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In vivo expression of a conditional TGF- β 1 transgene: No evidence for TGF- β 1 transgene expression in SM22 α -tTA transgenic mice

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Abstract

Transforming growth beta-1 (TGF- β 1) appears to play a critical role in the regulation of arterial intimal growth and the development of atherosclerosis. TGF- β 1 is expressed at increased levels in diseased arteries; however, its role in disease development remains controversial. Experiments in which TGF- β 1 is overexpressed in the artery wall of transgenic mice could clarify the role of TGF- β 1 in the development or prevention of vascular disease. However, constitutive overexpression of a TGF- β 1 transgene in the mouse artery wall is embryonically lethal. Therefore, to overexpress TGF- β 1 in the artery wall of adult mice, we generated mice that were transgenic for a conditional, tetracycline operator (tetO)-driven TGF- β 1 allele. These mice were viable, and when crossed with mice expressing a tetracycline-regulated transactivator (tTA) in the heart, expressed the TGF- β 1 transgene in a cardiac-restricted and doxycycline-dependent manner. Nevertheless, breeding of the tetO-TGF- β 1 transgene into three lines of mice transgenic for a smooth muscle-targeted tTA (SM22 α -tTA mice; reported elsewhere to transactivate tetO-driven alleles in smooth muscle cells of large arteries) did not yield expression of the TGF- β 1 transgene. Moreover, tTA expression was not detected in aortae of the SM22 α -tTA mice. Transgenic mice that express tTA at high levels in vascular smooth muscle and reliably transactivate tetO-driven transgenes would be useful for deciphering the role of TGF- β 1 (or other proteins) in normal arterial physiology and in the development of arterial disease. Currently available SM22 α -tTA mice were not useful for this purpose. Generation of higher-expressing lines of SM22 α -tTA mice appears warranted.

Keywords

Smooth muscle cells; Transforming growth factor beta-1; SM22 α ; α MHC; Conditional transgenesis; Doxycycline

1. Introduction

Transforming growth factor- β 1 (TGF- β 1) plays important roles in cardiovascular development and disease. TGF- β 1 appears to affect cardiac development, cardiac hypertrophy and fibrosis, arterial intimal growth, and atherosclerosis in both native and transplanted vessels [1]. A more precise elucidation of the roles of TGF- β 1 in these processes will likely deepen our understanding of normal development and physiology and may also provide clues for developing novel therapies for cardiovascular diseases.

Gene knockout and overexpression studies, performed in mice, are powerful approaches for elucidating biological roles of genes. Gene knockout studies aimed at manipulating TGF- β 1

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signaling have been both productive and problematic. Disruption of TGF- β 1 signaling in mice by germ line deletion of the genes encoding TGF- β 1 (*tgfb1*) or either of the two TGF- β 1 receptors (*tgfb1r1* and *tgfb1r2*) established critical roles for TGF- β 1 in immune regulation and early vascular development but also caused embryonic and perinatal lethality [2-4]. Thus, it has not been possible to determine the consequences of loss of TGF- β 1 expression or signaling in cardiovascular tissues of adult mice.

Germ line overexpression of TGF- β 1 in cardiovascular tissues has also been both productive and problematic. Constitutive cardiac overexpression of TGF- β 1 in mice, using the α -myosin heavy chain (α MHC) promoter, was compatible with survival to adulthood and revealed a potential role for TGF- β 1 in cardiac fibrosis [5]. However, constitutive overexpression of TGF- β 1 in murine smooth muscle cells (SMC), using the SM22 α promoter, caused early embryonic lethality [6]. Thus, it has not been possible to determine the consequences of chronic overexpression of TGF- β 1 in the vasculature of adult mice. This is unfortunate, because mice with increased vascular expression of TGF- β 1 would be an attractive experimental setting for testing hypotheses regarding the role of vascular TGF- β 1 expression in normal arterial physiology and in the development or prevention of arterial disease.

Conditional transgenic approaches offer powerful means for bypassing embryonic lethality and achieving overexpression in otherwise normal adult mice. The most widely used conditional transgenic approach involves use of a tetracycline-regulatable activator of transcription (tTA) to activate expression of a transgene that includes the tetracycline operator (tetO) [7]. Administration of tetracycline or an analogue during development prevents the tTA from activating tetO-regulated transgene expression; withdrawal of tetracycline postnatally allows the tTA to activate tetO-regulated transgene expression [8]. We used this conditional transgenic approach to attempt to bypass the embryonic lethality of SM22 α -TGF- β 1 transgenic mice and generate adult mice that overexpress TGF- β 1 in the artery wall. We intended to use these mice to unravel the role of vascular TGF- β 1 expression in arterial injury, experimental atherosclerosis, and organ transplantation. We began by generating mice with a conditional, tetO-driven TGF- β 1 allele. We verified that expression of this allele could be activated in tTA-expressing adult mouse hearts in a tissue-specific, pharmacologically controlled manner. We then obtained lines of mice transgenic for a SM22 α -tetracycline transactivator allele (SM22 α -tTA). These mice were reported, elsewhere, to drive expression of tetO-driven alleles specifically in SMC of large arteries [9-11]. However, in our hands, the SM22 α -tTA allele did not drive expression of the tetO-TGF- β 1 allele in adult murine vascular SMC in vivo.

2. Materials and Methods

2.1 Transgenic Mice

Three lines of mice expressing the tTA from a fragment of the SM22 α promoter (SM22 α -tTA; C57BL/6 background) [9] were generously provided to us by Dr. Mansoor Husain (University of Toronto, Ontario, Canada). These three lines, each descended from an independent founder, were designated as lines "19", "21", and "36". SM22 α -tTA mice expressed the tTA in a tissue-specific pattern at increasing levels (21 < 19 < 36; personal communication, M. Husain). Because of a concern regarding potentially leaky transgene expression and embryonic lethality [6], we initially requested and received lines 19 and 21. In spring 2000, when we reported difficulties in detecting transactivation of a tetO-regulated allele in mice from these two lines, two mice from line 36 were promptly and kindly provided to us. It is our understanding that line 36 is the line used in the published studies that report use of these SM22 α -tTA mice [9-11] and that data for these published studies were generated between 1998 and 2003 (personal communication, Dr. M. Husain). The majority of our expression studies on line 36 were carried out on first-generation offspring of the two mice we received in spring, 2000. Mice expressing the tTA from the cardiac α -myosin heavy chain gene promoter (α MHC-tTA;

93% FVB/N background) were obtained from Dr. Glenn Fishman (NYU, New York, NY) [12]. Mice transgenic for a tetO-driven β -galactosidase (β -gal) allele ("Ro1" mice; FVB/N background) were obtained from Dr. Bruce Conklin (Gladstone Institutes, San Francisco, CA) [13]. Mice transgenic for a SM22 α - β -gal transgene (C57BL/6 background) were obtained from Dr. Li Li (Wayne State University, Detroit, MI) [14]. The SM22 α promoter sequence in the SM22 α - β -gal transgene in Dr. Li's mice is the same sequence used by Dr. Husain's group to generate the SM22 α -tTA transgenic mice [9]. All mice were genotyped by Southern analysis of tail-tip DNA. The α MHC-tTA and SM22 α -tTA transgenic mice were genotyped by hybridization of digested tail DNA to a 833 bp *Xba*I-*Sph*I fragment of plasmid pUHD17-1 [15], which contains tTA sequences. This probe detected a 1.8 kb band in *Eco*RI-digested DNA of α MHC-tTA mice and a 4.5 kb band in *Hind*III-digested DNA of SM22 α -tTA transgenic mice. No other tTA-transgenic mice were present in our transgenic mouse colony.

We generated mice that were transgenic for a conditional, tetracycline-regulated TGF- β 1 allele. The transgene included a rat TGF- β 1 cDNA driven by a tetO-containing promoter. To begin to construct this transgene, we obtained two plasmids, one containing a 5' rat TGF- β 1 cDNA fragment and the other containing an overlapping 3' rat TGF- β 1 cDNA fragment (Dr. Anita Roberts, National Cancer Institute, Bethesda, MD). We obtained the plasmid pUH10-3 [13], containing the tetO upstream of a minimal CMV promoter from Dr. Bruce Conklin. The 5' and 3' TGF- β 1 fragments were ligated in pBluescript to generate a complete 1173 bp rat TGF- β 1 cDNA. This plasmid was used as a substrate for site-directed mutagenesis (QuikChange, Stratagene, La Jolla, CA) to convert G to C at position 668 and T to A at position 673. These changes convert two cysteine residues to serine residues and, by analogy with simian and porcine TGF- β 1 [16,17], generate a constitutively active TGF- β 1 molecule. The mutated rat TGF- β 1 cDNA was excised and inserted at the *Xba*I site of pUH10-3, downstream of the tetO and CMV promoter, to yield the plasmid pUH10-3-TGF- β 1. This plasmid was digested with *Alw*44I and *Bgl*II to release the 2.7 kb tetO-TGF- β 1 construct (Fig. 1). This construct was injected into C57BL/6 $\times$ SJL F1 hybrid zygotes, which were implanted into pseudopregnant mothers. Offspring were screened by Southern analysis of *Bam*HI-digested tail tip DNA, using a 1.4 kb *Pvu*II-*Xho*I fragment of pUH10-3-TGF- β 1 as a probe.

Mice were housed in specific-pathogen-free facilities and all animal protocols were approved by the Institutional Animal Care and Use Committees. To prevent premature expression of the conditional TGF- β 1 allele, mice doubly transgenic for tetO-TGF- β 1 and either the SM22 α -tTA or α MHC-tTA alleles were fed either doxycycline-containing chow (Bioserve, Laurel, MD) or doxycycline-containing water (1 mg/ml; Sigma, St Louis, MO) until initiation of an experiment. Pregnant females potentially carrying doubly transgenic embryos were also fed the doxycycline-containing chow or water.

2.2 Cell lines

HeLa cells stably expressing the tTA protein and control, nontransfected HeLa cells were obtained from Dr. Mark Goldsmith (Gladstone Institutes, San Francisco, CA).

2.3 Measurement of TGF- β 1 protein and mRNA

TGF- β 1 protein was measured by ELISA (Promega, Madison, WI) of mouse plasma or media conditioned by explanted mouse tissues. This assay detects either total or active TGF- β 1 depending on whether samples are acid activated before the assay. All samples were assayed in duplicate. Mouse blood was collected by ocular bleed into an EDTA-containing tube. Plasma was obtained by centrifugation and saved at -80°C . Organ explant cultures were performed by placing the entire aorta, heart, spleen, left kidney, or a single lobe of the liver in MEM without phenol red. After 20 hr, the medium was frozen for later ELISA. TGF- β 1 mRNA was detected by northern analysis. RNA was extracted with Trizol (Invitrogen, Carlsbad, CA). Blots

of total RNA were hybridized with a 651 bp *Bsr*G1 fragment of TGF- β 1 cDNA (excised from plasmid pUHG10-3-TGF- β 1).

2.4 Detection of tTA expression

Western analysis to detect tTA expression was performed on extracts of hearts, aortae, and cultured cells. Extracts were obtained by placing tissues in 200 μ l (hearts) or 100 μ l (aortae) of lysis buffer (0.1% Triton X-100, 100 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10% SDS, 5 mg/ml sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride) and grinding with a Polytron homogenizer at maximum speed for 30 s. Protein was extracted from cultured cells by incubation with lysis buffer for 30–60 min at 4 °C, with rocking. Extracts were spun in a microcentrifuge at 4 °C for 30 min and supernatants collected. Protein concentration in the supernatant was measured with the BCA assay (Pierce, Rockford, IL). Equal amounts of protein were separated by 10% SDS-PAGE (Pre-cast gel; Bio-Rad, Hercules, CA), transferred onto nitrocellulose, and probed with a rabbit anti-VP16 polyclonal antiserum (Clontech, Mountain View, CA; Cat. #3844-1). Bound antibody was detected with secondary antibodies coupled to horseradish peroxidase and chemiluminescence (ECL kit, Amersham, Piscataway, NJ).

We used northern analysis to detect expression of tTA and β -gal mRNA. To improve the sensitivity of northern analysis, we pooled RNA from aortae of five SM22 α -tTA mice and five SM22 α - β -Gal mice. RNA from two α MHC-tTA hearts (not pooled) and a single nontransgenic heart was also analyzed by northern blotting for expression of tTA. RNA was prepared with the RNeasy kit (Qiagen, Valencia, CA). Ten μ g of each sample was electrophoresed, blotted, and probed with a 833 bp *Xba*I-*Sph*I fragment of pUHD17-1 [15].

2.5 Detection of β -gal expression

β -gal activity in extracts of aortic segments (two per animal) was measured with a chemiluminescence assay [18], with normalization to total protein (BCA assay, Pierce). β -gal mRNA was detected by northern analysis of aortic extracts, using a 624 bp *Hpa*I fragment of the *E. coli* β -gal gene as a probe.

2.6 Histologic analysis of tissues

One year after withdrawal of doxycycline, five mice doubly transgenic for SM22 α -tTA $\times$ tetO-TGF- β 1 and three age-matched controls (single-transgenic SM22 α -tTA mice, also off doxycycline) were euthanized and their kidneys, liver, hearts, innominate arteries, aortic roots, aortic arches, and descending aortae embedded in paraffin, sectioned, and stained. Sections of aortae were examined at the level of the root, arch, and at 4 evenly spaced steps between the arch and bifurcation. Hearts were sectioned at three levels (base, mid, and apex). Single sections were cut of kidney and liver. Sections cut at all levels of each organ were stained with hematoxylin and eosin. Serial sections from the aortic roots, arches, innominate arteries, and descending aortae were also stained with Movat pentachrome. Serial sections from the hearts were stained with Masson trichrome.

2.7 Statistical analysis

Data are given as mean $\pm$ SEM or (for data not normally distributed) median with 25–75% range. We used the t-test to investigate the statistical significance of differences between means of normally distributed groups and the Mann-Whitney rank-sum test to investigate differences between groups that were not normally distributed.

3. Results

3.1 A conditional TGF- β 1 allele is transactivated in a tissue-specific and doxycycline-dependent manner

We obtained six tetO-TGF- β 1 transgenic founders. Four of the founders transmitted the transgene in Mendelian fashion. To begin to test the performance of the tetO-TGF- β 1 alleles, we bred tetO-TGF- β 1 transgenic mice from these four founder lines with mice transgenic for a α MHC-tTA allele that is proven to upregulate expression from tetO-regulated transgenes [12]. Doubly transgenic mice in two of the four lines showed upregulation of plasma total TGF- β 1 levels after withdrawal of doxycycline (Fig. 2, and data not shown). To continue to investigate the performance of the tetO-TGF- β 1 allele, one of these two lines of tetO-TGF- β 1 transgenic mice (line “T11”) was bred further with the α MHC-tTA mice.

Northern analysis of organs removed from doubly transgenic α MHC-tTA $\times$ tetO-TGF- β 1 mice on and off doxycycline as well as nontransgenic controls confirmed that the tetO-TGF- β 1 transgene was expressed only in the hearts of doubly transgenic mice off doxycycline (Fig. 2A). Moreover, plasma total TGF- β 1 levels increased in doubly transgenic mice within 1 week after doxycycline withdrawal and continued to rise for at least 5 weeks (Fig. 2B). The elevated plasma TGF- β 1 appeared to result from transgene expression in the heart because increased total and active TGF- β 1 expression was detected in explant cultures of hearts but not of other organs removed from doubly transgenic mice off doxycycline [for total TGF- β 1 data, see Fig. 2C; active TGF- β 1 was detected in explant cultures of hearts of doubly transgenic mice off doxycycline (210 ± 58 pg/heart/20 hr; $n = 4$) but not in any of the cultures of control hearts (< 60 pg/heart/20 hr; $n = 6$)]. When doubly transgenic mice that had been off doxycycline were restarted on doxycycline-containing chow, plasma total TGF- β 1 levels decreased to baseline levels within two weeks (Fig. 2D). Although active TGF- β 1 was detected in heart explant cultures of doubly transgenic α MHC-tTA $\times$ tetO-TGF- β 1 mice off doxycycline, no active TGF- β 1 was detected in plasma of any mouse, most likely due to binding of TGF- β 1 to plasma proteins that precludes detection of TGF- β 1 without acid activation [19].

3.2 No evidence for transactivation of the conditional TGF- β 1 allele by SM22 α -tTA

To obtain temporally regulated TGF- β 1 expression in vascular SMC of adult mice, we bred mice of tetO-TGF- β 1 line T11 with mice from each of the three lines carrying the SM22 α -tTA transgene (lines 21, 19, and 36; see above). All SM22 α -tTA transgenic mice were confirmed by Southern analysis to have the appropriate size tTA-containing restriction fragment in their genomic DNA (see Methods; data not shown). From ten days to four weeks after withdrawal of doxycycline from doubly transgenic SM22 α -tTA $\times$ tetO-TGF- β 1 mice, both plasma and aortic explant cultures were assayed for active and total TGF- β 1. No active TGF- β 1 was detected in the plasma or aortic explant cultures of any mouse. Total TGF- β 1 was detected in all samples; however, there was no evidence of upregulation of systemic or aortic total TGF- β 1 expression in any of the three lines of SM22 α -tTA $\times$ tetO-TGF- β 1 mice, when compared to control plasmas and explant culture media assayed in parallel (Table). Moreover, in contrast to the robust expression of TGF- β 1 transgene mRNA in hearts of doubly transgenic α MHC-tTA $\times$ tetO-TGF- β 1 mice off doxycycline (Fig. 2A), northern analysis of aortae from eighteen SM22 α -tTA $\times$ tetO-TGF- β 1 mice (nine mice from line 19 and nine mice from line 36, all off doxycycline for at least three weeks) did not reveal expression of the TGF- β 1 transgene (data not shown).

3.3 No evidence for tTA expression in SM22 α -tTA mice

Because our tetO-TGF- β 1 transgene was highly expressed in hearts of α MHC-tTA $\times$ tetO-TGF- β mice (Fig. 2), we hypothesized that lack of tetO-TGF- β 1 expression in SM22 α -tTA $\times$ tetO-TGF- β 1 mice was due to inadequate expression of the tTA from the SM22 α -tTA allele.

Consistent with this hypothesis, western analysis of aortae of mice from all three lines of SM22 α -tTA mice did not show expression of tTA (Fig. 3A and data not shown). In contrast, tTA protein was easily detected by western analysis of HeLa cells stably transfected with tTA and hearts of α MHC-tTA mice (Fig. 3A and data not shown). Moreover, northern analysis of aortae of SM22 α -tTA mice from line 36—reportedly the highest-expressing of the three SM22 α -tTA lines—showed no tTA transcripts; whereas, tTA transcripts were abundant in a heart harvested from a α MHC-tTA mouse (Fig. 3B).

3.4 No evidence for transactivation of a conditional β -galactosidase (β -gal) allele by SM22 α -tTA

We considered that absence of TGF- β 1 transgene expression in SM22 α -tTA $\times$ tetO-TGF- β 1 mice might somehow be due to resistance of our tetO-TGF- β 1 allele to transactivation by the SM22 α -driven tTA and that a different tetO-driven allele might be successfully transactivated by the SM22 α -driven tTA. We therefore bred mice from SM22 α -tTA line 21 with “Ro1” mice, which are transgenic for a tetO-driven β -gal transgene that is proven to be transactivated in vivo by a α MHC-driven tTA [13]. Ten days after doxycycline withdrawal, aortae were harvested from doubly transgenic SM22 α -tTA $\times$ Ro1 mice. Two segments of each aorta were assayed for β -gal activity and compared to segments of aortae harvested from nontransgenic mice and from SM22 α -tTA $\times$ Ro1 mice on doxycycline. Segments of aortae from SM22 α - β -gal transgenic mice were also included both as a positive control for β -gal expression and to verify the feasibility of artery wall transgene expression from the SM22 α promoter. β -gal activity in segments of aortae of doubly transgenic SM22 α -tTA $\times$ Ro1 mice off doxycycline did not differ from β -gal activity in either doubly transgenic mice on doxycycline or in nontransgenic mice (Fig. 4). In contrast, β -gal activity in aortic segments from SM22 α - β -gal mice was substantially higher. Moreover, a β -gal transcript was present in aortae of SM22 α - β -gal mice (Fig. 3C); whereas, no tTA transcripts were detected in aortae of SM22 α -tTA mice (see above; Fig. 3B). Because we detected neither expression of tTA nor transactivation of tetO-TGF- β 1 in aortae of SM22 α -tTA lines 19 and 36 (see above), these two lines were not crossed with the Ro1 mice.

3.5 No vascular phenotype in SM22 α -tTA $\times$ tetO-TGF- β 1 transgenic mice

Although we could not detect expression of either tTA or TGF- β 1 in aortae of SM22 α -tTA $\times$ tetO-TGF- β 1 mice, we considered that both tTA and TGF- β 1 transgene expression might nevertheless be present, at levels below the limits of detection of our assays. We therefore examined histologic sections of organs of five doubly transgenic SM22 α -tTA (line 36) $\times$ tetO-TGF- β 1 mice that were off doxycycline for one year and compared them to sections taken from organs of three age-matched singly transgenic SM22 α -tTA mice, also off doxycycline. H & E and Movat pentachrome-stained sections taken from the aortic roots, aortic arch, descending aorta, and innominate arteries of each mouse were examined for presence of a neointima, medial thickness, and extent of proteoglycan, elastin, and collagen staining. H & E and Masson trichrome-stained sections of hearts were examined for cellularity, matrix accumulation, and extent of collagen staining. H & E-stained sections of livers and kidneys were examined for cellularity and matrix accumulation. One of the five SM22 α -tTA $\times$ tetO-TGF- β 1 mice had multifocal mononuclear cellular infiltrates in the liver and kidney, but not in the heart or aortic wall. Otherwise, all sections taken from SM22 α -tTA $\times$ tetO-TGF- β 1 mice appeared normal and were indistinguishable from sections taken from the control mice (Fig. 5).

4. Discussion

We generated transgenic mice designed to express TGF- β 1 in a tissue-specific and time-dependent manner (tetO-TGF- β 1 mice). We verified tissue-specific, regulated expression of the TGF- β 1 transgene in the tetO-TGF- β 1 mice by breeding them with α MHC-tTA mice,

withdrawing dietary doxycycline, and documenting heart-specific, doxycycline-regulated TGF- β 1 expression (Fig. 2). However, our attempts to achieve SMC-specific, doxycycline-regulated TGF- β 1 expression by breeding the tetO-TGF- β 1 mice with three independent lines of SM22 α -tTA mice were not successful. We conclude that these three lines of SM22 α -tTA mice do not reliably transactivate all tetO-regulated alleles in aortic SMC.

We considered several explanations for why the doubly transgenic SM22 α -tTA $\times$ tetO-TGF- β 1 mice did not express any detectable TGF- β 1 after doxycycline withdrawal. Our experimental data exclude some of these explanations. First, it is possible that our tetO-TGF- β 1 transgene integrated only at transcriptionally silent areas of the genome, in which transactivation by the tTA is essentially absent. Indeed two of the four lines of tetO-TGF- β 1 mice we tested showed no transactivation of the tetO-TGF- β 1 allele when bred with α MHC-tTA mice. However, line T11 (the line of tetO-TGF- β 1 mice that we bred with all three of the SM22 α -tTA lines) had high, doxycycline-responsive expression of the tetO-TGF- β 1 transgene (Fig. 2). This observation argues strongly that—in line T11, the line of tetO-TGF- β 1 mice that we bred with SM22 α -tTA mice—the tetO-TGF- β 1 transgene is integrated in an area of the genome that is accessible to the tTA protein. A second possibility is that the SM22 α promoter is simply too weak to drive significant levels of tTA transgene expression. However, this possibility is refuted by others' data [14] as well as our own results (Figs. 3C and 4) confirming that the SM22 α promoter can drive significant expression of a β -gal transgene in mouse aortae, by our report of embryonic lethality in SM22 α -TGF- β 1 transgenic mice [6], and by others' use of the SM22 α promoter to drive efficient expression of Cre recombinase in aortic SMC of transgenic mice [20], an observation reproduced in our own laboratory (data not shown). A third possibility is that the SM22 α -tTA transgene is expressed in SMC of all three lines of SM22 α -tTA mice and that the tTA transactivates the tetO-TGF- β 1 allele, but that the expression levels of both tTA and TGF- β 1 are below the limit of detection of our assays. We cannot completely exclude this possibility; however, the lack of β -gal expression in aortae of SM22 α -tTA $\times$ Ro1 mice (Fig. 4) and the complete absence of a vascular phenotype one year after doxycycline withdrawal—compared to the dramatic effects of vascular TGF- β 1 overexpression during far shorter time periods in other animal models [21,22]—argue strongly that no significant expression of either the tTA or TGF- β 1 transgenes is present in aortae of SM22 α -tTA $\times$ tetO-TGF- β 1 mice.

Our results contrast with three reports in which these SM22 α -tTA mice were reported to drive significant tetO-regulated transgene expression [9-11]. In the first report, doxycycline-responsive transactivation of a tetO-regulated rat vascular chymase transgene was documented by RT-PCR, by immunoprecipitation of HA-tagged rat vascular cell chymase protein from pooled extracts of 10 mouse aortae, and by assays for chymase activity in explanted, cultured aortic SMC [9]. tTA-mediated transactivation of the chymase transgene, presumed to occur after doxycycline withdrawal from doubly transgenic mice, resulted in aortic medial thickening, abundant aortic SMC proliferation, elevated blood pressure, enhanced phenylephrine-induced constriction and impaired methacholine-induced relaxation of mesenteric arteries. These impressive phenotypic changes suggest that substantial chymase transgene expression levels were achieved in vivo. In the second report, tissue-specific, doxycycline-responsive transactivation of a tetO-regulated β -gal transgene was documented by histochemical staining [10]. Tissue-specific, doxycycline-responsive transactivation of a tetO-regulated dominant negative cMyb molecule was documented by immunohistochemistry and RT-PCR. Mice with tTA-mediated transactivation of the dominant negative c-Myb had less SMC proliferation, neointima formation, medial hyperplasia, and arterial remodeling after wire injury of the carotid artery. In the third report, tissue-specific, doxycycline-responsive transactivation of a tetO-regulated plasma membrane calcium ATPase transgene was documented by RT-PCR and western blotting of aortic homogenates [11]. Mice with tTA-

mediated transactivation of the calcium ATPase transgene had altered vascular reactivity and blood pressure.

Thus, three published studies report impressive effects of SM22 α -tTA-regulated transgene expression on the arterial phenotype. However, in contrast to the initial report of the α MHC-tTA mice [12], none of these three studies document tTA expression *in vivo* and none provides a robust, quantitative analysis of the reproducibility with which expression of tetO-regulated transgene-derived proteins is increased in individual animals. Although in all three cases significant tTA expression in vascular SMC appears to have occurred, the absence of northern or western analyses documenting and quantifying tTA expression is unfortunate. Other investigators contemplating using these SM22 α -tTA mice would likely benefit from knowing both the level of vascular tTA expression that is required for effective transactivation and the extent and range of tetO-driven transgene expression that should be expected in individual animals.

It remains unclear to us why, using the same SM22 α -tTA mice reported in these three studies, we were unable to find evidence of either tTA expression or transactivation of a tetO-responsive allele that is activated efficiently by a different, tissue-specific tTA (Fig. 2). Possible explanations for the discrepancy between our experience and the performance of the SM22 α -tTA mice in the three above-referenced studies include: 1) We received the wrong mice. This seems unlikely, in view of the documented transgenesis of all three lines of our SM22 α -tTA mice for tTA (which is not an endogenous murine gene) and the presence of a tTA-containing restriction fragment of the expected size on Southern blots of genomic DNA from all three lines. 2) There could have been multiple integration sites of the SM22 α -tTA transgene in the founder mice, including both silent and expressed integrants. If the integration sites were on different chromosomes or widely separated on the same chromosome, they could segregate during meiosis and the unfortunate propagation of a transcriptionally silent transgene would not always be detectable by Southern analysis. Although this is possible, it seems unlikely to have occurred with all three SM22 α -tTA lines. 3) The SM22 α promoter sequence could have been silenced by methylation. Again, although this is possible, it is our impression that methylation-mediated transgene silencing is found most commonly with viral-derived promoters and in association with bacterial sequences [23,24]. It would be atypical for a mammalian promoter such as SM22 α to be silenced by methylation; however, some of the DNA sequences encoding the tTA are derived from bacteria and others are from herpesvirus [15]. Close association of the SM22 α promoter with these sequences could potentially promote methylation. Nevertheless, it is unclear why the SM22 α promoter would be silenced by methylation in all 3 lines of SM22 α -tTA mice in our laboratory, but not in mice reported in others' studies in which this promoter is linked either to tTA or to other viral or bacterial DNA sequences [9-11,14,20]. 4) SM22 α -tTA expression is age-dependent and we erred by experimenting on older mice in which age-related loss of SM22 α -tTA expression had occurred. However, reports of experiments using the SM22 α -tTA mice do not provide any suggestions that age-dependent loss of tTA transgene expression is of concern. One article reports experiments on SM22 α -tTA mice aged 10–12 weeks [9], whereas the two other articles do not mention the ages of experimental mice [10,11]. The negative gene expression data reported here (Figs. 3-4 and Table) were obtained from mice aged 7 weeks to 6 months, with much of the data from mice aged 7 – 12 weeks. It is therefore unlikely that age-related loss of SM22 α -tTA transgene expression could explain all of our negative results. The most likely explanation for the disappointing performance of the SM22 α -tTA mice in our experiments remains inadequate expression of the tTA. Because we did not ever document tTA expression in our laboratory, we could not attempt to determine a mechanism through which tTA expression was lost in these lines of mice. Commentary on the discrepancy between our experience with the SM22 α -tTA mice and others' [9-11] has been published recently [25,26].

In conclusion, presently available SM22 α -tTA mice [9-11] may be useful reagents; however, they cannot be expected to transactivate all tetO-responsive alleles, including tetO-responsive alleles that are transactivated by other tissue-specific tTAs. We look forward to future reports from other investigators, in which these SM22 α -tTA mice transactivate other conditional, tetO-responsive alleles. We also look forward to generation of new lines of SM22 α -tTA mice that, by virtue of documented, higher expression of tTA in vascular SMC, can transactivate a broad range of tetO-driven conditional transgenes in SMC of large arteries. Such mice, bred with our tetO-TGF- β 1 lines, would be useful reagents for use in experiments that unravel the role of TGF- β 1 in vascular development, physiology, and disease.

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Abbreviations:

α MHC, (alpha myosin heavy chain); β -gal, (β -galactosidase); SMC, (smooth muscle cell); tetO, (tetracycline operator); TGF- β 1, (transforming growth factor-beta 1); tTA, (tetracycline transactivator).

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Fig. 1.

A conditional TGF-β1 transgene. The transgene contains the tetracycline operator (tetO), a minimal cytomegalovirus (CMV) promoter, and a rat TGF-β1 cDNA in which codons 223 and 225 were mutated to encode serines instead of cysteines.

Fig. 2.

Heart-specific, doxycycline-responsive expression of a conditional TGF-β1 allele. **A**, Northern analysis of endogenous and transgenic TGF-β1 mRNA. Organs are from a nontransgenic mouse off doxycycline and doubly transgenic αMHC-tTA × tetO-TGF-β mice either off or on doxycycline. Top: the endogenous TGF-β1 mRNA (2.4 kb) is expressed primarily in the spleen. The TGF-β1 transgene (only 1.4 kb, due to a truncated 3' untranslated region) is expressed only in the heart of the doubly transgenic mouse off doxycycline. The positive control is RNA from 293 cells infected with an adenovirus expressing the TGF-β1 transgene. Bottom: ethidium-stained gel. RNA size markers are at far left. **B**, Elevated plasma total TGF-β1 after doxycycline withdrawal in doubly transgenic αMHC-tTA × tetO-TGF-β mice. Doubly transgenic mice (open circles; n = 3–4) were taken off doxycycline at week 0 and plasma total TGF-β1 was measured by ELISA at weekly intervals. The control group (filled circles; n = 3–4) includes nontransgenic mice off doxycycline (n = 1–2) and doubly transgenic mice on doxycycline (n = 2). Values from the two control groups did not differ significantly and were therefore pooled. **C**, Heart-specific upregulation of TGF-β1 secretion after doxycycline withdrawal. Organs from doubly transgenic mice (DTG off; n = 4) were removed and placed in explant culture ten days after doxycycline withdrawal. Control organs (Cont; n = 6) were from nontransgenic mice off doxycycline (n = 3) and doubly transgenic mice on doxycycline (n = 3). Values from the two control groups did not differ significantly and were therefore pooled. Conditioned medium was assayed for total TGF-β1 by ELISA. For each organ, the median value of the control group was set at 100% and the other values are expressed in relation to this value. Bars indicate median and 25–75% range in each group. Similar results were obtained for active TGF-β1 (see Results). **D**, Rapid loss of TGF-β1 expression after resuming doxycycline treatment. Total plasma TGF-β1 was measured in nontransgenic mice off doxycycline (n = 4) and in doubly transgenic αMHC-tTA × tetO-TGF-β mice off doxycycline and after resuming doxycycline (n = 6). Bar heights are means; error bars are SEM.

Fig. 3.

No tTA protein or mRNA detected in aortae of SM22α-tTA mice. A, Western analysis. Equal amounts of protein from extracts of HeLa cells (untransfected or stably transfected with tTA) and of aortae harvested from mice either transgenic (Tg) for the SM22α-tTA allele or nontransgenic (non-Tg) were electrophoresed, blotted, and probed with an antibody to tTA. Size markers (left) are in kilodaltons. B-C, Northern analysis. B, RNA from hearts and aortae of mice that were either nontransgenic or transgenic (Tg) for the αMHC-tTA, SM22α-tTA, or SM22α-β-gal alleles was electrophoresed, blotted, and probed for expression of tTA. The presence of multiple tTA transcripts in αMHC-tTA hearts was reported previously [12]. C, The two right lanes shown in panel B were stripped and reprobed for expression of β-gal. A transcript is detected in RNA from the SM22α-β-gal aortae (arrow). The ethidium-stained gels are depicted at the bottom of B and C.

Fig. 4.

No inducible β -gal expression in aortae of SM22 α -tTA $\times$ tetO-LacZ mice. Segments of aorta (2 per mouse) were removed from nontransgenic mice, from doubly transgenic SM22 α -tTA $\times$ tetO-lacZ mice both on dietary doxycycline and 10 days after withdrawal of dietary doxycycline, and from transgenic SM22 α -LacZ mice. Data points are values from individual aortic segments; horizontal bars are group medians.

Fig. 5.

Normal arterial and cardiac histology in SM22 α -tTA $\times$ tetO-TGF- β 1 mice. Sections are from doubly transgenic SM22 α -tTA $\times$ tetO-TGF- β 1 mice or singly transgenic controls. All mice were off of doxycycline for one year. A–D, Sections of aortae; E–H, sections of hearts. A, B, E, F: Hematoxylin and eosin stain; C, D: Movat pentachrome stain; G, H: Masson trichrome stain. Insets are views of same slide at higher magnification. All size bars are 100 μ m.

TableNo evidence of TGF- β 1 transgene expression in SM22 α -tTA $\times$ tetO-TGF- β 1 mice.

Line	Genotype	Plasma total TGF- β 1 (ng/ml)	Total TGF- β 1 in aortic explant culture (pg/20 hrs)
21	SM22 α -tTA $\times$ tetO-TGF- β 1 (off doxycycline) [*]	8.7 $\pm$ 1.9 (n = 5)	760 $\pm$ 100 (n = 3)
	Controls [‡]	8.5 $\pm$ 1.3 (n = 6)	600 $\pm$ 71 (n = 5)
19	SM22 α -tTA $\times$ tetO-TGF- β 1 (off doxycycline) [*]	4.8 $\pm$ 0.93 (n = 7)	410 $\pm$ 51 (n = 7)
	Controls [‡]	6.2 $\pm$ 0.39 (n = 10)	510 $\pm$ 32 (n = 10)
36	SM22 α -tTA $\times$ tetO-TGF- β 1; (off doxycycline) [*]	2.6 and 3.2 (n = 2)	250 and 220 (n = 2)
	Controls [‡]	3.4 $\pm$ 0.52 (n = 3)	360 $\pm$ 29 (n = 3)

^{*} Plasma samples to be assayed for TGF- β 1 were taken from doubly transgenic mice that had stopped doxycycline for 2 weeks (line 21), 10 days (n = 4) or 3 weeks (n = 3) (line 19), and 3 weeks (line 36). Aortae were explanted from doubly transgenic mice that had stopped doxycycline for 4 weeks (line 21), 10 days or 3 weeks (line 19) and 3 weeks (line 36).

[‡] Controls for each line were assayed in parallel with experimental mice (i.e., doubly transgenic mice off doxycycline) from the same line. The controls comprise essentially equal numbers of doubly transgenic mice (from the same SM22 α -tTA line as the experimentals) on doxycycline and nontransgenic mice off doxycycline. We pooled these two control groups because their plasmas and aortic explants always had similar amounts of TGF- β 1 (data not shown).