Rapamycin Inhibits Smooth Muscle Cell Proliferation and Obstructive Arteriopathy Attributable to Elastin Deficiency

Wei Li,* Qingle Li,* Lingfeng Qin, Rahmat Ali, Yiping Qyang, May Tassabehji, Barbara R. Pober, William C. Sessa, Frank J. Giordano, George Tellides

Objective—Patients with elastin deficiency attributable to gene mutation (supravalvular aortic stenosis) or chromosomal microdeletion (Williams syndrome) are characterized by obstructive arteriopathy resulting from excessive smooth muscle cell (SMC) proliferation, mural expansion, and inadequate vessel size. We investigated whether rapamycin, an inhibitor of the cell growth regulator mammalian target of rapamycin (mTOR) and effective against other SMC proliferative disorders, is of therapeutic benefit in experimental models of elastin deficiency.

Approach and Results—As previously reported, Eln−/− mice demonstrated SMC hyperplasia and severe stenosis of the aorta, whereas Eln+/− mice exhibited a smaller diameter aorta with more numerous but thinner elastic lamellae. Increased mTOR signaling was detected in elastin-deficient aortas of newborn pups that was inhibited by maternal administration of rapamycin. mTOR inhibition reduced SMC proliferation and aortic obstruction in Eln−/− pups and prevented medial hyperlamellation in Eln−/− weanlings without compromising aortic size. However, rapamycin did not prolong the survival of Eln−/− pups, and it retarded the somatic growth of juvenile Eln−/− and Eln+/− mice. In cell cultures, rapamycin inhibited prolonged mTOR activation and enhanced proliferation of SMC derived from patients with supravalvular aortic stenosis and with Williams syndrome.

Conclusion—mTOR inhibition may represent a pharmacological strategy to treat diffuse arteriopathy resulting from elastin deficiency. (Arterioscler Thromb Vasc Biol. 2013;33:00-00.)

Key Words: aorta & elastin & mTOR & smooth muscle cell & Williams syndrome

Obstructive arteriopathy is characteristic of patients with elastin deficiency caused by heterozygous mutation of the ELN gene in a disorder termed familial supravalvular aortic stenosis (SVAS; OMIM No. 185500) or by partial deletion of chromosome 7 resulting in heterozygous loss of 26 to 28 genes, including an ELN allele, in a condition termed Williams syndrome (WS; OMIM No. 194050). Although stenoses may occur in any medium-or large-sized artery, it typically affects the ascending thoracic aorta sparing its root that contains the aortic valve—hence the term supravalvular. The obstructive lesions result from medial hypertrophy attributable to increased proliferation of smooth muscle cells (SMC) together with an increased number of thinner-than-normal elastic lamellae. Lumen loss is exacerbated by underdevelopment of the affected vessels. This combination of pathological factors was described by Williams in his original description of the eponymous disease as “the obstruction was due in part to hypertrophy of the media just above the sinuses of Valsalva and in part to constriction of the aortic wall at the same level.”

A targeted mutation of Eln in mice has recapitulated key features of SVAS and WS arteriopathy that varies according to gene dosage, that is, severe stenoses attributable to excessive SMC proliferation and markedly underdeveloped aortas in homozygous animals versus no focal obstruction but modestly smaller diameter aortas with increased number of thinner elastic lamellae in heterozygous animals. The molecular mechanisms linking loss of elastin to excess SMC proliferation remain poorly understood. The impact of arterial disease in SVAS and WS is variable and ranges from life-threatening malperfusion problems in infants to asymptomatic lesions in elderly subjects, although many patients have modest-to-significant obstructive arterial lesions. Current therapy for focal arterial obstruction in these patients includes surgical reconstruction often with prosthetic patches and, for certain indications, percutaneous balloon dilatation with or without stent placement. However, there is currently no treatment for diffuse arterial lesions or to address the underlying abnormality of SMC hyperplasia in SVAS and WS.

Cellular proliferation is associated with activation of the mammalian target of rapamycin (mTOR) complex 1 that signals via downstream effectors, including p70-S6 kinase (S6K), ribosomal protein S6, and 4E-binding protein 1, to regulate...
protein translation and cell growth. Pharmacological inhibitors of mTOR, such as rapamycin, have potent antiproliferative effects in addition to immunosuppressive properties. In clinical trials, rapamycin-coated stents decrease intimal proliferation and restenosis in atherosclerotic coronary arteries, and rapamycin reduces the incidence of intimal thickening in coronary arteries of rejecting cardiac allografts. The therapeutic benefit of mTOR inhibitors in arteriosclerosis is ascribed to their potent antimitogenic effect. We hypothesized that inhibition of SMC proliferation by rapamycin will also prevent obstructive arteriopathy mediated by elastin deficiency, and we tested this strategy in experimental systems of SVAS and WS.

Materials and Methods
Details of Materials and Methods are available in the online-only Data Supplement.

Results
Murine Models of Elastin-Deficient Aortopathy
To investigate pathogenetic mechanisms and therapeutic opportunities in SVAS and WS, we used an established murine model in which a targeted mutation of exon 1 disrupts the Eln allele. As previously reported, pups homozygous for loss of Eln died by 3 days of birth with severe stenosis of the thoracic aorta sparing its root (Figure 1A), whereas heterozygote animals survived long term characterized by modestly decreased aortic diameter (Figure 1B) with an increased number of medial elastic lamellae (Figure 1C). Ultrastructural analysis of aortas from adult Eln haploinsufficient mice showed the elastic laminae to be thinner structures, whereas SMC morphology seemed normal (Figure I in the online-only Data Supplement). Thus, newborn and mature animals offer different arteriopathy end points to assess depending on gene dosage.

Increased mTOR Signaling in Elastin-Deficient Aortas Is Inhibited by Rapamycin
We examined for dysregulation of mTOR signaling, because this pathway plays a crucial role in SMC proliferation in other obstructive arterial diseases. mTOR activity was evaluated by phosphorylation of an effector, S6K. In newborn pups, the expression of phospho-S6K was markedly increased in individual Eln−/− aortas, and a similar change of lesser magnitude was noted in Eln+/− aortas (Figure 2A). We also pooled aortas from multiple newborn pups to obtain sufficient material to confirm increased activation of several other effectors of this signaling pathway, including mTOR, S6, and 4E-binding protein 1 (Figure 2B). Because aortic abnormalities are known to develop at E17.5 in this experimental model, we reasoned that effective pharmacological treatment would require in utero delivery. Given that repeated fetal administration is extremely invasive, we pursued translacental delivery of rapamycin. We confirmed that maternal administration of rapamycin at 2 mg/kg IP daily during late gestation from E16.5 to E18.5 or 19.5 (treated pregnant mice often delivered at E19 and occasionally at E20) inhibited phospho-S6K expression in the aortas of neonates on the day of birth (Figure 2C). Further rapamycin administration to lactating mothers continued to suppress mTOR signaling in the aortas of offspring until weaning at 3 weeks postpartum (Figure 2D).

Rapamycin Reduces SMC Proliferation and Aortic Obstruction in Eln−/− Mice
To assess the effects of mTOR inhibition on the obstructive arterial phenotype, rapamycin was administered to gravid Eln−/− females (mated with Eln−/− males) for the last 3 days of pregnancy, and the pups were euthanized on the day of birth for vascular analyses. SMC proliferation was assessed by uptake of the synthetic nucleoside, BrdU, administered to pregnant mice at E17.5, the time point at which medial

Figure 1. Murine models of elastin-deficient aortopathy. Mice heterozygous for a targeted mutation in Eln were interbred, and the aortas of their progeny were analyzed. A, Intraluminal blue dye injected via the left ventricle showed severe stenosis (arrows) of the thoracic aorta, with relative sparing of the aortic root, in Eln−/− pups compared with their Eln+/− and Eln+/+ littermates at P0.5 (bar, 1 mm). B, Ultrasound analysis demonstrated a smaller distal ascending aorta diameter (blue lines) in Eln−/− mice compared with Eln+/− adult littermates at 30 weeks, and the results from multiple litters are shown in the histogram (n=5–7; **P<0.01, t test). C, Elastin-Van Gieson histological stains of distal ascending aorta cross-sections (bar, 100 µm) revealed an increased number of elastic lamellae in Eln−/− versus Eln+/− adult littermates at 30 weeks and results from multiple litters are shown in the histogram (n=6; **P<0.01, Mann-Whitney test of rank transformed data).
hyperplasia attributable to elastin deficiency is known to occur. As expected, aortas from Eln−/− and Eln+/− pups had more numerous BrdU-labeled medial cells than Eln+/+ littermates from vehicle-treated animals. Treatment with rapamycin significantly diminished SMC proliferation in the ascending aortas of all littermates, such that medial BrdU frequency was significantly diminished SMC proliferation in the ascending aorta of Eln−/−, Eln+/−, and Eln+/+ littermates with the signal intensity normalized to that of β-actin shown in the histogram (n=2; *P<0.05, 1-way ANOVA).

Rapamycin Prevents Medial Hyperlamellation Without Compromising Aortic Size in Eln−/− Mice

We next tested the effects of mTOR inhibition on the more modest arterial phenotype resulting from Eln hemizygosity. To avoid inoculation of the delicate pups, rapamycin or vehicle was administered to their mothers from E16.5 of pregnancy and throughout lactation until weaning at P21. Anesthetized weanlings were examined by ultrasound. Rapamycin treatment decreased aortic diameter in Eln−/− offspring, but did not further diminish the degree of ascending aorta and aortic arch obstruction in Eln−/− pups (Figure 3B). In further experiments in which the animals were not euthanized but monitored for survival, the mortality of Eln−/− pups from rapamycin-treated mice was indistinguishable from that of vehicle-treated controls despite the increase in aortic lumen area (Figure 3C).

Changes in cross-sectional media area, a measure of medial mass, were also in parallel to those of aortic size (Figure 4D).

Rapamycin Inhibits mTOR Signaling and Proliferation of Human SVAS and WS Cells

Finally, we tested the effects of mTOR inhibition in our cultured SMC. The activation of mTOR effectors induced by 20% serum for 36 to 48 hours. Similarly, a prolonged duration of mTOR activation was found in SV AS and WS than control cells before treatment with PDGF (ie, 24 hours after serum withdrawal). Further time course experiments revealed a paradoxical partial rebound in mTOR signaling was found in 180 minutes stimulated mTOR signaling to a similar extent in serum-deprived SMC from patients with SV AS and WS versus control cells. We established that PDGF at 10 ng/mL was an appropriate near-maximal dose to use for comparisons (Figure II in the online-only Data Supplement). At this dose, treatment with PDGF for 15 to 180 minutes stimulated mTOR signaling to a similar extent in serum-deprived SMC from patients with SVAS and WS versus controls (Figure 5A).

Although early signaling events were comparable, we noted that the basal expression of phospho-S6K and phospho-S6 in human SVAS and WS versus controls (Figure 5B). Similarly, a prolonged duration of mTOR signaling was found in both patient and control cells after extended serum deprivation for 36 to 48 hours. Similarly, a prolonged duration of mTOR signaling was found in SVAS and WS versus controls after serum withdrawal (Figure III in the online-only Data Supplement).

Figure 2. Increased mammalian target of rapamycin (mTOR) signaling in elastin-deficient aortas is inhibited by maternal administration of rapamycin. mTOR activity in the offspring of heterozygous parents was assessed by Western blotting. A, Expression of phospho-p70-S6 kinase (S6K) in individual aortas of P0.5 Eln+/+, Eln−/−, and Eln−/− littermates with the signal intensity normalized to that of β-actin shown in the histogram (n=2; *P<0.05, 1-way ANOVA). B, Expression of several mTOR signaling mediators in pooled aortas (n=5–15) of Eln−/−, Eln−/−, and Eln−/− pups at P0.5. C, Expression of phospho-S6K in P0.5 Eln−/− pups born to mothers treated with either vehicle or rapamycin at 2 mg/kg IP daily from E16.5 to E18.5 with normalized values shown in the histogram (n=3; *P<0.05, f test). D, Expression of phospho-S6K and phospho-S6 in 3-week-old Eln−/− weanlings born to mothers treated with either vehicle or rapamycin at 2 mg/kg IP daily from E16.5 to P21 with normalized values shown in the histogram (n=2; **P<0.01, 1-way ANOVA).
and 6B). Rapamycin at 10 ng/mL also prevented mTOR signaling after serum withdrawal and treatment with PDGF (Figure IV in the online-only Data Supplement). As previously reported, growth curve analyses over 8 days demonstrated greater proliferation of SVAS and WS cells than control SMC in response to 20% serum. Rapamycin at 10 ng/mL markedly reduced serum-dependent proliferation of both SVAS and WS cells, albeit to a lesser extent compared with control SMC (Figure 6C and 6D). Higher doses of rapamycin up to 100 ng/mL were only modestly more effective in suppressing residual SMC proliferation and did not achieve complete growth quiescence. Rapamycin at 10 ng/mL also greatly diminished the numbers of SMC treated with PDGF in the presence of low or optimal concentrations of serum, although effects on cell survival rather than on cell proliferation cannot be distinguished under the former conditions of serum depletion (Figure IV in the online-only Data Supplement). The inhibitory effect of rapamycin on cell growth was likely independent of autophagy-mediated cell death, as induction of autophagy pathways in SMC was only seen at rapamycin doses many orders of magnitude higher than those required to suppress mTOR signaling and cellular proliferation (Figure V in the online-only Data Supplement) corroborating previous similar findings in this cell type. Moreover, rapamycin at the relevant in vitro concentrations of 1 to 100 ng/mL did not induce SMC death as assessed by cell membrane integrity (Figure V in the online-only Data Supplement). To enhance our observations from only 2 patients with elastin deficiency and to determine whether the findings apply to cell types other than SMC, fibroblasts were obtained from the skin of an additional patient with WS. As previously reported, WS fibroblasts proliferated faster than control cells during the log phase of growth. Similar to the effects on SMC, rapamycin also markedly reduced the proliferation of both WS and control fibroblasts (Figure VI in the online-only Data Supplement).

**Discussion**

We find that mTOR signaling in vessel wall cells is increased by genetic loss of elastin and that mTOR inhibition by rapamycin reduces SMC proliferation and aortic obstruction in elastin-deficient mice in vivo and decreases the excessive growth of cultured cells from patients with SVAS and WS in vitro. These results suggest that pharmacological therapy may be possible for generalized arteriopathy of patients with SVAS and WS in addition to the current mechanical means of treatment for focal lesions.
Our data demonstrate an important role for mTOR signaling in medial hypertrophy resulting from elastin deficiency, although additional mitogenic pathways are not excluded. Similarly, mTOR activity is critical for the proliferation of SMC in arteriosclerosis. The antiarteriosclerotic effects of rapamycin include direct suppression of intimal and medial SMC proliferation independent of its immunosuppressive properties on leukocytes. Of relevance, rapamycin has also been shown to inhibit SMC proliferation and medial thickening of the pulmonary artery induced by hypoxia in mice. A direct relationship between loss of elastin and increased cellular proliferation has been previously reported for human and murine SMC. However, these studies did not describe molecular mechanisms by which elastin regulates cell division. In this initial report, we do not identify intermediaries leading from elastin deficiency to activation of the nutrient-sensing and growth factor-dependent mTOR pathway, nor do we uncover the basis for prolonged mTOR signaling in SVAS and WS cells.

Figure 4. Rapamycin prevents medial hyperlamellation without compromising aortic size in Eln− mice. Vehicle or rapamycin at 2 mg/kg was administered intraperitoneally daily to gravid Eln− mice from E16.5 to E18.5-E19.5, and maternal treatment was continued throughout lactation from P0 to P21. Eln− and Eln+/− weanlings were analyzed at 3 weeks of age. A, Representative ultrasound images of the distal ascending aorta diameter (blue lines) and the results from multiple animals are shown in the histograms (n=3–5; *P<0.05, t test). B, Body weight of the animals (n=3–5; **P<0.05, ***P<0.01, t test). C, Representative elastin-Van Gieson histological stains of elastic lamellae (black appearance) in distal ascending aorta cross-sections (bar, 50 µm) and the number of layers from multiple mice are shown in the histograms (n=3–5; **P<0.01, Mann–Whitney test of rank transformed data). D, Media area (between internal and external elastic laminae) was calculated by morphometric evaluation of distal ascending aorta cross-sections (n=3–5; *P<0.05, t test).

Our current studies are examining the activity of upstream mediators and regulatory phosphatases. Although direct effects of elastin on mTOR signaling cannot be excluded, we speculate that indirect hemodynamic and arterial stress responses may also be involved. Elastin has a structural role in large arteries, and its genetic deficiency in mouse models is known to result in aortic stiffness and systemic hypertension that is alleviated by reduction of NADPH-oxidase activity. However, cell intrinsic factors also play a role as both the current and previous studies demonstrate increased SMC proliferation in vitro independent of hemodynamic factors. A site-sensitivity of the vasculature is suggested by the preferential development of severe stenosis in the aorta rather than the pulmonary trunk starting at E17.5 when fetal blood pressure is equal in both systemic and pulmonary conduits at this stage. Epigenetic factors are also suggested by the sparing of the aortic root whose SMC originate from cardiac precursors that differ from the origins of the remainder of the thoracic aorta. The higher proliferative rate of cultured
dermal fibroblasts from patients with SVAS and WS in this and previous work implies that the abnormal cellular phenotype is not restricted to elastin-producing vascular SMC and that adventitial fibroblasts may also play a role in elastin-deficient arteriopathy via paracrine signaling to the medial layer.

The more atypical arteriopathy manifestations resulting from elastin deficiency of increased elastic lamellae and vessel underdevelopment are not as well studied as stenosis and hypertension sequelae. An increased number of elastic lamellae in the aortic wall have been previously interpreted as secondary to the increased proliferation of SMC, but direct proof was lacking. Our work with rapamycin shows that the medial hyperlamellation phenotype can be rescued by inhibiting SMC mitogenesis starting at late gestation. The relative sparing of aortic growth in Eln<sup>−/−</sup> mice by rapamycin is fortuitous because any gain in lumen size by suppressing medial hypertrophy may be lost by further inward vascular remodeling. We have previously observed that rapamycin allows outward vascular remodeling, while effectively inhibiting intimal and medial SMC proliferation in an experimental model of arteriosclerosis. A similar sparing of coronary artery outward vascular remodeling by rapamycin is apparent in the data set from a clinical trial in patients who had cardiac transplant. The reasons for this beneficial drug profile are unknown, but may pertain to rapamycin-insensitive mTOR complex 2 signaling, which regulates the cytoskeleton and spatial aspects of growth.

Yet mTOR inhibition also failed, at least partially, in certain key end points of our study. Rapamycin did not completely

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**Figure 5.** Prolonged mammalian target of rapamycin (mTOR) signaling in smooth muscle cells (SMC) from patients with supravalvular aortic stenosis (SVAS) and Williams syndrome (WS). A, Representative western blotting for mTOR signaling effectors in serum-deprived SMC from the coronary arteries of a patient with SVAS (left) or from the ascending aorta of a patient with WS (right) and from corresponding vessels of age-matched controls in response to platelet-derived growth factor (PDGF)-BB at 10 ng/mL for 15 to 180 minutes. Phospho-p70-S6 kinase (S6K) and phospho-S6 normalized to β-actin are shown for the patient with SVAS (performed in duplicate) vs 4 controls (left) or for the patient with WS vs 3 controls (right). B, Similar analysis of PDGF-BB–mediated mTOR signaling for a longer duration of 6 to 48 hours in serum-deprived SMC from the patient with SVAS vs 4 controls (left) or from the patient with WS vs 3 controls (right).
suppress the division of cultured SMC from patients with SVAS and WS. Moreover, rapamycin did not completely relieve aortic obstruction nor did it prolong the survival of Eln−/− pups. We cannot exclude rapamycin toxicity contributing to the death of Eln−/− pups, although this is unlikely to occur within the observed time frame of a few days. Toxicity was not related to single allele deletion as equivalent growth retardation occurred in Eln+/− and Eln+/+ mice, which is of relevance to patients with elastin haploinsufficiency diseases. Explanations for the modest efficacy of rapamycin in the setting of elastin deficiency include inadequate drug dosage, mTOR-independent mitogenic pathways, or that death occurs from residual aortic obstruction and nonvascular causes, such as cardiac or respiratory failure.21,22 We did not measure drug levels in pups, but pregnant animals were administered doses of rapamycin known to be effective for transplacental delivery in mice.16 The inhibition of aortic mTOR activity, the prevention of medial hyperlamellation, and somatic growth retardation all argue for successful dosing. It is possible that the concentration of rapamycin required to inhibit SMC proliferation may be higher than that for other effects.

We have previously found that higher doses of rapamycin at 1.5 mg/kg per day were required to suppress SMC mitogenesis rather than 0.5 mg/kg per day for immunosuppressive effects on alloreactive T cells in the same humanized mouse model of graft arteriosclerosis.13,21 This observation is of concern for proposed trials in children with generalized arteriopathy because rapamycin can have serious side-effects in pediatric transplant recipients even at the dose used for immunosuppression.24 Besides our study, somatic growth retardation by rapamycin has been observed in mouse embryos16 and in young rats.25 This phenomenon in children is controversial with one study finding adverse effects of rapamycin on growth of pediatric transplant recipients even at the dose used for immunosuppression.26 Whereas another study found normal linear growth in a similar cohort.27 On the other hand, similar doses of mTOR inhibitors seem to be better tolerated and not cause growth retardation in children without organ transplants or the concurrent use of multiple other immunosuppressive agents, for example, in the treatment of astrocytomas associated with tuberous sclerosis.28 Before proceeding with trials of mTOR inhibitors for diffuse arteriopathy of children with SVAS or WS, it is prudent to perform further investigations to both mitigate undesired effects and to assess whether the survival of experimental animals can...
be prolonged. To that end, we have embarked on a new study to
determine for synergism of mTOR inhibitors with other classes
of drugs that inhibit SMC proliferation and to use postweaning
Eln<sup>−/−</sup> mice partially rescued by expression of a transgene for
human ELN.28

We conclude that mTOR inhibition by rapamycin is a prom-
ising strategy to reduce SMC proliferation and aortic obstruction
attributable to elastin deficiency, but that improvement in
therapeutic efficacy without significant somatic growth retardation
should be sought before initiating clinical trials for
obstructive arteriopathy in children with SVAS and WS.

Sources of Funding

This work was supported by the Kiev Foundation and the Williams
Syndrome Association (W.C. Sessa, F.J. Giordano, G. Tellides) and
The Wellcome Trust (M. Tassabehji).

Disclosures

None.

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rescue of elastin insufficiency in mice by the human elastin gene: implica-

Significance

Elastin haploinsufficiency results in supravalvar aortic stenosis and Williams syndrome characterized by arterial obstructive disease. Lumen loss is attributable to smooth muscle cell proliferation, increased layers of the arterial wall, and smaller diameter vessels. Focal lesions are amenable to surgical or percutaneous interventions, whereas multiple lesions are difficult to manage. We find increased signaling of the mitogenic mammalian target of rapamycin pathway in elastin-deficient murine aorta and in elastin haploinsufficient vascular smooth muscle cells isolated from patients with supravalvar aortic stenosis and with Williams syndrome. In these experimental systems of arterial elastin deficiency, treatment with the mammalian target of rapamycin inhibitor, rapamycin, reduces aortic obstruction in vivo and decreases the excessive proliferation of cultured cells in vitro. Mammalian target of rapamycin inhibition represents a promising pharmacological strategy to treat severe and diffuse arterial obstructive disease attributable to elastin deficiency.
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Arterioscler Thromb Vasc Biol. published online March 14, 2013;
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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http://atvb.ahajournals.org/content/early/2013/03/14/ATVBAHA.112.300407

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Supplemental Figure I: Ultrastructural detail of murine aortic wall. Thoracic aortas from adult male (upper and lower panels) and female (middle panels) $Eln^{+/+}$ and $Eln^{+/-}$ littermates were examined by transmission electron microscopy. (A) Multilayered electron-lucent elastic laminae (arrows) alternated with more electron-dense SMC (bar=10 µm). (B) SMC chromatin-containing nuclei in longitudinal section (arrows) with surrounding cytoplasm (bar=2 µm). (C) Elastic laminae with less central amorphous elastin but more peripheral microfibrils (arrows) in elastin-deficient aorta (bar=2 µm).
Supplemental Figure II: PDGF dose response in human SMC. Representative western blotting for mTOR signaling effectors in serum-deprived SMC from the coronary arteries of a patient with SVAS or an aged-matched control in response to treatment with PDGF-BB at various concentrations for 30 min. Phospho-S6K and phospho-S6 normalized to β-actin are shown for the SVAS patient (performed in duplicate) vs. 2 independent controls.
Supplemental Figure III: Prolonged mTOR signaling in elastin-deficient human SMC after serum withdrawal. Representative western blotting for mTOR signaling effectors in SMC from the coronary arteries of a patient with SVAS (left panel) or from the ascending aorta of a patient with WS (right panel) and from corresponding vessels of control age-matched individuals after changing the cell culture medium supplemented with 20% FBS to no serum for 0-48 hr. Phospho-S6K and phospho-S6 normalized to β-actin are shown for the SVAS patient vs. 3 controls (left panels) or for the WS patient vs. 3 controls (right panels).
Supplemental Figure IV: Rapamycin abrogates residual serum-derived and PDGF-mediated mTOR signaling of elastin-deficient human SMC. (A) Representative western blotting for mTOR signaling effectors in ascending aorta SMC from a patient with WS and an age-matched control after serum withdrawal and treatment with rapamycin at different doses for 24 hr. Phospho-S6K and phospho-S6 normalized to β-actin are shown in the histograms for the WS patient vs. 3 controls. (B) Similar analysis of serum-deprived SMC in response to treatment with PDGF-BB and/or rapamycin at different doses for 30 min. (C) Growth of coronary artery SMC from a SVAS patient vs. 3 age-matched controls in response to PDGF-BB at 10 ng/mL with or without rapamycin at 10 ng/ml under low (0.5%; left panel) or optimal (20%; right panel) concentrations of serum. Complete withdrawal of serum for the duration required to assess SMC growth was not possible due to poor viability of the cells. The cells were plated at 2x10^4 cells/well and counted after 4 d (n=4-8 replicates from each subject, *P<0.05, **P<0.01, ***P<0.001, one-way ANOVA).
Supplemental Figure V: Sensitivity of human SMC to suppression of mTOR signaling or induction of autophagy by rapamycin. (A) Representative western blotting for markers of autophagy (ratio of LC3A-I to LC3A-II) and mTOR signaling (phosphorylation of S6K and S6) in coronary artery SMC from a SVAS patient and an aged-matched control treated with PDGF-BB at 10 ng/mL in 0% FBS for 48 hr in the presence of varying concentrations of rapamycin from $10^{-1}$ to $10^{4}$ ng/mL. Microtubule-associated protein 1 light chain 3 alpha, also known as LC3A, occurs in two molecular forms. LC3A-I is cytosolic, whereas LC3A-II binds to autophagosomal membrane and correlates with the number of autophagosomes and is essential for their function. An increase in LC3A-II occurred at 5 orders of magnitude higher concentrations of rapamycin than the de-phosphorylation of S6K and S6. (B) SVAS SMC were cultured in 0.5% FBS or 20% FBS with different doses of rapamycin from 1-100 ng/mL for 48 hr. Dead cells were identified by Annexin V labeling due to phosphatidylserine membrane translocation and loss of membrane integrity with uptake of 7-AAD dye. Representative dot plots and a histogram of 3 replicates are shown (**$P<0.01$ vs. 0.5% FBS; $P$ not significant for rapamycin-treated vs. 20% FBS, one-way ANOVA).
Supplemental Figure VI: Rapamycin inhibits the proliferation of elastin-deficient human fibroblasts. (A) Growth curves of WS (n=1) vs. control (n=2) fibroblasts plated at 3x10⁴ cells/well in response to 10% serum ± rapamycin at 10 ng/mL for 0-8 d (n=6-8 replicates from each subject; ***P<0.001, one-way ANOVA). (B) Under the same conditions, rapamycin decreased both the number of nuclei (blue color) and their frequency of BrdU uptake (brown color) over 24 h in WS and control fibroblast cultures.
Materials and Methods

Human cells

Human studies were approved by the Human Investigation Committee of Yale University School of Medicine and the New England Organ Bank.

Coronary arteries were obtained from the explanted hearts of 9 organ donors or patients undergoing transplantation for non-ischemic cardiomyopathy who were between 22 to 64 years old (mean age of 46.3±4.8 years) and from a 39 year old cardiac transplant recipient with SVAS. Direct sequencing of ELN in the SVAS patient found a heterozygous mutation for a 4-base pair nucleotide insertion in exon 9 (c.448_449ins GTAT) predicted to result in a frameshift and premature termination codon in exon 10 with documented nonsense-mediated decay of the affected allele [1].

Ascending aortas were obtained from 3 organ donors who were between 25 and 59 years old (mean age of 40.7±9.9 years) and at autopsy from a 46 year old WS patient. Chromosome microarray (Agilent 180K) of the WS patient performed in the Cytogenetic Laboratory at Yale demonstrated a 1.407 Mb deletion at 7q11.23 (chr7:72,726,572-74,133,332.GRCh37/hg19) from TRIM50 to GTF2I, including ELN.

Foreskins were obtained after circumcision from 3 children in the first decade of life, one of whom had WS. Chromosome microarray of the WS child confirmed a 1.413 Mb deletion at 7q11.23 (chr7:72,726,572-74,133,332.GRCh37/hg19) from TRIM50 to GTF2I, including ELN [2].

The cells were isolated by explant outgrowth from minced artery and skin specimens. Vascular SMC and dermal fibroblasts were serially cultured in M199 medium with 20% fetal bovine serum (FBS) or DMEM medium with 10% FBS, respectively. The medium was supplemented with L-glutamine at 2 mmol/L, penicillin at 100 U/mL, and streptomycin at 100 µg/mL (Invitrogen). Cells at passages 3-6 were analyzed.

Animals

Mouse studies were approved by the Institutional Animal Care and Use Committee of Yale University School of Medicine. Eln+/- mice were obtained from Dr. D. Li (University of Utah) and have been previously described [4]. The animals were backbred to a C57BL/6J background for >10 generations. Offspring of heterozygote mates were studied and littermate controls were used except when their mothers were treated with either DMSO vehicle vs. rapamycin (Calbiochem) in which case different litters were compared. Certain pregnant mice also received BrdU (Sigma-Aldrich).

Imaging

In euthanized P0.5 pups, the aorta containing blood or flushed with Evans blue via the left ventricle was examined using a dissecting microscope with camera attachment (Olympus). In lightly isoflurane-anesthetized 3 and 30 wk old mice, transthoracic B-mode images of the thoracic aorta in longitudinal and transverse planes were obtained using a Vevo 770 high frequency ultrasound system (VisualSonics). Maximum transverse dimensions of the distal ascending aorta were measured at end-systole.

Histology and immunohistochemistry

The animals were exsanguinated and perfused with 4% paraformaldehyde via the left ventricle at a pressure of 80 mmHg. The distal ascending aorta was excised, post-fixed, embedded in paraffin, and 5 µm-thick transverse sections were stained with elastin-Van Gieson using standard techniques. Morphometric evaluation of the histological sections was performed using ImageJ software (http://rsbweb.nih.gov/ij/) by outlining the internal and external elastic laminae to calculate media area (between the two perimeters), vessel area (within the outer perimeter), and media thickness (difference of the two radii). Alternatively, the sections were
heat denatured and labeled with an antibody to BrdU (Abcam) or isotype-matched, nonbinding immunoglobulin. Binding of secondary antibodies (Jackson ImmunoResearch) was detected with DAB peroxidase substrate kits (Vector Laboratories), counter-stained with hematoxylin (Sigma-Aldrich), and imaged using an Axioskop2 microscope (Carl Zeiss).

Western blotting

For signaling studies, SMC were serum-deprived for 24 hr prior to treatment with PDGF-BB (R&D Systems) and/or rapamycin. Protein was extracted from aortas or cells using RIPA lysis buffer containing protease inhibitors (Thermo Scientific) with phos-stop (Roche) and boiled in SDS sample buffer for 6 min. Equal amounts of protein per sample were separated by SDS-PAGE, transferred electrophoretically to a nitrocellulose membrane (Bio-Rad Laboratories), and blotted with antibodies to phospho-mTOR (Ser2481), mTOR, phospho-S6K (Thr421/Ser424), S6K, phospho-S6 (Ser235/236), S6, phospho-4E-BP1 (Ser65), 4E-BP1, and LC3A (all from Cell Signaling Technology), or β-actin (Sigma-Aldrich), followed by horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch). Bound antibody was detected with Western Lightning Plus-ECL (Perkin Elmer). The blotting membrane was cut to allow for detection of both phosphorylated proteins and β-actin from the same sample, whereas total proteins were detected after stripping and reprobing the blotting membrane. In comparing multiple samples, the expression of phospho-proteins was normalized to that of β-actin.

Electron microscopy

Thoracic aorta was flushed in situ with saline followed by 3% glutaraldehyde buffered with 0.1M sodium cacodylate (pH 7.4), cut into 2 mm rings, postfixed in 3% glutaraldehyde in 0.1M sodium cacodylate (pH 7.4) at room temperature for 2 h, and then treated with osmium tetroxide, tannic acid, and uranyl acetate. After dehydration through a graded series of methanol and infiltration with Epon, the tissue was embedded in Epon, cut into 60 nm sections, and counterstained with methanolic uranyl acetate and lead citrate. The sections were examined in a FEI Tecnai BioTWIN transmission electron microscope at 80 kV using iTEM software.

Proliferation assays

SMC and fibroblasts were plated at sub-confluent densities in 2 cm² 24-well plates and rested overnight. The cells were treated with 1% DMSO or rapamycin for 0-8 days with fresh serum-supplemented medium and drug changes every 2 days. In some experiments, the cells were treated with PDGF-BB under low or optimal serum conditions. The cells were collected after mobilization with 0.25% trypsin and 0.5 mM EDTA and counted using a Coulter counter (Beckman Coulter). Alternatively, 1 mM BrdU (BD Pharmingen) was added to cell culture slides for 24 hr, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, denatured with 2 M HCL for 10 min, and BrdU uptake was assessed by immunohistochemistry.

Flow cytometry

Adherent and floating SMC from rapamycin-treated cultures were pelleted together and assayed for Annexin-V labeling and 7-amino-actinomycin D (7-AAD) uptake using a FACSsort (BD Biosciences).

Statistical analysis

Data represent mean±SEM. Comparisons between two groups were by t-test, between more than two groups by one-way ANOVA, for counts of elastic lamellae by Mann-Whitney test of rank transformed data, and between survival curves by logrank test. P values were two-tailed and values <0.05 were considered to indicate statistical significance. Data were analyzed using Prism 4.0 software (GraphPad).
References