Vascular Smooth Muscle Emilin-1 Is a Regulator of Arteriolar Myogenic Response and Blood Pressure

Gaia Litteri,* Daniela Carnevale,* Alessandra D’Urso, Giuseppe Cifelli, Paola Braghetta, Antonio Damato, Dario Bizzotto, Alessandro Landolfi, Francesco Da Ros, Patrizia Sabatelli, Nicola Facchinello, Angelo Maffei, Dino Volpin, Alfonso Colombatti, Giorgio M. Bressan, Giuseppe Lembo

Objective—Emilin-1 is a protein of elastic extracellular matrix involved in blood pressure (BP) control by negatively affecting transforming growth factor (TGF)-β processing. Emilin1 null mice are hypertensive. This study investigates how Emilin-1 deals with vascular mechanisms regulating BP.

Methods and Results—This study uses a phenotype rescue approach in which Emilin-1 is expressed in either endothelial cells or vascular smooth muscle cells of transgenic animals with the Emilin1<sup>−/−</sup> background. We found that normalization of BP required Emilin-1 expression in muscle cells, whereas expression of the protein in endothelial cells did not modify the hypertensive phenotype of Emilin1<sup>−/−</sup> mice. We also explored the effect of treatment with anti-TGF-β antibodies on the hypertensive phenotype of Emilin1<sup>−/−</sup> mice, finding that neutralization of TGF-β in Emilin1 null mice normalized BP quite rapidly (2 weeks). Finally, we evaluated the vasoconstriction response of resistance arteries to perfusion pressure and neurohumoral agents in different transgenic mouse lines. Interestingly, we found that the hypertensive phenotype was coupled with an increased arteriolar myogenic response to perfusion pressure, while the vasoconstriction induced by neurohumoral agents remained unaffected. We further elucidate that, as for the hypertensive phenotype, the increased myogenic response was attributable to increased TGF-β activity.

Conclusion—Our findings clarify that Emilin-1 produced by vascular smooth muscle cells acts as a main regulator of resting BP levels by controlling the myogenic response in resistance arteries through TGF-β. (Arterioscler Thromb Vasc Biol. 2012;32:2178-2184.)

Key Words: Emilin1 • myogenic response • systemic hypertension • transforming growth factor • vascular smooth muscle

The Emilin/Multimerin family of extracellular matrix proteins (for nomenclature see online-only Data Supplement material) comprises of 4 members with a common multidomain structure consisting of an amino-terminal elastin microfibril interface domain, a central sequence with high probability of coiled-coil conformation, and a carboxy-terminal C1q-like domain.1,2 All 4 proteins are expressed in the cardiovascular system, however, with significant differences concerning their fine distribution.1,3,4 While Emilin-1 is expressed by endocardium and right ventricle myocytes and by cells of the entire blood vessel wall (endothelial cells [ECs], smooth muscle cells [SMCs] and adventitial fibroblasts), the other genes have a more restricted expression pattern: Emilin2 is active in myocardium, whereas Mmnn1 and Mmnn2 are expressed only by ECs in blood vessels and, in addition, in platelets and endocardial cells, respectively.

The function of these proteins in the cardiovascular system has not been fully disclosed. One prominent function of Emilin-1 is the regulation of systemic blood pressure (BP).5 The protein, through its elastin microfibril interface domain, inhibits transforming growth factor (TGF)-β biosynthesis by blocking the proteolytic cleavage of the proTGF-β precursor into the latency associated peptide/TGF-β complex. The increase in TGF-β signaling induced by Emilin-1 deficiency results in systemic hypertension, accompanied by narrowing of arterial tree and structural alterations of the wall of elastic arteries.5,6

In spite of this important information on alterations induced by Emilin-1 deficiency, it is still unknown which cell types mediate protein effects. In particular, it has not been investigated whether hypertension is causally related to abnormal behavior of vascular SMCs (VSMCs) or ECs. Gaining insight into this issue not only would add new knowledge to the biology...
of Emilin-1 but could also be of potential relevance in investigations addressing the function of EMILIN1 gene in human hypertension. In fact, recent association studies have suggested that specific haplotypes of EMILIN1 are useful genetic markers of essential hypertension in Japanese men and that the interaction of age and genotype variation of specific single nucleotide polymorphisms might increase the risk of hypertension in a northern Han Chinese population.6,7

Here, we have addressed the question of the cellular context of Emilin-1 activity using a phenotype rescue approach by expressing the protein in either ECs or VSMCs or in all cells of the arterial wall of Emilin-1−/− mice. Our results indicate that Emilin-1 expression in VSMCs is specifically required for BP control and further clarify that the protein regulates the arteriolar myogenic response through TGF-β.

**Materials and Methods**

**Animals**

Transgenic mice were generated by pronuclear microinjection of DNA into fertilized eggs using the constructs described in Figure 1. Animals were anesthetized with a single intraperitoneal injection of avertin (250 mg/kg) and killed by cervical dislocation. All procedures involving animals conformed to the Directive 2010/63/EU of the European Parliament and have been approved by the Ethics Review Board of our Institutions (University of Padova, approval reference number 63TER/2010, and IRCCS Neuromed). The procedures used for the synthesis of constructs, the generation and breeding of lines, and the characterization of transgenic mouse lines using immunohistochemistry, electron microscopy, histology, and echocardiography are described in detail in the Methods section of the online-only Data Supplement.

**Evaluation of BP**

BP was evaluated using implanted radiotelemetry pressure transducer or noninvasively by tail cuff plethysmography in conscious animals.

**Vascular Function Evaluation**

Mesenteric arteries were isolated from mice euthanized with an overdose of sodium pentobarbital (250 mg/kg), placed in a pressure myograph in Krebs solution and myogenic response and contraction to phenylephrine and angiotensin II analyzed as described in the Methods section in the online-only Data Supplement.

**Infusion of Antibodies to TGF-β**

Neutralizing antibodies to TGF-β (0.5 μg/kg per day; R&D Systems Inc., Minneapolis, MN, diluted in PBS) or preimmune IgG were infused for 24 days into Emilin1−/− and eNOS−/− mice or the corresponding wild-type control littermates through osmotic minipumps (Alzet model 2004, Durect Corporation, Cupertino, CA) implanted subcutaneously on the right side of the back of the mice. BP was evaluated before and during treatment. The antibody dose used corresponds to the lowest dose effective in reducing BP of Emilin1−/− mice to normal levels, as determined in preliminary experiments.

**Statistical Analysis**

For BP measurements, vascular reactivity parameters and percentage of phospho Smad3 (P-Smad3)-positive nuclei data are expressed as mean±SEM. Multiple comparisons were evaluated by 1-way ANOVA for factorial design or by 2-way ANOVA for repeated measures, accordingly to study design, followed by Bonferroni post hoc test.

**Results**

**Transgenes Expression Levels**

The constructs used for tissue-specific expression of Emilin1 cDNA are sketched in Figure 1. The expression of transgenic mouse cDNA transcripts relative to the mRNA levels produced from 1 copy of endogenous Emilin1 gene is reported in the Table for the different lines. For further studies, the lines with the highest expression in aorta were chosen for constructs with the Emilin1 and SM22a promoters (lines Emilin1-Emilin1.73 and SM22a-Emilin1.165, respectively). Among the various Tie2-Emilin1 lines, 2 were chosen: one (number 73) among the high expressing and the other (number 71) among the low expressing ones.

The levels of Emilin1 mRNA produced in the aorta of some transgenic mouse lines were comparable with those of the
endogenous gene. This was the case for the Emilin1–Emilin1.73 line, with about the same expression of 1 chromosomal gene copy, and the Tie2–Emilin1.71 line. The latter produced about one half of the mRNA expressed by 1 copy of endogenous gene; however, it should be considered that the normal gene is synthesized by both ECs and VSMCs, while the Tie2 transgene is active only in ECs. Assuming an equivalent production of Emilin1 mRNA by ECs and VSMCs on a per cell basis, and a VSMCs/ECs number ratio of =5 (the number of VSMC layers in aorta is 5–6, while there is only 1 EC layer), the calculated actual excess of transgene Emilin1 mRNA compared with 1 normal gene copy in ECs is 2.5 times, a range close to the physiological levels expressed by wild-type mice. On the other hand, the level of expression of transgenic Emilin1 mRNA attained with the SM22a promoter was low in aorta; however, the immunofluorescence analysis (see below) showed a stronger positive staining for Emilin1 in resistance arteries when compared with aorta, suggesting a higher level of expression of the protein in vessels that give a major contribution to BP regulation.

Transgenes Expression Patterns

The appropriate tissue-specific expression of the transgenes carrying the mouse Emilin1 cDNA was tested by immunohistochemistry in an Emilin1−/− background. The expression of the transgenic Emilin1 promoter in embryos reproduced the complexity expected from the distribution of endogenous gene products (Figure I in the online-only Data Supplement).1,3,9 Matching of this promoter activity with that of the endogenous gene was investigated in mice harboring the human EMLIN1 cDNA in Emilin1−/− embryos. As shown in Figure II in the online-only Data Supplement, distribution of the transgenic protein extensively overlapped with that produced from the chromosomal gene. Analysis of adult blood vessels also revealed a good correspondence of transgenic and endogenous protein deposition in both conductance and resistance arteries (Figure III in the online-only Data Supplement). Expression of the Tie2 promoter was strictly confined to ECs in both the mouse lines investigated, namely Tie2–Emilin1.71 (Figure IV in the online-only Data Supplement) and Tie2–Emilin1.73 (data not shown). As for the SM22a line, the transgenic protein was poorly expressed during development (data not shown), while it could be detected in adult blood vessels where labeling was not associated with the endothelium (Figure V in the online-only Data Supplement and Table). Taken together, these data show that the transgenes generated with the Tie2 and SM22a promoters correctly target the expression of Emilin1 cDNA in ECs and VSMCs, respectively, while the construct containing Emilin1 promoter directs production of recombinant protein to the whole arterial wall.

To test whether expressed transgenes were functional, we searched for reversal of blood vessels morphological alterations induced by Emilin1 deficiency. Although no ultrastructural defects were detected in resistance arteries (data not shown), aorta showed irregular outline and fragmentation of elastic lamellae, frequent blebbing, and detachment of ECs from the subendothelial extracellular matrix and atrophic, sometimes necrotic, VSMCs surrounded by enlarged extracellular space and disrupted adhesions of VSMCs to the elastic lamellae5 (Figure VI in the online-only Data Supplement). The presence of the Emilin1–Emilin1.73 transgene reversed the mutant morphology to a normal-looking ultrastructure of the vessel wall (Figure VI in the online-only Data Supplement). In particular, alterations of ECs and VSMCs dropped from 32% and 14% to 0.4% and 0.3%, respectively (Table I in the online-only Data Supplement). Re-expression of Emilin1 in the endothelium (line Tie2–Emilin1.71) considerably reduced alterations of ECs (Table I and Figure VI in the online-only Data Supplement) and also attenuated the defects of the media to some extent (Table I and Figure VI in the online-only Data Supplement). The morphology of the internal

### Table. Characterization of Transgenic Mouse Lines Generated With Different Emilin1 cDNA Constructs

<table>
<thead>
<tr>
<th>Transgenic Mouse Line*</th>
<th>Transgene Copy Number</th>
<th>Aorta</th>
<th>Heart</th>
<th>Lung</th>
<th>Bladder</th>
<th>Kidney</th>
<th>Brain</th>
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</thead>
<tbody>
<tr>
<td>Emilin1–Emilin1.39</td>
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<td>0.02</td>
<td>0.06</td>
<td>0.05</td>
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<td>n.d.</td>
<td>n.d.</td>
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<td>1.6</td>
<td>1.6</td>
<td>—</td>
<td>0.05</td>
</tr>
<tr>
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<td>0.2</td>
<td>0.12</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
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<td>0.1</td>
<td>0.5</td>
<td>0.5</td>
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<tr>
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<td>0.5</td>
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<td>2.6</td>
</tr>
<tr>
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<td>2.6</td>
<td>5</td>
<td>12</td>
<td>1.2</td>
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<td>4</td>
</tr>
<tr>
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<td>1.5</td>
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<td>3.5</td>
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<td>1</td>
<td>2.5</td>
<td>4</td>
<td>0.2</td>
<td>0.5</td>
<td>0.5</td>
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<tr>
<td>Tie2–Emilin1.136</td>
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<td>n.d.</td>
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<tr>
<td>SM22a–Emilin1.15</td>
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<td>—</td>
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<tr>
<td>SM22a–Emilin1.165</td>
<td>6</td>
<td>0.1</td>
<td>—</td>
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<td>—</td>
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<td>—</td>
</tr>
</tbody>
</table>

n.d. indicates not detectable; —, not done.

* Tie2 and the SM22a promoters drive expression in endothelial cells and smooth muscle cells, respectively,11,12 while the Emilin1 regulatory region is active in both cell types.9

† The relative amount of mRNA from the transgene and the endogenous gene was assessed by reverse transcription-polymerase chain reaction in transgenic mice with the Emilin1−/− background, because of the presence of a SalI restriction site that distinguishes the transgene cDNA (see Methods).
elastic lamella was slightly improved, whereas the alterations of media elastic lamellae remained (Table I in the online-only Data Supplement). A similar result was obtained with the line Tie2-Emilin1.73 (data not shown). Production of Emilin-1 only in VSMCs (SM22a-Emilin1.165; Emilin1−/−) significantly reduced the abnormal morphology of the media, although not as efficiently as the Emilin1-Emilin1 transgene (Table I and Figure VI in the online-only Data Supplement). Interestingly, it also ameliorated ECs alterations and improved internal elastic lamella morphology (Table I and Figure VI in the online-only Data Supplement). These data show that the transgenic proteins are functional.

**Transgene Expression in VSMCs, but Not ECs, Rescues the Hypertensive Phenotype of Emilin1−/− Mice**

As shown in Figure 2 and in Figure VII in the online-only Data Supplement, telemetric and tail cuff monitoring of hemodynamics indicated that Emilin1−/− mice displayed higher BP, confirming our previous observation.2 Expression of transgenic Emilin1 cDNA under Emilin1, Tie2, or SM22a promoters in the wild-type background did not have any influence on BP (data not shown). On the contrary, when cDNA was expressed in the Emilin1 null background, the effect on BP depended on the cell type in which the promoter was active. In particular, BP was normalized when the VSMC-specific SM22a promoter (line SM22a-Emilin1.165) was used, but not when the transgene was driven by the EC-specific Tie2 promoter (lines Tie2-Emilin1.71 and Tie2-Emilin1.73) (Figure 2 and Figure VII in the online-only Data Supplement). Furthermore, we showed that, as expected, the Emilin1−/− mice hypertensive phenotype was rescued when the transgene was driven by the Emilin1 promoter (Figure 2 and Figure VII in the online-only Data Supplement). Interestingly, the percentage of P-Smad3-positive VSMCs nuclei, a marker for TGF-β signaling, followed a pattern of variation similar to that of hypertension, being higher in Emilin1−/− and Tie2-Emilin1.73; Emilin1−/− lines and comparable to controls in Emilin1-Emilin1.73; Emilin1−/− and SM22a-Emilin1.165; Emilin1−/− mouse lines (Figure VIIIA in the online-only Data Supplement). Instead, the P-Smad3-positive nuclei of ECs increased not only in the Emilin1 knockout but also in the line SM22a-Emilin1.165; Emilin1−/−, while they remained similar to controls in lines Tie2-Emilin1.73 and Emilin1-Emilin1.73; Emilin1−/− (Figure VIII in the online-only Data Supplement). Because the difference among groups was rather low (20%–25%), global P-Smad2 was also assayed by immunoblotting in Emilin1-deficient and control arteries to assess the maximum variation. The average induction was ≈50% (Figure VIIIIB and VIIC in the online-only Data Supplement). The difference obtained with the 2 methods is likely attributable to higher background of the former (immunoperoxidase) or lower background of the latter (immunoblotting) methods, respectively. Overall, the above data strongly suggest that Emilin-1 must be expressed in VSMCs to modulate BP levels and further link hypertension of Emilin1−/− animals to increased TGF-β signaling.

**Anti-TGF-β Treatment Rescues Emilin1−/− Hypertensive Phenotype**

It was previously found that inactivation of 1 TGF-β1 allele rescued the hypertensive phenotype of Emilin1−/− mice.4 This result, however, could be the consequence of the reversal of a vascular developmental defect induced by increased TGF-β signaling because of lack of Emilin-1 or an enduring alteration of VSMCs behavior triggered by enhanced TGF-β activity. Treatment of Emilin1−/− mice with neutralizing antibodies to TGF-β should distinguish between these 2 possibilities, as a vascular developmental defect is expected to be irreversible, whereas a functional alteration of VSMCs is not. When a neutralizing anti-TGF-β antibody was administered to Emilin1−/− mice, a BP lowering effect was already evident after 6 days, followed by a complete normalization of BP within 15 days (Figure 3). The BP effect of anti-TGF-β antibody was not detected in nitric oxide synthase-deficient mice, a different model of hypertension, indicating that the antihypertensive effect was selective for Emilin1−/− mice (Figure 3). The effectiveness of the neutralizing antibody in lowering TGF-β signaling was indicated by the reduction of percentage of P-Smad3-positive nuclei in VSMCs in both Emilin1-deficient and wild-type mice, although the difference did not reach statistical significance in the latter group (Figure IX in the online-only Data Supplement). The conclusion coming from the above data is that increased TGF-β activity is continuously required for maintenance of the hypertensive phenotype of Emilin1−/− mice.

**Emilin-1 in VSMCs Is a Regulator of Arteriolar Myogenic Response Through TGF-β**

Our previous data suggested that hypertension is a primary abnormality in Emilin1−/− mice, attributable to increased peripheral vascular resistance.4 Resistance arteries have the
specific property, called arteriolar myogenic response, to constrict in response to stepwise increases in perfusion pressure. The arteriolar myogenic response is known to be mediated by Ca\(^{2+}\) handling in VSMCs and is a key element for BP maintenance. Thus, we examined the effect of increasing the intra-arterial pressure in 2 different resistance arteries, mounting in a pressure myograph second-order mesenteric arteries and gracilis muscle arteries from "Emilin1−/−" and "Emilin1+/+" mice. The myogenic response was calculated for each pressure step as the difference between passive and active diameters (measured in Ca\(^{2+}\) free and in normal physiological salt solution, respectively). Despite slightly reduced passive diameters in a Ca\(^{2+}\)-free bathing solution, "Emilin1−/−" arteries had much narrower diameters in the presence of Ca\(^{2+}\) when compared with control arteries (Figure X in the online-only Data Supplement), indicating increased myogenic response in the mutant arteries (Figure 4A and Figure XI in the online-only Data Supplement). Interestingly, the SMC-specific SM22α-Emilin1 transgene (in addition to the "Emilin1−/−"-Emilin1-construct) restored to normal levels the elevated myogenic response developed by "Emilin1−/−" arteries, whereas the EC-specific Tie2-Emilin1 transgene maintained an elevated myogenic response (Figure 4). Thus, the same transgenes that rescued hypertension of "Emilin1−/−" mice also reversed the abnormal vascular myogenic response in mutant vessels, suggesting a direct association between increased myogenic response and development of hypertension.

Then, we examined the response of resistance arteries to neurohumoral vasoconstrictors. In contrast to myogenic tone, amplitudes of the contraction were comparable between "Emilin1−/−" and "Emilin1+/+" mesenteric arteries, for both phenylephrine and angiotensin (Figure XII A and XII B in the online-only Data Supplement). Moreover, neither the SMC- nor the EC-specific Emilin1 transgenes affected the contractile response to vasoactive agents in "Emilin1−/−" arteries (Figure XII A and XII B in the online-only Data Supplement).

The uncoupling of agonist-induced vasoconstriction from the hypertensive phenotype of "Emilin1−/−" mice suggests a major role of arteriolar myogenic response in determining higher BP levels in resting conditions. We therefore investigated whether decrease of BP of "Emilin1−/−" mice by antibodies to TGF-β was also accompanied by the reduction of myogenic response. As shown in Figure 5A, anti-TGF-β antibody infusion were other than the ones in resistance arteries, we evaluated cardiac and large vessel function, finding no changes induced by the treatment. In particular, cardiac function was comparable among groups (Table II in the online-only Data Supplement), as was arterial pulse wave velocity of aorta and carotids (Table III in the online-only Data Supplement), an index of arterial stiffness, thus excluding that BP lowering effect could be ascribed to changes in vascular compliance.

To finally disclose whether the consequence of neutralizing antibodies on myogenic tone was attributable to a direct effect of TGF-β on resistance arteries or to indirect in vivo effects, we applied directly the antibody on mesenteric arteries during myogenic response measurements. As shown in Figure 5B, the treatment of mesenteric arteries of "Emilin1−/−" with neutralizing antibody to TGF-β rescued the increased myogenic response to perfusion pressure.

**Discussion**

To gain insight into the mechanism of the "Emilin1−/−" phenotype, characterized by systemic hypertension, we used a phenotype rescue approach comprising re-expression of the protein in specific vascular cell types. The message coming from our study is that Emilin-1 expressed in VSMCs controls BP by regulating the arteriolar myogenic response to mechanical stress. The maintenance of increased myogenic tone is dependent on continuous higher levels of TGF-β activity in VSMCs, as indicated by the rescue of the hypertensive phenotype and enhanced myogenic tone by lowering TGF-β signaling using neutralizing antibodies.
TGF-β signaling may therefore be because of the high background of the method, which has the advantage of giving separate information for VSMCs and ECs, or the lower activation of Smad3 compared with Smad2, that were the targets in immunoperoxidase and Western blotting experiments, respectively. One may also wonder about the finding that anti-TGF-β treatment reduced BP only to wild-type levels in Emilin1−/− mice and had no significant effect in control animals. However, it should be pointed out that the dose of antibodies infused into animals was the lowest dose effective in normalizing BP, among several tested in trial experiments.

The rescue of the hypertensive phenotype of mutant mice using TGF-β neutralizing antibodies prompted us to look for functional alterations of mutated vessels that could be responsible for the hypertensive phenotype. Indeed, our previous data, showing narrower vessels in Emilin1−/− mice, suggested that the hypertensive phenotype was likely caused by developmental perturbation of vessel size.4 However, the present work clarifies that the narrowing in lumen size of Emilin1−/− vessels is recruited by the arteriolar myogenic response that is the functional contractile response to perfusion pressure. Indeed, we found that resistance arteries of Emilin1−/− mice exhibited an increased myogenic response to mechanical stress induced by stepwise pressure increase, in experimental setting completely devoid of any neurohumoral influence. Interestingly, the phenotype rescue experiment with SMC- and EC-specific transgenes revealed that the increased myogenic response of Emilin1−/− mice was dependent on the lack of Emilin-1 in VSMCs, in line with what was observed for the hypertensive phenotype. It is noteworthy to emphasize that arteriolar myogenic response to perfusion pressure is a pivotal element in BP control and is a peculiar feature of VSMCs.10 We should also note that in our previous data we found that lumen of Emilin1−/− arteries was reduced when compared with control and that this effect was more prominent with steady increase in perfusion pressure.4

We also demonstrated that the increased myogenic response of Emilin1−/− arteries is dependent on TGF-β signaling, because treatment with neutralizing antibody, both in vivo and ex vivo, was effective to restore this response to normal levels. Altogether, previous and present data demonstrate that lack of by ex vivo treatment with neutralizing antibodies to TGF-β. Normalization of myogenic tone of Emilin1−/− vessels by ex vivo treatment with neutralizing antibodies to TGF-β. Resistance arteries from wild-type and Emilin1−/− mice were mounted in a pressure myograph and neutralizing antibody to TGF-β or PI IgG added to the buffer (0.01 ng/mL). After 30 minutes of incubation, the myogenic response was measured. *P<0.05 vs Emilin1−/−+PI IgG.

**Figure 5.** Dependence of myogenic response on transforming growth factor (TGF)-β activity in resistance arteries. A, Anti-TGF-β treatment abolished the altered myogenic tone of Emilin1−/− arteries. Neutralizing antibodies to TGF-β or preimmune IgG (PI IgG) were administered for 24 days to control and mutant mice. *P<0.01, Emilin1+/+ vs Emilin1−/−+PI IgG; §P<0.001, Emilin1−/−+PI IgG vs Emilin1−/−+anti-TGF-β. B, Normalization of myogenic tone of Emilin1−/− vessels in normalizing BP, among several tested in trial experiments.

The first finding of our work suggests that, for regulation of BP, Emilin-1 is strictly required in VSMCs. This deduction is straightforward in the light of 2 considerations. On one hand, the experimental setup used was appropriate to address the question regarding the cell type mainly involved in the regulation of BP by Emilin-1. Indeed, the regulatory sequences used to drive protein expression in the null mutant background exhibited a high degree of cell type specificity in both conductance and resistance arteries. On the other hand, BP measurement showed that the hypertensive phenotype of Emilin1−/− mice was only rescued by transgenes including VSMCs as expression targets (ie, those with Emilin1 and SM22a regulatory sequences). On the contrary, EC-specific production of recombinant Emilin-1 did not rescue BP mutant phenotype, although the expression levels of mRNA achieved in the 2 Tie2-Emilin1 lines tested were comparable to or higher than that of the endogenous gene.

In our previous article, we demonstrated that lowering TGF-β levels by a genetic approach, obtained by crossing Emilin1−/− with Tgfb1−/− mice, is sufficient to rescue the vascular defects and the hypertensive phenotype of Emilin1−/− animals.3 However, that strategy did not allow us to distinguish between a structural and a functional effect of TGF-β on BP control. Here, we report that the hypertensive phenotype is dependent on functional alterations induced by TGF-β activity, as afforded by BP lowering effects of pharmacological anti-TGF-β treatment. Compared with the genetic approach, the method used here tells us not only that TGF-β regulation is key to Emilin1−/− hypertension but also that increased TGF-β activity must be continuously operating to maintain higher BP. The difference of TGF-β signaling between wild-type and Emilin1 mutant vessels, assessed by the percentage of P-Smad3–positive nuclei after immunoperoxidase staining, may appear too small (20%–25%) to account for a considerable change in resistance vessel function (15 mm Hg–20 mm Hg difference in BP). Using immunoblotting to P-Smad2 on a relevant number of arterial extracts, variation of TGF-β signaling was found to be about double (50% higher). The small difference detected with immunoperoxidase staining may therefore be because of the high background of the method, which has the advantage of giving separate information for VSMCs and ECs, or the lower activation of Smad3 compared with Smad2, that were the targets in immunoperoxidase and Western blotting experiments, respectively. One may also wonder about the finding that anti-TGF-β treatment reduced BP only to wild-type levels in Emilin1−/− mice and had no significant effect in control animals. However, it should be pointed out that the dose of antibodies infused into animals was the lowest dose effective in normalizing BP, among several tested in trial experiments.
Emilin-1 in VSMCs, by deranging TGF-β signaling, increases the gain of vasoconstriction to perfusion pressure, thus resulting in higher BP levels. Because a main role in myogenic vascular contraction is played by Ca\(^{2+}\) influx into cells, it is conceivable that the role of Emilin-1 in VSMCs may be to control Ca\(^{2+}\) regulation through TGF-β signaling.

On the contrary to what was observed for myogenic response, the unaltered vasoconstriction to neurohumoral agents was comparable in Emilin1\(^{-/-}\) and control vessels and remained unaffected by re-expression of either SMC- or EC-specific transgenes in Emilin1\(^{-/-}\) animals, suggesting negligible contribution of this factor to hypertension of Emilin1 mutants and highlighting a major role of myogenic response for the establishment of resting BP levels. In conclusion, the present study clarifies that Emilin-1 produced by VSMCs, by modulating the bioavailability of TGF-β, acts as a main regulator of resting BP levels by controlling the myogenic response in resistance arteries.

Sources of Funding
The work was supported by grants from Agenzia Spaziale Italiana (DCMC Program, workpackage 181119, to G.M. Bressan), Telethon (Project number GGP06066, to G.M. Bressan), the Italian Ministero dell’Istruzione dell’Università e della Ricerca (PRIN 2007, to G.M. Bressan), and the Italian Ministero della Salute (2006, to G. Lembo).

Disclosures
None.

References
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Arterioscler Thromb Vasc Biol. 2012;32:2178-2184; originally published online July 19, 2012; doi: 10.1161/ATVBAHA.112.254664

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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SUPPLEMENTAL MATERIAL

Vascular Smooth Muscle Emilin-1 is a regulator of arteriolar myogenic response and blood pressure

Litteri et al.: Vascular smooth muscle function in Emilin1−/− hypertension

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Nomenclature

The following Emilin/Multimerin gene and proteins symbols have been used in this manuscript:

<table>
<thead>
<tr>
<th>Emilins gene and protein symbols</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Emilin1</em></td>
</tr>
<tr>
<td><em>EMILIN1</em></td>
</tr>
<tr>
<td>Emilin-1</td>
</tr>
<tr>
<td>EMILIN-1</td>
</tr>
<tr>
<td>Emilin1/EMILIN1</td>
</tr>
<tr>
<td>Emilin-2</td>
</tr>
<tr>
<td>Multimerin-1</td>
</tr>
<tr>
<td>Multimerin-2</td>
</tr>
</tbody>
</table>

For nomenclature of the entire Emilin/Multimerin gene family see http://www.genenames.org/genefamily/emilin.html.
Supplemental Methods

DNA constructs

Mouse Emilin1 cDNA was derived by fusion of two fragments obtained by RT-PCR from total RNA purified from C57BL/6NCrl mouse embryos (13.5 days post coitum). cDNA first strand was synthesized with SuperScript III Reverse Transcriptase (Invitrogen) and the two fragments amplified with Expand High Fidelity PLUS PCR System (Roche) using the following primers: CTCTAAGGGCAGCAAGGAA (forward) and TGGCTCCTAGCACTTGC (reverse) for the 5’ fragment; GAGAACGTATGCCTCCTAT (forward) and GGCCCCTGTAAGATAAGTTTG (reverse) for the 3’ fragment. The two fragments, that extend from base -28 to +1150 (length 1178) and from base + 982 to +3093 (length 2112) from the translation start site and include a SalI site at +1079, were digested with SalI and fused by cloning into pGEM-T Easy (Promega).

The full length Emilin1 cDNA was then removed by EcoRI and inserted into the StuI site of pCS2 vector after blunting the ends with Klenow enzyme.

The derivation of the human cDNA was reported previously. For the synthesis of the Emilin1-Emilin1 construct, a 16.8 kb HindIII/HindIII fragment, here called K17, was subcloned from a 135 kb BAC, derived from a 129/SvJ genomic library (Genome Systems, Inc.), into the vector pBlueScript II KS+ (Addgene). The fragment contains the entire Emilin1 gene, the 5’- and 3’-flanking sequences and portions of the contiguous 2310016E03Rik and Khk genes (Figure 1). A fragment extending from the BamHI site at -402 to -1 from the transcription start site was amplified by PCR from K17 and inserted into the BamHI and XbaI sites of pBlueScriptIIKS+. A fragment of K17 extending from the XhoI to the BglII sites (+79 to +2650) was cloned 3’ to the 403 bp fragment leaving Clal and NotI sites between the two fragments. In the next step, the Emilin1 cDNA with the polyadenylation signal sequence was excised from pCS2 and introduced between these two sites. Finally, the Emilin1 cDNA with the polyadenylation signal sequence was excised from pCS2 and introduced between these two sites. The Emilin1 cDNA and the sequence form +79 to the BamHI site +1300 was inserted into K17 from which the BamHI/BamHI fragment (nucleotides -402 to +1300) had been removed, thus producing the Emilin1-Emilin1 construct.

The synthesis of the Tie2-Emilin1 construct started with the cloning into pBlueScriptIIKS+ of two fragments derived from a construct donated by Dr. T. Sato. The Emilin1 cDNA was then cloned between these two fragments. The 2.1 kb HindIII/HindIII fragment at 5’ contained the Tie2 promoter, while the 9.8 kb Nael/SalI fragment at 3’ included the Tie2 enhancer that activates transcription in adult endothelial cells (ECs). The SM22a-Emilin1 construct was derived by inserting a 2.1 kb fragment including the SM22a promoter sequence (gift of Dr. JM Miano) in front of the Emilin1 cDNA. All constructs were sequenced in both directions to verify correct cloning.

Generation and characterization of transgenic mice

Transgenic mouse lines were produced from B6D2F1 females mated with B6D2F1 males (Charles River Italy) by pronuclear DNA microinjection using standard procedures. The mice were genotyped by PCR of DNA purified from tail biopsies using the following primers: forward primers, 5’TTCAGCCTCATTCTGACCACACT3’ and 5’GCTACCCCTCCTGAGGTTTGC3’, matching sequences within the Emilin1 and the EMILIN1 cDNAs, respectively; reverse primer, 5’TTTTCAGCTCATTCTAGTTG3’, derived from the SV40 polyadenylation signal sequence.

The number of copies of the transgene was determined by exploiting the presence of a C/A polymorphism in exon 4 (in the cDNA sequence this polymorphism maps at position +1083 from the translation initiation site). The presence of A introduces a SalI site in most mouse strains with the exception of the 129 strain, in which a C is present (tested strains were...
C57BL/6NCrl, DBA, CD1 and 129S2/SvPasCrlf). To develop the transgene in a background lacking the SalI site, founders were crossed with 129S2/SvPasCrlf mice (Charles River Italy). Aliquots of tail DNA were amplified by PCR (forward primer: 5'-TCTTGCTCAGTGTGCCTGAC-3'; reverse primer: 5'-CAGCTTTTCTAGCCGTCTCTG-3') and the resulting fragment (445 bp) digested with SalI, a treatment that splits the molecular species derived from the transgene into two fragments (270 and 175 bp), while leaving unaltered the one generated from the endogenous gene. Densitometric quantitation of the intensity of the three bands (Kodak 1D Image Analysis Software) allows calculation of the relative abundance of the transgene compared with one copy of the endogenous gene. The number of copies was calculated using the formula I_{270} + I_{175}/(I_{445}/2) for Tie2-Emilin1 and SM22a-Emilin1 lines and I_{270} + I_{175}/(I_{445} - (I_{270} + I_{175}))/2 for the Emilin1-Emilin1 lines to take into account the presence of the additional exon 4 sequence lacking the SalI site in the transgene (I_{number} is the intensity, in arbitrary units, of the dsDNA band with the corresponding nucleotide length).

Transgenic founders were mated with Emilin1−/− animals that were the product of 10 or more backcrossings with C57BL/6NCrl, Charles River Italy. The resulting Emilin1 heterozygous mutants were intercrossed to derive mice with different genotypes, i.e. with or without the transgene and Emilin1+/+ or Emilin1−/−. Similarly, transgenic mice were mated with eNOS−/− mice and animals derived from eNOS heterozygous intercrosses were used for experiments.

Total RNA was extracted from organs from different transgenic mice with the 129S2/SvPasCrlf background and heterozygous for the Emilin1 null mutation using TRIzol reagent (Gibco-BRL). The relative proportion of mRNA transcribed from the normal Emilin1 gene and from the transgenes was calculated after RT-PCR with the above primers, digestion with SalI and densitometry of the resulting bands. This was possible as the Emilin1 null allele does not express any stable mRNA species.2

As important note to the above procedures, it should be stressed that crosses of transgenic lines with 129 mice were used only to measure the gene copy number and the expression levels of transgenes relative to the endogenous gene, while BP measurements and characterization of vascular reactivity were carried out exclusively in mice developed in the C57BL/6NCrl background. This point is relevant, as BP varies in different mouse genetic backgrounds.

**Blood Pressure Monitoring**

To telemetrically monitor arterial blood pressure (BP), the PA-C10 pressure transmitter (Data Sciences International, St Paul, USA.) was implanted into a mouse under anesthesia with ketamine/xylazine. The pressure-sensing catheter was inserted into the femoral artery, and the transmitter body was placed in a subcutaneous pouch on the back. Following the surgery, the mice were allowed to recover for at least 1 week. Radio signals from the implanted transmitter were captured by the Physiotel RPC-1 receiver (Data Sciences International), and the data were stored online using the Dataquest Ponemah 4.9 data acquisition system (Data Sciences International). BP was monitored in 10-sec episodes at 5-min intervals for 7 days.

**Ultrasound analysis**

Echocardiographic analysis was performed in mice anesthetized with tribromoethanol (175 mg/kg), using a Vevo 2100 device equipped with 18-38MHz linear-array transducer with a digital ultrasound system (Visualsonics inc., Canada). A left ventricular M-mode tracing was obtained using the 2D parasternal short axis imaging as a guide. End-diastolic interventricular septum (IVSd), posterior wall thicknesses (LVPWd), and left ventricular internal diameter (LVIDd) were measured. Relative wall thickness (RWT), ejection fraction (EF%) and fractional shortening (FS%) were calculated according to standard formulas. The E/E’ ratio
was used to assess diastolic dysfunction. E wave peak was measured placing PW Doppler on mitral valve in 4 chamber view. E’ wave peak was obtained measuring Tissue Doppler peak on the mitral annulus in 4 chamber view.

For arterial stiffness evaluation, we determined both mean and peak arterial pulse wave velocity on ascending and descending aorta and in right and left carotid arteries.

**Vascular function evaluation**

Second-order mesenteric arteries or gracilis muscle arterioles were dissected free of connective tissue from anesthetized mice and mounted onto two glass micropipettes (80 or 60 µm, respectively) in a vessel chamber and pressurized from 25 to 125 mmHg using a pressure myograph (DMT Instrumentation). Cannulated arterial segments were submerged in 10 mL of Krebs solution (NaCl 114.13 mM, KCl 4.69 mM, MgSO₄·7H₂O 2.51 mM, KH₂PO₄ 1.18 mM, NaHCO₃ 25 mM, C₆H₁₂O₆ 5.99 mM, CaCl₂ 2.5 mM) pH 7.4, oxygenated with 5% CO₂ and 95% O₂. Vessels were equilibrated for 30 minute at 50 mmHg.

To enable the calculation of arterial myogenic tone, response to pressure was measured (25 mmHg increments, starting at 25 mmHg) with vessels maintained at each pressure step for 20 min. At the end of the experiment, the vessel was perfused with Ca²⁺-free buffer and the pressure steps were repeated. Myogenic tone was calculated as \[\frac{(\text{passive diameter} - \text{active diameter})}{\text{passive diameter}} \times 100\].

Concentration-response curves were performed for PE (from \(10^{-9}\) mol/L to \(10^{-5}\) mol/L) and for AngII (from \(10^{-9}\) mol/L to \(10^{-5}\) mol/L). Data are expressed in µm as (diameter of the vessel in the presence of agonist – AD in the absence of agonist) at 75 mmHg.

**Immunohistochemistry**

Cryostat sections (7 µm) from 14.5 days old embryos and aorta or second branch mesenteric artery from 3 months old mice were treated for 1 h at room temperature with 5% normal goat serum in phosphate buffered saline (PBS) and then incubated for 2 h at room temperature or overnight at 4°C with the primary antibody diluted in the same buffer. After washing in PBS, the secondary antibody was applied for 1 h at room temperature. The slides were washed in PBS, mounted in 80% glycerol in PBS and observed in a Zeiss Axioplan microscope equipped with epifluorescence optics or in a Bio-Rad confocal microscope. A rabbit polyclonal antibody to mouse Emilin-1 (gift of Dr. D. Forrest) and mouse monoclonal antibody 6A5 to human EMILIN-1 were used as primary reagents. Secondary reagents included Cy2- or Cy3-conjugated goat antibody against rabbit or mouse IgG (Jackson Immuno Research). Some sections were counterstained with Cytox green (Invitrogen) (0.167 µM solution).

For immunoperoxidase staining with antibodies to P-Smad3, second branch mesenteric arteries were dissected with the adjacent tissues and immediately placed in a solution of 10% paraformaldehyde (Sigma P6148) in PBS. After 24 hours of fixation at 4°C they were extensively washed and processed for paraffin embedding. 5-7 µm cross section were cut and placed on Superfrost Plus glass slides (Thermo Scientific). The retrieval of the antigen was performed by means of a 20 minutes treatment in a steamer at the approximate temperature of 110°C in the presence of 10 mol/L citrate buffer, pH 6.0. The sections were subsequently treated with a mixture of methanol and oxygen peroxide (0.3%) for 30 minutes and a solution of 10% goat serum in PBS. The antibody [Epitomics Phospho Smad3 (pS423/425), rabbit monoclonal antibody cat. # 1880-1] was diluted 1:400-1:800 in 5% goat serum in PBS and applied for 12 hours at 4°C. After extensive washing the sections were incubated with a rabbit IgG specific secondary antibody conjugated with horseradish peroxidase (Vectastain ABC system, PK-6101, Vector Laboratories) according to the protocol indicated by the company. The staining was obtained incubating the sections with a solution of diaminobenzidine (0.05%) and hydrogen peroxide (0.03%) in 50 mol/L Tris Buffer pH 7.5,
for 1 hour at room temperature in the dark. Nuclei were stained with SYTOX green dye (Invitrogen 0.2 µg/ml in water) for 15 minutes at room temperature. The photographs were acquired with a Zeiss Axiophot Microscope equipped with a Leica DC500 digital camera.

**Electron microscopy**

Aortas were dissected from 3 months old mice (2 males for each group) and samples were fixed with 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.4) overnight, washed in cacodylate buffer overnight, and treated with 2% tannic acid in cacodylate buffer for 1h a 4°C. All samples were dehydrated with ethanol and embedded in Epon E812. Ultrathin sections were stained with lead citrate and uranyl acetate, and observed in a Philips EM 400 transmission electron microscope. For quantitative evaluation of altered ECs and vascular smooth muscle cells (VSMCs), all cells present in a section were examined and the percentage of abnormal cells calculated. At least 3 sections 100 µm apart from the two aortas were analyzed.

**Immunoblotting**

The mesenteric arterial tree was freed of connective and adipose tissue, large arterial trunks removed and smaller arteries (first and higher order branches) frozen in liquid nitrogen, pulverized in a mortar in the presence of liquid nitrogen and extracted in RIPA buffer (20 mM Tris-HCl pH7.5, 150 mM NaCl, 1 mM EGTA, 1% NP40, 1% sodium deoxycholate) containing protease and phosphatase inhibitors (Roche, 30 µL/mesenteric artery). After 30 min incubation on ice and centrifugation, protein content of the supernatant was determined by the Protein Assay kit (Pierce). 20-40 µg were analyzed by immunoblotting using the rabbit polyclonal anti-Smad2(pSpS465/467) antibody (Cell Signaling Technology).
### Supplemental Table I. Rescue of morphological alterations of Emilin1<sup>−/−</sup> aorta by expression of Emilin1 cDNA in different cells of the arterial wall.*

<table>
<thead>
<tr>
<th></th>
<th>No transgene</th>
<th>Emilin&lt;sup&gt;−&lt;/sup&gt;-Emilin1.73</th>
<th>Tie2&lt;sup&gt;−&lt;/sup&gt;-Emilin1.71</th>
<th>SM22a&lt;sup&gt;−&lt;/sup&gt;-Emilin1.165</th>
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<tbody>
<tr>
<td>Alterations of internal elastic lamella</td>
<td>+ + + -</td>
<td>- - -</td>
<td>+ + -</td>
<td>+ + + -</td>
</tr>
<tr>
<td>Alterations of elastic lamellae of the media</td>
<td>+ + + -</td>
<td>- - -</td>
<td>+ + + -</td>
<td>+ - -</td>
</tr>
<tr>
<td>Alterations of endothelial cells</td>
<td>32±10.53</td>
<td>0.36±0.54†</td>
<td>2.5±1.5†</td>
<td>0.62±0.41†</td>
</tr>
<tr>
<td>Alterations of smooth muscle cells</td>
<td>13.86±5.03</td>
<td>0.34±0.57‡</td>
<td>7.3±1.65§,</td>
<td></td>
</tr>
</tbody>
</table>

* Thoracic aorta was dissected from Emilin1<sup>−/−</sup> mice carrying the indicated transgenes (defined in Figure 1) and processed for examination in the electron microscope. Each group comprised three mice. The alterations considered are described in detail elsewhere and included:

i) For elastic lamellae: interruptions, indentations and irregular profile.

ii) For endothelial cells: blebbing and detachment of the cells from the subendothelial layer.

iii) For smooth muscle cells: disarrangement of cell-elastic lamella connections, rounding of cell shape and presence of enlarged organelles, cell atrophy/necrosis.

The alterations of elastic lamellae were classified on an arbitrary scale from absence/very rare alterations (- - - - ) to very frequent alterations (++++), while the cell alterations are reported as the percentage of abnormal cell on the total number of cells examined (mean ± sd). The number of total cells examined is given in parentheses.

†, p< 0.01 vs. no-transgene;
‡, p< 0.001 vs. no-transgene;
§, p< 0.05 vs. no-transgene;
||, p< 0.05 vs. Emilin1-Emilin1.73.
### Supplemental Table II. Echocardiographic analysis of mice treated with neutralizing antibodies to TGF-β.*

<table>
<thead>
<tr>
<th>Group</th>
<th>IVSd</th>
<th>LVPWd</th>
<th>LVIDd</th>
<th>EF</th>
<th>E/E’</th>
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<tbody>
<tr>
<td>Emilin1+/+ PI IgG</td>
<td>0.77 ±</td>
<td>0.77 ±</td>
<td>3.62 ±</td>
<td>74.14 ±</td>
<td>20.76 ±</td>
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<td></td>
<td>0.01</td>
<td>0.01</td>
<td>0.09</td>
<td>0.68</td>
<td>0.38</td>
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<tr>
<td>Emilin1/- PI IgG</td>
<td>0.80 ±</td>
<td>0.79 ±</td>
<td>3.66 ±</td>
<td>76.29 ±</td>
<td>20.73 ±</td>
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<tr>
<td></td>
<td>0.02</td>
<td>0.03</td>
<td>0.05</td>
<td>0.96</td>
<td>0.70</td>
</tr>
<tr>
<td>Emilin1+/+ AB to TGF-β</td>
<td>0.77 ±</td>
<td>0.77 ±</td>
<td>3.64 ±</td>
<td>74.97 ±</td>
<td>20.84 ±</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>0.02</td>
<td>0.03</td>
<td>0.97</td>
<td>0.52</td>
</tr>
<tr>
<td>Emilin1/- AB to TGF-β</td>
<td>0.79 ±</td>
<td>0.78 ±</td>
<td>3.58 ±</td>
<td>75.94 ±</td>
<td>20.00 ±</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>0.01</td>
<td>0.04</td>
<td>0.73</td>
<td>0.36</td>
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</table>

*Anti-TGF-β antibodies (AB) or preimmune (PI) IgG were administered to animals (5/group) for 24 days and echocardiographic analysis performed on each of them.

### Supplemental Table III. Pulse wave velocity analysis on carotid artery and aorta.*

<table>
<thead>
<tr>
<th>Group</th>
<th>Left common carotid artery</th>
<th>Right common carotid artery</th>
<th>Ascending aorta</th>
<th>Descending aorta</th>
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<tbody>
<tr>
<td></td>
<td>Mean Vel</td>
<td>Peak Vel</td>
<td>Mean Vel</td>
<td>Peak Vel</td>
</tr>
<tr>
<td>Emilin1+/+ PI IgG</td>
<td>0.16 ±</td>
<td>0.30 ±</td>
<td>0.17 ±</td>
<td>0.28 ±</td>
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<td>Emilin1/- PI IgG</td>
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<td>0.14 ±</td>
<td>0.21 ±</td>
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<tr>
<td>Emilin1+/+ AB to TGF-β</td>
<td>0.14 ±</td>
<td>0.25 ±</td>
<td>0.15 ±</td>
<td>0.26 ±</td>
</tr>
<tr>
<td>Emilin1/- AB to TGF-β</td>
<td>0.14 ±</td>
<td>0.24 ±</td>
<td>0.14 ±</td>
<td>0.25 ±</td>
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</tbody>
</table>

*The same animals of Table II were used.
Supplemental References


Supplemental Figure Legends

Supplemental Figure I. Expression of Emilin1-Emilin1.73 transgene in the embryo. 14.5 days old embryos carrying the transgene in the Emilin1 null background were stained by immunofluorescence. The staining pattern conforms to that previously described for the Emilin1 gene. In Brain (B), the antibody labels small blood vessels (arrows). Abbreviations: a, aorta; e, esophagus; sc, spinal cord; v, atrioventricular valve; vc, vertebral column. Bars: A and D, 50 µm; B and E, 100 µm; C, 200 µm; F, 400 µm.

Supplemental Figure II. Co-distribution of the protein produced from the chromosomal gene and from the transgene. Sections from 14.5 days old mouse embryos of the line Emilin1-EMILIN1.41 in the Emilin1+/- background were processed for double immunofluorescence with a mouse monoclonal antibody against human EMILIN-1 and with a rabbit polyclonal antibody to mouse Emilin-1 and observed for the distribution of the human protein (A, D and G), the mouse protein (B, E and H) and both proteins at the same time (C, F and I). A-C) Aorta and pulmonary artery. Bar, 100 µm. D-F) Brain. Small blood vessels are stained. Bar, 50 µm. G-I) Lung. Bar: 100 µm.

Supplemental Figure III. Expression of recombinant protein in adult blood vessels of transgenic line Emilin1-Emilin1.73;Emilin1+. Sections derived from 3 months old wild-type mice (A and B), mice carrying the transgene Emilin1-Emilin1.73 in the Emilin1 null background (C and D) and Emilin1 null mice (E and F) aorta (A, C and E) and second branch mesenteric artery (B, D and F) were stained by immunofluorescence with antibodies to mouse Emilin-1 and observed for the distribution of the human protein. A-C) Aorta and pulmonary artery. Bar, 100 µm. D-F) Brain. Small blood vessels are stained. Bar, 50 µm. G-I) Lung. Bar: 100 µm.

Supplemental Figure IV. Expression of recombinant protein in blood vessels of transgenic line Tie2-Emilin1.73;Emilin1+. Sections were prepared from mice carrying the transgene Emilin1-Emilin1.73 in the Emilin1 null background (D-I) and Emilin1 null mice (A-C) aorta and (E and F) second branch mesenteric artery from 3 months old animals. Nuclei were counterstained with Cytox green. The protein is localized only at the basal surface of ECs. l, lumen of vessel. Bars: A, 100 µm; B, 50 µm; C, 10 µm; D, 5 µm.

Supplemental Figure V. Expression of recombinant protein in blood vessels of transgenic line SM22a-Emilin1.165;Emilin1+. Sections were prepared from 3 months old mice carrying the transgene in the Emilin1 null background and stained by immunofluorescence with antibodies to mouse Emilin-1 (red). Elastic lamellae are identified by green autofluorescence. A) aorta. B) Second branch mesenteric artery. Note absence of staining at the endothelial side of the internal elastic lamella (iel) (compare with control staining of Online Figure IIIA and IIIB). l, lumen of vessel. Bar: 10 µm.

Supplemental Figure VI. Electron micrographs showing the effect of expression of different transgenes on the morphological alterations induced by Emilin1 deficiency. Aortas were dissected from Emilin1+/- mice carrying no transgene (A-C) or harboring Emilin1-Emilin1.73 (D-F), Tie2-Emilin1.71 (G-I) or SM22a-Emilin1.165 transgenes (J-L), and processed for electron microscopy. Different details were observed in the samples, including the endothelium, the internal elastic lamella and the first VSMCs layer at low (A, D, G, J) and at a higher magnification (B, E, H, K), and VSMCs and elastic lamellae of the media (C, F, I, L). Bar = 1 µm. Magnification is the same for micrographs in each row.
Supplemental Figure VII. Blood pressure determination of different transgenic mouse lines by plethysmography. The hypertensive phenotype of Emilin1⁻/⁻ mice (Emilin1⁺/⁻;No transgene) was rescued when Emilin-1 was expressed in the entire arterial wall (Emilin1⁻/⁻;Emilin1.73;Emilin1⁺/⁻) or only in VSMCs (SM22α-Emilin1.165;Emilin1⁺/⁻), but not when it was expressed only by ECs (Tie2-Emilin1.71;Emilin1⁺/⁻ and Tie2-Emilin1.73;Emilin1⁺/⁻). 5 animals were examined in each group. *, p< 0.05 vs. Emilin1⁺/⁺;No transgene.

Supplemental Figure VIII. TGF-β activity in resistance arteries from different transgenic mouse lines. A) Second branch mesenteric arteries were stained by immunoperoxidase with antibodies to P-Smad3 and the percentage of positive nuclei was determined in VSMCs and ECs. *, p< 0.05 vs. wild-type (Emilin1⁺/⁺;No transgene); #, p< 0.001 vs. Tie2 promoter containing line; §, p< 0.05 vs. Emilin1 promoter containing line. B) Representative P-Smad2 immunoblot analysis on mesenteric arteries extracts from wild-type (WT) and Emilin1⁻/⁻ (KO) mice. C) Relative levels of P-Smad2 in WT and KO mesenteric arteries. Data were derived from immunoblots obtained from 8 separate experiments in each of which different mice were used.

Supplemental Figure IX. Neutralizing antibodies to TGF-β reduce growth factor’s activity in VSMCs of resistance arteries. Mice (3 months old) were treated with either preimmune (PI) IgG or neutralizing antibodies (AB) to TGF-β in PBS for 24 days (see Figure 4) using minipumps. Second branch mesenteric arteries were processed for immunoperoxidase staining with antibodies to P-Smad3 and the percentage of positive nuclei determined. *, p< 0.05; ***, p< 0.001.

Supplemental Figure X. Pressure-diameter correlation in second branch mesenteric arteries. The diameter of vessel was measured at the indicated applied pressures in Ca²⁺-containing and in Ca²⁺-free buffer, thus deriving values for active diameter (AD) and passive diameter (PD), respectively. *, p< 0.05 vs. Emilin1⁻/⁻ PD (Two-way repeated measures ANOVA).

Supplemental Figure XI. Myogenic response in gracilis muscle artery. The measure was carried out as described in the legend to Figure 4. *, p< 0.001, Emilin1⁻/⁻ vs. wild-type.

Supplemental Figure XII. Response of vessels to neurohumoral factors. Second order mesenteric arteries mounted in a pressure myograph were maintained at an intraluminal pressure of 75 mmHg and stimulated with PE (A) or AngII (B). Reduction of diameter of arteries from mice with different genotype did not differ significantly among mice with different genotype. The results obtained with the Emilin1-Emilin1.73 transgene are not shown.
Supplemental Figures

Litteri et al., Supplemental Figure I
Litteri et al., Supplemental Figure V
Litteri et al., Supplemental Figure VI

<table>
<thead>
<tr>
<th>No transgene</th>
<th>Emilin1-Emilin1.73</th>
<th>Tie2-Emilin1.71</th>
<th>SM22-Emilin1.165</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>D</td>
<td>G</td>
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<td>I</td>
<td>L</td>
</tr>
</tbody>
</table>
Litteri et al., Supplemental Figure VII

Blood pressure (mm Hg)

Emilin1^{+/+}; No transgene
Emilin1^{-/-}; No transgene
Emilin1^{-/-}; Emilin1.73
Tie2-Emilin1.73
Emilin1^{-/-}; Emilin1.71
Tie2-Emilin1.71
SM22α-Emilin1.165
Emilin1^{-/-}; Emilin1.71

* indicates significant difference from control group.
Litteri et al., Supplemental Figure VIII

A

![Bar chart showing P-Smad3+ nuclei distribution across different genotypes](image)

B

![Western blot analysis](image)

C

![Graph showing P-Smad2/3 fold induction](image)
P-Smad3+ nuclei (%)

Emilin1+/+

Emilin1−/−

Litteri et al., Supplemental Figure IX
Litteri et al., Supplemental Figure X

![Graph showing internal diameter (µm) vs. mmHg for different conditions](image-url)
Litteri et al., Supplemental Figure XI

![Graph showing the relationship between mmHg (y-axis) and % Myogenic tone (x-axis) for Emilin1^+/+ and Emilin1^-/- groups.](Image)
Litteri et al., Supplemental Figure XII

A

\[ \Delta \text{Internal diameter (\textmu m)} \]

-50 -40 -30 -20 -10 0

\( 10^{-9} \) \( 10^{-8} \) \( 10^{-7} \) \( 10^{-6} \) \( 10^{-5} \)

- Phenylephrine (M)

- \( Emilin1^{+/+}; \) No transgene
- \( Emilin1^{-/-}; \) No transgene
- \( Tie2-Emilin1.71; \) Emilin 1 \( ^{-/-} \)
- \( SM22a-Emilin1.165; \) Emilin1 \( ^{-/-} \)

B

\[ \Delta \text{Internal diameter (\textmu m)} \]

-20 -15 -10 -5 0

\( 10^{-9} \) \( 10^{-8} \) \( 10^{-7} \) \( 10^{-6} \) \( 10^{-5} \)

- Angiotensin II (M)

- \( Emilin1^{+/+}; \) No transgene
- \( Emilin1^{-/-}; \) No transgene
- \( Tie2-Emilin1.71; \) Emilin 1 \( ^{-/-} \)
- \( SM22a-Emilin1.165; \) Emilin1 \( ^{-/-} \)