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Promoters to study vascular smooth muscle: mistaken identity?

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Abstract

Smooth muscle cells (SMCs) are a critical component of blood vessel walls that provide structural support, regulate vascular tone, and allow for vascular remodeling. These cells also exhibit a remarkable plasticity that contributes to vascular growth and repair but also to cardiovascular pathologies, including atherosclerosis, intimal hyperplasia and restenosis, aneurysm, and transplant vasculopathy. Mouse models have been an important tool for the study of SMC functions. The development of smooth muscle-expressing *Cre*-driver lines has allowed for exciting discoveries, including recent advances revealing the diversity of phenotypes derived from mature SMC transdifferentiation *in vivo* using inducible $CreER^{T2}$ lines. We review SMC-targeting Cre lines driven by the *Myh11, Tagln,* and *Acta2* promoters, including important technical considerations associated with these models. Limitations that can complicate study of the vasculature include expression in visceral SMCs leading to confounding phenotypes, and expression in multiple non-smooth muscle cell types, such as *Acta2-Cre* expression in myofibroblasts. Notably, the frequently employed *Tagln*/*SM22α-Cre* driver expresses in embryonic heart but can also confer expression in non-muscular cells including perivascular adipocytes and their precursors, myeloid cells, and platelets, with important implications for interpretation of cardiovascular phenotypes. With new *Cre*-driver lines under development and the increasing use of fate mapping methods, we are entering an exciting new era in SMC research.

Introduction

Smooth muscle cells

Smooth muscle cells (SMCs) make up the muscular layer of arteries and veins and are essential in regulation of vascular tone and blood pressure. Under physiological conditions, SMCs are differentiated and quiescent, but in response to injury, SMCs de-differentiate,

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characterized by proliferation, migration, and loss of contractile protein expression. While the remarkable phenotypic plasticity of SMCs in the vessel wall is important for wound healing and growth, SMCs also play a role in pathophysiology. SMC de-differentiation is central to vascular pathologies such as atherosclerosis, restenosis, and aneurysms [1, 2]. Phenotypically modulated SMCs also contribute to the formation of fibrous cap, neointima, and foam cells in atherosclerotic plaques [2]. SMCs are integral to the vascular reactivity and remodeling changes that contribute to the pathophysiology of hypertension and its complications [3]. Thus, it is critical to elucidate the mechanisms by which SMCs are regulated.

Differentiated SMCs are characterized by a large repertoire of genes encoding a variety of contractile/cytoskeletal proteins, including α-smooth muscle actin (ACTA2), smooth muscle myosin heavy chain (MYH11; formerly known as SM-MHC), calponin (CNN1), transgelin (TAGLN; formerly known as SM22α), h-caldesmon (CALD1) and smoothelin (SMTN) [4]. These genes have been important markers in the study of SMC, and the regulatory regions of several of these genes have been used to drive expression of Cre recombinase. We will discuss the pros and cons of each in this review.

The Cre/*lox* system

The Cre/*lox* site-specific recombination system allows generation of cell-type specific and conditional deletion of a specific gene. Cre, a 38 kDa bacteriophage enzyme, has been shown to catalyze recombination between two 34 bp *lox* sites[5]. The Cre protein creates a DNA loop and then either excises or inverts the looped segment depending on the orientation of the *loxP* sites [6, 7]. Initially, the method was developed to function efficiently in yeast to cause recombination on chromosomes and was subsequently adapted for use in mammalian cells and mouse models[6, 8]. The Cre/*lox* site-specific recombination system is now well established for generation of conditional cell type-specific deletion or overexpression of specific genes, allowing for sophisticated mouse models in which to study cardiovascular disease. DNA regions flanked by *loxP* sites are often referred to as "floxed". This Cre/*lox* system has been elegantly summarized in the prior article in this series on endothelial-specific Cre drivers [9]. An important variation in this technology is the fusion of Cre with a mutant estrogen receptor (Cre-ER^{T2}) to allow tamoxifen-inducible cell type-specific gene expression or deletion. Feil et al. constructed two mutants of the estrogen receptor that either contain G400V/M543A/L544A or G400V/L539A/L540A in their ligand binding domain. These two mutants are termed as T1 and T2 respectively. The T2 mutant version of ER is strongly activated by tamoxifen and has been widely used in mouse genetic studies [10]. Tamoxifen treatment induces translocation of Cre-ER^{T2} from the cytosol to the nucleus where it can induce DNA recombination between 34 bp *loxP* sites [11].

As reviewed and highlighted in this review, a strategy that tends to yield faithful *Cre* expression involves inserting *Cre* recombinase into the gene of interest in a bacterial artificial chromosome to express in mice. The limitations involve random site of integration and variable number of gene copies. Song et al. highlight the use of gene targeting by homologous recombination in embryonic stem cells or by using CRSPR/Cas9 methods to ensure a single copy of a *Cre* recombinase gene is expressed from an endogenous gene locus

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[7]. There are also now different types of recombinases and several variants of lox sites that can be used together to create more controlled recombination events. Song et al. elegantly describe breeding strategies to avoid unwanted recombination and methods to overcome such issues if they arise [7]. Proper breeding strategies, genotyping protocols, and control experiments should help investigators in *Cre*-dependent gene manipulations and interpret their data appropriately.

There are additional general important considerations when using Cre/*lox* approaches. Several studies have highlighted the potential for differential sensitivities to Cre mediated recombination in distinct targeted floxed alleles[12]. Liu et al investigated the recombination correlation between several floxed alleles induced by Cre-expressing mouse lines and determined that factors including potential methylation, distance between *loxP* sites, sequences flanking the *loxP* sites and the level of Cre activity per cell can contribute to differences in Cre-mediated recombination [12]. Accordingly, investigators should carefully document the expression pattern of new Cre-driver mice with a reporter (*lacZ*, mTmG, YFP, etc). Similarly, the extent of recombination and degree of deletion should be evaluated using qPCR or Southern blotting strategies. For inducible Cre lines, optimization of the timing and dosing of tamoxifen may be required. Additional important “best practices” include maintaining the Cre mice in a hemizygous manner (for transgenes) and as heterozygous (for Cre-knockin) to avoid problems such as mutagenesis or loss of endogenous gene function. Refreshing breeders periodically can also help minimize genetic drift, and periodic re-evaluation of Cre patterns and deletion efficiency should be performed. Validating findings with multiple SMC Cre drivers, as was successfully employed by Herring et al (see below in *Myh11-Cre* section), is an additional rigorous approach[13].

In reviewing the different approaches to generate Cre driver lines, it is apparent that fidelity in replicating endogenous gene expression patterns improves with larger promoter fragments, with enhanced recapitulation generally obtained from constructs generated from bacterial artificial chromosome (BAC) segments or with knock-in of the Cre recombinase into the endogenous locus. While these Cre-based strategies have opened new frontiers in SMC research, targeting Cre expression specifically to SMCs has proven challenging as many of these smooth muscle markers are expressed, at least transiently, in other cell types, including fibroblasts and myofibroblasts (detailed in following sections). Furthermore, expression of target genes in visceral smooth muscle can produce gastrointestinal phenotypes that complicate or preclude analysis of vascular phenotypes (discussed in the concluding section). Herein, we review smooth muscle-targeting Cre driver lines and highlight the advantages and caveats of each. We additionally summarize some of the recent paradigm changing discoveries made possible by this technology.

Myh11-Cre—Of the aforementioned smooth muscle markers, MYH11 is widely considered the most specific for the smooth muscle lineage and the most definitive marker of SMC differentiation. Joe Miano, Eric Olson and colleagues were the first to report *in situ* hybridization analysis of *Myh11* transcript expression and concluded that *Myh11* was highly specific to SMCs in embryonic and adult mice [14]. Subsequently, a transgene consisting of the 4.2 kb of the 5’ flanking region of the rat *Myh11* gene, 88 bp of the first exon (which is untranslated), an additional 11.5 kb of the first intron followed by the *lacZ* reporter gene was

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used to generate transgenic mice [15]. These *Myh11-lacZ* mice displayed specific transgene expression in vascular and non-vascular SMCs; however, there was wide variability in lacZ expression both within and between specific vessels [15]. Taking advantage of the Cre-lox system, the Owens and Kotlikoff labs generated mouse lines that expressed Cre recombinase driven by a fragment of the *Myh11* promoter to induce recombination in SMCs [16, 17]. Unfortunately, subsequent studies suggested that these Cre lines also induce recombination in the germ line [18, 19].

More recently, a transgenic mouse carrying a *Myh11*-driven inducible Cre was generated through a distinct cloning strategy by the Offermanns group [20]. A fusion protein of the Cre recombinase with the modified estrogen receptor binding domain (CreER^{T2}) was cloned into the initial coding ATG of the mouse *Myh11* gene carried by a BAC [20]. Tamoxifen treatment then promotes Cre-mediated recombination and subsequent expression or excision of the target gene [11]. In their initial characterization of *Myh11-CreER^{T2}* mice also carrying the ROSA26R-lacZ reporter [21], Wirth et al. reported inducible Cre-mediated beta-galactosidase (β-Gal) activity exclusively in SMCs in the tissues examined, including in blood vessels in multiple organs and in stomach, colon, and bladder visceral SMCs [20]. The use of the large genomic segment in the BAC likely confers this expression pattern replicating that of endogenous *Myh11* with higher fidelity. It should be noted, however, that expression from this construct has not yet been thoroughly characterized during embryogenesis.

The *Myh11-CreER^{T2}* mice generated by the Offermanns lab are available from Jackson Laboratories (B6.FVB-Tg(*Myh11-cre/ER^{T2}*)1Soff/J) and have proven to be a windfall for diverse studies evaluating the role of gene products in SMCs and the fate of SMCs during development and disease[22]. This Cre driver was used in two studies that determined that the majority of neointimal cells in models of vascular injury are derived from mature medial SMCs. These rigorous findings provided consensus in the field on the question of neointimal origin as the results were validated by two different labs using two distinct reporters (LacZ[13] vs mTmG)[23] as well as two different injury models (femoral wire denudation[23] and carotid ligation[13]). Furthermore, Herring *et al* additionally confirmed the finding with the *Acta2-CreER^{T2}*[13](see later section for description of this Cre line).

Work from several laboratories has employed lineage tracing with the *Myh11-CreER^{T2}* line to uncover paradigm-shifting new roles for SMC in atherosclerosis. Gary Owens and colleagues demonstrated that tamoxifen induction of *Apoe$^{(-/-)}$, Myh11-CreER^{T2}, ROSA26R$^{(YFP/YFP)}$* mice results in efficient marking of medial SMCs in large arteries. Notably, their lineage tracing approach revealed that the contributions of SMCs to atherosclerotic plaques had been underestimated, as >80% of SMC-derived cells in plaques could not be identified by immunostaining for SMC contractile protein markers after high fat diet feeding [24]. In plaques, mature YFP^{+} SMC were found to have lost their classical SMC markers and express markers of other cell types, including macrophages or stem cells [24]. More recent work from the Greif lab found that pre-existing SMCs are recruited into atherosclerotic plaques, first coating the cap of the plaque and subsequently invading the core [25]. Importantly, these investigations provide evidence that SMCs change fate during atherogenesis. Further mechanistic experiments, which include introducing a floxed allele for the *Kruppel-like factor (klf) 4* into *Myh11-CreER^{T2}* lineage tracing mice, demonstrate

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that KLF4 plays a key role in pathological SMC transitions in atherosclerosis [24] as well as in cancer [26]. Fate mapping studies using the *Acta-CreER^{T2}* and *Klf4$^{flox/flox}$* mice also identified a key role for KLF4 in SMC transitions in pulmonary hypertension (see *Acta2-Cre* section below)[27].

Although *Myh11* transcript and the *Myh11-CreER^{T2}* transgene were originally reported to be specific to SMCs, data indicating expression in non-SMC populations are beginning to come to light in distinct contexts. For instance, the Greif lab recently showed that tamoxifen induction of *Myh11-CreER^{T2}, ROSA26R$^{(mTmG/mTmG)}$* mice also marked ACTA2^{-} cells in the alveoli of the adult lung [28]. In addition, hypoxia-induced alveolar ACTA2^{+} myofibroblasts express MYH11; indeed, in mice exposed to hypoxia (FiO_2 10%) for 21 days, ~85% of alveolar ACTA2^{+} myofibroblasts express MYH11 [28]. In a recent study from another group, the authors suggest that in the adult mouse lung, MYH11 is expressed in SMCs as well as at least a subset of pericytes and that these pericytes are YFP^{+} in tamoxifen-induced *Myh11-CreER^{T2}, ROSA26R$^{(YFP/YFP)}$* mice [26]. In the lungs of these mice, 10% of YFP^{+} cells were found to be negative for MYH11. While these may be derived from dedifferentiated pericytes or SMC, their origin is not definitively known. This study found that KLF4 upregulation also mediates phenotypic switching in YFP^{+} perivascular cells, which contribute to a pro-metastatic microenvironment [26].

An important consideration is that the *Myh11-CreER^{T2}* transgene initially incorporated into the Y chromosome which has limited investigations with this transgene to male mice. This limitation is profound as SMCs are key players in cardiovascular development and disease, and cardiovascular disease is the leading cause of death in women. Notably, the laboratory of Zhihua Jiang recently reported that through breeding they have obtained a colony of *Myh11-CreER^{T2}* mice – presumably by translocation to the X chromosome - in which the transgene is inherited by both male and female mice [29–31]. Importantly, this colony may markedly enhance the study of SMCs in the development and disease of female mice.

Tagln-Cre—Transgelin (TAGLN), formerly known as SM22α, is an actin binding protein that regulates SMC contraction and serves as a marker of the differentiated phenotype. Accordingly, *Tagln/SM22α-Cre* drivers have been commonly used to study smooth muscle-specific expression. While these were originally referred to and published as "*SM22α-Cre*", we will refer to them in this review using the current official nomenclature "*Tagln-Cre*". These Cre drivers have been widely employed despite the well-known limitation that Tagln is transiently expressed in cardiac myocytes during embryonic development. By mid-gestation, *Tagln* gene expression becomes restricted to visceral and vascular SMCs. Olson and colleagues initially determined that a 455 bp fragment of the mouse *SM22α* promoter was sufficient to drive lacZ reporter activity in SMCs *in vivo* but did not completely recapitulate endogenous expression patterns [32]. The first *Tagln-Cre* lines were made by the Feil group, using a transgenic or knock-in approach. The knock-in *Tagln-Cre-ER^{T2}* line was superior in recombination to the transgenic line, but efficiency was very low in vascular SMC compared to visceral SMC [33]. Another early attempt created a transgenic *Tagln-Cre* with a 1.4 kb fragment of the *Tagln* promoter [34]. A ROSA26R-LacZ reporter indicated embryonic expression in heart, aorta, and umbilical vessels, but also in the head and tail regions of the embryo at E10.5. Conditional deletion of *Srf* with this *Tagln-Cre* proved to be

embryonic lethal, identifying a key role for *Srf* in heart and vascular development [34]. The Liaw lab also took two approaches incorporating a 1.4 kb fragment of the *Tagln* promoter to make a transgenic *Tagln-Cre* and a Tet-on inducible *Tagln-rtTA-Cre*. When crossed to five reporter lines, these mice showed very high variability in recombination [35].

An additional transgenic line was generated employing a longer 2.8 kb fragment of the mouse *Tagln* promoter to drive Cre expression. The Cre mRNA was detected at high levels in aorta, intestine, and uterus similar to endogenous *Tagln*. When crossed with ROSA-26R-lacZ reporter mice, efficient recombination was demonstrated in hepatic and pulmonary arteries, while non-muscle cells in these organs did not express β-Gal [36]. This line (now congenic) is available from the Jackson Laboratories (B6.Cg-Tg(*Tagln-cre*)1Her/J) [36] and has been used by many investigators.

The Parmacek group used a similar strategy to construct Tagln-Cre using –2775-bp to +39-bp of the *Tagln* promoter which was similarly expressed in SMC during development and in adult tissues [37]. The expected expression was found in the vascular wall of cranial vessels, the bronchial arch arteries, umbilical artery as well as the developing heart at E9.5, and in adult vascular and visceral smooth muscle. While arterial SMC were labeled with nearly 100% efficiency, as expression was also noted in the adult ventricular myocardium, this driver line did not faithfully recapitulate Tagln expression in the adult.[37]

Chen and colleagues employed a distinct approach, knocking the Cre recombinase coding sequence into the endogenous *Tagln* locus in frame just prior to the *Tagln* initiation site (*Tagln-Cre^{KI}*) [38]. In this approach two targeted clones were injected into C57BL/6 blastocysts to produce chimeras, which were then further crossed with C57BL/6 female mice to generate heterozygous *Tagln-Cre^{KI}* mice. A homozygous knockin of *Cre* may cause inadvertent deletion of endogenous gene. When crossed to ROSA26R LacZ or eGFP reporter mice, expression from this Cre faithfully recapitulated expression of Tagln in adult tissues, including arteries, veins, bladder and gastrointestinal tract, with no expression in skeletal muscle. Strikingly, however, this *Tagln-Cre^{KI}* did not drive expression in embryonic SMC or cardiac myocytes at E9-E16.5, but could be detected in neonates after birth. While this does not mimic endogenous expression in this regard, this line could potentially circumvent adverse effects of gene expression changes driven by the Tagln-Cre during development. This line has also been available through Jackson Laboratories (B6.129S6-*Tagln$^{tm2(cre)Yec}$*/J) [38] and is widely published.

Non-smooth muscle expression of Tagln

Some early studies that predated development of *Tagln-Cre* lines detected Tagln expression in non-SMC or cardiac myocyte tissues. These include TAGLN expression in cultured human fetal lung myofibroblasts [39], rabbit bladder serosal myofibroblasts [40], and porcine adventitial myofibroblasts following vascular injury [41]. Subsequently, other studies have revealed that *Tagln-Cre* lines direct expression in other cell types. One study by Shen and colleagues used both *Tagln-Cre* lines available at Jackson Laboratories and bred them individually to a strain to transgenically express a dominant negative mastermind-like-GFP fusion protein (DNMAML-GFP) [42]. This allowed use of GFP signal to trace cells labeled by each Cre, with the caveat of potentially confounding effects of overexpression of a factor

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that inhibits canonical Notch-mediated transcription on the pattern of GFP expression. While GFP was detected in the expected SMC-rich tissues including aorta, bladder, stomach, and uterus, very high expression was also noted in the immune cell-rich spleen. Subsequent FACS and labeling approaches revealed that the Tg(*Tagln-cre*)1Her/J line expressed in a portion of circulating neutrophils and monocytes. Genotyping for a recombined allele also revealed Cre activity in peripheral blood and peritoneal macrophages of the Chen lab *Tgln2-Cre^{KI}* line[42]. While this study is not definitive due to the introduction of DNMAML, it raised the provocative possibility that *Tagln-Cre* lines could drive expression in cells other than smooth muscle and cardiac myocytes. Work from other labs, including our own, discussed below, supports this notion.

Importantly, megakaryocytes, which give rise to platelets, also express TAGLN [43]. Diane Krause's lab noted that in mice with a tdTomato to GFP Cre-reporter (mTmG)[44] (Jax Gt(ROSA)26Sor$^{tm4(ACTB\text{-}tdTomato,\text{-}EGFP)Luo}$/J), the *Tagln-Cre* transgene (Tg(*Tagln-cre*)1Her/J) causes from 30–60% of platelets to become GFP^{+}. In Figure 1, the tdTomato and GFP fluorescence of peripheral blood platelets is shown for WT mice, mTmG mice without Cre recombinase, and mTmG mice crossed with *Tagln-Cre*. Note that in the mouse with the *Tagln-Cre*, over half of the platelets in the peripheral blood are Tomato^{-} and GFP^{+}, indicative of Cre-mediated recombination in megakaryocytes. Thus, in mice with a gene of interest deleted using the *Tagln-Cre*, loss of gene expression in platelets could impact the phenotypes observed. This is particularly notable as this Cre driver is frequently used to study vascular SMC responses in models such as wire denudation to induce intimal hyperplasia, a process heavily influenced by platelets. Given the caveats of *Tagln-Cre*-driven expression in myeloid cells and platelets, bone marrow transplantation experiments would be an important approach to determine the extent to which observed phenotypes are attributable to SMC versus hematopoietic cells. Additionally, Varberg and colleagues recently demonstrated fetal *Tagln* expression in the vascular progenitor endothelial colony-forming cells (ECFCs)[45]. This study revealed that upregulation of Tagln in these ECFCs in response to gestational diabetes led to abnormalities in vasculogenesis, suggesting another potential factor to consider when using *Tagln-Cre* constructs in developmental studies.

Another notable Tagln expression pattern was uncovered when the Chen lab employed their *Tagln-Cre* knock-in line in order to delete PPARγ selectively in SMCs. The previously unappreciated expression of Tagln in a perivascular adipose precursor fortuitously led to very exciting and unexpected observations due to a complete lack of development of perivascular adipose tissue (PVAT) in these mice [46]. PVAT was found to be highly similar to brown adipose tissue (BAT), demonstrating a thermogenic effect to regulate intravascular temperature. PVAT was also revealed as an important source of prostacyclin such that loss of PVAT led to endothelial dysfunction. Another intriguing possibility generated by this study is the existence of a common precursor that gives rise to both PVAT and vascular SMC. While "off target" gene expression driven by the *Tagln-Cre^{KI}* precluded a clear analysis of PPARγ function in SMC in this study, this unexpected Tagln expression pattern revealed multiple fascinating novel insights into the functions of PVAT and vascular homeostasis.

Another intriguing and serendipitous discovery was made using the *Talgn-Cre* [36] to delete IκB kinase β (IKKβ) in SMC. The *Tagln-Cre*-IKKβ$^{f/f}$ (Tg(*Tagln-cre*)1Her/J) mice were

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resistant to atherosclerosis in the *LDLR*$^{-/-}$ background, but were surprisingly also found to be resistant to diet-induced obesity, hepatic steatosis, and exhibited improved glucose homeostasis [47]. Crossing the *Talgn-Cre* to *Rosa26*^{EGFP} revealed that *Talgn-Cre* is also active in primary adipose stromal vascular cells [47]. Interestingly, deficiency of IKKβ diminished the ability of these cells to differentiate, leading to accumulation of adipocyte precursor cells in adipose tissue, and revealed a mechanistic link between IKKβ and β-catenin [47].

Data from Kathleen Martin's lab reveal bright GFP expression in the aortic media in *Tagln-Cre-mTmG* reporter mice (Tg(*Tagln-cre*)1Her/J strain), as well as in the perivascular adipocytes (Figure 2). It is now appreciated that adipose precursors reside in a perivascular niche [48]. The multiple studies mentioned herein suggest or directly indicate Tagln expression in adipogenic, vascular precursors as well as mature hematopoietic cells. This is an important caveat to consider when using *Tagln-Cre* drivers. The functional consequences of expression of Tagln in stem cells are unknown, but suggest an interesting new avenue for further investigation.

Acta2-Cre—Smooth muscle α-actin (ACTA2) is a key smooth muscle contractile protein whose expression increases with differentiation, but lower level expression is maintained even in "synthetic" phenotype SMC. Indeed, this marker is not a definitive marker of the smooth muscle lineage as it is expressed in many other cell types, most notably, fibroblasts and myofibroblasts. Multiple labs have generated *Acta2-Cre* lines. These have been primarily employed for the study of myofibroblasts and fibrosis, but under appropriate conditions, may have utility for SMC lineage tracing as described below.

The first *Acta2-Cre* line was generated in 1996 using a 4.7 kb fragment of the human *ACTA2* gene (–891 to +3828, containing promoter region, exon 1, intron 1, and 14 bp of exon 2). Crossing to a β-Gal reporter, they reported expression only in the heart, aorta, and diencephalon in early stage embryos [49]. Another early *Acta2-Cre* line was developed by Wu *et al.*, using a fragment of the *Acta2* gene spanning –1070 to +2582 bp including the first exon and part of first intron. When crossed to *ROSA26R-LacZ*, β-Gal expression was observed as expected in SMC-rich tissues including arteries, veins, and airways, and this reporter line was used to characterize mechanisms of bleomycin-induced pulmonary fibrosis [50].

Metzger and colleagues generated a transgenic tamoxifen-inducible *Acta2-CreER*^{T2} line using a large segment of the mouse *Acta2* gene in a BAC [51]. This construct was found to drive expression in both vascular and visceral smooth muscle with limited expression in cardiomyocytes [51]. This line (Tg(*Acta2-cre/ER*^{T2})Pcn) and has been the most utilized of all published Acta2-Cre drivers to date with at least 23 citations. The Greif laboratory has employed this line to study changes in the muscularization of lung arterioles in response to hypoxia, a model of the pathological hypermuscularization in pulmonary hypertension (PH) [28]. *Acta2-CreER*^{T2}, *ROSA26R*$^{mTmG/mTmG}$ mice were used to induce GFP labeling of mature SMCs in pulmonary arterioles. By assessing these vessels over a time course following hypoxia treatment, it was determined that GFP^{+} vascular SMCs present in the normal adult were the source of nascent distal muscularization [28]. This study also made the surprising observation that the vast majority of alveolar myofibroblasts that arise in response

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to hypoxia are not derived from ACTA2$^+$ cells. Conversely, the *Myh11-Cre-ER^{T2}* line crossed to *ROSA26R$^{mTmG/mTmG}$* revealed that some GFP labeled cells near the lung arterioles lacked SMC markers in the absence of hypoxia. Thus, in this particular organ, vascular bed, and time point, *Acta2-Cre-ER^{T2}* was a more rigorous marker for SMCs than *Myh11-Cre-ER^{T2}*[27]. Further studies using the *Acta2-CreER^{T2}* crossed to a multi-color reporter line (*ROSA26R$^{Rainbow/+}$*) extended these PH findings, revealing a clonal origin of SMCs in hypermuscularized distal arteriolar regions [27].

In addition to studies in pulmonary arteries, the *Acta2-CreER^{T2}* (Tg(*Acta2-cre/ER^{T2}*)#Pcn) was used in a groundbreaking study assessing vasomotion in brain vessels. By comparing different Cre reporters and immunostaining approaches, it was determined that ACTA2 was expressed in arterioles but not in capillary-associated pericytes. *In vivo* live imaging revealed that SMC but not pericyte constrictions are critical in pathological responses to brain ischemia [52]. The *Acta2-CreER^{T2}: ROSA26R* transgenic line was also used for lineage tracing in a recent study which noted that myoepithelial cells in the proximal trachea also express ACTA2.[53]Another transgenic *Acta2-Cre* line employing 5.2kb of the mouse *Acta2* gene, including 2.4kb of promoter as well as exon 1, intron 1 and part of exon 2, has been generated by the Kalluri lab [54, 55]. An *Acta2-RFP* reporter line demonstrated RFP expression in SMCs and rare interstitial cells in the kidney [43], and the *Acta2-Cre* crossed to a YFP reporter was employed in a study of myofibroblasts in kidney fibrosis [44]. This *Acta2-Cre* (B6.FVB-Tg(*Acta2-cre*)1Rkl/J), backcrossed to C57BL/6J) [44] and the reporter line ACTA2-RFP (B6.FVB-tg(*Acta2-DsRed*)1Rkl/J) [43] are available from The Jackson Laboratory.

Grcevic and colleagues produced and applied an *Acta2-CreER^{T2}* crossed to a reporter line to identify ACTA2$^+$ mesenchymal progenitors within bone marrow and adipose tissue with osteogenic potential [56]. Stappenbeck and colleagues used the BAC approach to generate an independent *Acta2-CreER^{T2}* line and crossed it to an mTmG reporter, generating strong GFP labeling of SMC in the gastrointestinal tract and demonstrating that loss of *Acta2* gene expression in a mouse colonic injury model was due to SMC death as opposed to dedifferentiation [57].

Summary and Conclusions

In reviewing Cre driver lines used to study smooth muscle, we have noted that expression in non-SMCs is a common caveat which needs to be considered when designing and interpreting studies. While all of the current SMC-targeting Cre driver lines have pros and cons, we highlight the need to select the most appropriate Cre for studies, depending on the processes one is attempting to model. Target organ biology, vascular bed, visceral SMC, and spatiotemporal considerations are all key variables to consider.

The *Myh11-CreER^{T2}* is currently regarded as the most specific for SMCs, yet expression in SMC-like cells, such as pericytes, have been noted in the lung. Platelet-derived growth factor receptor-β and neuron-glial 2 are well known pericyte markers. However, these markers are also expressed in other cell types, including subsets of SMCs, thereby limiting the utility of mice carrying a Cre driven by the promoter of one of these genes. A Cre that specifically

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labels pericytes would be a major advancement for the field. Analysis of single cell transcriptomic studies of pericytes is yielding candidate genes, and promoters of these genes will need to be assayed as to whether they can be used to specifically drive Cre expression in pericytes[58].

The *Tagln-Cre* lines, while very widely used, are now appreciated to be expressed in cardiac myocytes, adipocytes and their precursors in the vascular adventitia, megakaryoctyes, platelets, and myeloid cells. This complex expression pattern requires caution when evaluating phenotypes as these cell types may collectively influence cardiovascular physiology and pathophysiology through cardiometabolic and inflammatory effects. Bone marrow transplant, platelet transfusion, or complementary experiments employing myeloid- or platelet-targeting Cre drivers are potential strategies to address myeloid and platelet contributions in *Tagln-Cre* mice. The perivascular adipose expression is more difficult to address. Murine PVAT transplants are challenging but may be a potential alternative in some models [59]. The *Acta2-Cre* lines strongly express in SMC,but may be confounded by myofibroblast and stem cell expression [56]. Depending on the context, an *Acta2-CreER*^{T2} approach may have utility for the study of vascular [2852] or visceral [46] SMC as described above.

A limitation to SMC-targeting Cre driver lines is the potential to label both vascular and visceral SMCs. When these tools are used to overexpress or delete genes that may cause a phenotype in non-vascular SMCs, there is potential for disruptive respiratory, genitourinary, and/or gastrointestinal complications that may preclude evaluation of the role of the gene in the vasculature. Examples include inducible deletion of *Serum response factor (Srf)* with the inducible *Tagln-CreER*^{T2} (Feil lab) which led to cachexia and death due to chronic intestinal pseudo-obstruction [60,61]. Lethal gastrointestinal phenotypes were also observed with *Myh11-CreER*^{T2}-mediated deletion of *Srf* [62] or *Dicer* [63]. Promoters that distinguish between vascular and visceral, or arterial and venous SMC expression would be useful reagents for in vivo studies. In an early transgenic lacZ reporter driven by 445 bp of the Tagln promoter was found to express in embryonic vascular smooth, cardiac, and skeletal muscle, but notably did not express in venous or visceral SMC[32]. A subsequent study identified the converse reagent using a 370 bp fragment of the telokin gene. This telokin construct was reported to be visceral-specific in vivo, while chimeras between this telokin sequence and the Tagln sequence were found to modulate the vascular vs visceral expression patterns[64]. A smooth muscle Cre driver line with preferential activity for vascular SMCs is currently under development (personal communication, Joe Miano). It is possible that additional SMC-specific or -restricted enhancers may be discovered as well, especially with the advent of genome-wide epigenetic investigations in SMC.

Some of the limitations associated with temporal patterns in Cre expression may be circumvented by using inducible constructs such as the *Cre-ER*^{T2}. For example, induction in adult mice can avoid the developmental expression of *Tagln-Cre* in the heart. The ability to induce recombination at a specific point in time is an essential hallmark of lineage tracing studies. The permanent marking and tracing of mature, differentiated SMCs with a Cre-driven reporter allows these cells to be identified as SMCs even after downregulating their classic contractile markers beyond detection. While SMCs are well known to comprise

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fibrous caps, lineage tracing has identified key roles for SMCs in the initial formative processes of atherosclerotic lesions [25], as well as their previously unappreciated role in giving rise to macrophage-like and stem cell-like cells [24]. This strategy also identified primed SMCs that are source of hypermuscularization in PH [2728]. Such fate mapping approaches, when rigorously applied, will continue to expand our knowledge of the physiological consequences of SMC plasticity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

ACTA2	Smooth muscle alpha actin
BAC	bacterial artificial chromosome
ER	estrogen receptor
ER^{T2}	tamoxifen-inducible mutant estrogen receptor
GFP	green fluorescent protein
lacZ	encodes β-galactosidase
MYH11	Smooth muscle myosin heavy chain
PVAT	perivascular adipose tissue
RFP	red fluorescent protein
SMCs	Smooth muscle cells
SRF	serum response factor
TAGLN	Transgelin (also known as SM22α)
YFP	yellow fluorescent protein

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Highlights:

We summarize smooth muscle-expressing Cre lines and their advantages and caveats.

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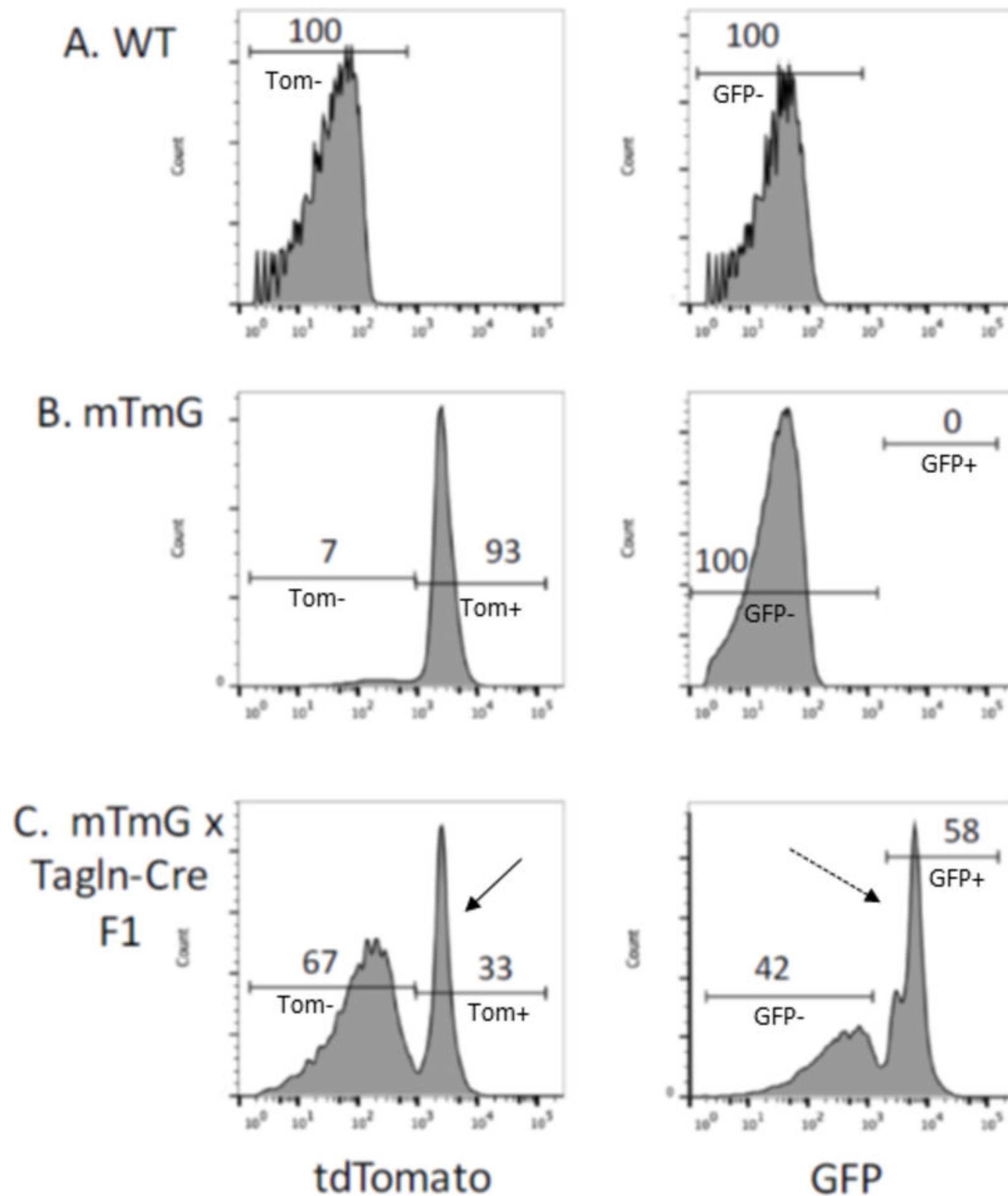

Figure 1. Flow cytometric analysis reveals Tagln-Cre mediated switch from tdTomato to GFP in a majority of platelets.

FACS analysis of platelets isolated from platelets from WT (A), mTmG (B) and Tagln-Cre x mTmG F1 (C) mice for tdTomato red (left) and GFP (right). The cutoff for positive signal is based on analysis of WT (A) nonfluorescent platelets. In 8 week old Tagln-Cre x mTmG mice (C), 33% of platelets are positive for tdTomato expression (solid arrow) while 58% are positive for GFP expression (dashed arrow) after Cre-mediated recombination.

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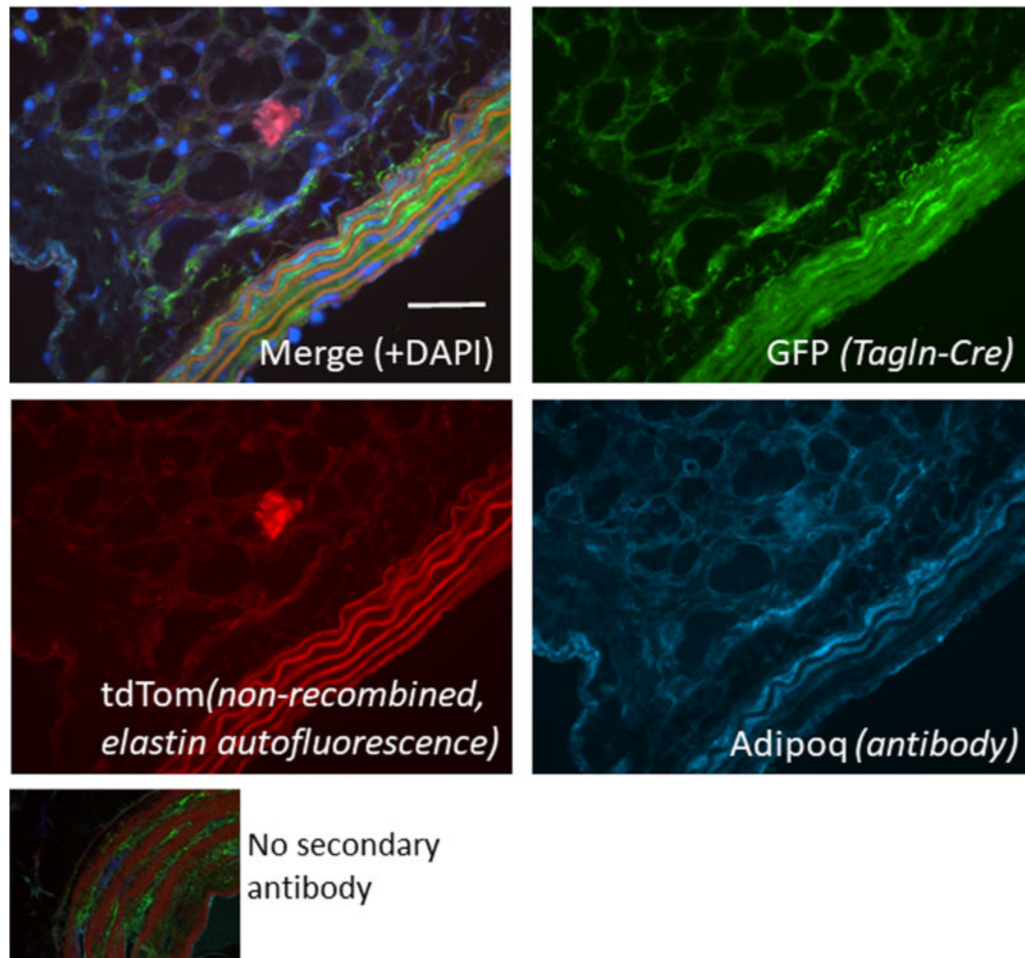

Figure 2. *Tagln-Cre-mTmG* labels the aortic media and perivascular adipose tissue.

Confocal spinning disk microscopy of a section of aorta from a male *Tagln-Cre-mTmG (Tg(Tagln-cre)1Her/J)* mouse that was immunostained with an antibody to mouse Adiponectin (#1119 R&D Systems, 1:10 dilution) and secondary antibody (Cy5, Cyan). Merged image at top left, individual channels for GFP, tdTomato, and Cyan also shown. Scale bar = 50 mm. GFP (green) indicates cells in which Tagln-Cre is active, while tdTomato (red) indicates cells in which there is no Cre-induced recombination. DAPI (dark blue) staining indicates nuclei. A negative control with no secondary antibody is shown at bottom left. Note that adiponectin, a marker of differentiated adipocytes, strongly costains the perivascular adipocytes, as well as some medial SMCs, which we have shown to express adiponectin [65].

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Table 1.

Commonly employed SMC-expressing Cre driver lines.

These lines have been widely published (Jackson Laboratories (JAX) strain name in italics). All express in vascular and visceral smooth muscle. Advantages and limitiations of each Cre line have been discussed in the main text and are summarized here.

Mouse strain, Reference	Advantages	Limitations
Myh11-CreER^{T2} [20] Offermanns laboratory JAX: *B6.FVB-Tg(Myh11-cre/ERT2)1Soff/J*	• BAC construct faithfully recapitulates endogenous expression • Inducible	• Also marks pericytes in the lung • Y chromosomal transgene limits study to males • (Jiang lab variant line expresses in male and female [29–31])
Tagln-Cre [36] Herz laboratory JAX: *B6.Cg-Tg(Tagln-cre)1Her/J*	• Expresses appropriately in SMC • B6 Congenic	• Also expresses in myeloid cells, platelets, and perivascular adipocytes and their precursors, and ECFCs • Not inducible
Tagln-Cre^{KI} [38] Chen laboratory JAX: *B6.129S6- Tagln$^{tm2(cre)Yec}$/J*	• Knock-in line expresses appropriately in adult SMC • Does not express in embryonic SMC or cardiac myocytes	• Also expresses in perivascular adipocytes and adipose precursors • (Not determined whether also expresses in myeloid cells or platelets) • Not inducible
Acta2-Cre-ER^{T2} [51] Metzger laboratory Tg(*Acta2-cre/ER^{T2}*)Pcn (NOT at JAX.)	• BAC-derived, appropriate SMC expression • Inducible • Validated for lineage tracing in atherosclerotic plaques and pulmonary arterioles	• Expresses in myofibroblasts, myoepithelial cells *(normal expression of endogenous Acta2 beyond SMC)*