

Statins Reduce Thoracic Aortic Aneurysm Growth in Marfan Syndrome Mice via Inhibition of the Ras-Induced ERK (Extracellular Signal-Regulated Kinase) Signaling Pathway

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Background—Statins reduce aneurysm growth in mouse models of Marfan syndrome, although the mechanism is unknown. In addition to reducing cholesterol, statins block farnesylation and geranylgeranylation, which participate in membrane-bound G-protein signaling, including Ras. We dissected the prenylation pathway to define the effect of statins on aneurysm reduction.

Methods and Results—*Fbn1*^{C1039G/+} mice were treated with (1) pravastatin (HMG-CoA [3-hydroxy-3-methylglutaryl coenzyme A] reductase inhibitor), (2) manumycin A (MA; FPT inhibitor), (3) perillyl alcohol (GGPT1 and -2 inhibitor), or (4) vehicle control from age 4 to 8 weeks and euthanized at 12 weeks. Histological characterization was performed. Protein analysis was completed on aortic specimens to measure ERK (extracellular signal-regulated kinase) signaling. In vitro *Fbn1*^{C1039G/+} aortic smooth muscle cells were utilized to measure Ras-dependent ERK signaling and MMP (matrix metalloproteinase) activity. Pravastatin and MA significantly reduced aneurysm growth compared with vehicle control (n=8 per group). In contrast, PA did not significantly decrease aneurysm size. Histology illustrated reduced elastin breakdown in MA-treated mice compared with vehicle control (n=5 per group). Although elevated in control Marfan mice, both phosphorylated c-Raf and phosphorylated ERK1/2 were significantly reduced in MA-treated mice (4–5 per group). In vitro smooth muscle cell studies confirmed phosphorylated cRaf and phosphorylated ERK1/2 signaling was elevated in *Fbn1*^{C1039G/+} smooth muscle cells (n=5 per group). *Fbn1*^{C1039G/+} smooth muscle cell Ras-dependent ERK signaling and MMP activity were reduced following MA treatment (n=5 per group). Corroborating in vitro findings, MMP activity was also decreased in pravastatin-treated mice.

Conclusions—Aneurysm reduction in *Fbn1*^{C1039G/+} mice following pravastatin and MA treatment was associated with a decrease in Ras-dependent ERK signaling. MMP activity can be reduced by diminishing Ras signaling. (*J Am Heart Assoc.* 2018;7:e008543. DOI: 10.1161/JAHA.118.008543.)

Key Words: aneurysm • cell signaling • Marfan syndrome • vascular biology

Marfan syndrome (MFS) is a heritable connective tissue disorder (*FBN1* [fibrillin 1] mutation) that commonly affects the cardiovascular, ocular, and musculoskeletal systems. Aortic root aneurysm formation, with subsequent dissection and rupture, remains the leading cause of death.^{1,2} Over the past decade, investigators have learned that

enhanced TGF- β (transforming growth factor- β) signaling, primarily via the noncanonical ERK (extracellular signal-regulated kinase) pathway, plays a key pathophysiological role, although the downstream events are not completely understood.³ Although prophylactic surgical aortic root replacement continues to be the primary therapeutic

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Clinical Perspective

What Is New?

- Using a murine model of Marfan syndrome, the novel findings from this study include the following: (1) Statins reduce aneurysm formation primarily via inhibition of Ras prenylation, (2) Ras prenylation reduction decreases downstream ERK (extracellular signal-regulated kinase) signaling, and (3) statin-mediated attenuation of Ras signaling correlates with inhibition of ERK-dependent MMP (matrix metalloproteinase) activation.

What Are the Clinical Implications?

- Although only prophylactic surgical replacement of the aortic root increases life expectancy in patients with Marfan syndrome, perhaps the optimal medical regimen to slow aneurysm growth or delay surgery should include drugs that block various locations within the TGF- β (transforming growth factor β) signaling pathway, including β -blockers, losartan, and statins.

treatment, several clinical trials have looked at the efficacy of various medical agents in slowing aneurysm growth, including β -blockers and angiotensin receptor blockers (losartan), whereas HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase inhibitors (statins) have been studied in animal models.^{4,5}

Statins, a class of drugs originally utilized to reduce serum cholesterol, reduce aneurysm formation in a Marfan mouse model, although the mechanism remains unknown.⁶ Statins have also been shown to reduce the ascending aortic aneurysm growth rate in humans, including in bicuspid aortic valve-associated aortopathies.^{7,8} Statins reduce cholesterol by inhibiting the enzyme HMG-CoA reductase, the rate-limiting step in the pathway that converts mevalonate to cholesterol. Statins exert other beneficial pleiotropic effects independent of their effects on cholesterol levels, including reduction in inflammation and MMP (matrix metalloproteinase) activity.⁸⁻¹¹ Moreover, HMG-CoA reductase inhibition results in the decrease of the 15- and 20-carbon hydrocarbon chain isoprenoids, farnesyl and geranylgeranyl, respectively (Figure 1).¹² Posttranslational modification of small guanine nucleotide-binding proteins (G-proteins), such as Ras and Rho, with isoprenoids is required for membrane localization.^{12,13} G-proteins are GTPases that cycle between active GTP-bound and inactive GDP-bound forms and are crucial for various cellular functions, including cell maintenance, motility, secretion, and proliferation. Membrane association allows the small G-proteins to associate with relevant membrane-bound proteins to permit downstream signaling. Ras-related proteins

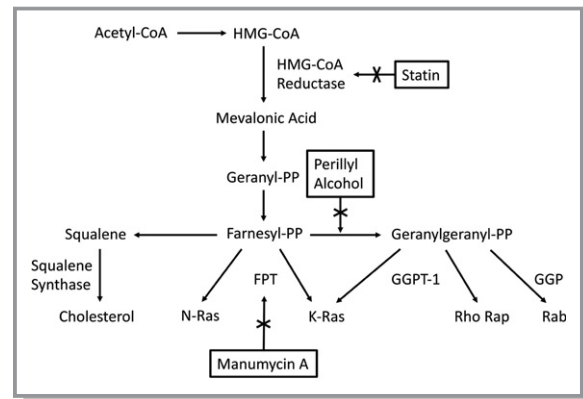


Figure 1. Schematic of the HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase pathway. The level of blockade within the pathway by each inhibitor is boxed. CoA indicates coenzyme A; FPT, farnesyl protein transferase; GGPT, geranylgeranyl protein transferase.

are typically farnesylated, whereas Rho proteins are geranylgeranylated.¹³

In this study, by systemically dissecting the prenylation pathway, we sought (1) to compare the efficacy of HMG-CoA reductase inhibition and selective isoprenoid blockade on aneurysm prevention in an MFS mouse model, (2) to establish whether the beneficial effects of isoprenoid inhibition on aneurysm reduction can be attributed to alterations in small G-protein signaling in the aorta, and (3) to help further elucidate the role of TGF- β signaling during aneurysm formation. Identification of a more targeted pathway would provide a theoretical framework for the development of targeted therapeutics aimed at slowing aneurysm growth.

Methods

The data and analytic methods will be made available to other researchers for purposes of reproducing the results or replicating the procedure. The data will be maintained at the Stanford Thoracic Aortic Surgery Research Laboratory and available upon request.

Experimental Animals

Animal protocols were approved by the Administrative Panel on Laboratory Animal Care at Stanford University. The protocols followed the National Institutes of Health and US Department of Agriculture *Guidelines for the Care and Use of Animals in Research*. Experiments were performed with equal numbers of male and female *Fbn1*^{C1039G/+} mice and C57BL/6J littermate wild-type (WT) controls. *Fbn1*^{C1039G/+} mice were kindly donated by Dr Harry C. Dietz, Johns Hopkins University School of Medicine.

Animal Treatment Groups

Fbn1^{C1039G/+} mice (4 weeks old) were treated subcutaneously with either (1) pravastatin (HMG-CoA reductase inhibitor, 100 mg/kg per day); (2) manumycin A (MA; FPT inhibitor, 2.5 mg/kg every other day); (3) perillyl alcohol (PA; GGPT1 and -2 inhibitor, 5.0 mg/kg IP every other day); or (4) vehicle control from age 4 to 8 weeks. Animals were euthanized at age 12 weeks (n=8 per group).

Echocardiography

Transthoracic echocardiography was performed at age 4 weeks (baseline) and then at 6, 8, 10, and 12 weeks of age on mice sedated with 2% inhaled isoflurane (2-chloro-2-[difluoromethoxy]-1, 1, 1-trifluoro-ethane) delivered via nose cone. The aorta was imaged in the parasternal long-axis view using a Vevo-2100 echo (Visualsonics). The aortic diameter was measured 3 times (outer edge to outer edge) at the largest portion of the aortic root/ascending aorta by 2 blinded investigators. These 6 measurements were then averaged to represent the single data point for the animal. Statistical analysis was performed using the averaged measurements from different animals.

Histology

The ascending aorta was dissected and fixed in 4% paraformaldehyde. The aorta was embedded in Tissue-Tek OCT Compound Histomount (Sakura). The sample was sliced at 4- μ m cross-sections and stained with the Accustain Elastin Verhoeff's Van Gieson kit (Sigma Aldrich), according to the manufacturer's instruction. The aorta was imaged at $\times 40$ magnification using a Leica DM4000B microscope. For quantification, the average number of elastin breaks per elastic lamina using the whole circumference was measured by a pathologist blinded to genotype and treatment arm. Three consecutive sections from each animal were graded and the average used for that individual animal was calculated. Statistical analysis was performed using the averaged measurements from 5 different animals.

Protein Assay

Protein from tissue or cells was extracted using radioimmunoprecipitation assay lysis buffer, and protein concentration was determined through a bicinchoninic acid assay, according to manufacturer's instructions (Thermo Scientific Pierce). Protein phosphorylation and expression were analyzed by Wes (ProteinSimple). Samples were mixed with Simple Western sample buffer (ProteinSimple; concentration 1.0 μ g/ μ L for in vivo studies, 0.2 μ g/ μ L for in vitro studies),

reduced, and denatured. The following antibodies and concentrations were used: phosphorylated ERK1/2 (pERK1/2; 1:50), ERK 1/2 (1:50), phosphorylated c-Raf (pRaf1; 1:150), Raf1 (Raf1 proto-oncogene; 1:150), and vinculin (1:150). All antibodies were purchased from Cell Signaling Technology. Secondary antibodies and chemoluminescent substrate are dispensed in microliter volumes into designated wells in a low-volume 25-well assay plate. Wes carried out all assay steps automatically. Quantification by densitometry was performed using the area of targeted proteins (pRaf1 and pERK1/2) and normalized to vinculin as a loading control. Results are expressed as fold change compared with littermate WT control. Experiments included the following groups: WT, n=4; *Fbn1* vehicle control, n=5; *Fbn1* MA, n=5; *Fbn1* PA, n=4.

Smooth Muscle Cell Drug Treatment Studies, In Vitro

Aortic smooth muscle cells (SMCs) were derived from 4-week-old *Fbn1*^{C1039G/+} and C57BL/6J WT littermate control mice. SMCs were cultured in SMC media (SmGM-2 Bullet Kit; Lonza) and starved for 24 hours for the protein assay and for 48 hours for both the Ras activation and MMP (matrix metalloproteinase) activity assays. SMCs were treated with (1) MA (100 nmol/L), (2) PA (800 μ mol/L), (3) tipifarnib (5 μ mol/L), or (4) vehicle control for 45 minutes, followed by mouse recombinant TGF- β (5 ng/mL). Experiments included 4 or 5 samples per group.

Ras Activation Assay, In Vitro

Ras activity was measured by the G-Lisa Ras Activation Assay Biochemistry kit (Cytoskeleton). *Fbn1*^{C1039G/+} and WT SMCs were starved for 48 hours and then treated with mouse recombinant TGF- β (5 ng/ μ L) for 10 minutes. SMC protein was extracted using cell lysis buffer and immediately frozen in liquid nitrogen (stored at -80°C). Ras activity was measured according to the manufacturer's recommended protocol. Experiments included 4 or 5 samples per group.

MMP Activity Assay (Zymography)

In vitro

Fbn1^{C1039G/+} SMC (2.0×10^5 cells/mL) were starved for 48 hours and then treated with MA (100 nmol/L), PA (800 μ mol/L), or vehicle control for 45 minutes. Mouse recombinant TGF- β (5 ng/ μ L), with or without isoproterenol (0.1 μ mol/L), was added to 1 mL condition medium and incubated for 48 hours. Following drug treatment, SMC culture supernatants were collected and concentrated using Amicon Ultra-4 centrifugal filters (Ultracel-30K; MilliporeSigma). MMP zymography assays were performed using the Gelatin-Zymography kit (Cosmo Bio). Concentrated

supernatant (10 μ L) was placed into each well and MMP2 activity was measured, according to the manufacturer's instructions. Experiments included 5 samples per group.

In vivo

Fbn1^{C1039G/+} mice (4 weeks old) were treated with either (1) pravastatin or (2) vehicle control from ages 4 to 8 weeks. Animals were euthanized at age 12 weeks (n=5 per group). The aortic root/ascending aortas were harvested and protein collected. MMP zymography assays were performed using the Gelatin-Zymography Kit, as noted. Protein samples (6 μ g) were placed into each well, and MMP2 activity was measured according to the manufacturer's instructions. MMP2 activity levels were compared with MMP2 markers and then expressed as fold difference versus littermate WT control mice.

Statistical Analysis

Statistical analysis was performed using SPSS 119.0 (IBM Corp). Data are presented as mean \pm SD. When comparing aortic diameter growth pattern between treatment groups, a

linear mixed effects model was used. The Mann–Whitney and Kruskal–Wallis nonparametric tests were used to compare non-normally distributed data, with data expressed as median and interquartile range. Significance of individual differences was evaluated using the Bonferroni or Steel–Dwinn correction for multiple comparisons. A value of $P < 0.05$ was considered statistically significant.

Results

Effect of Prenylation Inhibitors on Aneurysm Formation in Marfan Mice

Ascending aortic diameters in *Fbn1*^{C1039G/+} and C57BL/6J WT littermate control mice were measured using transthoracic echocardiography at 4, 6, 8, 10, and 12 weeks of age (Figure 2A, n=8 per group). By 12 weeks, the aortic diameter in *Fbn1*^{C1039G/+} mice reached 1.77 \pm 0.05 mm in comparison to 1.39 \pm 0.03 mm in WT littermate controls ($P=0.05$). Pravastatin treatment (HMG-CoA reductase inhibitor) significantly reduced aneurysm growth in *Fbn1*^{C1039G/+} mice

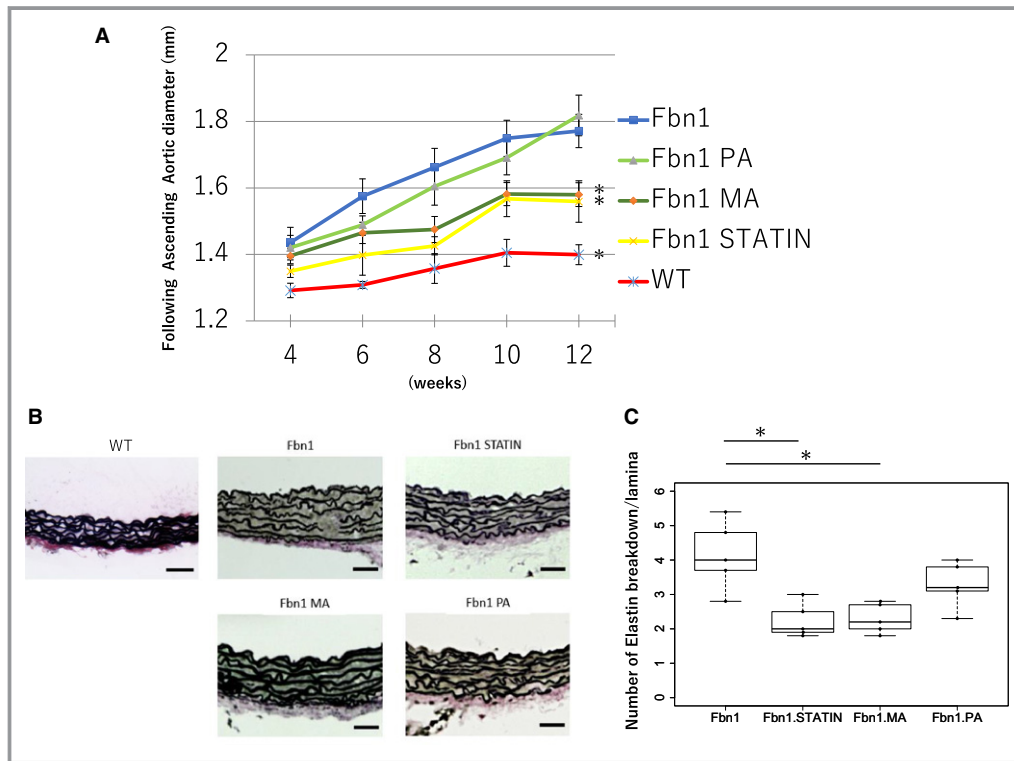


Figure 2. Aortic aneurysm development and elastin remodeling in *Fbn1*^{C1039G/+} mice treated with statins or prenylation inhibitors. **A**, Ascending aortic diameter (mm) in *Fbn1*^{C1039G/+} mice compared with *Fbn1*^{C1039G/+} mice treated with pravastatin (STATIN), manumycin A (MA), or perillyl alcohol (PA), using transthoracic echocardiography (n=8 per group). **B**, Representative elastin histological staining (Elastin Verhoeff's Van Gieson) of ascending aorta from *Fbn1*^{C1039G/+} mice and *Fbn1*^{C1039G/+} mice treated with STATIN, MA, or PA at 12 weeks. Scale bars represent 50 μ m. **C**, Average number of elastin lamina breaks per lamellae in ascending aorta (360°) of *Fbn1*^{C1039G/+} mice and *Fbn1*^{C1039G/+} mice treated with STATIN, MA, or PA at 12 weeks. Results presented as mean \pm SD. * $P \leq 0.05$. Fbn1 indicates fibrillin 1; WT, wild type.

(1.56±0.03 mm, $P=0.003$). To better define the role of specific isoprenoids in statin-induced Marfan aneurysm reduction, selective prenylation blockade was used. MA (FPT inhibition) significantly reduced aneurysm formation in *Fbn1*^{C1039G/+} mice (1.55±0.06 mm, $P=0.011$) compared with untreated vehicle control *Fbn1*^{C1039G/+} animals. In contrast, PA (GGPT1 and -2 inhibition) did not significantly alter aneurysm formation (1.81±0.06 mm, $P=0.46$).

Histological characterization with Elastin Verhoeff's Van Gieson staining (Figure 2B and 2C, n=5 per group) revealed severe elastin fragmentation during aneurysm formation in *Fbn1*^{C1039G/+} mice (4.2±0.6 breaks per lamina) compared with WT littermate controls (0.4±0.2 breaks per lamina) at 12 weeks ($P=0.02$). Correlating with reduced aneurysm growth, pravastatin and MA significantly reduced elastin breakdown in *Fbn1*^{C1039G/+} mice (2.2±0.5 breaks per lamina, $P=0.01$; and 2.3±0.4 breaks/lamina, $P=0.01$, respectively). In contrast, we did not detect any difference in elastin breakdown in PA-treated *Fbn1*^{C1039G/+} mice (3.3±0.6 breaks per lamina).

MA (Ras Inhibition) Reduces ERK Signaling in *Fbn1*^{C1039G/+} Marfan Mice

Investigators previously reported that TGF- β -dependent ERK signaling plays an important role in aneurysm formation in *Fbn1*^{C1039G/+} mice.³ Because MA reduces Ras activity, a known ERK activator, we hypothesized that prenylation inhibitors reduce aneurysm formation by impeding ERK signaling. The Ras downstream proteins, pRaf1 and pERK1/2 were measured with Wes (Figure 3; WT, n=4; *Fbn1* vehicle control, n=5; *Fbn1* MA, n=5; *Fbn1* PA, n=4). Both pRaf1 and pERK1/2 were significantly elevated in *Fbn1*^{C1039G/+} mice compared with WT littermate control (pRaf1: 8.8±0.8, $P=0.01$; pERK1/2: 3.5±0.2, $P=0.01$). Corresponding to reduced aneurysm growth, pRaf1 and pERK1/2 were significantly reduced in MA-treated *Fbn1*^{C1039G/+} mice compared with *Fbn1* vehicle control (pRaf1: *Fbn1* MA, 4.1±0.9; *Fbn1* vehicle control, 8.8±0.8 [$P=0.02$]; pERK1/2: *Fbn1* MA, 2.3±0.1; *Fbn1* vehicle control, 3.5±0.2 [$P=0.003$]). Correlating with absence of therapeutic benefit, PA did not significantly reduce pRaf1 or pERK1/2 compared with untreated *Fbn1*^{C1039G/+} mice (*Fbn1* PA: pRaf1: 6.6±0.8, $P=0.4$; pERK1/2: 2.9±0.1, $P=0.5$).

Statin and MA Reduce MMP Activity by Blocking Ras-Dependent ERK Signaling

To define a mechanistic link between TGF- β -dependent ERK signaling and the protective effect of statins reported during Marfan mice aneurysm formation, we performed in vitro studies using aortic SMCs derived from *Fbn1*^{C1039G/+} mice. At baseline, WT and untreated *Fbn1*^{C1039G/+} SMC have similar

Ras, Raf, and ERK1/2 levels (Figure 4A–4C). We incubated *Fbn1*^{C1039G/+} SMCs with recombinant TGF- β or vehicle control and measured both Ras activity and ERK activation. Confirming our in vivo findings, TGF- β treatment significantly increased pRaf1 and pERK1/2 signaling in *Fbn1*^{C1039G/+} SMC (data not shown; n=5 per group, $P=0.03$). Then, to determine whether the prenylation inhibitors reduce Ras-dependent ERK activation, we quantified Ras, pRaf1, and pERK1/2 activation in TGF- β -stimulated *Fbn1*^{C1039G/+} SMCs following MA and PA treatment (Figure 4A–4C; n=5 per group). A significant reduction in Ras, pRaf1, and pERK1/2 activation was detected after MA but not PA treatment (Ras: *Fbn1* TGF- β , 1.94±0.29; *Fbn1* MA, 1.38±0.35, $P=0.04$; *Fbn1* PA, 1.51±0.26, $P=0.14$; pRaf1: *Fbn1* TGF- β , 1.79±0.32; *Fbn1* MA, 0.86±0.23, $P=0.01$; *Fbn1* PA, 1.26±0.38, $P=0.27$; pERK1/2: *Fbn1* TGF- β , 0.96±0.11; *Fbn1* MA, 0.36±0.08, $P=0.003$; *Fbn1* PA, 0.58±0.18, $P=0.12$). Even more, a similar reduction in Ras and pERK1/2 signaling was found after tipifarnib treatment (Figure 4D; n=4 per group), which is another competitive farnesyl transferase Ras inhibitor, like MA (pRaf1: *Fbn1* TGF- β , 1.25±0.16; *Fbn1* tipifarnib, 0.67±0.13, $P=0.03$; pERK1/2: *Fbn1* TGF- β , 0.96±0.11; *Fbn1* tipifarnib, 0.58±0.07, $P=0.03$). Finally, pERK1/2 activation was increased in both MA- and PA-treated *Fbn1*^{C1039G/+} SMCs following incubation with isoproterenol, a known ERK stimulator (Figure 4D; n=5 per group; pERK1/2: *Fbn1* MA, 0.41±0.04; *Fbn1* MA isoproterenol, 0.80±0.02, $P=0.01$; *Fbn1* PA, 0.59±0.09; *Fbn1* TGF- β PA isoproterenol, 1.18±0.06, $P=0.01$).

Proteolytic degradation of the extracellular matrix by MMPs participates in aneurysm formation.¹¹ The increase in elastin fragmentation evident on histological sections suggests that heightened proteolytic activity may explain aneurysm formation in *Fbn1*^{C1039G/+} mice. As biological proof of concept that Ras inhibition protects against extracellular matrix remodeling, MMP2 activity was measured by gelatin zymography in supernatant secreted by *Fbn1*^{C1039G/+} SMC (Figure 5; n=5 per group). MMP2 activity was significantly increased after TGF- β stimulation compared with untreated aortic SMCs (*Fbn1* TGF- β : 2.41±1.1-fold increase, $P=0.01$). This enhancement in MMP2 activity was significantly reduced following MA treatment (*Fbn1* TGF- β : 2.41±1.1; *Fbn1* MA: 0.97±0.41, $P=0.03$). Finally, increased MMP2 activity returned once MA-treated SMCs were incubated with isoproterenol (*Fbn1* MA: 0.97±0.41; *Fbn1* MA ISO: 2.48±1.36, $P=0.04$). As predicted, PA treatment did not significantly reduce MMP2 activity (data not shown).

After finding that Ras inhibition reduces MMP2 activity in vitro, we returned to our animal model and measured MMP activity levels in the ascending aorta of *Fbn1*^{C1039G/+} mice treated with either pravastatin or vehicle control. Corroborating the in vitro studies, MMP2 activity was significantly

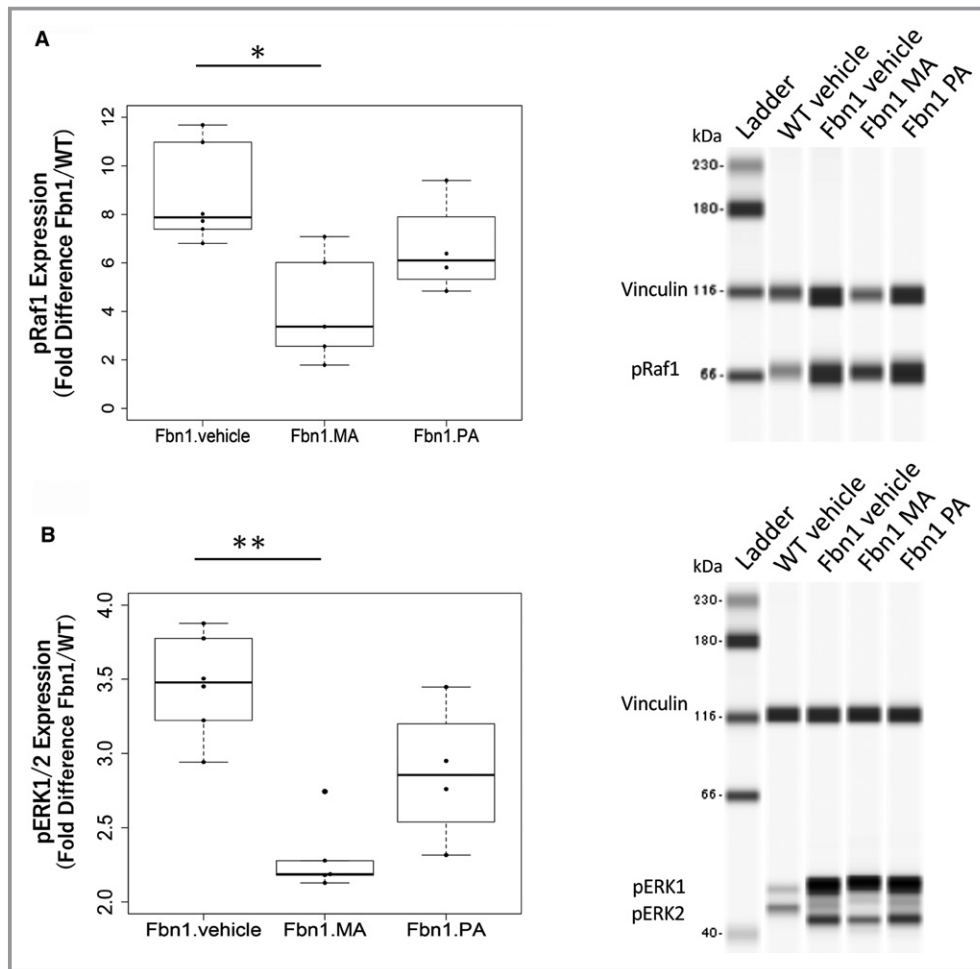


Figure 3. ERK (extracellular signal-regulated kinase) signaling in *Fbn1*^{C1039G/+} Marfan mice treated with prenylation inhibitors. **A**, WES of phosphorylated c-Raf (pRaf1) in ascending aortic specimens from *Fbn1*^{C1039G/+} Marfan mice treated with vehicle control (n=5), manumycin A (MA; n=5), or perillyl alcohol (PA; n=4) and euthanized at 12 weeks. Expressed as fold difference compared with littermate wild-type (WT) control mice (n=4). **B**, WES of phosphorylated ERK1/2 (pERK1/2) in ascending aortic specimens from *Fbn1*^{C1039G/+} Marfan mice treated with vehicle control (n=5), MA (n=5), or PA (n=4) and euthanized at 12 weeks. Expressed as fold difference compared with WT mice (n=4). **P*≤0.05. ***P*≤0.01. Fbn1 indicates fibrillin 1.

decreased in pravastatin-treated mice compared with *Fbn1*^{C1039G/+} vehicle control (pravastatin: 2.32±0.13; vehicle control: 4.91±0.81, *P*=0.01; Figure 6, n=5 per group).

Discussion

The pathophysiology of aneurysm formation in MFS is a complex multifactorial process involving vascular SMC apoptosis, reduced elastin production and stability, and enhanced extracellular matrix breakdown and remodeling.^{14–16} Understanding the molecular pathways that lead to aneurysm formation may translate into novel medical therapies directed at preventing or slowing aneurysm growth. Statins (HMG-CoA reductase inhibitors) are lipid-lowering agents that have beneficial pleiotropic, cholesterol-independent protective

effects, including anti-inflammatory, antioxidative, and antiproliferative properties.^{8–11} Utilizing HMG-CoA reductase and selective isoprenoid inhibitors, we have systematically dissected the prenylation pathway to better define the mechanism behind statin reduction of aneurysm formation in a murine model of MFS (Figures 1 and 7). The major findings of this study are as follows: (1) Statins reduce aneurysm formation primarily via inhibition of Ras prenylation, (2) Ras prenylation reduction decreases downstream ERK signaling, and (3) statin-mediated attenuation of Ras signaling correlates with inhibition of ERK-dependent MMP activation.

In the current study, we use a well-established Marfan mouse model that reproducibly develops ascending aortic aneurysms, recapitulating the pathology seen in human MFS. The *Fbn1* mutation in this model system leads to enhanced

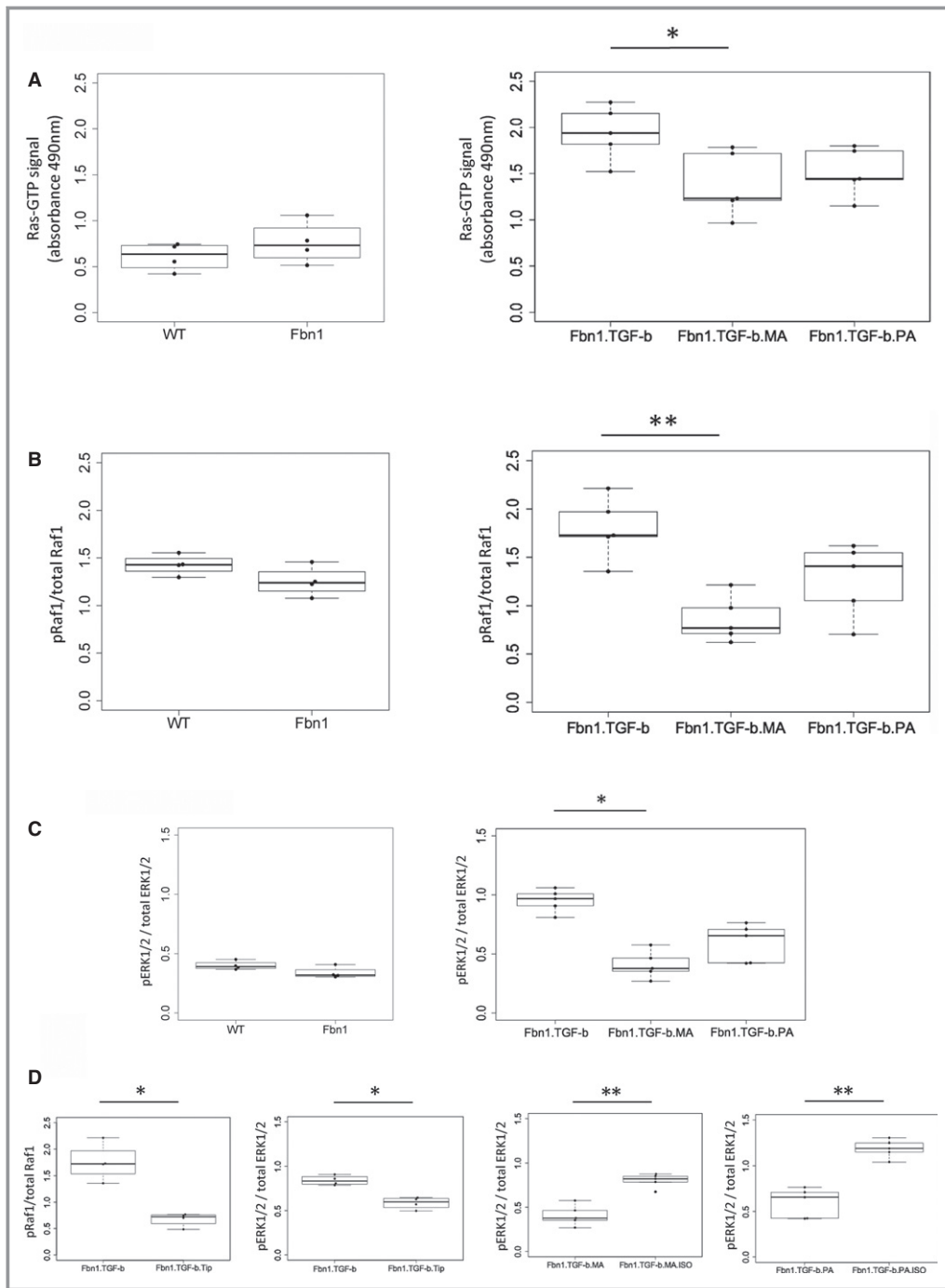


Figure 4. ERK (extracellular signal-regulated kinase) signaling in *Fbn1*^{C1039G/+} ascending aorta-derived smooth muscle cells (SMCs). **A**, Ras activity measured in *Fbn1*^{C1039G/+} SMCs treated with TGF-β (transforming growth factor β) alone, TGF-β and manumycin A (MA), or TGF-β and perillyl alcohol (PA). n=5 per group. **B**, Wes protein analysis of phosphorylated c-Raf (pRaf1) or phosphorylated ERK 1/2 (pERK 1/2) in ascending aorta-derived SMCs from *Fbn1*^{C1039G/+} Marfan mice treated with TGF-β alone, TGF-β and MA, or TGF-β and PA. n=5 per group. Expressed as fold difference compared with total c-Raf or pERK 1/2. **C**, Wes protein analysis of pRaf1 or pERK 1/2 in ascending aorta-derived SMCs from *Fbn1*^{C1039G/+} Marfan mice treated with TGF-β alone or TGF-β and tipifarnib. n=4 each group. Expressed as fold difference compared with total c-Raf or pERK 1/2. **D**, Wes protein analysis of pRaf1 or pERK 1/2 in ascending aorta-derived SMCs from *Fbn1*^{C1039G/+} Marfan mice treated with TGF-β and MA, TGF-β and MA plus isoproterenol (ISO), TGF-β and PA, or TGF-β and PA plus ISO. n=5 per group. Expressed as fold difference compared with total c-Raf or pERK 1/2. Results presented as mean±SD. *P<0.05. **P<0.01. Fbn1 indicates fibrillin 1; WT, wild type.

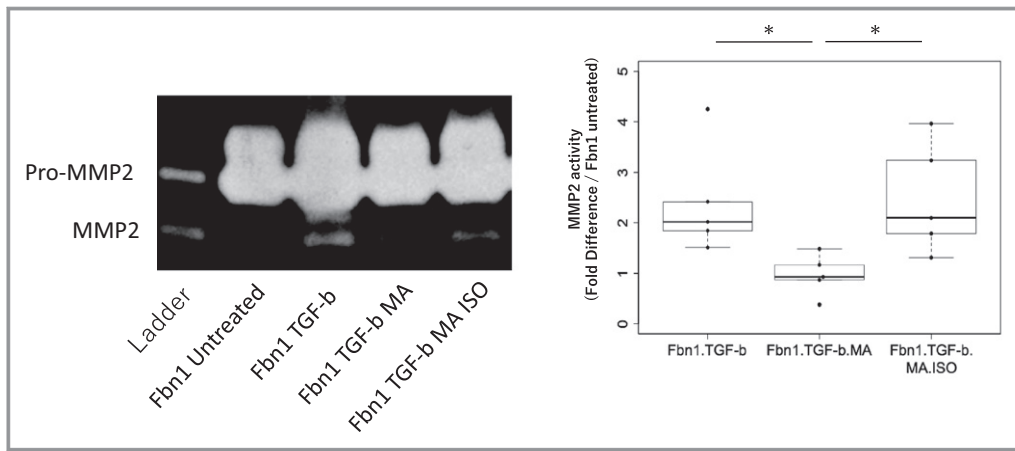


Figure 5. MMP (matrix metalloproteinase) activity level in *Fbn1*^{C1039G/+} ascending aorta-derived smooth muscle cells (SMCs) following manumycin A (MA) treatment. **A**, MMP zymography in *Fbn1*^{C1039G/+} SMCs treated with TGF-β (transforming growth factor β) alone, TGF-β and MA, or TGF-β and MA plus isoproterenol (ISO). n=5 per group. **B**, MMP activity expressed as fold difference comparing *Fbn1*^{C1039G/+} treated vs untreated SMCs. Results presented as mean±SD. *P<0.05. Fbn1 indicates fibrillin 1.

TGF-β signaling, acting predominantly via the noncanonical ERK pathway.^{2,17} Importantly, the molecular mechanisms by which excessive ERK signaling leads to aneurysm development remain unknown. In this study, we further delineated the pathway of ERK-mediated aneurysm formation in this model system, reporting that Ras-dependent ERK activation increases MMP activation. Statins have previously been proposed to slow abdominal aortic aneurysm growth, given their pleiotropic, anti-inflammatory actions rather than their lipid-lowering effects.^{18,19} Several previous studies have reported that inflammatory cells are major sources of MMPs during abdominal aortic aneurysm formation in both human and animal models of abdominal aortic aneurysm formation.^{20,21} Importantly, the absence of inflammatory cells in this Marfan model suggests that statins must reduce aneurysm formation through an alternative mechanism, likely

via the diminution of MMPs or other yet unmeasured elastases produced directly from SMCs. Nevertheless, we cannot rule out the possibility that statins enhance SMC survival and elastin production.

Prenylation inhibitors are utilized as a tool to further delineate the mechanism leading to TGF-β-mediated aneurysm formation in the *Fbn1*^{C1039G/+} mouse model. Our in vitro experiments infer that statins reduce aneurysm formation by inhibiting TGF-β-mediated Ras signaling, thereby downregulating ERK-induced MMP activity. Supporting this hypothesis, statin therapy also reduced MMP activity and aneurysm formation in *Fbn1*^{C1039G/+} ascending aortas in vivo. Notably, the detected reduction in MMP activity levels are subtle in both in vitro and in vivo model systems. Possible explanations include the following: (1) Statin treatment dosage or duration are suboptimal, (2) statins only partially block MMP activity,

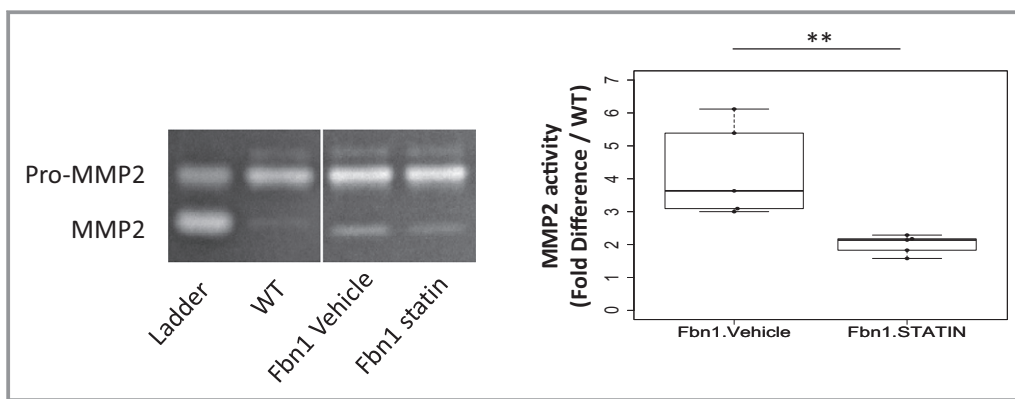


Figure 6. MMP (matrix metalloproteinase) activity level in *Fbn1*^{C1039G/+} ascending aorta following statin treatment, in vivo. **A**, MMP zymography in littermate wild-type (WT) control, *Fbn1*^{C1039G/+} with vehicle, or *Fbn1*^{C1039G/+} with pravastatin (STATIN). n=5 per group. **B**, MMP activity expressed as fold difference, *Fbn1*^{C1039G/+} vs WT. Results presented as mean±SD. **P<0.01. Fbn1 indicates fibrillin 1.

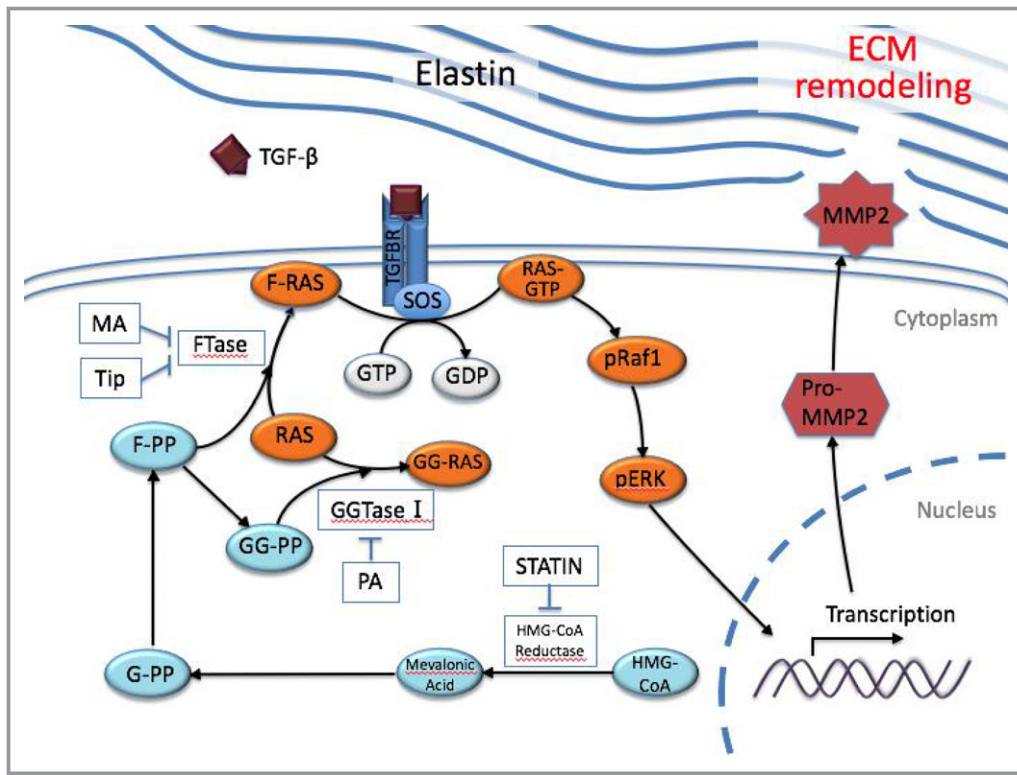


Figure 7. Statins and prenylation inhibitors in Marfan syndrome aneurysm development: hypothesized model. The GTPase Ras is a key regulator of TGF- β (transforming growth factor β)–dependent ERK (extracellular signal-regulated kinase) signaling. Ras modification with the isoprenoids, farnesyl (F-PP) and geranylgeranyl (GG-PP) allows membrane attachment. TGF- β signaling through a receptor serine/threonine kinase complex (TGFBR) results in Ras activation (Ras-GTP). In *Fbn1*^{C1039G/+} aortic root/ascending aorta, we hypothesize that TGF- β activates Ras, which triggers ERK signaling. ERK activation regulates a wide variety of targets involved in breakdown of the extracellular matrix (ECM), including the matrix metalloproteinases (MMPs). We believe statins indirectly reduce TGF- β –dependent ERK activation by preventing Ras modification with isoprenoids, thereby decreasing membrane association. HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; MA, manumycin A; PA, perillyl alcohol; pERK, phosphorylated ERK; pRaf1, phosphorylated c-Raf; STATIN, pravastatin; Tip, tipifarnib.

and (3) the MMP activity assay has limitations. Ras proteins are small GTPases that translocate from the cytoplasm to the plasma membrane following farnesylation by farnesyltransferase—essential for turning on downstream signaling pathways, including ERK—and then activate transcription factors.^{22,23} Analyzing the prenylation pathway, MA seems to be most effective at reducing aneurysm formation because it blocks farnesyltransferase (N-Ras, K-Ras). In contrast, we hypothesize that PA, which predominantly inhibits geranylgeranyl transferase 1 and 2 and, to a smaller extent, farnesyltransferase, is less effective at reducing aneurysm formation because it blocks the GTPases Rap and Rab and, to a minor extent, Ras.²⁴ An obvious study limitation is that the individual GTPases were not quantified; therefore, we cannot comment on the relative amounts that each were reduced. Using the same mouse model, McLoughlin et al initially described the attenuation of aortic dilatation by pravastatin but attributed the reduction to a yet undefined decreased in

protein production because they noted reduced rough endoplasmic reticulum histologically.⁶ Supporting our findings, Luan et al found that statins can inhibit vascular SMC secretion of MMP1, -2, -3, and -9 in vitro given an indeterminate posttranscriptional process.⁹

Approaches to target and inhibit Ras proteins are under way in many biotechnology and pharmaceutical companies, especially in the area of human cancers, RASopathies (neurofibromatosis type 1, Noonan syndrome, Noonan with Multiple Lentigenes [NSML], Cardio-Facio-Cutaneous (CFC), and Costello and Legius syndromes), and neurological disorders.^{25–28} There are challenges to blocking Ras signaling, including its physical structure, multiple feedback loops and signaling redundancy, and the fact it is a critical pathway for most normal cells. Statin therapy may be an innocuous strategy to reduce TGF- β –mediated Ras/ERK activation in Marfan patients, without the potentially harmful systemic affects (immunity and cell growth).^{4,6,29} Notably, although excessive TGF- β signaling

promotes aneurysm formation by increasing ERK activation in mice, whether this mechanism has fidelity with the human disease has not been firmly established. Furthermore, because TGF- β stimulates multiple signaling pathways, it is unclear which pathways result in aortic disease. Although only prophylactic surgical replacement of the aortic root effectively increases life expectancy in MFS patients, perhaps the optimal medical regimen to slow aneurysm growth or delay surgery should include drugs that block various locations within the TGF- β signaling pathway, including β -blockers, losartan, and statins. The answer is most likely to be found only with a large prospective randomized clinical trial.

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Disclosures

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