More than 50 years ago, smooth muscle cells (SMCs) of the carotid artery were shown to undergo dedifferentiation on ligation injury. Since this classical study, scores of research groups have used a variety of in vivo and in vitro model systems, as well as numerous clinical studies, to demonstrate the conversion of normally contractile vascular SMCs to a less differentiated state of proliferation, migration, and exuberant extracellular matrix secretion, a process generally referred to as phenotypic modulation or plasticity. Such changes in differentiated state of proliferation, migration, and exuberant vascular SMC phenotype are thought to underlie many vascular occlusive diseases. However, in a recent issue of *Nature Communications*, Tang et al provide data from in vitro cell culture, flow cytometry, and lineage tracing experiments, which they believe directly challenge this long-standing and widely accepted paradigm. Specifically, these authors contend that: (1) differentiated (SM myosin heavy chain [MHC], SM MHC+) vascular SMCs are incapable of proliferation either in vivo in response to injury or in vitro in cell culture; (2) within the media of mature blood vessels there exists a small population (<10%) of undifferentiated (SM MHC−) cells that activate markers of mesenchymal stem cells, including Sox17, Sox10, and S100β, and proliferate to completely reconstitute medial cells in response to vascular injury; and (3) these so-called medial-derived multipotential vascular stem cells (MVSC) also proliferate and express several mesenchymal stem cell markers when placed in cell culture and can be induced to differentiate into neural, chondrogenic, and SMC lineages with appropriate culture methods. The authors’ bold conclusion that “MVSC activation and differentiation, instead of SMC dedifferentiation, results in the proliferative and synthetic cells in the vascular wall,” if true, would have a huge impact on our understanding of the role of SMCs in vascular injury repair and disease. Because an implicit aspect of the authors’ claims is that previous studies have been conducted on MVSCs, not SMCs, it also potentially impacts conclusions from thousands of articles using cultured SMCs as model systems. However, close examination of their results, which are in direct contradiction to a large body of published research, reveals several misinterpretations, overstatements, and technical deficiencies that seriously undermine most of their major conclusions as discussed further in this Perspective.

Although extensive studies have identified mechanisms that control the process of SMC phenotypic switching in cultured cells as reviewed by Owens et al, there are still major ambiguities regarding the definitive identification of altered SMC phenotypes during vascular remodeling, including vascular injury–induced proliferation and atherosclerotic plaque progression, because a key feature of this process is the loss of expression of SMC-selective gene products, such as SM MHC and SM α-actin (SMA). As stated by Tang et al, “a widely accepted explanation is that SMCs have phenotypic plasticity and that mature or contractile SMCs can dedifferentiate into proliferative and synthetic SMCs. However, this dedifferentiation process has not been directly demonstrated by tracking the fate of mature or contractile SMCs.” Indeed, we support this statement, that is, that there is a critical need for definitive in vivo SMC lineage tracing studies, as emphasized in several recent reviews.

Although not explicitly stated in the methods, the SMC lineage tracing model system used in the Tang et al study was generated by crossing smMHCCreosGF mice, originally made by Michael Kotlikoff’s laboratory using an SM MHC promoter–enhancer provided by the Owens laboratory (which faithfully recapitulates expression of the endogenous SM MHC gene), with a Cre activatable indicator mouse strain, ROSA26-EGFP, both obtained from Jackson laboratories. The choice of an SM MHC–based model system was a good one, because SM MHC is the most specific marker of differentiated
SMCs identified to date. However, there is a major limitation of the model system of Tang et al.7 The model is not conditionally regulated and thus cannot rigorously lineage tag mature SMCs at a given time point. Therefore, the model does not offer the opportunity to validate that the labeling is completely SMC-specific, and ultimately address to which cell type progeny belongs. The lack of conditional SMC lineage tracing, along with deficiencies in experimental design and methods of data analyses, severely compromises the conclusions drawn by Tang et al. Indeed, a more compelling lineage tracing study of vascular SMCs performed previously by Nemenoff et al11 provided clear evidence that differentiated SMCs undergo phenotypic modulation in response to vascular injury using tamoxifen-inducible SM MHC-CreERT2/Rosa26-floxStop/βGal mice, thus directly contradicting the findings of Tang et al (see Figure 1A and 1C in Nemenoff et al11).

Because the lineage tracing systems differ in the Tang et al and Nemenoff et al studies, it is necessary to further scrutinize the discrepancies between these published results (represented in Figure 1 of this commentary). Tang et al7 show a notable lack of green fluorescent protein (GFP) staining 5 days after wire injury that they interpreted as loss of mature SMCs, although no formal demonstration of this implied conclusion was presented (eg, terminal deoxynucleotidyl transferase dUTP nick end labeling staining). Instead, the authors suggest total recellularization of the vascular wall by MVSCs (Figure 1A–1F herein, which is adapted from Figure 7A–7F in Tang et al). In contrast, Nemenoff et al11 showed that βGal+ SMCs downregulate SMαA and contribute to neointima formation at 7 days after femoral artery wire injury (Figure 1G–1L). Nemenoff et al11 also demonstrated that a fraction of β-Gal+ SMCs are BrdU+ within the intima and media 3 weeks after injury, consistent with the prevailing dogma wherein mature SMCs undergo injury-induced SMC phenotypic switching with onset of cell proliferation. Tang et al7 proposed that the discrepancies in their results and those of Nemenoff et al11 were because of the acquisition of βGal expression in MVSCs after prolonged vascular remodeling. However, this is highly unlikely because the conditionally regulated lineage tracing system used by Nemenoff et al11 should not activate βGal in MVSCs after vascular injury because tamoxifen was not present. Consistent with this, the tamoxifen serum half-life in mice is 12 hours,12 and Nemenoff et al11 completed their tamoxifen injections 1 week before injury. As such, we conclude that proliferating MVSCs could not acquire βGal expression and that, in fact, mature SMCs participate in vascular remodeling after injury. One possible explanation is that the extent of wire injury in the Tang et al7 studies may have been very severe, leaving behind no resident GFP+ SMC in the media (compare Figure 1A versus Figure 1D), thus precluding their ability to analyze the role of SMC phenotypic modulation in vascular remodeling and neointima formation. Indeed, the failure to perform SM MHC imaging and characterization of the mechanisms responsible for loss of medial SMCs at early

![Figure 1. Synthetic smooth muscles contribute to vascular remodeling after wire injury. A to F. Lineage tracing data at 5 days after carotid wire injury using a noninducible smooth muscle myosin heavy chain (SM MHC)-Cre/eGFP floxed-stop Rosa GFP mouse, adapted from Tang et al. After wire injury, no smooth muscle cells (SMCs) can be detected in the media (D), precluding the ability to analyze the role of SMC phenotypic modulation. The media is replaced by cells expressing S100b (E). G to L. Lineage tracing data at 7 days after femoral artery wire injury using a tamoxifen-inducible SM MHC-CreERT2/Rosa26-floxStop/βGal mouse, adapted from Nemenoff et al. After wire injury, intimal βGal+SMα-actin (SMαA+) SMCs (arrows) and βGal+SMαA– phenotypically modulated SMCs (open arrowheads) are observed, including BrdU+ LacZ+ proliferating cells (see Figure 1C of Nemenoff et al supporting the role of synthetic SMCs in vascular remodeling and neointima formation). Lines delineate the arterial media. M to T. Lineage tracing data at 7 days after carotid wire injury using SM-MHC-LacZ, SM22a-LacZ, or SM22agc-LacZ (mutated G/C repressor) reporter mice, adapted from Regan et al. After wire injury, SMCs dedifferentiate as evidenced by no positive staining in SM-MHC-LacZ mice SM22a-LacZ. Medial and intimal phenotypically modulated SMCs are identified by LacZ+ staining after injury in the SM22agc-LacZ (mutated G/C repressor) reporter mice, further supporting the role of synthetic SMCs in vascular remodeling and neointima formation. Arrows denote internal elastic lamina, and arrowheads denote external elastic lamina.](http://circres.ahajournals.org/lookup/suppl/doi:10.1161/CIRCRESAHA.113.300323/-/DC1/figure1.jpg)
time points after injury is a major limitation of the Tang et al17 studies. Furthermore, based on the Methods section, it appears that Tang et al17 may not have properly fixed carotid arteries before optimal cutting temperature compound embedding, which is required to prevent soluble GFP from leaking.13,14 As such, it is also possible that improper fixation methods used by Tang et al17 resulted in markedly reduced sensitivity to detect GFP+ SMCs, including those that proliferate or contribute to long-term repair of vascular injury.

We agree with Tang et al17 that “…the fate of mature or contractile SMCs” requires direct in vivo lineage tracing. Although Nemenoff et al13 did not provide data elucidating the ultimate fate of phenotypically modulated SMCs, neither did Tang et al,7 despite their claims. Surprisingly, Tang et al only presented data at a single 5-day time point after injury in their smMHC−/− LacZ reporter Rosa26-EGFP mice (Figure 7D and Online Figure XIV in Tang et al17), and then, for reasons that are not clear, they switch to use of nonlineage tracing rats for all subsequent time points. Because the authors do not analyze cell fates beyond 5 days of injury and because their lineage tracing mouse is noninducible such that any cell that transiently expresses SM MHC will activate their lineage tracing gene and be labeled, they cannot draw any conclusions as to whether or not resident differentiated medial SMCs contributed to vascular remodeling after wire injury. In addition, it is unclear whether Tang et al17 performed high-resolution confocal analyses with Z-stack imaging as is necessary for rigorous lineage tracing as is reviewed in Hoofnagle et al.15,16 Furthermore, many of the images are of low magnification and provide poor resolution of individual cells as required for definitive lineage tracing, and immunofluorescence tissue staining is inconsistent across samples. For example, the staining for SMαA (compare Online Figure I with Figure 6E) and enhanced GFP (EGFP) (compare Figure 3A with Figure 7A) varies drastically between figures. Finally, despite the major focus of the article being on analysis of SM MHC−/− MVSCs, many of the key figures fail to show staining for this critical marker.

There are also numerous other previous studies that contradict the key findings of Tang et al17 using independent experimental approaches and animal models. As a result of space constraints, we can only describe a few examples of these reports, and we apologize to the many groups whose space constraints, we can only describe a few examples of these reports, and we apologize to the many groups whose

staining was not observed at 7 days after injury in an SMrαALacZ, SM MHC LacZ, or SM22α LacZ mice (Figure 4B, 4F, and 4J in Regan et al17 and Figure 1N, 1P, and 1R herein). Similarly, Wamhoff et al18 showed a high frequency of proliferating intimal SMCs within lesions of Western diet–fed ApoE−/− mice using the SM22α G/C repressor lineage tracing mouse model. Consistent with the preceding findings that SMCs undergo phenotypic transitions, SM22α Cre-dependent lineage tracing has provided evidence that SMCs give rise to osteochrondrogenic cells within calcified arterial media,19 as well as atherosclerotic lesions. These latter studies in more pathophysiologically relevant atherosclerosis models, compared with wire injury, provide additional compelling evidence for SMC phenotypic transitions.

Several classical studies completed 30 years ago also directly refute the major conclusions of Tang et al.7 First, innovative experiments by Thomas et al,21 conducted in hyperlipidemic pig models of atherosclerosis, used 3H-thymidine pulse labeling experiments followed by complete serial sectioning of the thoracic aortas and construction of ancestor tables based on grain counting. Their experiments relied on the simple principle that when a cell undergoes sequential rounds of cell division in vivo, there is sequential halving of grain counts. Remarkably, the results of Thomas et al21 provided compelling evidence showing that large numbers of medial SMCs undergo multiple rounds of cell division during development of experimental atherosclerosis. Second, the comprehensive studies of Clowes et al,22,23 as well as the seminal work of Stemerman and colleagues,24 provided clear evidence that a large number of medial SMCs are capable of proliferation after vascular injury. Briefly, Clowes et al22,23 performed 3H-thymidine labeling studies after balloon catheter–induced denudation of the rat carotid artery, and then determined growth fractions (ie, the fraction of initial cells that enter the cell cycle) at various time points after injury. Of major relevance to the focus of this commentary, 46% of medial SMCs entered the cell cycle within 48 hours after injury. Furthermore, experiments by Miano et al25 showed rapid induction of several early growth response genes in the medial layer of the rat aorta after balloon injury; immunolocalization studies confirmed such rapid activation in clusters of SMCs (much more than 10%) subjacent to the intima where platelet- and serum-derived factors first emerge after injury (Figure 4D in Miano et al25). Taken together, these studies cast serious doubt over the concept advanced by Tang et al17 that differentiated SMCs are postmitotic and that the proliferating population of cells after vascular injury are derived solely from a minor stem cell population.

Another limitation of the Tang et al17 study is a general failure to provide rigorous quantification of their data. Insufficient evidence is presented to substantiate their claim that 10% of medial cells are SM MHC−, such as performing flow cytometric assays, and showing high-resolution confocal images of histological sections clearly illustrating this purported cell population along with graphical data with sufficiently high replicates to make statistical inferences. Furthermore, their broad conclusion that there is a lack of proliferating SM MHC+ medial cells is based on showing 1 cell in vitro (Figure 1C in Tang et al17). Of greatest concern, they fail to show a single image with costaining for SM MHC and the proliferation marker

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Ki67 in their in vivo model, which is arguably the most important experiment for testing their stated hypothesis. For example, the data shown in Figure 6G, 6H, and 6I would have been far more informative had they stained for SM MHC rather than SM\(\alpha\)A, and also included Ki67 staining. Indeed, the results shown in Figure 2 herein, based on unpublished studies from the laboratories of Drs Weiser-Evans and Miano, clearly show that SM MHC+ SMCs do, in fact, enter the cell cycle 7 days after distinct modes of murine arterial injury, thus directly refuting one of the major conclusions of the Tang et al study. Importantly, the results shown in Figure 2 were derived from 2 separate laboratories using independent but complementary methodologies. Finally, there is a growing body of evidence from studies of SM MHC promoter-dependent conditional knockout mice that are highly inconsistent with the conclusions of Tang et al that mature SM MHC+ medial SMCs are incapable of phenotypic transitions. Just 2, of many examples, include the studies of Nemenoff et al\(^{11}\) and Offermanns et al,\(^{26}\) showing that SMC-specific conditional knockout of phosphatase and tensin homolog or the \(\alpha\)-subunits of \(G_{\alpha_i}\) (or \(G_{\alpha_j}\)), respectively, resulted in profound changes in SMC phenotype.

Taken together, previous studies clearly refute the major conclusions of the Tang et al\(^{11}\) article and clearly establish that in vivo SMCs can exhibit a modulated phenotype after vascular injury or in experimental atherosclerosis that includes dedifferentiation, rapid induction of numerous early growth response genes, proliferation, and neointima migration, as well as other phenotypic transitions. In light of this body of evidence, what conclusions, if any, can be drawn from the in vitro data of Tang et al?\(^{17}\) In their attempt to establish primary SMC cell lines using both enzymatic digestion and tissue explant methods, they serendipitously established MVSC cell lines that were capable of differentiating into ectodermal and mesodermal lineages. Tang et al\(^{17}\) conclude that the proliferative, synthetic SMCs studied by most laboratories until now are actually derived from these MVSCs. Although it is plausible that current cell lines of synthetic SMCs may be derived from or may contain a proportion of MVSCs, there is a much simpler explanation for the results of Tang et al that may render them of questionable significance because of several critical deficiencies in their SMC isolation and culture methods long appreciated by most SMC laboratories. First, although the author’s claim that MVSCs do not arise from the adventitia, it is virtually impossible to effectively remove the adventitia from large vessels without a predigestion step followed by careful microdissection that allows one to peel off the adventitial layer like a sock. Tang et al\(^{17}\) apparently failed to use this predigestion step to remove the adventitia, which especially with small rodent vessels can result in cultures highly contaminated with adventitial fibroblasts and adventitial Sca1+ stem cells because these cells show much higher plating efficiencies and growth rates compared with SMCs. Although the MVSCs described by Tang et al\(^{17}\) are Sca1−, it is known that expression of this gene is rapidly downregulated once cells are placed in culture.\(^{27}\) The preceding problems, particularly if compounded with use of nonoptimized methods for isolating and culturing SMCs, result in derivation of cultured cells virtually devoid of SMCs. Although the data presented in Figure 3B indicate that the cell isolates derived by Tang et al\(^{17}\) are primarily GFP+ at time zero, the cells that actually adhere to the culture dish and give rise to cultures 24 hours later are weakly stained for SM\(\alpha\)A (Figure 6A; described as SM\(\alpha\)A low by authors), which is in marked contrast to the results of experienced SMC laboratories where >90% of plated medial cells continue to express SMC markers for many days in culture.\(^{3}\) Indeed, Blank et al\(^{28}\) showed that although enzymatically dissociated SMCs rapidly suppress synthesis of SMC marker genes, SM\(\alpha\)A and SM MHC are long-lived proteins so that they remain detectable well after cells have undergone phenotypic switching and initiated proliferation in culture. Second, it is well documented that it takes over 1 week for SMCs to adhere, proliferate, and migrate using the tissue explant method,\(^{29–33}\) which was used extensively in the Tang et al\(^{17}\) study. Thus, the authors cannot exclude the possibility that after 3 days of culture, adventitial cells, not a novel medial MVSC cell population, accounted for the SM MHC− GFP− cells. Third, whereas we do not doubt that Tang et al\(^{17}\) have isolated and identified a population of progenitor/stem cells capable of differentiating into multiple cell types in vitro, further controls and validation studies are needed to determine whether they are truly derived from the media and are distinct from the multipotential adventitial/perivascular stem cells previously described by several groups.\(^{37,12–36}\) However, based on the marker expression patterns, they appear to be distinct from the stem cells identified by Peault and coworkers\(^{33,35}\) but similar to those characterized by Klein et al,\(^{32}\) which were also CD44+ CD29+ CD31− CD34− CD146− and of adventitial origin. Definitive resolution of this issue will require the following: (1) use of a conditionally regulated SMC lineage tracing system such as that used by Nemenoff et al,\(^{11}\) combined with high-resolution confocal analyses of the subsequent fate.

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**Figure 2.** Smooth muscle myosin heavy chain-positive (SM MHC+) smooth muscle cells (SMCs) replicate after arterial injury. **A**, Section of femoral artery of SM MHC-CreERT2/Rosa26-floxStop\(-\)Gal reporter mouse 7 days after wire injury (data provided by the laboratory of Dr Mary C. Weiser-Evans; see Nemenoff et al\(^{11}\) for additional methodological details). **Arrowheads** denote internal elastic lamina, and **arrows** indicate proliferating BrdU+ SMC. **B** and **C**, Ten-micrometer thick reconstructed Z-stacked confocal microscopy images from a wild-type mouse carotid artery 7 days after ligation injury (unpublished data provided by the laboratory of Dr Joseph M. Miano). Most medial cells are SM MHC+ (green) with many exhibiting clear Ki67 staining (magenta). Nuclei are stained with 4',6-diamidino-2-phenylindole. The bar in **B** is 50 μm. Boxed region in **B** is shown at higher magnification in **C**, with **arrows** pointing to obvious SM MHC+/Ki67+ medial SMCs and **arrowheads** indicating weakly stained SM MHC+/Ki67+ cells. The bar in **C** is 10 μm.
of mature SMCs at multiple time points after vascular injury or induction of disease, as well as determination of the fate and properties of mature SMCs in culture using techniques optimized for survival of the SMCs in vitro; and (2) development of methods to directly lineage tag the putative MVSC population in vivo followed by rigorous quantitative fate mapping studies both in vivo and in vitro.

In summary, there are serious deficiencies that undermine most of the major conclusions of Tang et al.7 Contrary to their statements, there is compelling evidence that mature SMCs are not terminally differentiated and are capable of transitions in phenotype, including cell proliferation and loss of differentiation markers. However, the Tang et al studies do reemphasize the importance of definitive characterization of the origins of SMC cultures and the urgent need for more rigorous and complete SMC lineage tracing studies in vivo. Indeed, at present there is a scarcity of studies defining the ultimate fate of mature SMCs in different models of vascular remodeling (repair) and disease, and further investigations are needed in this important area.8 Finally, although several groups have identified a perivascular origin of mesenchymal stem cells,33–36 it remains to be determined whether synthetic SMCs represent a reservoir of stem cells capable of transdifferentiation in vivo in different models of injury repair. Indeed, should this prove to be true, it would have major therapeutic implications, including identification of potential approaches to augment the stem cell–like properties of SMCs for purposes such as plaque stabilization, augmentation of vascular remodeling, stabilization of the tumor vasculature, and promoting overall tissue regeneration and repair.

Disclosures
None.

References


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