c-Myb–Dependent Smooth Muscle Cell Differentiation

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Abstract—Both in vitro and in vivo studies have implicated the c-Myb transcription factor in vascular smooth muscle cell (SMC) proliferation and hematopoiesis. However, its role in differentiation and maturation of contractile, as opposed to proliferating, SMCs have not been investigated. Here we demonstrate that c-myb−/− embryonic stem cells (ESCs) are incapable of producing embryoid bodies (EBs) with spontaneously contracting SMCs but can differentiate into contracting cardiomyocytes unimpaired. Quantitative real-time RT-PCR revealed that whereas mesodermal differentiation was unaffected, myocardin, a critical determinant of SMC differentiation, became upregulated at day 7 in wild-type, but not in c-myb−/− EBs. SMC-specific genes smoother muscle α-actin, SM22α and smooth muscle myosin heavy chain reached peak expression levels by day 15 of differentiation and were 2- to 3-fold higher in wild-type as compared with c-myb−/− derived EBs. Similarly, fluorescence-activated cell-sorting analysis confirmed significantly different proportions of smooth muscle α-actin–positive cells in wild-type (26.8±0.7%) versus c-myb−/− (12.3±0.4%) EBs. Temporal induction of these SMC-specific markers preceded and paralleled contractile SMC appearance and predicted the relative (in)ability of c-myb−/− and wild-type ESC lines to generate EBs with contracting SMCs. Importantly, data from EBs faithfully predicted a significant reduction in c-myb−/− cell contribution to SMC lineage in vivo, in chimeric embryonic day 11.5 embryo and adult aortas relative to brain and skin chimerism, respectively. Moreover, the visceral SMC population in chimeric embryos was nearly devoid of c-myb−/− cells. Our data are the first to implicate c-Myb in SMC differentiation from precursor stem cell–derived populations, reinforcing its potential role in phenotypic modulation of SMCs and vascular disease. (Circ Res. 2008;102:0-0.)

Key Words: smooth muscle cell differentiation c-Myb ES cell

In vascular smooth muscle cells (SMCs), seemingly disparate and highly specialized functions of contractility and vessel repair are achieved through phenotypic plasticity.1 However, such plasticity makes SMCs susceptible to adverse phenotypic modulation involving proliferation and chemotaxis, with decreased expression of contractile proteins.2 Presently, few specific factors are known to promote or obstruct specification of multipotent mesenchymal cells to the SMC lineage and maturation to the contractile form.

c-Myb is prominently involved in proliferation of mature SMCs by facilitating G1-to-S phase cycle transitions through inositol 1,4,5-trisphosphate receptor type-1 and plasma membrane Ca2+-ATPase (PMCA),1–6 and dominant-negative c-Myb (Myb-Engrailed) has been shown to inhibit SMC proliferation and arterial remodeling following carotid artery injury.7 Although these studies support the role of c-Myb in SMC proliferation, they do not discount the potential effects of c-Myb on SMC development and on the differentiation capacity of vessel-resident subpopulations of SMC progenitors or circulating bone marrow–derived stem cells.8–10 Indeed, c-Myb has been implicated in hematopoietic differentiation through studies in erythroleukemia cell lines as well as differentiating c-myb−/− embryonic stem cells (ESCs).11–13 In vivo, an increase in c-myb−/− cell contribution to SMC lineage is brought about by c-Myb in a calcineurin-independent manner.14 Given these parallel observations, we hypothesized a role for c-Myb in differentiation and maturation of the contractile SMC phenotype.
ESC-derived c-myb-null mice are able to survive until embryonic day (E)15, at which point they perish because of failed hematopoiesis. However, whether or not subtle defects in cardiovascular development, especially with respect to SMC function, were present in c-myb<sup>-/-</sup> embryos, was never specifically examined. Here we demonstrate, using an in vitro model of SMC differentiation, that c-myb<sup>-/-</sup> ESC-derived EBs show (1) complete absence of spontaneous SMC-like contractility and (2) decreased expression of SMC-specific markers. We also demonstrate that c-myb<sup>-/-</sup> cells are impaired in their ability to contribute to SMC lineages in vivo.

**Materials and Methods**

**ESC and EB Culture**

Four mouse ESC lines, G4<sup>1,7</sup>, RT<sup>3,8</sup> CCE<sup>3,9</sup> and c-myb<sup>-/-</sup><sup>11,16</sup> (CCE background), were cultured on monolayers of primary mouse embryonic fibroblasts and maintained in DMEM for ESCs (ES-DMEM) background), were cultured on monolayers of primary mouse embryonic fibroblasts and maintained in DMEM for ESCs (ES-DMEM) with 15% FBS (Wisent, St-Bruno, Quebec, Canada) in the presence of leukemia inhibitory factor, as previously described.

To induce differentiation, cells were trypsinized, pelleted, and resuspended in ES-DMEM containing 20% FBS, without leukemia inhibitory factor. Cell suspension was diluted to 2×10<sup>4</sup> cells/mL and used to make EBs via the hanging drop method (30 µL each). Later (1.5 to 2 days), EBs were transferred individually to 96-well Ultra Low Cluster plates (Corning Costar) and cultured in suspension for an additional 5 days. At day 7, EBs were plated individually onto 0.1% porcine gelatin-coated (Sigma-Aldrich, Oakville, Ontario, Canada) 24-well plates (BD Bioscience, Mississauga, Ontario, Canada) and treated for 5 days with 10 nmol/L all-trans-retinoic acid (Sigma-Aldrich) and 0.5 mmol/L dibutyryl cyclic AMP (Sigma-Aldrich) or all-trans-retinoic acid alone, as described by others.

Subsequently, attached EBs were kept in normal differentiation medium (20% FBS, no leukemia inhibitory factor) with daily replacement.

**Generation of Novel Transgenic ESCs**

A linearized SM–myosin heavy chain (SM-MHC)-Cre-ires-EGFP construct<sup>22</sup> was coelectroporated with a supercoiled phosphoglycerate kinase–puromycin construct<sup>13</sup> into G4 mouse ESCs harboring a chicken β-actin promoter-driven, floxed-stop, element-containing reporter gene for DsRed-MST (Figure 1A).<sup>17</sup> Colonies resistant to transient puromycin selection were expanded and screened for Cre by genomic PCR. Clones incorporating the transgene construct were subjected to SMC-directed differentiation using the EB model. Clones displaying Cre-mediated excision of the floxed-stop element and robust DsRed expression in areas of SMC-like contraction were identified. Enhanced green fluorescent protein (EGFP) expression was detected by staining with anti-EGFP-Alexa488 (Molecular Probes). Representative fluorescent, live video recordings were captured with Zeiss Axiovert 200m/L (Zeiss).

**RNA Isolation and Real-Time Quantitative RT-PCR**

EBs were rinsed in PBS and harvested via trypsinization in case of attached cultures. Total cellular RNA was isolated using the PicoPure RNA Isolation Kit (Arcturus Bioscience, Inc, Mountain View, Calif) and stored at −80°C. PolyA<sup>+</sup> RNA was converted to cDNA using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Burlington, Ontario, Canada). Real-time PCR was performed using the ABI Prism 7700 sequence detection system (Applied Biosystems, Streetsville, Ontario, Canada). Mouse-specific primers for GAPDH (5'-GCA-TGG-CCT-TCC-GTG-TTC-3'), 5'-ATG-ATC-CAC-GCA-GGT-TTC-3'), smooth muscle (SM)-actin,<sup>24</sup> SM22α,<sup>24</sup> myocardin (5'-GAA-ATC-GTT-GTC-GTA-C-3'), and brachyury (5'-AAC-GAG-ATG-GTT-GTC-ACC-AAG-ACC-C-3'), myocardin (5'-CAT-GAA-GTC-CAG-CAG-AGA-G-3') were designed with Primer3 (Massachusetts Institute of Technology) to span at least 2 different exons and checked against cross homology with BLAST (National Center for Biotechnology Information). Primers for atrial natriuretic factor (ANF) were obtained from the literature.<sup>25</sup> For detection of SM-MHC, a TaqMan Gene Expression Assay was used (Mm0043031_m1; Applied Biosystems). Each reaction mixture consisted of first-strand cDNA template, 50 nmol/L primer pair or 1.25 µL of primer probe, and 12.5 µL of SYBR Green or TaqMan master mix (Applied Biosystems) in a total 25-µL volume. The following cycling conditions were used: 95°C for 10 minutes, followed by 50 cycles of 95°C for 15 seconds and 60°C for 60 seconds (or 58°C for myocardin, brachyury, and ANF). Subsequently, each sample underwent dissociation curve analysis to examine primer target specificity. Representative PCR products were size analyzed on agarose gel.

**Fluorescence-Activated Cell-Sorting Analysis**

Day 18 EBs were dissociated to single-cell suspension using trypsin (0.25%) with gentle agitation, followed by mechanical shearing through a 20-gauge needle. Subsequently, cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 in 2%
FBS/PBS, and stained with monoclonal mouse anti–SMα-actin–Cy3 antibody (1:200; Sigma-Aldrich) for 30 minutes at room temperature, followed by flow cytometric analysis using MoFlo High Performance Cell Sorter (Dako Cytomation, Mississauga, Ontario, Canada).

Chimera Generation
A linearized construct composed of chicken β-actin promoter-driven β-galactosidase (β-gal) transgene with nuclear-localizing signal was coelectroporated with supercoiled phosphoglycerate kinase–puromycin construct into c-myb−/− ESCs. Clones exhibiting 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) staining at ESC stage were put through the EB differentiation assay to select those with ubiquitous β-gal expression. Four such clones were tested for their ability to generate SMC contractions, which was found lacking, similar to parental ESCs. The clone with consistently high β-gal activity was subsequently used for in vivo analysis. Outbred, wild-type (WT) ICR 8 cell embryos were used as hosts for diploid aggregations with c-myb−/− (NLS-β-gal−) ESCs. Aggregates were cultured overnight and transferred into pseudopregnant female C57BL/6. E11.5 embryos and those exhibiting blue eyes, indicative of chimerism, were used for further analyses. Alternatively, c-myb−/− ESCs were injected into WT C57BL/6 blastocysts and allowed to reach full term. Adult chimeras, 12 to 18 weeks of age, were assessed for extent of agouti coat color.

Immunofluorescent and X-Gal Staining
Aortas were dissected, rinsed with ice-cold PBS, fixed overnight in 4% paraformaldehyde, cryoprotected in 30% sucrose, embedded in OCT, and stored at −80°C until further processing. Ten-micron cryosections were obtained for immunostaining. To distinguish host- and donor-derived cell contributions, sections were blocked with 5% goat serum, stained with rabbit polyclonal anti-neomycin antibody (Cortex Biochem, San Leandro, Calif; 1:100), followed by anti-rabbit-Cy2 secondary antibody (Jackson Immunoresearch Laboratories Inc, West Grove, Pa), and counterstained with Hoechst (Molecular Probes, Eugene, Ore). For β-gal detection, E11.5 embryo organs were extracted, fixed for 30 minutes in 0.2% glutaraldehyde on ice, and incubated at 37°C overnight in 1 mg/mL X-gal stain.

Data Acquisition and Statistical Analysis
The number of EBs containing spontaneous contractile areas21,26 was recorded for each group at select time points (40 to 500 EBs per group per time point). High-frequency beats (>30 per minute; generally 50 to 120 per minute) were classified as cardiac, whereas low-frequency beats (<10 per minute; generally ~5 per minute), moving as a sheet of longitudinally oriented cells, were designated as SM-like. Identity of the latter was also confirmed using ESCs exhibiting SM-specific EGFP and DsRed expression. The data were expressed as percentages of total number of EBs examined and tested for statistically significant differences (P<0.05) using the χ² probability test. Furthermore, we repeated the quantitative assessment of contractility up until day 18 of differentiation in 3 independent experiments in which scoring was conducted in blinded fashion. Real-time PCR data were analyzed using the standard curve method for relative quantification (User Bulletin 2, Applied Biosystems, 2001). GAPDH was used as a reference for sample-to-sample comparisons and to account for template input. All expression levels were ratio-normalized to c-myb−/− ESCs. Analysis of accompanying fluorescence-activated cell-sorting (FACS) data were performed on Summit v4 software (Dako Cytomation, Mississauga, Ontario, Canada).

Adult mouse aorta images were captured at ×60 magnification using the Olympus Fluoview 1000 confocal microscope (Olympus America Inc, Melville, NY). Aortas from WT C57BL/6 and neo/NI Red2/−/− mice were used as controls. By counting neo/NI cells (c-myb−/−/H11021) and Hoechst-labeled nuclei within chimeric sections, the ratios of c-myb−/− versus WT aortic SMCs were determined. Percentage average was calculated from a minimum of 4 different sections. Similarly c-myb−/− cell contribution to chimeric embryo organs was scored based on extent of X-gal stain per unit area using Image J software (NIH, from which percentage average and relative ratios were calculated.

Statistical analyses: ANOVA, t test, and χ² were performed using SPSS 11.0. All final results were displayed as means±SE. Significance was defined as P<0.05.

Results
SM-Like Contractions in EBs Correspond to Areas of SM-MHC–Directed Reporter Gene Expression
Treatment of WT EBs with all-trans-retinoic acid and dibutyryl cyclic AMP led to formation of low-frequency (~5 per minute), spontaneously contracting areas with SM-like attributes from day 18 of differentiation. To examine the identity of cells within these contracting areas we generated transgenic ESC lines with SM-specific reporter expression (Figure 1A).21,26 We introduced a SM-MHC promoter-driven bicistronic Cre recombine and EGFP expression construct22 along with a puromycin-resistance construct23 into G4 mouse ESCs harboring a Cre-activatable reporter gene for red fluorescent protein (DsRed) (Figure 1A).17 Initially, 9 of 96 puromycin-resistant clones tested positive for the presence of Cre. Of these, 7 displayed DsRed expression in cells within areas of SM-like contraction, beginning at day 18 of EB-based differentiation (Video in the online data supplement, available at http://circres.ahajournals.org). Generally, live cells exhibited strong DsRed but weak EGFP expression. Immunofluorescent studies of select clones confirmed DsRed and EGFP colocalization. However, some EGFP+ cells lacked DsRed, likely because of delayed transcription requiring Cre-mediated excision of a floxed-stop element preceding DsRed (Figure 1B through 1D).

Complete Absence of SM-Like Contractions in c-myb−/− EBs
Differentiating WT EBs of CCE background exhibited SM-like contractions at day 18 in 23±5.7% of cases (Figure 2A). Such contractions were analogous to those acquired by fluorescent microscopy using the DsRed-reporter-tagged G4 cells, slow and involving undulating sheets of longitudinally oriented cells.21,26 In contrast, no such activity was observed in c-myb−/− EBs of the same genetic background (0±0%; P<0.05; Figure 2A), suggesting that the SMC developmental
program becomes impaired in the absence of c-Myb. Interestingly, c-myb<sup>−/−</sup> ESCs exhibited enhanced developmental potential for the cardiac lineage, as measured by percentage of EBs containing high-frequency (≈50 to 120 per minute) cardiac-like pulsations at day 24 (WT, 11.6±8.4%; c-myb<sup>−/−</sup>, 52.9±9.5%; *P<0.05; Figure 2B).

**Reduced Myogenic and SM-Specific Gene Expression in c-myb<sup>−/−</sup> EBs**

At select time points, WT and c-myb<sup>−/−</sup> EB cultures were assessed for the transcript levels of SM-specific markers. Myocardin, a potent transcriptional activator of SM- and cardiac-specific gene expression, was rapidly upregulated in WT EBs starting at day 7 of differentiation and peaked at day 12 but not in c-myb<sup>−/−</sup> EBs (Figure 3A). Temporal analysis revealed that high mRNA levels of myocardin were transient, such that by day 18, they declined to day 7 levels. Consistent with the absence of SM-like contractions (Figure 2A), the magnitude of SM-specific gene expression in c-myb<sup>−/−</sup> EBs was significantly lower than that recorded in WT EBs (Figure 3B through 3D). Notably, SMα-actin induction was clearly reduced at days 12, 15, and 18. SM22α, a marker of more mature SMCs, exhibited lower expression at days 15, 18, and 24 in c-myb<sup>−/−</sup> EBs. SM-MHC, which was SM lineage-restricted and associated with fully differentiated SMCs, was negligible in c-myb<sup>−/−</sup> EBs while being highly induced in WT EBs at days 12, 15, 18, and 24, resulting in significant differences at all of these time points. Accordingly, in the case of intermediate and advanced markers of SMC differentiation, SM22α and SM-MHC, significant differences between the 2 groups persisted until day 24. Importantly, induction of SM-selective gene expression peaked at day 15 (Figure 3) and preceded the appearance of SM-like contractions at day 18 in WT-derived EBs (Figure 2A), with SM-MHC upregulated last (Figure 3C).

**SM-Specific Gene Expression in Other ESC Lines Also Predicted the Appearance of SM-Like Contractions**

As a proof of principle, we examined further whether increases of greater magnitude in SM-specific gene expression augment the prevalence of SM-like contractile activity. To this end, we examined another WT ESC line of distinct genetic background, namely R1. We quantified the incidence of spontaneous SM-like contractile areas as compared with mRNA levels of early and late SM markers in R1 versus CCE WT EBs. R1-derived EBs showed a trend toward higher levels of SM marker expression than did CCE-derived EBs; in particular, SM-MHC expression at day 12 was significantly higher (*P<0.05, n=3). Significantly greater occurrence of SM-like contractions in R1 as compared with CCE EBs at day 24 (*P<0.05). Taken together, these data suggest that the propensity for increased SMC-specific gene expression can form the basis for developing SM-like contractions.

**Decreased Percentage of SMα-Actin–Positive Cells in c-myb<sup>−/−</sup> EBs**

To corroborate our results regarding mRNA levels, we extended our analysis of the effects of c-myb ablation by FACS analysis for SMα-actin. At day 18 of differentiation, the percentage of SMα-actin<sup>+</sup> cells in dissociated WT EBs was approximately twice that in c-myb<sup>−/−</sup> EBs (26.8±0.7%; 12.3±0.4%, *P<0.001; Figure 4).

**Mesodermal and Cardiac Marker Gene Expression**

To establish whether mesoderm induction was impaired in c-myb<sup>−/−</sup> EBs, we quantified mRNA levels of a mesoderm-associated transcription factor brachyury. Lack of c-Myb did not diminish brachyury expression at the early stages of EB differentiation, SM22<sup>−</sup> and SM-MHC upregulated last (Figure 3C).
Discussion

Protein and mRNA levels of SMα-actin, SM22α, SM-MHC, smoothelin, h-caldesmon, and calponin, which are decreased in synthetic SMCs within atherosclerotic lesions and neointima, have been used extensively as indicators of SMC phenotype and extent of differentiation.1 In our model, we chose a panel of such markers to demonstrate that lack of c-Myb–impaired SMC differentiation from ESCs but did not completely block this process. This was evidenced by a temporal increase in SM-specific gene expression in WT EBs, with significantly lower levels observed in c-myb−/− EBs (Figure 3). Based on flow cytometric analysis of SMα-actin–positive cells, a reduction in c-myb−/− SMC number further underscored differences between WT EBs and those lacking c-Myb. In addition, c-myb−/− EBs were unable to generate spontaneous SM-like contractions (Figure 2A), indicating that although some SMCs are present, they lack a critical parameter of contractile function. Notably, we demonstrated that SM-like contractions coincide with SM-MHC promoter activity, based on live fluorescent microscopy of DsRed+ cells (Figure 1 and supplemental Video), and that such functional phenotype closely parallels SM-specific gene expression in our model, as verified by analysis of 2 ESC lines of distinct genetic backgrounds.

Importantly, our in vivo analysis of chimeric tissues demonstrated that c-myb−/− ESCs maintain some potential to contribute to SMC lineage. Clearly this validates the findings of Mucenski et al, in which c-myb−/− embryos were able to survive until E15, when the cardiovascular system is already well developed.16 However, when compared with WT cells, c-myb−/− mutants are less likely to differentiate into SMCs, both in vitro and in vivo, and fail to develop into functional SMCs in vitro. Significant impairment of the ability of c-myb−/− mutants to make fully differentiated SMCs.

The presence of reduced numbers of SMCs in our model, rather than their complete absence, suggests that c-Myb–independent mechanism(s) can suffice for differentiation of some, but not all, SM lineages, analogous to recent studies using myocardin knockout ESCs.27 c-Myb begins to play a significant role in SMC differentiation before myocardin upregulation at day 7. Indeed, we have located 3 putative c-myb−/− cells to make fully differentiated SMCs.

Alternatively, lack of c-Myb may also compromise SMC progenitor expansion, in a manner comparable to its effects on hematopoietic lineages.33 Based on some studies, c-Myb can function as an activator of immature gene expression and a suppressor of terminal differentiation in a number of lineages.34 Recently, it was demonstrated that c-Myb is involved in suppression of MyoD transcriptional activity in skeletal myoblast precursors.35 Consequently, we cannot
exclude a possible role for c-Myb in promoting SMC progenitor growth.

c-Myb threshold levels may be strictly regulated at key stages of SMC differentiation, as they are in hematopoiesis, where constitutive c-myb transgene rescue fails to restore the normal B-cell differentiation process. Moreover, our data suggest that c-Myb ablation begins to impair SMC differentiation well before any SM-specific genes are expressed. Consequently, the use of SM-specific c-myb knockout precludes analysis of the effects of c-Myb on very early SMC progenitor commitment and expansion. Accordingly, both stage-specific knockout and rescue experiments would bene-

Figure 5. c-myb−/− cell participation in embryogenesis. X-Gal stain was used to detect c-myb−/− (β-gal−) cells and establish their contribution to the developing heart (A), intestines (B), aorta (C), and brain (D). Knockout cells efficiently competed for participation in cardiogenesis but showed poor contribution to vascular and visceral SMCs, relative to brain tissue chimerism. Only 1 of 7 intestines showed visible patches of blue cells (B3). White scale bar: 1 mm; black bar: 0.5 mm (C) and 0.25 mm (D). Percentage average of X-gal stain per unit area (>0.75 mm²) was determined (E through G) and also expressed as mean ratios relative to brain (H).
fit from the use of inducible systems to achieve coordinate control of c-myb-transgene expression.

In our model, c-myb+/− EBs exhibit greater incidence of cardiac-like beating as compared with WT EBs but are unable to produce spontaneous SM-like contractions (Figure 2). Similarly in vivo c-myb+/− cell contribution to the heart greatly exceeds that of the aorta or intestine (Figure 5). We speculate that the absence of c-Myb might allow for competitive differentiation between muscle lineages, preferentially favoring cardiac muscle. In fact, c-Myb overexpression has been shown to lead to cardiac and skeletal muscle degeneration.37 Recently, a common progenitor for cardiomyocytes and SMCs has been identified,38 and ESCs can be induced to differentiate into both cell types by the use of retinoic acid during a similar time window. The physical interaction between the retinoid receptors and the Myb oncoprotein has been well established in hematopoietic lineages but may have tissue-restricted nuances based on the distribution of the 6 retinoid receptors and their alternative splice variants.39,40 The use of all-trans-retinoic acid to influence the differentiation process may make our model especially susceptible to effects of c-Myb deficiency.

We show that myocardin, SMα-actin, and SM22α mRNA become induced by day 7 in WT EBs, although the later developmental marker SM-MHC is only amplified by day 12 (Figure 3), thus mimicking to some extent the time course of embryonic development. Expression levels of these early SM marker genes, SMα-actin and myocardin, become attenuated at late stages of differentiation, whereas SM22α and SM-MHC remain elevated. This appears to indicate that increasing numbers of SMC precursors continue to acquire more “advanced” marker expression as they progress through further stages of differentiation. Because our gene profiles were normalized to a globally expressed housekeeping gene, and because in attached EB cultures, the total cell number continues to increase41 while SMCs have matured, we observed an apparent decrease in SM-specific gene expression levels. However, this does not indicate a reduction in absolute SMC numbers or a loss of differentiated phenotype but rather the variable rates of proliferation and differentiation of the multiple cell lineages present within an EBs.

In conclusion, the present study is the first to demonstrate the importance of the c-Myb transcription factor in SMC differentiation, both in vitro and in vivo. Interestingly, global ablation of c-myb allowed for effects to manifest themselves as early as day 7 of differentiation, highlighting the need to consider early events critical to the emergence of SMC precursors. Furthermore, our in vitro findings were corroborated by the observation that c-myb+/− cells have diminished potential for SMCs, but not cardiomyocyte, differentiation in vivo. Finally, we have definitively correlated the presence of spontaneous ESC-derived SM-like contractile areas in vitro with reporter expression under the control of SM-MHC promoter. Such direct promoter–reporter readout is valuable for monitoring SMC precursor responses to changes in molecular, chemical, and physiological environment, consequently helping to implicate the molecular pathways and conditions responsible for SMC differentiation and phenotypic modulation.

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**Disclosures**

None.
References

21. Sinha S, Hooffnagle MH, Kingston PA, McCanna ME, Owens GK. Trans-
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