libitum*. All protocols were approved by the Animal Resource Centre at the University Health Network (AUP#1032, 1034, 1605).

**Carotid wire denudation injury**
Carotid wire injury was performed as previously described<sup>2,3</sup>. Mice were anesthetized, then their left common carotid artery (CCA) was exposed. A temporary ligature on the proximal end of the left CCA and on the internal carotid artery was used to stop the flow of blood. A permanent ligation was made on the external carotid artery 2-3 mm distal to the bifurcation of the left CCA. A small incision was then made in the external carotid artery and a wire inserted to denude the endothelium in three passes. After removing the wire, a second permanent ligation was made proximal to the incision towards the bifurcation of the CCA. Both temporary ligatures were then removed to restore blood flow. The right CCA served as the uninjured control artery. Mice were allowed to recover for 8-, 14- or 28-days following injury, at which time animals were sacrificed and left and right CCA collected for histology, flow cytometry or RNA extraction. To assess proliferation, mice were injected with 125 mg/kg bromodeoxyuridine (BrdU) 7.5-, 13.5- and 27.5-d post-injury.

**Histology**
For Masson’s trichrome staining, ~5mm of the CCA and ~1.5mm of both external and internal carotid arteries were carefully dissected and fixed overnight in 4% paraformaldehyde (PFA) before being processed for embedding in paraffin. Serial 5 μm transverse sections were stained with Masson’s trichrome. Brightfield images were taken on a Leica DMLB microscope, captured with an Infinity2-2 CCD camera (Lumenera). Morphometry was performed for sections that were determined to be 100, 200 and 300 μm proximal to the bifurcation of the CCA. Lumen area (a), area within medial elastic lamina (b), and area within outer elastic lamina (c) were measured with ImageJ software (NIH). Intima area was calculated as b – a; media was calculated as c – b; intima:media (I/M) ratio calculated as (b–a)/(c–b).

For immunofluorescence, tissues were fixed in 4% PFA, rinsed with PBS, then cryoprotected with 20% sucrose in PBS and frozen in OCT. 5 μm transverse cryosections were dried at room temperature for 10 minutes, fixed for 15 minutes in 2% PFA, rinsed 3 times in 1x PBS, then blocked for 1 hour in 2% normal donkey serum in PBS. Sections were then incubated with rat anti-Sca1 (1:100; BD Biosciences 553333) and rabbit anti-Calponin 1 (1:200; Abcam ab46794) overnight. For staining controls, isotype control antibodies for anti-Sca1 (rat IgG<sub>2A</sub>, 1:100; Abcam Ab18450) and anti-Calponin 1 (rabbit IgG, 1:200; Abcam ab172730) were used. Sections were then rinsed 3 times in 1x PBS, then incubated donkey anti-rat Cy3 (1:400; Jackson ImmunoResearch 712-165-150) and donkey anti-rabbit Cy5 (1:400; Jackson ImmunoResearch 712-175-152) secondary antibodies for 2 hours at room temperature, protected from light. Nuclei were counterstained with 10 μg/ml Hoechst 33258 in ddH<sub>2</sub>O for 10 minutes before slides were mounted with 50:50 glycerol:PBS and stored at -20C° until imaging.
Images were captured on an Olympus VS120 fluorescence slide scanner. Adventitial Sca1+ nuclei were counted in ImageJ.

Flow cytometry
To prepare tissues for flow cytometry, left and right CCA of mice were carefully dissected, and enzymatically dispersed as previously described4. Vessels were cut into small pieces and subject to enzymatic dispersion in 450 U/ml collagenase I (Sigma-Aldrich C0130), 125 U/ml collagenase XI (Sigma-Aldrich C7657), 60 U/ml DNase I (Sigma-Aldrich DN25), 60 U/ml hyaluronidase (Sigma-Aldrich H3506) in 20 mM HEPES buffer (Mediatech 25-060-C1) for in a heated shaker at 37 C° and 900 rpm for 30 minutes. Aortic arches were partially digested (5 minutes at 37 C° and 900 rpm) and adventitia was microdissected away from the medial and intimal layers, then minced and completely digested for another 25 minutes to specifically examine adventitial cell populations. After digestion, vessel samples were washed in 1x PBS and passed through a 40 μm filter. Blood samples were collected in heparinised capillary tubes, lysed with 1x RBC lysis buffer (BioLegend 42301), then washed in 1x PBS. Single cell preparations were stained for 45 minutes before being fixed with Cytofix buffer (BD Biosciences). Antibodies used were as follows: anti-Sca1-PECy7 (eBiosciences 25-5981-82), anti-CD34-A700 (eBiosciences 56-0341-82), anti-Flk1-APC (eBiosciences 17-5821-80), anti-cKit-FITC (eBiosciences 11-1171-82) or anti-cKit-APC (BD Biosciences 553356), anti-CD45-eF450 (eBiosciences 48-0454-82), anti-CD3-PE (BD Biosciences 555275), anti-CD31-PE (BD Biosciences 553373), anti-CD11b-PE (BD Biosciences 55331), anti-CNN1-PE (BD Biosciences 553673), anti-CD11b-PE (BD Biosciences 55331), anti-Ter119-PE (BD Biosciences 553673), anti-NK1.1-PE (BD Biosciences 553165), anti-B220-PE (BD Biosciences 553090).

Cell proliferation was assessed using the FITC BrdU flow kit (BD Biosciences 559619) according to manufacturer protocol. Apoptosis was determined using annexin V staining (BD Biosciences 563973) according to manufacturer protocol. For CNN1 and ACTA2 staining, cells were fixed and permeabilized using Cytofix/Cytoperm buffer (BD Biosciences 554714) for 30 minutes on ice, and were stained with anti-ACTA2 (1:100) and anti-CNN1 (1:100) for 45 minutes on ice protected from light. Samples were washed 3x with Perm/Wash buffer, then stained with secondary antibodies for 45 minutes on ice, protected from light. Samples were run on a LSR II (BD Biosciences), and then subsequently analyzed by FlowJo software (v.10; Flowjo). Gating was determined through the use of fluorescence minus one (FMO) or isotype controls where appropriate.

Adventitial cell culture and differentiation
Mice were anesthetized and flushed with 10 ml ice cold 1x PBS via the left ventricle. Left and right CCA were carefully dissected, and a modified adventitial cell isolation protocol5 was used. Arteries were digested for 5 minutes at 37 C° in 0.2 U/μl collagenase III (Sigma Aldrich C0255), 0.1 mg/ml elastase IV (Sigma Aldrich E0258), 0.5 mg/ml soy trypsin inhibitor (Sigma Aldrich T6522), 25 mM HEPES pH 7.4, 1mg/ml BSA, 100 U/ml penicillin, 100 μg/ml Streptomycin, 2.5 μg/ml amphotericin B (Sigma Aldrich A2942), 50 μg/ml gentamicin (Sigma Aldrich G1397) in 1x high glucose DMEM (Invitrogen 11995073). Adventitia was then microdissected away from the CCA, cut into small pieces, then completely digested for 25 minutes at 37 C° before being cultured in 1x high glucose DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml Streptomycin, 2.5 μg/ml amphotericin B, 50 μg/ml gentamicin, 100 μM β-mercaptoethanol, and 10 ng/ml leukemia inhibitory factor. Cells and adventitial fragments were allowed to grow to ~80-90% confluence, before magnetic activated cell sorting (MACS). Cells were washed 3x in 1x PBS, then trypsinized, centrifuged and resuspended in FACS buffer (1x PBS, 0.5% BSA). Cells were labelled with anti-Sca1-FITC (BD Biosciences 562058) for 15 minutes on ice, washed in 1x PBS, then resuspended in FACS buffer. Sca1+ cells were then incubated with anti-FITC microbeads (Miltenyi biotec 130-042-701) for 30 minutes on ice, washed in 1x PBS and resuspended in FACS buffer. Sca1+ cells were isolated by positive MACS selection using...
LS columns (Miltenyi biotec 130-042-401), and were passed through two columns to improve purification. Positively selected cells were then rinsed in 1x PBS and cultured in cell culture media.

For cell differentiation, media was changed to 0.2% FBS in 1x high glucose DMEM supplemented with 10 ng/ml TGFβ-1 (R&D Systems 7666-MB-005) to induce VSMC differentiation.

**RNA isolation and qRT-PCR analysis**
RNA was extracted from cells and CCA using a PicoPure RNA isolation kit (Life Technologies) according to manufacturer protocol. RNA concentration was obtained using a Nanodrop ND100 spectrophotometer. cDNA was synthesized using qScript cDNA SuperMix (Quanta BioSciences) according to manufacturer protocol. Transcripts were then detected using specific primers (see Supplemental Table SI) using SYBR Green Master (Roche) detected on a LightCycler 480 (Roche). B2M was used as the housekeeping gene; values were compared using the $2^{-\Delta\Delta Ct}$ method. Samples were run in triplicate and averaged.

**Adenoviral overexpression of myocardin**
Adenoviral constructs to over-express mouse myocardin (Adeno-Myocd; ADV-265349) or a GFP control vector (Adeno-GFP; ADV-1060) were purchased from Vector Biolabs. Isolated WT and c-myb<sup>h/h</sup> adventitial Sca1<sup>+</sup> VSMC progenitor cells were infected overnight, and then differentiated in VSMC differentiation media for 7 d prior to RNA isolation and subsequent qRT-PCR analysis.

**HEK cell transfection and luciferase**
c-Myb-dependent activation of a myocardin promoter was determined by co-transfection of a myocardin promoter-luc construct (0.5μg/well), pRL-TK (renilla luciferase 0.025μg/well, Promega) and a mouse p75 c-Myb over-expression vector<sup>6,7</sup> or empty vector control (0.5μg/well) in HEK293 cells using jetPrime (Polyplus-Transfection) according to manufacturer protocol. Luciferase luminescence was read on a Pherastar FS (BMG Labtech). Data were normalized to renilla luciferase expression and expressed as relative luciferase units (RLU).

**Constructs**

**Hypomorphic (hypo) c-Myb:** To create a hypomorphic mouse c-Myb over-expression vector, a mouse p75 c-Myb over-expression vector was mutated in the transactivation domain of c-Myb (Hypo: TGTTGACATCAGGAGC to TGTTGACCACAGGAGC) using site directed mutagenesis with primers shown in Supplemental Table II and Q5 Site-Directed Mutagenesis kit (New England Biolabs) according to manufacturer protocol. The resultant hypomorphic cMyb vector contains the same mutation as the c-myb<sup>h/h</sup> mouse<sup>1</sup>.

**mR3-3 transactivation mutant c-Myb:** An additional mutant c-Myb, mR3-3, (a gift from Dr. Lipsick<sup>8</sup>) known to have defective transactivation of c-Myb target genes was used to confirm that c-Myb transactivates a myocardin-luc reporter.

**Site-directed mutagenesis:** To delete c-Myb binding sites (MBS) identified within relevant regions of the core myocardin promoter<sup>9</sup>, MBS1 (ΔMBS1: TAACTCCC to TccgTCCC), MBS2 (ΔMBS2: GAACAGCC to GccgAGCC), MBS3 (ΔMBS3: CAGCGGTC to CccgGGTC), and MBS8 (ΔMBS8: CAACCTTTT to CccgTTTT) were mutated using primers shown in Supplemental Table II and Q5 Site-Directed Mutagenesis kit (ΔMBS 1 and ΔMBS8) or QuickChange II Site-Directed Mutagenesis kit (Agilent) (ΔMBS2 and ΔMBS3). After mutagenesis, each clone was confirmed by DNA sequencing.
Chromatin immunoprecipitation
Chromatin immunoprecipitation (ChIP) was performed on Sca1⁺ adventitial progenitor cells using the ChIP-IT express enzymatic kit (ActivMotif 53009) according to manufacturer protocol. Chromatin was collected from Sca1⁺ cells either pre or 1-d post TGFβ-1 induction of VSMC differentiation. Antibodies against c-Myb (Santa Cruz sc-516), RNA polymerase II (Santa Cruz sc-900) and whole rabbit serum were used for immunoprecipitation. Isolated gDNA fragments were then analyzed by qRT-PCR as described above. Primers used are listed in Supplemental Table I.

Supplemental Information References:
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<th>Gene Name</th>
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Vessel injury → \( \uparrow \text{cKit}^+\text{Sca1}^+ \) progenitors → c-Myb dependent progenitor cell proliferation

Differentiation

\( \uparrow \text{c-myb} \) → Binding of c-Myb to myocardin promoter → Activation of myocardin transcription → VSMC differentiation

- \( \text{Sca1}^+\text{CD45}^-\text{Lin}^- \) Vessel-resident adventitial VSMC progenitor cell
- Other adventitial stem/progenitor cells
- Vascular smooth muscle cell (VSMC)
- Endothelial cell