TRA2β controls Mypt1 exon 24 splicing in the developmental maturation of mouse mesenteric artery smooth muscle

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Submitted 5 September 2014; accepted in final form 17 November 2014

Zheng X, Reho JJ, Wirth B, Fisher SA. TRA2β controls Mypt1 exon 24 splicing in the developmental maturation of mouse mesenteric artery smooth muscle. Am J Physiol Cell Physiol 308: C289–C296, 2015. First published November 26, 2014; doi:10.1152/ajpcell.00304.2014.—Diversity of smooth muscle within the vascular system is generated by alternative splicing of exons, yet there is limited understanding of its timing or control mechanisms. We examined splicing of myosin phosphatase regulatory subunit (Mypt1) exon 24 (E24) in relation to smooth muscle myosin heavy chain (Smhmhc) and smoothelin (Smtn) alternative exons during maturation of mouse mesenteric artery (MA) smooth muscle. The role of transformer 2β (Tra2β), a master regulator of splicing in flies, in maturation of arterial smooth muscle was tested through gene inactivation. Splicing of alternative exons in bladder smooth muscle was examined for comparative purposes. MA smooth muscle maturation began after postnatal week 2 and was complete at maturity, as indicated by switching to Mypt1 E24 and Smtn E20 splice variants and 11-fold induction of Smhmhc. Similar changes in bladder were complete by postnatal day 3. Splicing of Smhmhc E6 was temporally dissociated from Mypt1 E24 and Smtn E20 and discordant between arteries and bladder. Tamoxifen-induced smooth muscle-specific inactivation of Tra2β within the first week of life but not in maturity reduced splicing of Mypt1 E24 in MAs. Inactivation of Tra2β causing a switch to the isoform of MYPT1 containing the COOH-terminal leucine zipper motif (E24−) increased arterial sensitivity to cGMP-mediated relaxation. In conclusion, maturation of mouse MA smooth muscle begins postnatally and continues until sexual maturity. TRA2β is required for specification during this period of maturation, and its inactivation alters the contractile properties of mature arterial smooth muscle.

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Sequencing of human and model animal genomes and subsequent studies reveal that tissue diversity is mostly generated by posttranscriptional mechanisms, including alternative splicing of exons (29). Smooth muscle of the mammalian vascular system is phenotypically diverse. Smooth muscle of the large arteries (conduit vessels) expresses a pure slow contractile gene program, while the smaller resistance arteries (SRAs), where blood flow and pressure are regulated, generally express parts of the slow and fast contractile gene programs underlying their mixed contractile properties (reviewed in Ref. 4). Portal venous smooth muscle is unique, in that it expresses a pure fast gene program underlying its phasic contractile activity. The splicing of alternative exons of pre-mRNAs coding for contractile proteins in each of these vascular tissues is unique, yet there is little understanding of the mechanisms that control this aspect of the generation of vascular smooth muscle phenotypic diversity (reviewed in Refs. 4 and 6).

Our laboratory has used the alternative splicing of exon 24 (E24) of myosin phosphatase regulatory subunit (Mypt1) as a model for the study of smooth muscle phenotypic diversity. Splicing of E24 is highly tissue-specific, developmentally regulated, and evolutionarily conserved and modulates in disease (reviewed in Ref. 2). E24 is predominantly skipped in the tonic smooth muscle of the large arteries and included in the phasic smooth muscle of the portal vein and intestines and predominately included in SRAs. Prototypical phasic smooth muscle, such as portal vein (18) and intestines (5, 12), switches from E24 to E24 as part of postnatal implementation of the fast smooth muscle gene program. We recently demonstrated that rat mesenteric SRAs undergo a similar process of maturational switching in E24 that begins after postnatal week 2 and is not complete until near sexual maturity (20).

The first goal of the current study was to define developmental maturation of exon splicing in mouse mesenteric SRA smooth muscle. Maturation of Mypt1 E24 splicing was compared with splicing of smooth muscle myosin heavy chain (Smhmhc) E6 and smoothelin (Smtn) E20, smooth muscle-specific transcripts that undergo tissue-specific splicing of the indicated exons (11, 28). Our second goal was to define the role of the splicing regulatory factor transformer 2β (TRA2β, also known as Sfrs10) in splicing-dependent maturation of mouse SRA smooth muscle. TRA2β is an atypical member of the SR family of RNA-binding proteins and orthologous to TRA proteins, which function as master regulators of sex determination in Drosophila through the regulated alternative splicing of exons in Doublesex and Fruitless (reviewed in Ref. 23). TRA2β binds in a sequence-specific manner to Mypt1 E24 and transactivates its splicing in vitro (24). In a previous study, we used Sm22Cre-mediated conditional knockout (cKO) of TRA2β to demonstrate its role in splicing of Mypt1 E24 in intestinal smooth muscle (5). Our previous study did not examine vascular maturation and was limited by embryonic lethality of homozygous cKO. In the current study we use tamoxifen-inducible Smhmhc-CreERT2 (30) to test the role of TRA2β in Mypt1 E24 splicing and SRA maturation at different stages of development. For comparative purposes, we examined alternative exon splicing-dependent maturation and the effect of Tra2β inactivation in the bladder, a pure phasic smooth muscle tissue.

http://www.ajpcell.org 0363-6143/15 Copyright © 2015 the American Physiological Society C289
MATERIALS AND METHODS

Animals. Mice were used according to the National Institutes of Health “Guide for the Care and Use of Laboratory Animals,” and all experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Maryland, Baltimore. Wild-type C57BL/6 mice were used in the development study. The tamoxifen-inducible smooth muscle-specific Cre transgenic (Smmhc-CreERT2) mouse line (30) was crossed with Tra2β E4 floxed (Tra2β<sup>fl</sup>) mice (13) and maintained on a C57BL/6 genetic background. Only male mice were used for cKO, as Smmhc Cre<sup>ERT2</sup> is integrated on the Y chromosome. Cre activity was induced by tamoxifen injection (30 mg·kg<sup>−1</sup>·day<sup>−1</sup> ip for 3 days), and tissues were collected 1–2 mo after the last injection. Genotyping was performed as previously described (8, 13). Control groups included Cre<sup>−/−</sup> male mice treated with vehicle (sunflower seed oil) and Cre<sup>−/−</sup> female littermates treated with tamoxifen.

Measurement of mRNAs. Total RNA from mouse mesenteric arteries (MAs) and bladders was isolated using the PureLink RNA Mini Kit (Ambion) according to the manufacturer’s instructions. cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen) and random primers. Mypt1 E24 splice variants were amplified by conventional PCR using primers flanking the alternative exon, as previously described (5). The same method was used to amplify the Smmhc 3′ alternative exon E20 using the following primers: 5′-TTCTTCCCTGAGGCTTTTGA-3′ and 5′=-ATCATGTC-3′. PCR products were separated by gel electrophoresis, band intensities were quantified using a LI-COR Odyssey CT scanner, and the ratio E6-skipped:non-isoform-specific Smmhc mRNAs were measured by qPCR with predesigned TaqMan probes. Percent Smmhc E6 inclusion was calculated using the comparative threshold (2<sup>−ΔΔCt</sup>) method, in which AC<sub>T</sub> between E6<sup>+</sup> and Smmhc was divided by the sum of differences between E6<sup>+</sup> and E6<sup>−</sup> vs. Smmhc. This method of measuring Smmhc E6 was validated using smooth muscle tissues known to be E6<sup>+</sup> and E6<sup>−</sup> (not shown). Conventional PCR could not be used to estimate E6 inclusion because of contamination by non-muscle myosin heavy chain (data not shown). Predesigned TaqMan probes were used to measure non-isoform-specific mRNAs of Mypt1, Smmhc, and Smtn and Smtn isoform B and normalized to cyclophilin A, which was invariant between samples. Relative mRNA levels were determined using the 2<sup>−ΔΔCt</sup> method and are reported as fold change.

Western blotting. Mouse MAs and bladders were homogenized in a 10× volume of lysis buffer containing 125 mM Tris·HCl (pH 6.8), 20% sucrose, 10% SDS, and 1% proteinase inhibitor cocktail (catalog no. P8340, Sigma) in a Bullet blender (Next Advance). Proteins (10 μg) were separated on 4–15% Tris-glycine gels (catalog no. 456-CTCCACCTCCAC-3′. PCR products were separated by gel electrophoresis, band intensities were quantified using a LI-COR Odyssey imager, and percent exon inclusion was calculated. Smmhc E6-included mRNA was measured by quantitative PCR (qPCR) using the custom-designed TagMan probes 5′-GGTGGGATCCTCCCCACAAG-3′ and 5′-GCTCCCCGTAGGCAAAAGAT-3′ and the probe 5′-CAGCATTACGCAAGTG-3′ from Life Technologies. Smmhc E6-skipped and non-isoform-specific Smmhc mRNAs were measured by qPCR with predesigned TaqMan probes. Percent Smmhc E6 inclusion was calculated using the comparative threshold (2<sup>−ΔΔCt</sup>) method, in which AC<sub>T</sub> between E6<sup>+</sup> and Smmhc was divided by the sum of differences between E6<sup>+</sup> and E6<sup>−</sup> vs. Smmhc. This method of measuring Smmhc E6 was validated using smooth muscle tissues known to be E6<sup>+</sup> and E6<sup>−</sup> (not shown). Conventional PCR could not be used to estimate E6 inclusion because of contamination by non-muscle myosin heavy chain (data not shown). Predesigned TaqMan probes were used to measure non-isoform-specific mRNAs of Mypt1, Smmhc, and Smtn and Smtn isoform B and normalized to cyclophilin A, which was invariant between samples. Relative mRNA levels were determined using the 2<sup>−ΔΔCt</sup> method and are reported as fold change.

Fig. 1. Maturation changes in contractile mRNAs in mouse mesenteric arteries (MAs). MAs from the entire arterial arcade were isolated on postnatal days (PND) 3, 7, 14, 28, and 56, and total RNA was purified. A and C: alternative exon splice variants of myosin phosphatase regulatory subunit (Mypt1) [ exon 24 (E24)-positive (E24<sup>+</sup>) and E24<sup>−</sup>], 371 and 340 bp, respectively] and smoothelin (Smtn) (E20<sup>+</sup> and E20<sup>−</sup> 293 and 128 bp, respectively) were measured by conventional PCR using primers flanking the alternative exons. Exon-included and exon-skipped cDNA products were separated by gel electrophoresis and directly quantified. Data are reported as percent exon inclusion (signal of exon-included divided by total signal). B: smooth muscle myosin heavy chain (Smmhc) E6 inclusion was determined by quantitative PCR (qPCR) using TaqMan probes specific to the E6<sup>−</sup> transcript. Data are presented as percent exon inclusion. D–F: total message level of Mypt1, Smmhc, and Smtn were measured by real-time PCR, normalized to cyclophilin A, and reported as fold change relative to PND3 or PND7. Smmhc probes were selected to amplify all Smmhc transcripts (Smmhc total) or to selectively amplify the Smtn B (vascular) isoform (F). Fold change is shown for each transcript relative to its level at PND7. *P < 0.05 vs. all others (A–E) and other Smmhc groups (F). †P < 0.05 vs. PND7 (A), PND3 and PND7 (D), and PND7 and PND14 (F). ‡P < 0.05 vs. PND3, PND7, and PND14 (D). Values are means ± SE; n = 3 for all groups. Vertical lines indicate positions where the image of a single gel was cropped for purposes of presentation.
1085, Bio-Rad) at 90 V for 1.5 h and transferred to nitrocellulose membranes at 25 V for 2 h. Blocking buffer (catalog no. 927-40000, LI-COR) was used to block membranes and dilute antibodies. The following primary antibodies were used: rabbit polyclonal antibody recognizing the COOH-terminal leucine zipper (LZ) motif present in MYPT family members MYPT1, p85, and M21 (1:3,000 dilution) (19), rabbit polyclonal TRA2B antibody (Ab66901, Abcam; 1:1,000 dilution), and rabbit polyclonal serine/arginine-rich splicing factor 3 (SFRS3) antibody (Ab73891, Abcam; 1:3,000 dilution). IRDye 800CW and 680LT goat anti-rabbit and goat anti-mouse IgG secondary antibodies (1:10,000 dilution) were purchased from LI-COR. Bands were scanned in the Odyssey system, quantified in Image Studio 3.0 (LI-COR), and normalized against p85 or SFRS3.

Vascular function. A 2-mm length of first-order MA (MA1) was dissected and placed in an ice-cold physiological saline solution containing (mM) 119 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 25 NaHCO3, 1.2 NaH2PO4, 0.027 EDTA, and 5.5 glucose at pH 7.4 with 95% O2-5% CO2 bubbled into the solution. Arteries were mounted on a wire myograph (model 610M, Danish Myo Technology, Aarhus, Denmark) for measurement of isometric force generation. Starting tension was applied by stretching the vessel to IC90, as previously described (15). Vessels were then primed by two separate additions of 10 μM phenylephrine (PE) to the bath. Once the vessels were primed, a 20-min incubation period was allowed prior to the first experiment. Vessels were activated by 10 μM PE (Sigma), and, after steady-state force was achieved, arteries were exposed to cumulative concentrations (1 nM–100 μM) of 8-bromo-cGMP (8-Br-cGMP, Sigma). Force was continuously recorded and measured at steady state. Vasorelaxation data are presented as percentage of maximum force remaining at each concentration of 8-Br-cGMP.

RESULTS

MA smooth muscle maturation. MAs express predominantly the Mypt1 E24- variant (slow isofrom) at postnatal day (PND) 3 (PND3) and PND14 (Fig. 1A). From PND14 to PND56, there is a progressive switch to the Mypt1 E24+ variant associated with a 3.5-fold increase in total Mypt1 mRNA levels (Fig. 1D). Smmhc mRNA increased ~11-fold from PND14 to PND56, indicating accretion of differentiated smooth muscle in the MAs (Fig. 1E). In contrast to Mypt1 E24, the level of inclusion of Smmhc E6 (the fast isofrom) was higher at PND3 and subsequently decreased and was maintained at ~10% of transcripts (Fig. 1B). The alternative splicing of Smtn 3’ alternative exon E20 followed a pattern similar to that of Mypt1 E24, although in the opposite direction, with a relatively high level of inclusion of the alternative exon that switched to exclusion from PND14 to PND56 (Fig. 1C). Smtn mRNA variants are also generated by alternative transcriptional start sites giving rise to A (visceral or fast) vs. B (vascular or slow) isoforms (22, 28). These variants differ by a unique upstream transcriptional start site in Smtn A, generating a unique (first) exon and a shorter transcript. In the MA, Smtn B mRNA increased

Statistical analysis. Values are means ± SE. SigmaPlot software (SYSTAT, Chicago, IL) was used for statistical testing and graph generation. Student’s t-test was used for vascular contractility assays. One-way ANOVA and Student’s t-test were used for real-time PCR comparisons. P < 0.05 was considered statistically significant.

Fig. 2. Maturational changes in contractile mRNAs in mouse bladder. Bladders were isolated at embryonic day 17 (E17) and PND1, PND7, PND21, and PND56, and total RNA was purified. mRNAs were measured as described in Fig. 1 legend. A–C, Mypt1 E24, Smmhc E6, and Smtn E20 splice variants. D–F, Mypt1, Smmhc, Smtn B, and Smtn total mRNAs measured by qPCR, normalized to cyclophilin A, and reported as fold change relative to E17. *P < 0.05 vs. others (A–F). †P < 0.05 vs. total Smtn at E17 and PND3 (F). ‡P < 0.05 vs. E17 Smtn B (F). Values are means ± SE; n = 3 for all groups. Vertical lines indicate positions where the image of a single gel was cropped for purposes of presentation.
2.6-fold from PND14 to PND21 and then decreased so that, by PND56 (maturity), it was <50% of the level at PND7 (Fig. 1F). Total Smtn mRNA levels did not change significantly during development. We were unable to measure Smmhc A, as there is only a single unique exon, but the nearly fivefold decrease in Smmhc B with no significant change in total Smmhc from PND21 to PND56 suggests a corresponding increase in the Smmhc A (fast) isoform.

**Bladder smooth muscle maturation.** In contrast to the SRAs, Mypt1 was already ~50% E24+ in the embryonic day 17 (E17) bladder (Fig. 2A) and, by PND3, had attained the mature level of ~80% E24+ associated with a threefold increase in total Mypt1 mRNA (Fig. 2D). Total Smmhc increased ~3.2-fold between E17 and PND3, indicative of accretion of differentiated smooth muscle in the bladder (Fig. 2E). In the mature bladder, Smmhc is nearly exclusively E6+ (fast isoform). As with the SRAs, the developmental switch of bladder to E6+ was not temporally correlated with the switch in Mypt1 E24 splicing and other markers of smooth muscle differentiation (Fig. 2B). Smtn E20 splice variants in the bladder followed the temporal pattern of Mypt1 E24, with intermediate levels of alternative splicing at E17 that switched to predominance of exon skipping by PND3 (Fig. 2C). Total Smtn was increased at PND21 and PND56, likely reflecting an increase in Smtn A as Smtn B decreased during maturation (Fig. 2F), as previously reported for visceral smooth muscle (28).

**TRA2β is required for developmental induction of Mypt1 E24 splicing in mesenteric SRA.** To test the role of TRA2β in control of exon splicing in SRA smooth muscle, Smmhc-CreERT²/Cre mice were crossed into the Tra2βflox (flox) line of mice. Treatment of Cre+/floxf/f mice with tamoxifen (30 mg·kg⁻¹·day⁻¹ ip for 3 days) at all ages tested, from 1 day to 18 wk of age, resulted in efficient recombination at the Tra2β locus (Fig. 3A). Mice that were Cre+//floxf/f but not treated with tamoxifen had a low level of background recombination (controls assayed at 18 and 4 wk of age), while Cre−/floxf/f mice treated with tamoxifen had no recombination (controls treated with tamoxifen at 6 days and 1 day of age). In mice that were Cre−/floxf/f and treated with tamoxifen at 6 days of age, TRA2β protein in the MAs at 8 wk of age was reduced to ~25% of that of controls (Fig. 3B). Tamoxifen administered at 1 or 6 days of age significantly reduced Mypt1 E24 splicing in SRAs measured at 4 or 8 wk of age (maturity), respectively (Fig. 4A). In contrast, Tra2β inactivation had no significant effect on splic-

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**Figure 3.** Tamoxifen treatment causes efficient knockdown of transformer 2β (TRA2β). Smmh-CreERT²/Tra2βf/f conditional knockout (cKO) mice were treated with tamoxifen (Tx) at 1 day, 6 days, 28 days, and 18 wk of age to deplete TRA2β specifically in smooth muscle cells (cKO). Tissues were harvested from the mice for analysis at 4, 8, 12, and 24 wk of age. Cre+ mice treated with vehicle were used as controls in 4- and 18-wk groups; Cre− mice treated with tamoxifen were used as controls in the 1- and 6-day groups. A: genomic DNA was purified from aortas of the mice and amplified by PCR with primers spanning floxed exon 4, and products were separated by gel electrophoresis as described in METHODS. Intact Tra2β allele yielded a ~1,200-bp product, which, after recombination, was reduced to 256 bp. Percent recombination was calculated by dividing intensities of the 2 bands. B: Western blot of mesenteric arterial (MA) and bladder lysates from control and cKO mice quantified with antibodies to TRA2β (40 kDa) normalized against the splicing factor SRp20 (20 kDa) or cyclophilin A (Cyclo A, 18 kDa), respectively (n = 3 each).
There was no change in the LZ variant is associated with a 1.3-fold increase in the MYPT1 isoform coded for by the E24 splice variant at 5) or time to half-relaxation (206) (Fig. 6). Next, MA1s were activated with PE (10 5 M; Fig. 6). For the relaxation of PE (10 5 M), maximal reduction in force of MA1s more sensitive to the relaxing effects of PE (10 5 M) or time to half-relaxation (206) (Fig. 6). For the relaxation of PE (10 5 M), maximal reduction in force of MA1s was more sensitive to the relaxing effects of PE (10 5 M) or time to half-relaxation (206) (Fig. 6).

**Fig. 4.** Smooth muscle-specific cKO of TRA2β reduces Mypt1 E24 splicing in MAs. Smmhc-CreERT2;Tra2βfl/fl (cKO) mice were treated with tamoxifen as described in Fig. 3 legend. Mypt1 E24, Smmhc E6, and Smtn E20 splice variants were measured as described in Fig. 1 legend. A: Mypt1 E24 splicing is suppressed in the MA with cKO of TRA2β beginning at 1 day and 6 days but not at 28 days and 18 wk of age. B and C: cKO of TRA2β beginning at 6 days of age in MA reduces splicing of Mypt1 E24 but has no effect on Smmhc E6 or Smtn E20 (B) and in bladder has no effect on Mypt1 E24, Smmhc E6, or Smtn E20 splicing (C). *P < 0.05. Values are means ± SE; n = 3.

**Effect of Tra2β inactivation on protein expression.** A polyclonal antibody that specifically recognizes the LZ motif in MYPT1 and family members p85 and M21 was used to measure changes in MYPT1 isoforms during developmental maturation and after cKO of TRA2β. Because p85 is the third member of the MYPT family and is generated from a separate gene (Ppp1R12c), M21 is generated from a unique transcriptional start site within Mypt2 (Ppp1R12b). The normal developmental switch of mouse MA to the Mypt1 E24 splice variant is associated with a reduction of the MYPT1 LZ isoform coded for by the E24 splice variant (Fig. 5A). cKO of TRA2β at 6 days of age resulting in a reduction of the Mypt1 E24 splice variant is associated with a 1.3-fold increase in the MYPT1 LZ isoform coded for by the E24 splice variant at 5) or time to half-relaxation (206) (Fig. 6). Next, MA1s were activated with PE (10 5 M; Fig. 6). For the relaxation of PE (10 5 M), maximal reduction in force of MA1s was more sensitive to the relaxing effects of PE (10 5 M) or time to half-relaxation (206) (Fig. 6). For the relaxation of PE (10 5 M), maximal reduction in force of MA1s was more sensitive to the relaxing effects of PE (10 5 M) or time to half-relaxation (206) (Fig. 6). For the relaxation of PE (10 5 M), maximal reduction in force of MA1s was more sensitive to the relaxing effects of PE (10 5 M) or time to half-relaxation (206) (Fig. 6).
In this study we have demonstrated the developmental maturation of mouse MA smooth muscle and the role of the splicing factor Tra2β. Particularly salient features of this process include its relatively late timing, i.e., after postnatal week 2 and not complete until the animal is near maturity and much later than maturation of the bladder, suggesting local, not systemic, programming factor(s). The process of smooth muscle maturation may be considered in terms of smooth muscle differentiation, for which Smmhc and Smtn are reliable indicators, and smooth muscle phenotypic (phasic vs. tonic) diversification, for which Mypt1 E24 and Smmhc E6 splice variants are reliable markers (reviewed in Refs. 4 and 17). In the SRA, the >10-fold increase in Smmhc from PND7 to PND56 is consistent with accretion of differentiated smooth muscle in the vessel wall. This is consistent with a study of rat jejunal (mesenteric) artery that showed that, at birth, this 350-μm diameter artery consists of a single layer of partially differentiated smooth muscle cells that is adjacent to the endothelium and surrounded by five cell layers of undifferentiated mesenchymal cells that begin to differentiate over the following 2 wk (31), as well as our recent study showing a similar time course for differentiation in rat MAs (20). The switch from Mypt1 E24- to E24+ between PND14 and PND56 is a marker of the fast (phasic) gene program. This developmental switch from a slow to a fast gene program occurs in numerous tissues destined for a phasic phenotype, including intestine (5, 12) and portal vein (18) (reviewed in Ref. 4), suggesting a generalized phenomenon; however, the timing varies, with the bladder the first and the MAs the last to switch. An exception to this rule is Smmhc E6 splicing in the MAs, which at PND3 is 40% E6+, representing the fast isoform, which by PND7 has dropped to 10%, i.e., a fast-to-slow transition, where it is maintained to adulthood. This ratio of Smmhc 10% E6+ is much lower than the 50–80% E6+ reported in other studies of small arteries (3, 19). The prior studies used conventional PCR to measure Smmhc E6+ variants, which, upon further examination, appear to include both the smooth muscle and nonmuscle splice variants. In the current study we used custom-designed TaqMan probes that specifically span the Smmhc E5–E6 junction and, thus, provide a more accurate estimate of its minor contribution to the myosin pool, while a role for nonmuscle myosin in smooth muscle contractility has been suggested (32).

Although the importance of the generation of phenotypic diversity from alternative splicing of exons is well recognized (14), understanding of the control mechanisms in smooth muscle and other muscle types remains limited (4, 10). In the current study we used a tamoxifen-inducible smooth muscle-specific Cre for inactivation of Tra2β during smooth muscle postnatal maturation and into adulthood. This overcome limitations of the previous study of Tra2β inactivation using SM22Cre (5); such limitations include 1) embryonic lethality of homozygous inactivation and 2) potentially confounding effects of inactivation of Tra2β in the heart and smooth muscle tissues early in development. In the current study, using the inducible Cre with a more selective smooth muscle promoter, we show that splicing of Mypt1 E24 is reduced by Tra2β inactivation when the period of SRA maturation is targeted but not thereafter. As the Cre-induced Tra2β recombination efficiency is similar at the different stages, we interpret these data to indicate that Tra2β is required for the developmental programming of Mypt1 E24 splicing, but not for its maintenance. However, we cannot exclude alternative explanations, including idiosyncrasies of conditional gene knockout and compensation in the adult by other SR family members such as Tra2α. cKO of TRA2β at PND1 or PND6 caused a partial reduction in splicing of Mypt1 E24, consistent with combinatorial control of its splicing, as suggested by previous studies (2, 24), as is true of most alternative exons. cKO of TRA2β did not affect the splicing of other alternative exons examined in this study, including Smmhc E6 and Smtn E20. This is not surprising, given that this and prior studies (19, 33) have demonstrated dissociation in the time course of developmental switching of the alternative exons, as well as variation in their tissue-specific expression. Defining the full complement of alternative exons regulated by TRA2β will require high-throughput.
studies of exon abundance and TRA2β binding, as performed for the identification of alternative exons developmentally regulated by TRA2β in neural cells (7, 25).

cKO of TRA2β in the postnatal period, causing a reduction of Mypt1 E24 splicing and a switch to the MYPT1 LZ/H11001 isoform, was associated with increased sensitivity of the mature mouse MA1 to the relaxing effect of 8-Br-cGMP. This result is consistent with the requirement for the COOH-terminal LZ motif of MYPT1 for cGMP-dependent protein kinase 1 (cGK1) LZ-mediated heterodimerization with MYPT1 and activation of myosin phosphatase, causing calcium desensitization (9, 12, 26). In a prior study of rats (20), we demonstrated that the maturational shift of the MA1 to the relaxing effect of 8-Br-cGMP. This is consistent with the requirement for the COOH-terminal LZ motif of MYPT1 for cGMP-dependent protein kinase 1 (cGK1) LZ-mediated heterodimerization with MYPT1 and activation of myosin phosphatase, causing calcium desensitization. In conclusion, we have demonstrated that postnatal maturation of the mouse SRA smooth muscle begins after postnatal week 2 and is not complete until maturity. The maturational increase in splicing of Mypt1 E24 and relative resistance of myosin phosphatase activation to the second messenger of NO, cGMP, specific to the small arteries, require the splicing factor TRA2β, as its conditional deletion during maturation results in loss of these properties. This SRA maturation is dysynchronous with other smooth muscle tissues, such as bladder, suggesting local programming factors. In a prior study in the rat, we demonstrated that postnatal maturation of SRAs, including Mypt1 E24 splicing, requires sympathetic neural signals (20). These two sets of observations have interesting parallels with Drosophila, in which Tra homologs are dose-dependent master regulators of sexual differentiation (reviewed in Ref. 23). In the fly, development of the male muscle of Lawrence, an abdominal mating muscle specific to the male, is under the control of innervation and TRA (1, 16, 27). Future studies will examine signaling pathways that might connect neural or other upstream inputs with TRA2β or other splicing factors in the control of the unique gene program of the SRA smooth muscle.

ACKNOWLEDGMENTS

The authors thank Alex Lloyd for technical assistance.

GRANTS

This work was supported by National Institutes of Health Grant R01 HL-066171 (to S. A. Fisher) and Grants T32 HL-072751 and T32 AR-007592 (to J. Reho).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

X.Z. and J.J.R. performed the experiments; X.Z. and J.J.R. analyzed the data; X.Z., J.J.R., and S.A.F. interpreted the results of the experiments; X.Z. prepared the figures; X.Z. and S.A.F. drafted the manuscript; X.Z., B.W., and S.A.F. edited and revised the manuscript; X.Z., J.J.R., B.W., and S.A.F. approved the final version of the manuscript; B.W. and S.A.F. developed the concept and designed the research.

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