SUPPLEMENTAL MATERIALS

Loss of Transforming Growth Factor Beta Signaling in Aortic Smooth Muscle Cells Causes Endothelial Dysfunction and Aortic Hypercontractility

Jay Zhu, Stoyan Angelov, Ilkay Alp Yildirim, Hao Wei, Jie Hong Hu, Mark W. Majesky, Frank V. Brozovich, Francis Kim, David A. Dichek

From the Departments of Medicine (S.A., I.A.Y., H.W., J.H.H., F.K., D.A.D.), Surgery (J.Z.), Pediatrics and Laboratory Medicine and Pathology (M.W.M.), University of Washington, Seattle, WA, the Center for Developmental Biology and Regenerative Medicine, Seattle Children's Research Institute, Seattle WA (M.W.M.), and the Department of Medicine, Mayo School of Medicine, Rochester, MN (F.V.B.).

Major Resources Tables

Animals (in vivo studies)

Species	Vendor or Source	Background Strain	Sex
Mus musculus	Karlsson S.	C57BL/6 *Tgfbr2*$^{flox/flox}$	Both
Mus musculus	Offermanns, S.	C57BL/6 *Myh11*CreER$^{T2\ +/0}$	Male
Mus musculus	The Jackson Laboratory	C57BL/6J	Both

Animal breeding

	Species	Vendor or Source	Background Strain	Other Information
Parent - Male	Mus musculus	Offermanns, S.	C57BL/6J *Myh11*CreER$^{T2\ +/0}$ *Tgfbr2*$^{flox/flox}$	Used to generate male experimental mice and female control mice
Parent - Female	Mus musculus	Karlsson, S.	C57BL/6J *Tgfbr2*$^{flox/flox}$	Used to generate male experimental and male and female control mice
Parent - Male	Mus musculus	Karlsson, S.	C57BL/6J *Tgfbr2*$^{flox/flox}$	Used to generate male control mice
Parent - Male	Mus musculus	The Jackson Laboratory	C57BL/6J	Used to generate mice for pilot studies of EC-specific western blotting
Parent - Female	Mus musculus	The Jackson Laboratory	C57BL/6J	Used to generate mice for pilot studies of EC-specific western blotting

Antibodies

Target antigen	Vendor or Source	Catalog #	Working concentration	Lot # (preferred but not required)
MYH11	Santa Cruz Biotechnology	SC-6956	0.2 µg/mL	
ACTA2	Abcam	Ab32575	0.025 µg/mL	
NOS3 (eNOS)	Cell Signaling Technology	32027	1:1000 dilution	
p-eNOS (Ser^{1177})	Cell Signaling Technology	9570	1:1000 dilution	
p-eNOS (Thr^{495})	Cell Signaling Technology	9574	1:1000 dilution	
p-MLC	Cell Signaling Technology	3675	1:1000 dilution	
p-MLC	Abcam	2480	1:1000 dilution	

MLC	Santa Cruz Biotechnology	28329	1:1000 dilution	
MLC	Cell Signaling Technology	3672	1:1000 dilution	
p-VASP	Cell Signaling Technology	3114	1:1000 dilution	
p-MYPT (Thr^{853})	Cell Signaling Technology	4563	1:1000 dilution	
p-MYPT (Thr^{696})	Cell Signaling Technology	5163	0.043 µg/mL	
CAV1	Cell Signaling Technology	12506	1:1000 dilution	
GAPDH	Santa Cruz Biotechnology	SC-20357	0.1 µg/mL	
Goat IgG	Santa Cruz Biotechnology	SC-2033	0.08 µg/mL	
Rabbit IgG	Santa Cruz Biotechnology	SC-2030	0.08 µg/mL	
Rabbit IgG	Cell Signaling Technology	CST-7074	0.065 µg/mL	
PECAM1 (Immunostaining)	R&D Systems	AF3628	1.3 µg/mL	
Mac2	Cedar Lane	CL8942AP	1 µg/mL	
Goat IgG	R&D Systems	BAF109	0.1 µg/mL	
Rat IgG	Vector	BA4001	0.25 µg/mL	
PECAM1 (Western)	R&D Systems	AF3628	0.5 µg/mL	
CDH5	Cell Signaling Technology	2500	0.113 µg/mLl	
VIM	Cell Signaling Technology	5741	0.045 µg/mL	
ACTB	Cell Signaling Technology	8457	0.037 µg/mL	

Table I. E_{max} values

				MALE TBR2$^{f/f}$				FEMALE TBR2$^{f/f}$		
Time point (weeks)	**Agonist**	**Units**		**mean**	**SEM**	**n**		**mean**	**SEM**	**n**
3	PE	mN		8.8	0.57	6		10	0.91	6
	ACh	% Relaxation		98.5	5.9	6		106	2.8	6
	SNP	% Relaxation		101	0.36	6		101	0.22	6

				TBR2$^{f/f}$				TBR2$^{SM\Delta}$		
Time point (weeks)	**Agonist**	**Units**		**Mean**	**SEM**	**n**		**Mean**	**SEM**	**n**
1	PE	mN		7.9	0.63	8		11	0.66	10
	ACh	% Relaxation		104	2.9	8		70	5.7	10
	SNP	% Relaxation		108	3.9	8		102	1.8	10
3	PE	mN		7.8	0.46	19		13	0.49	20
	ACh	% Relaxation		97	1.5	19		37	3.6	20
	SNP	% Relaxation		100	0.28	18		106	3.6	17
14	PE	mN		9.3	1.2	10		17	1.9	8
	ACh	% Relaxation		93	1.5	10		15	3.9	8
	SNP	% Relaxation		100	0.73	10		104	1.2	8

				TBR2$^{SM\Delta}$+vehicle				TBR2$^{SM\Delta}$+Bosentan		
Time point (weeks)	**Agonist**	**Units**		**mean**	**SEM**	**n**		**mean**	**SEM**	**n**
3	PE	mN		14	0.88	8		13	1.0	8

				TBR2$^{SM\Delta}$+vehicle				TBR2$^{SM\Delta}$+SQ29548		
Time point (weeks)	**Agonist**	**Units**		**mean**	**SEM**	**n**		**mean**	**SEM**	**n**
3	PE	mN		14	0.81	6		15	0.87	6

				TBR2$^{SM\Delta}$+vehicle				TBR2$^{SM\Delta}$+SOD/Catalase		
Time point (weeks)	**Agonist**	**Units**		**mean**	**SEM**	**n**		**mean**	**SEM**	**n**
3	ACh	% Relaxation		33.7	6.8	5		42.4	11	5

				Denuded TBR2$^{f/f}$				Denuded TBR2$^{SM\Delta}$		
Time point (weeks)	**Agonist**	**Units**		**mean**	**SEM**	**n**		**mean**	**SEM**	**n**
3	PE	mN		10	0.59	10		13	1.2	9

				L-NAME TBR2$^{f/f}$				L-NAME TBR2$^{SM\Delta}$		
Time point (weeks)	**Agonist**	**Units**		**mean**	**SEM**	**n**		**mean**	**SEM**	**n**
3	PE	mN		17	0.76	8		18	0.97	8

Values of E_{max} were measured for each aortic ring, and the mean and SEM were calculated for each group. n=number of individual mice. mN=milliNewtons; PE=phenylephrine; ACh=acetylcholine; SNP=sodium nitroprusside; L-NAME=N(ω)-nitro-L-arginine methyl ester; SOD=superoxide dismutase.

Table II. $-\text{LogEC}_{50}$ values

			MALE TBR2$^{f/f}$				FEMALE TBR2$^{f/f}$				
Time point (weeks)	**Agonist**		**mean**	**SEM**	**n**		**mean**	**SEM**	**n**		**P**
	PE		6.9	0.078	6		6.9	0.10	6		0.7
3	**ACh**		7.3	0.040	6		7.5	0.037	6		0.03
	SNP		7.6	0.068	6		7.7	0.058	6		0.4

			TBR2$^{f/f}$				TBR2$^{SM\Delta}$				
Time point (weeks)	**Agonist**		**mean**	**SEM**	**n**		**mean**	**SEM**	**n**		**P**
	PE		7.1	0.090	8		6.8	0.096	10		0.1
1	**ACh**		7.1	0.15	8		6.5	0.17	10		0.01
	SNP		8.1	0.15	8		8.0	0.20	10		0.3
	PE		6.7	0.056	19		7.2	0.12	20		0.01
3	**ACh**		7.5	0.038	19		6.6	0.13	20		< 0.001
	SNP		7.7	0.082	18		7.4	0.09	17		0.01
	PE		6.5	0.066	10		7.0	0.21	8		0.007
14	**ACh**		7.5	0.086	10		6.0	0.13	7		< 0.001
	SNP		7.8	0.056	10		7.6	0.15	8		0.07

			TBR2$^{SM\Delta}$+vehicle				TBR2$^{SM\Delta}$+Bosentan				
Time point (weeks)	**Agonist**		**mean**	**SEM**	**n**		**mean**	**SEM**	**n**		**P**
3	**PE**		7.2	0.22	8		7.6	0.16	8		0.1

			TBR2$^{SM\Delta}$+vehicle				TBR2$^{SM\Delta}$+SQ29548				
Time point (weeks)	**Agonist**		**mean**	**SEM**	**n**		**mean**	**SEM**	**n**		**P**
3	**PE**		6.9	0.19	6		7.5	0.13	6		0.03

			TBR2$^{SM\Delta}$+vehicle				TBR2$^{SM\Delta}$+SOD/Catalase				
Time point (weeks)	**Agonist**		**mean**	**SEM**	**n**		**mean**	**SEM**	**n**		**P**
3	**PE**		6.7	0.16	5		6.4	0.094	5		0.2

			Denuded TBR2$^{f/f}$				Denuded TBR2$^{SM\Delta}$				
Time point (weeks)	**Agonist**		**mean**	**SEM**	**n**		**mean**	**SEM**	**n**		**P**
3	**PE**		7.4	0.11	10		7.2	0.11	9		0.4

			L-NAME TBR2$^{f/f}$				L-NAME TBR2$^{SM\Delta}$				
Time point (weeks)	**Agonist**		**mean**	**SEM**	**n**		**mean**	**SEM**	**n**		**P**
3	**PE**		7.8	0.082	8		7.8	0.15	8		0.4

Values of $-\text{LogEC}_{50}$ were calculated for each aortic ring, and the mean and SEM values were then calculated for each group of rings. Groups were compared using either a 2-tailed Student's t-test or a Mann-Whitney rank-sum test (see Materials and Methods). n=number of individual mice. PE=phenylephrine; ACh=acetylcholine; SNP=sodium nitroprusside; L-NAME=N(ω)-nitro-L-arginine methyl ester; SOD=superoxide dismutase.

Table III. Primers used for PCR genotyping

***Myh11*-CreER^{T2}**	**Sequence**
1	5’ TGACCCCATCTCTTCACTCC 3'
2	5' AACTCCACGACCACCTCATC 3'
3	5' AGTCCCTCACATCCTCAGGTT 3'
Tgfbr2	
P3	5' TAT GGA CTG GCT GCT TTT GTA TTC 3'
P4	5' TGG GGA TAG AGG TAG AAA GAC ATA 3'

The *Myh11*-CreER^{T2} allele was detected using 3 primers (1–3). When present, the *Myh11*-CreER^{T2} allele yields a 280 bp amplicon; when absent, a 210 bp amplicon is generated. The *Tgfbr2*^{flox} allele was detected using primers P3 and P4. These primers produce a 575 bp amplicon when *Tgfbr2*^{flox} is present and a 422 bp amplicon when *Tgfbr2*^{wt} is present.

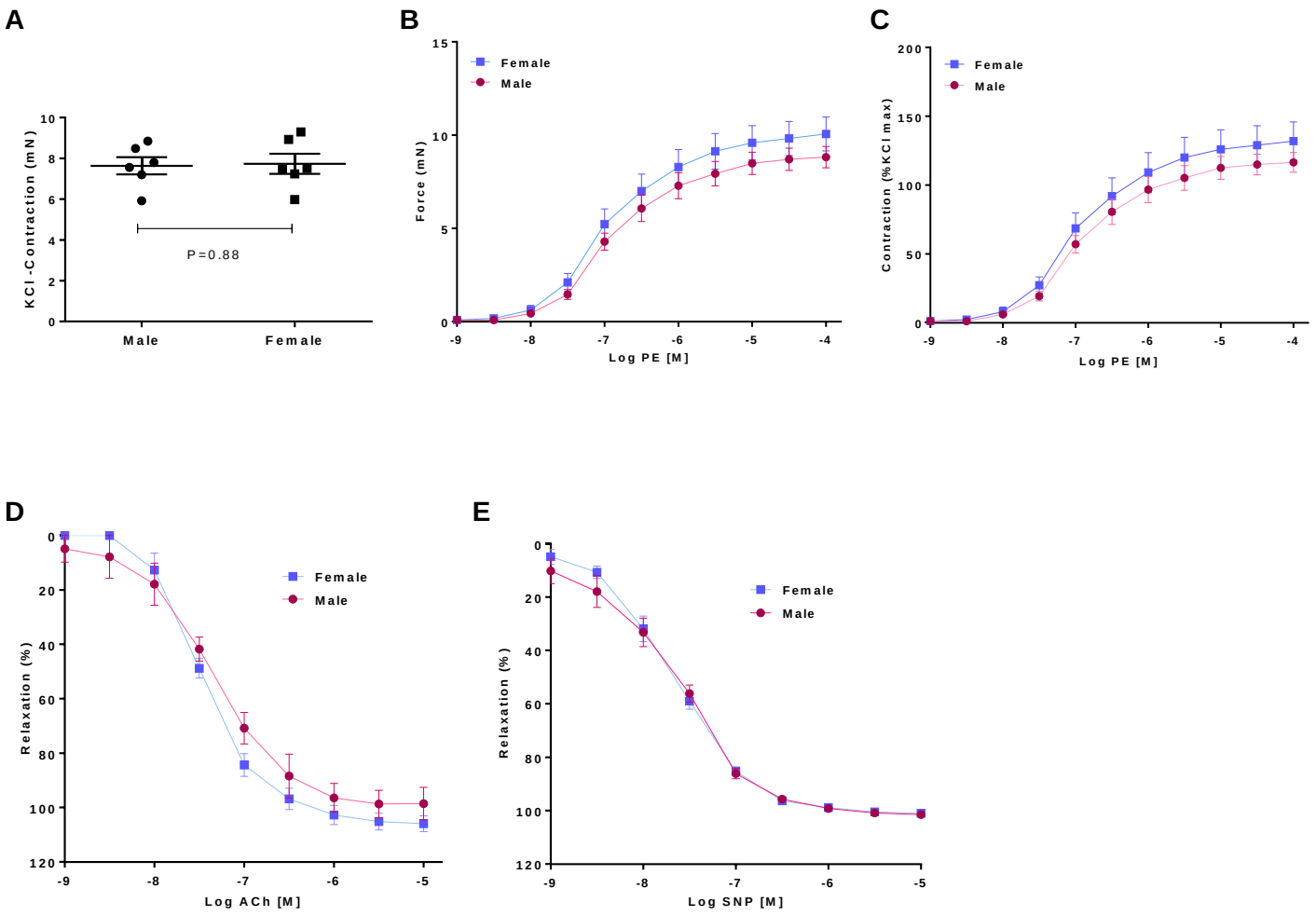

Figure I. Vasomotor properties of female and male *Myh11*-CreER$^{T2\ 0/0}$ *Tgfbr2*$^{f/f}$ control mice. We used female mice as controls in initial experiments and male mice in the remaining experiments (see Materials and Methods). Ascending aortic rings of female and male mice were treated with vasoactive agents including: (**A**), 40 mM potassium chloride (KCl); (**B**–**C**), phenylephrine (PE); (**C**), portrays results in (**B**), with normalization to maximal potassium chloride-initiated contraction for each segment. (**D**), acetylcholine (ACh); and (**E**), sodium nitroprusside (SNP). (**B**), Force is measured in milliNewtons (mN). (**A**–**E**), n=6 per group; *P*=0.5–1.0 for comparisons of the values of the 2 groups at all concentrations of PE, ACh, and SNP. For calculated –logEC_{50} values, see Table II.

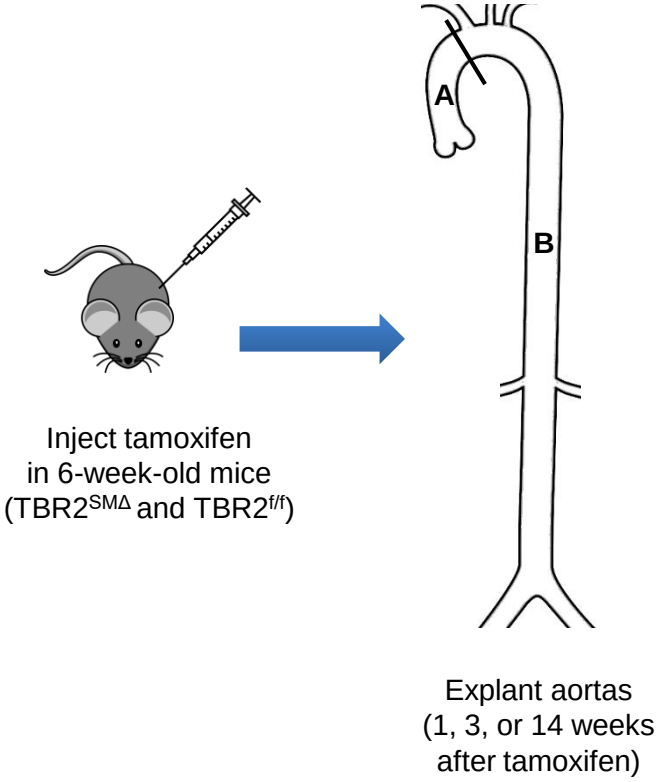

Figure II. Generation of experimental aortic tissue and allocation of segments for experiments. *Myh11*-CreER$^{T2\ +/0}$ *Tgfbr2*$^{f/f}$ and *Myh11*-CreER$^{T2\ 0/0}$ *Tgfbr2*$^{f/f}$ were injected with tamoxifen at 6 weeks of age to generate experimental mice with *Tgfbr2* deleted in SMC (TBR2$^{SM\Delta}$) and littermate control mice (TBR2$^{f/f}$). Aortas were removed 1, 3, or 14 weeks later. (**A**) Ascending aortas were used immediately for vasomotor studies or were fixed for histologic studies. (**B**) The remainder of the aorta was snap-frozen for protein extraction and western blotting. These studies were performed only on aortas removed 3 weeks after tamoxifen injections. In separate aortas, nitric oxide and superoxide were measured in thoracic aortic segments, and vascular permeability was measured in the thoracic aorta (see Materials and Methods).

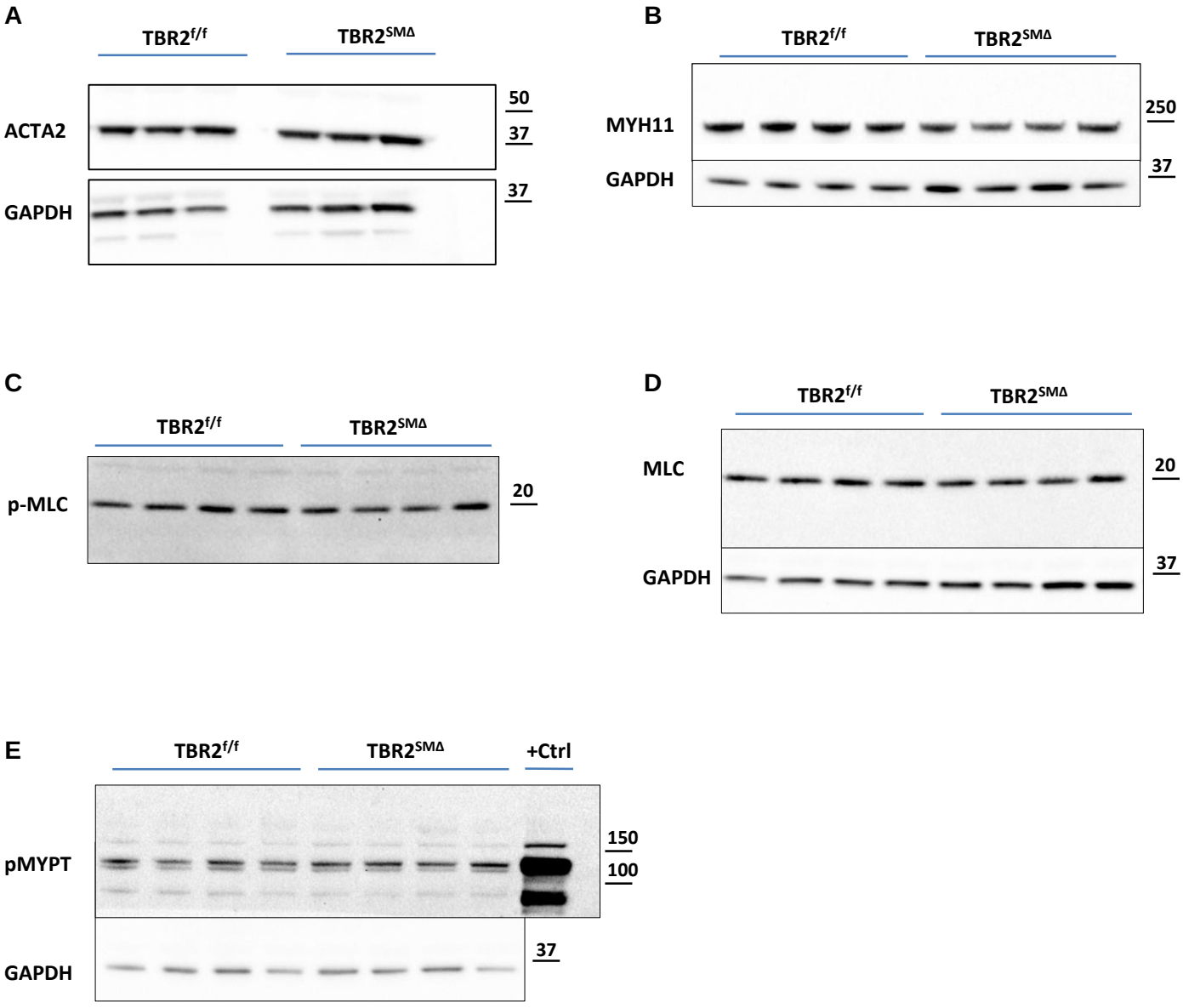

Figure III. No difference in levels of SMC contractile-apparatus proteins in aortic extracts of TBR2$^{f/f}$ and TBR2$^{SM\Delta}$ mice. Aortic proteins were extracted and analyzed by immunoblotting for: (**A**), smooth muscle actin (ACTA2); (**B**), smooth muscle myosin heavy chain (MYH11); (**C**), phosphorylated myosin light chain (p-MLC); (**D**), myosin light chain (MLC); and (**E**), phosphorylated myosin phosphatase (p-MYPT). (**A**, **B**, **D**, **E**). Blots were also probed for glyceraldehyde phosphate dehydrogenase (GAPDH), as a loading control. Results are representative of immunoblots of total of 7–15 samples per group for each protein (see Online Figure IV for quantitation). Each lane contains a sample from a different mouse. Size markers are in kDa. (E), +Ctrl=positive control sample provided by antibody supplier.

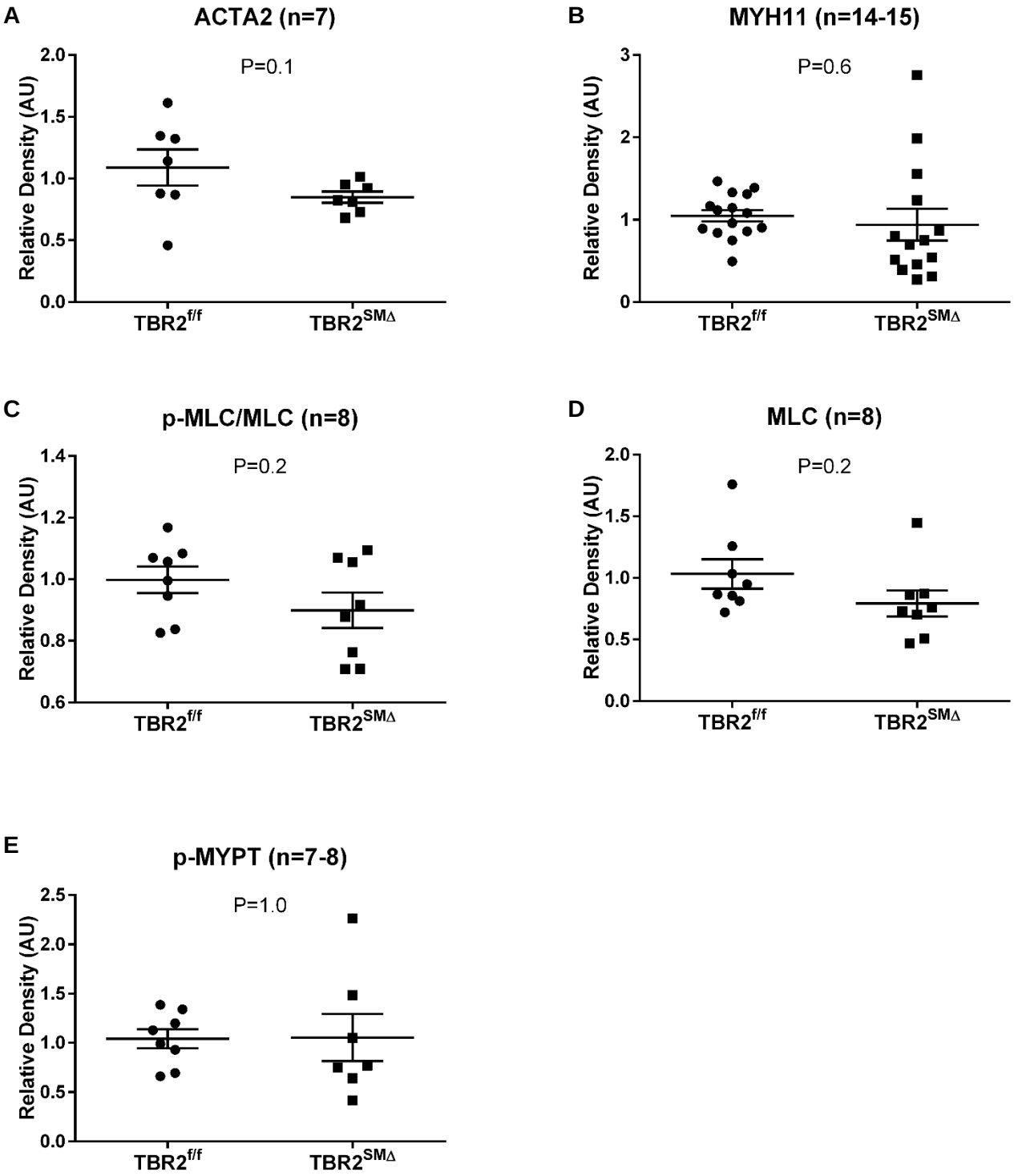

Figure IV. No differences in levels of SMC contractile-apparatus proteins in aortic extracts of TBR2$^{f/f}$ and TBR2$^{SM\Delta}$ mice. Protein levels were measured by densitometry of western blots (for example, Figure 1 and Online Figure III). Proteins include: (**A**) smooth muscle actin (ACTA2), (**B**) smooth muscle myosin heavy chain (MYH11), (**C**) phosphorylated myosin light chain (p-MLC), (**D**) myosin light chain (MLC), (**E**) phosphorylated myosin phosphatase (p-MYPT). Densitometry signals for these proteins were normalized to the density of the GAPDH signal in the same lane, except for p-MLC/MLC for which p-MLC signals were normalized to MLC signals from the same samples, on separate blots. (**A**–**E**), (AU=arbitrary units)

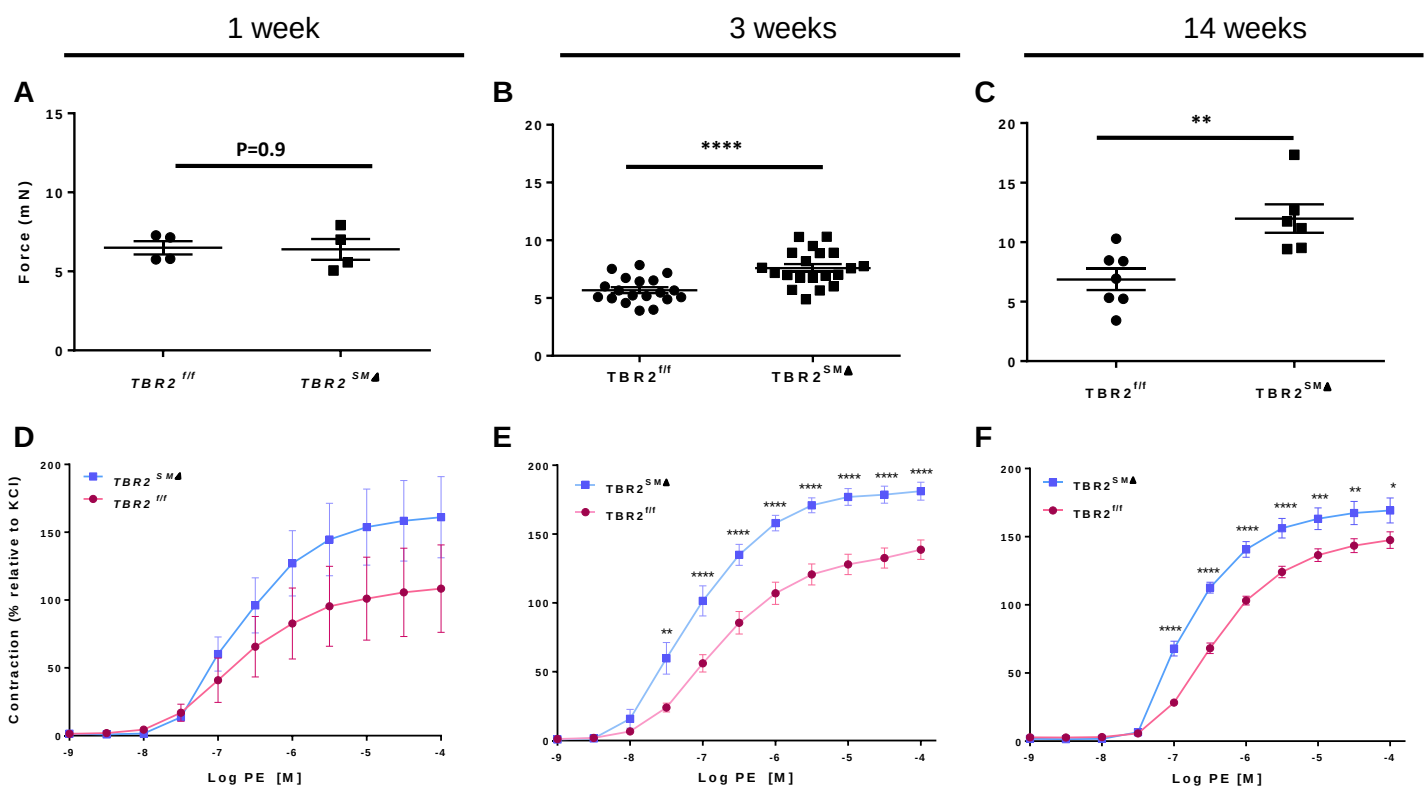

Figure V. Aortas of TBR2$^{SM\Delta}$ mice are hypercontractile to potassium chloride (KCl) and phenylephrine (PE). (**A**–**C**) Force in milliNewtons (mN) developed by aortic segments treated with 40 mM KCl. ((**D**–**F**), Force developed by aortic segments treated with increasing concentrations of PE, normalized as a percent of the force developed after treatment with 40 mM KCl. (**A**, **D**), n=4; (**B**, **E**), n=19–20; and (**C**, **F**), n=6–7. (**A**–**F**), Data are mean±SEM. **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001.

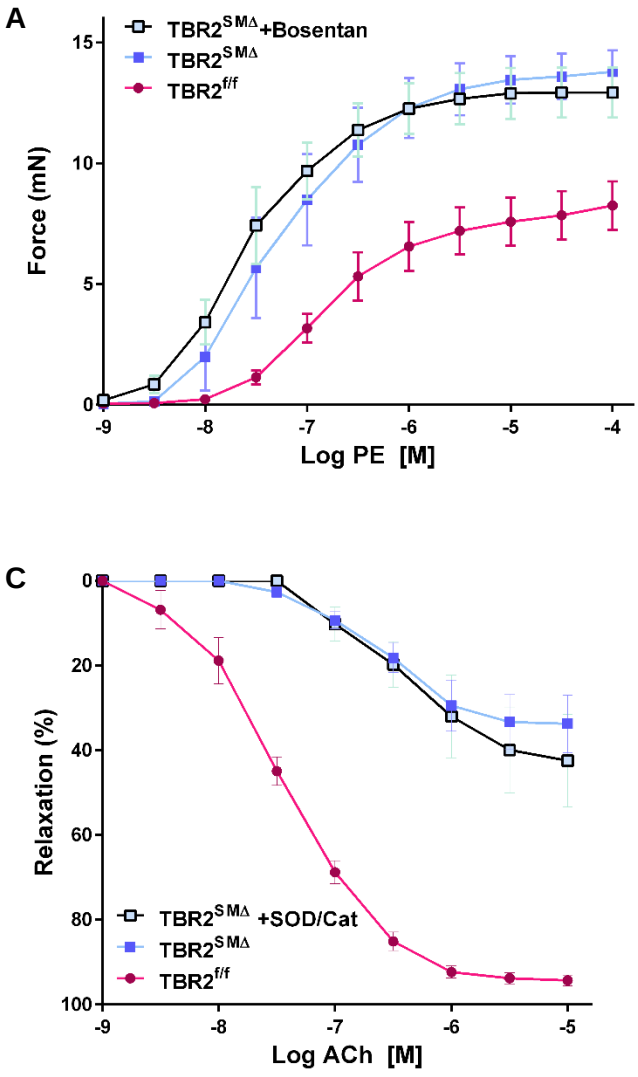

Figure VI. Ex vivo inhibition of endothelin, thromboxane, and reactive oxygen species does not reverse hypercontractility and endothelial dysfunction of TBR2$^{SM\Delta}$ aortas. Ascending aortic rings from TBR2$^{SM\Delta}$ mice were treated ex vivo with: (**A**), The endothelin-1 receptor antagonist bosentan; (**B**), The thromboxane A2 receptor antagonist SQ29548; or (**C**), Superoxide dismutase (SOD) and catalase (Cat). Rings from TBR2$^{f/f}$ mice are included as tamoxifen-treated wild-type controls. (**A**–**B**), force generation in milliNewtons (mN) in response to phenylephrine (PE; n=5–6). (**C**), relaxation in response to acetylcholine (ACh; n=5).

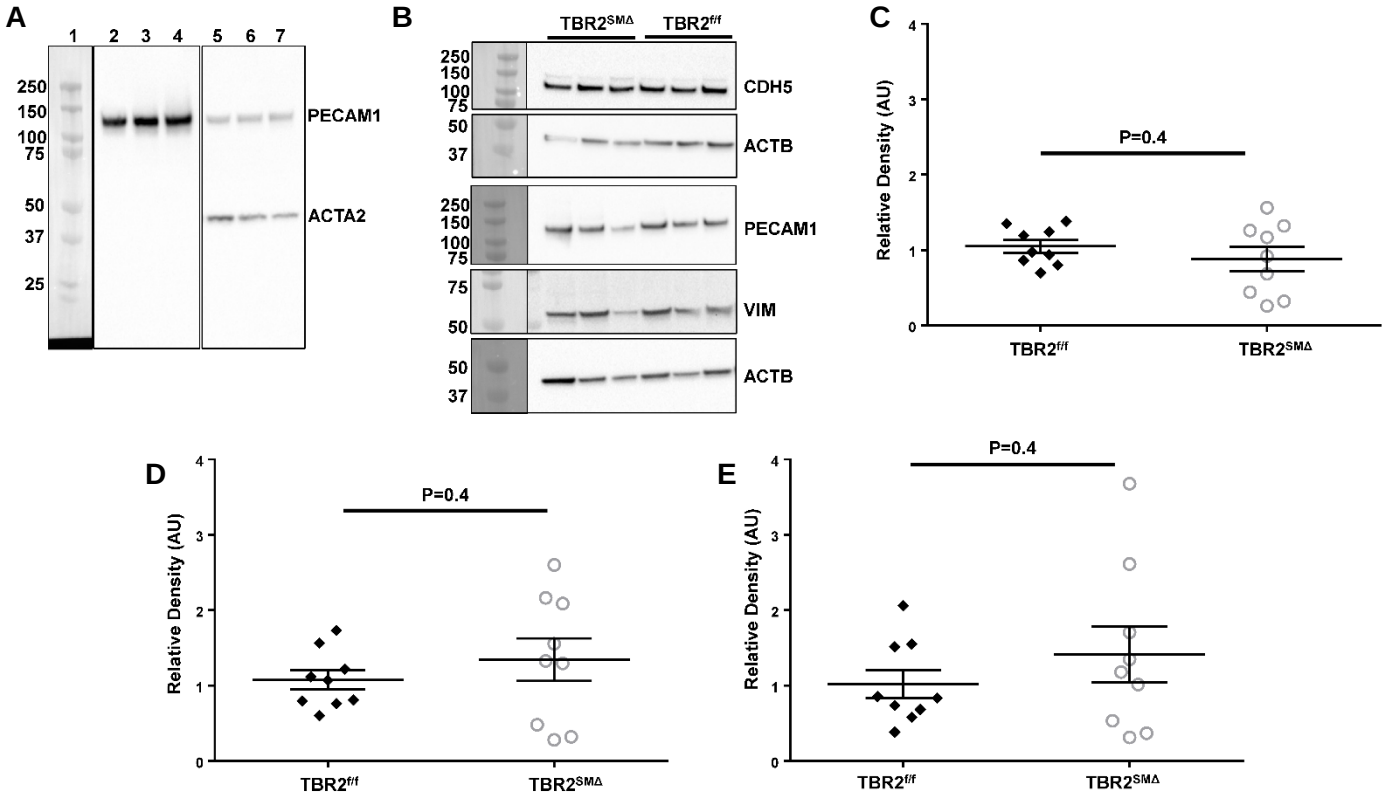

Figure VII. Absence of endothelial to mesenchymal transition in aortas of TBR2$^{SM\Delta}$ mice. (**A**) Western blots of lysates of aortic luminal cells (lanes 2-4) or lysates of remaining aortic tissue (lanes 5-7; from the same aortas as lanes 2–4), probed for detection of platelet endothelial cell adhesion molecule-1 (PECAM1) and smooth muscle actin (ACTA2). All aortas are from TBR2$^{WT/WT}$ mice. Lanes 2-4 and 5-7 are from the same blot and were probed together. (**B**) Representative blots of lysates of aortic luminal cells from TBR2$^{f/f}$ or TBR2$^{SM\Delta}$ mice, probed for cadherin-5 (CDH5), beta actin (ACTB), PECAM1, and vimentin (VIM). The top 2 images are from the same blot; the bottom 3 images are from a separate blot. (**C**–**E**) Densitometry analysis of (**B**) and other blots: (**C**)=PECAM1; (**D**)=CDH5; (**E**)=VIM. All values are normalized to ACTB signals in the same lane and are expressed as ratio to the mean TBR2$^{f/f}$ signal on same blot. (**A**–**B**) Molecular weights are in kiloDaltons. (**C**–**E**) Each point is a single aorta. Mean and SEM. (**C**–**D**) t-test with Welch's correction. (**E**) Student's t-test.

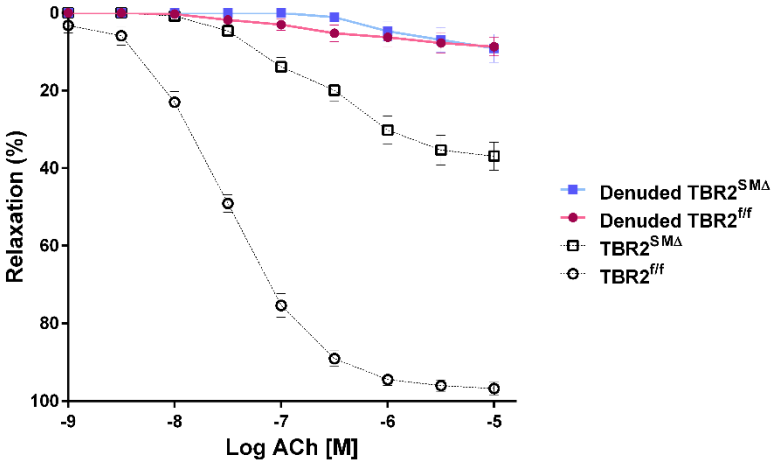

Figure VIII. Mechanically denuded aortic segments lack endothelium-dependent vasodilation. Ascending aortic segments were removed from TBR2$^{SM\Delta}$ and TBR2$^{f/f}$ mice, mounted on a wire myograph, and denuded of luminal endothelium by gentle abrasion with a steel wire. The segments were then precontracted with phenylephrine and treated with increasing concentrations of acetylcholine (ACh). Data are mean±SEM (n=9–10 for each group of denuded segments). Data from non-denuded segments (n=19–20) are reproduced from Figure 3, for comparison.

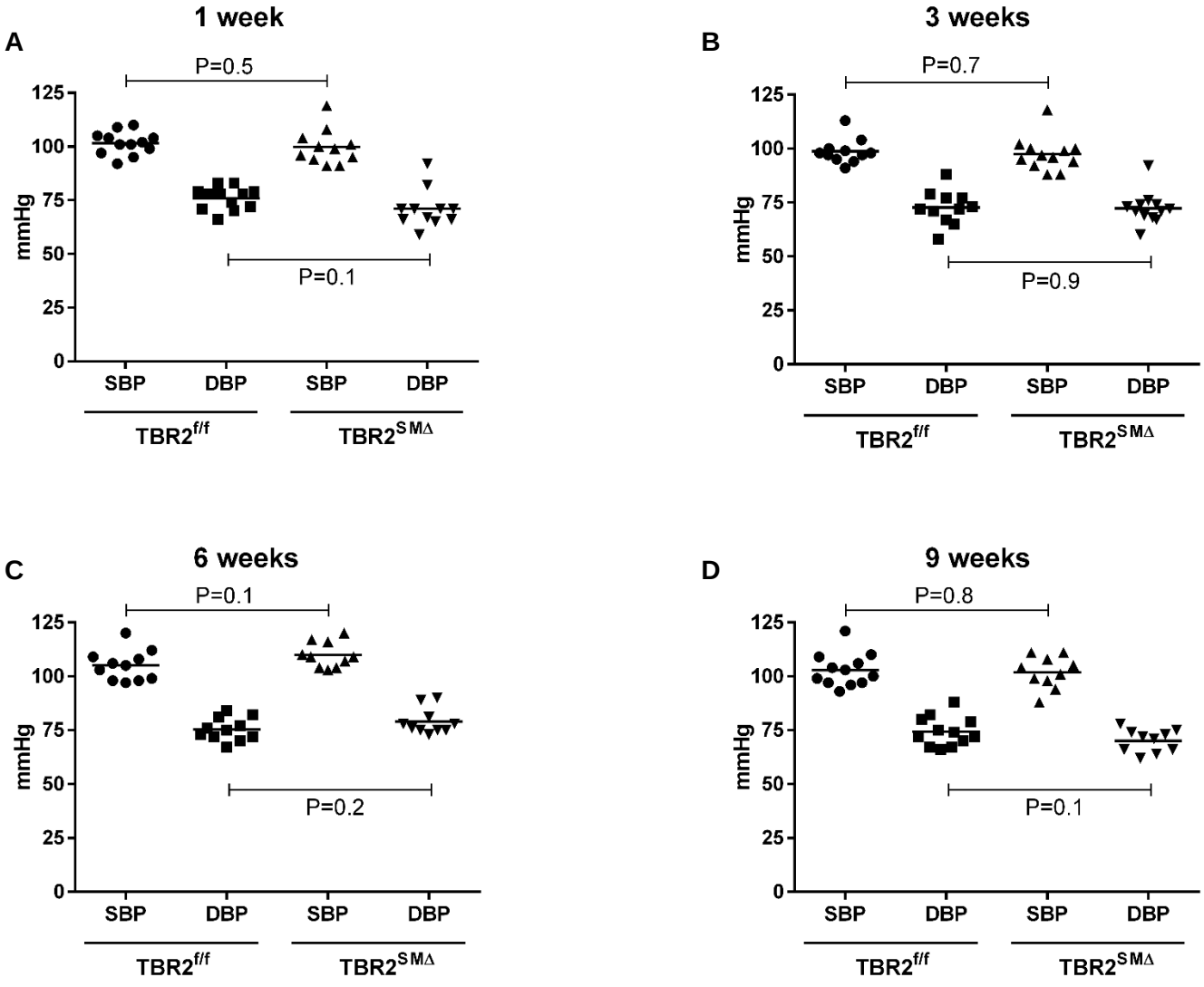

Figure IX. Systemic blood pressure is not altered in mice with SMC-specific deletion of *Tgfbr2*. (**A**–**D**), Blood pressure was measured noninvasively (see Materials and Methods) in TBR2$^{f/f}$ and TBR2$^{SM\Delta}$ mice 1, 3, 6, and 9 weeks after completing tamoxifen injections. SBP=systolic blood pressure; DBP=diastolic blood pressure. Data points are individual mice (n=10–12 per group); bars are group means.

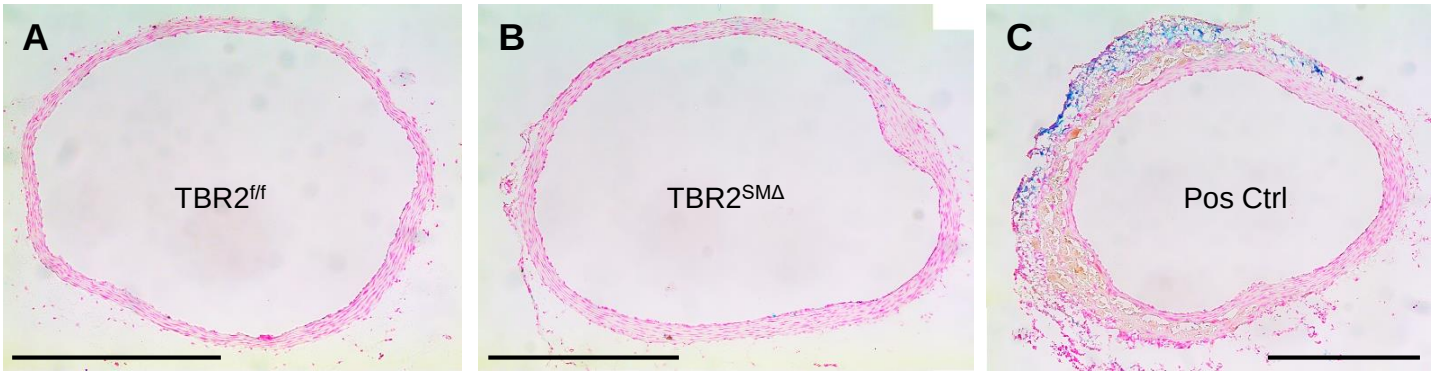

Figure X. No microscopic intramural hemorrhages in aortas removed 1 week after tamoxifen injection. Aortas without grossly visible hemorrhage were removed from (**A**), $TBR2^{f/f}$ and (**B**), $TBR2^{SM\Delta}$ mice, sectioned, and stained with Prussian blue. (**C**), Prussian blue-stained section from an aorta with grossly visible hemorrhage, included as a positive control. (**A**–**C**), size bars = 500 μm.

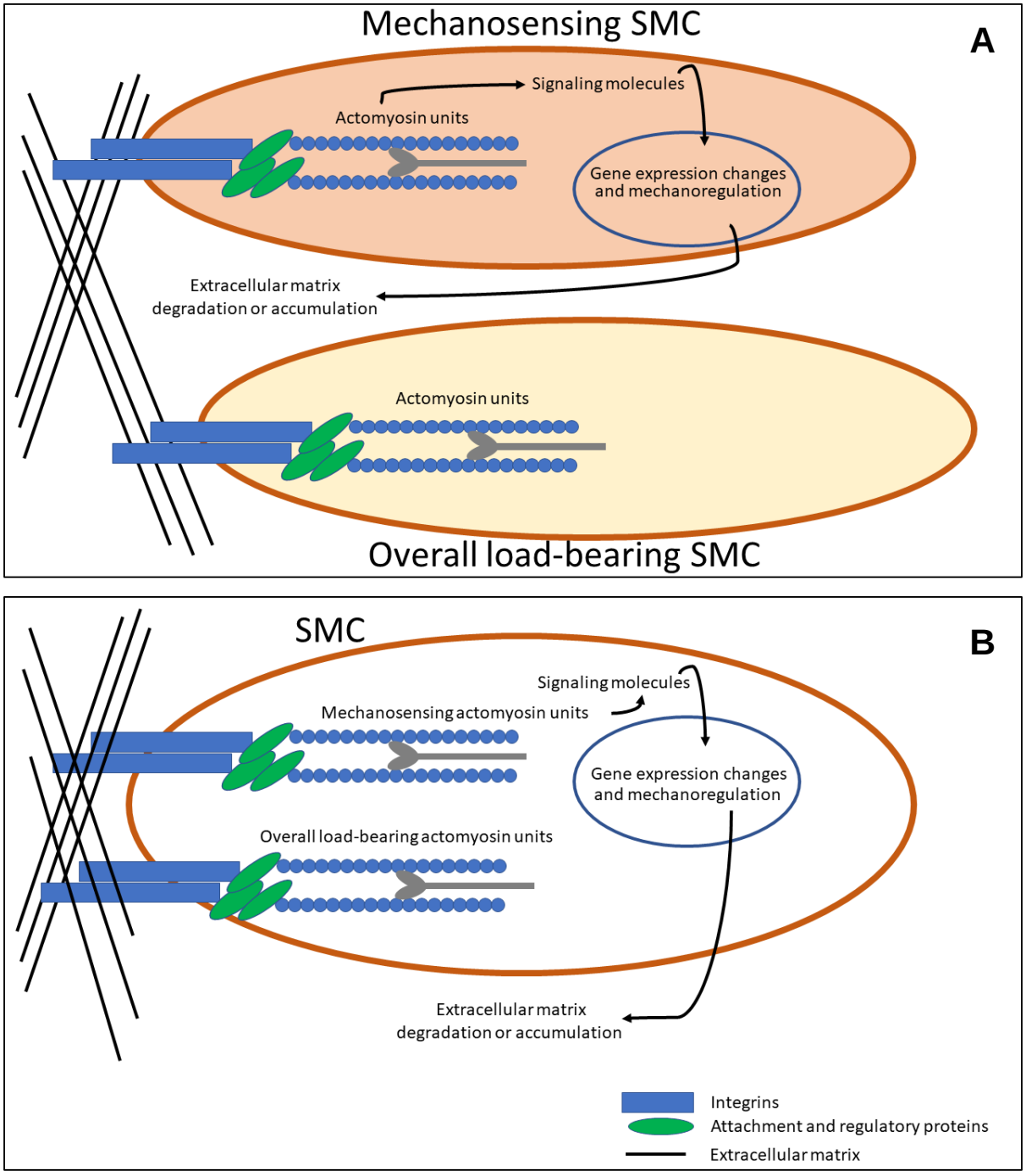

Figure XI. Intra- or inter-cellular heterogeneity could allow actomyosin-containing contractile units to contribute both to mechanosensing/mechanotransduction and to overall load bearing. Mechanosensing/mechanotransduction may be mediated by actomyosin-containing contractile units that sense and maintain stresses of ~5 kPa, whereas vessel caliber is controlled by SMC that generate stresses of ~100 kPa.[1] Both of these functions could be accomplished in the same vessel wall if: (**A**) different populations of SMC served these 2 roles; or (**B**) different actomyosin-containing contractile units in the same SMC served these 2 roles. If these 2 functions are distinct, dysfunction of actomyosin-containing contractile units that contribute to mechanosensing/mechanotransduction could lead to aneurysm formation without affecting overall load-bearing contractile function.

Reference

1. Humphrey JD, Schwartz MA, Tellides G, Milewicz DM. Role of mechanotransduction in vascular biology: Focus on thoracic aortic aneurysms and dissections. *Circ. Res.* 2015;116:1448–1461